

ISSN 2525-2461

Vietnam Journal of Science, Technology and Engineering

JUNE 2017 • VOL.59 NUMBER 2



Effects of elicitors on the growth and active compounds
of *Panax vietnamensis* cells in the bioreactor

CLASSIFICATION SCHEME

1. Mathematics and Computer Science

- 1.1. Mathematics
 - 1.2. Computer Science
 - 1.3. Computational Science
-

2. Physical Sciences

- 2.1. Physics
 - 2.2. Chemistry
 - 2.3. Engineering
-

3. Life Sciences

- 3.1. Agriculture
 - 3.2. Medicine
 - 3.3. Pharmacology
 - 3.4. Biology
 - 3.5. Biotechnology
-

4. Geosciences

- 4.1. Geography
 - 4.2. Geology
 - 4.3. Geophysics
 - 4.4. Oceanography
-

5. Nanoscience and Nanotechnology

- 5.1. Nanophysics
 - 5.2. Nanochemistry
 - 5.3. Nanobiotechnology
 - 5.4. Nanomedicine
 - 5.5. Nanoengineering
-

6. Environmental Sciences

- 6.1. Ecology
- 6.2. Climatology

A study of the magnetic properties of $\text{LaNi}_{5-x}\text{Si}_x$ alloys and their hydrides

Nhan Ba Dam^{1*}, Van Tuan Chu¹, Anh Tuan Do², Quyet Thang Le³

¹Department of Basic science, Hung Yen University of Technology and Education

²Faculty of Electronic and Electrical engineering, Hung Yen University of Technology and Education

³Faculty of Electronic and Electrical engineering, Quang Ninh University of Industry

Received 5 April 2017; accepted 8 June 2017

Abstract:

In this work, we study the influence of the substitution Si for Ni for the magnetic properties of $\text{LaNi}_{5-x}\text{Si}_x$ ($x = 0-0.5$) alloys. The structure of the alloy was tested by X-ray diffraction (XRD) measurements. The magnetic properties of the samples were carried out using a vibrating sample magnetometer (VSM) system. The results showed that when Ni was substituted for Si in alloys and after hydrogen absorption, $\text{LaNi}_{5-x}\text{Si}_x$ alloys retained their CaCu_5 structure. The susceptibility (χ) of the alloys decreased when substituting Ni by Si increased. The original bulk samples showed a Pauli paramagnetism state after hydrogen absorption transformed into a superparamagnetic state. The results obtained showed that although measurements of magnetic characteristics are relatively simple, quantitative information about the microscopic processes occurring in the material was used as the negative electrode in Ni-MH rechargeable battery.

Keywords: hydrogen absorption, LaNi_5 , magnetic properties, nano, Ni-MH batteries.

Classification number: 2.1

Introduction

An investigation of the R - T (R = rare earth, T = transition metal) interaction in $4f$ - $3d$ intermetallic compounds has been the subject of many theoretical and experimental studies [1, 2]. R - T compounds are known to show interesting magnetic properties associated with both localised moments of rare earth atoms and itinerant electrons of the $3d$ atoms [3, 4]. There is considerable interest in the electronic structure of LaNi_5 in regard to both its high capacity for hydrogen absorption and in its feature of having exchange-enhanced susceptibility [5-7]. LaNi_5 possesses the hexagonal CaCu_5 structure (space group $P6/mmm$) with one formula unit (f.u.) per unit cell [8-11].

The LaNi_5 compound is also considered to be at 4.2 K using an exchange-enhanced paramagnet, having a magnetic susceptibility of $\chi = 2.5 \times 10^{-3}$ emu/f.u. or 2.27×10^{-3} emu/f.u. [12]. Since no corrections for the possible presence of magnetic impurities have been made, the above values can be considered as upper limits. The estimated exchange-enhancement factor ($s = 5-6$) is lower than that reported for Laves-phase compounds [13, 14]. Thus, it is of interest to analyse the magnetic behaviour of systems which show exchange-enhancement factors intermediate between those reported for RCO_2 compounds and normal paramagnets [15, 16].

There has been a lot of research to improve the quality of the material in LaNi_5 systems, such as the replacement of La metal by Nd, Er, Y, etc. [17-21], and the replacement of Ni metal with Al, Cu, Fe, etc. [22-24]. Research is continuing to improve performance and extend the lifetimes of Ni-MH batteries. In this paper, we study the effect of the substitution of Si for Ni of the magnetic properties of alloys $\text{LaNi}_{5-x}\text{Si}_x$ used as the negative electrode in Ni-MH batteries recharge. The partial replacement of Ni using Si LaNi_5 materials acts as an antioxidant and increases the lifetime of Ni-MH batteries.

*Corresponding author: Email: damnhanba@gmail.com

Experimental

Reagents and MH electrode preparation

The $\text{LaNi}_{5-x}\text{Si}_x$ system alloys were prepared using an arc-melting method in an Ar gas atmosphere from the metal components at a purity of at least 99.9% or more. Here, a slight excess of La was added to compensate for the weight loss during arc melting. The samples were turned over and re-melted several times to attain good homogeneity. Powder samples were created by milling in an agate mortar for 30 minutes to a grain size of about 50 μm . Hydride samples were prepared using a process of charge-discharging per 10 cycles. Electrochemical measurements were conducted in a three electrodes system. The electrolyte was 1M LiOH and 6M KOH, and the positive electrode was $\text{Ni}(\text{OH})_2$, which is a negative electrode material in which we need to study. In charge-discharge capacity measurements, all electrodes are connected to a potential device called a Bi-Potentiostat 366A. The electrodes were fully charged (the over-charged ratio was approximately 30-50%) at the current density of 50 mA/g, and then discharged at the same current density to the cut-off potential of -0.7 V (versus SCE). Electrochemical measurement results have been shown in our previous studies.

Microstructure measurements

The crystalline structure and the phase impurities of the samples at room temperature were examined using X-ray powder diffractometer, using $\text{Cu-K}\alpha$ radiation. The obtained powder XRD patterns were analysed via means of a Rietveld refinement procedure using X'pert High Score Plus in order to determine the type of structure and the lattice parameters.

Magnetization measurements

Magnetic properties of the alloys $\text{LaNi}_{5-x}\text{Si}_x$ were measured using a vibrating sample magnetometer (VSM) in magnetic fields up to 1.3 T. The temperature dependence of the magnetization $M(T)$ of hydride samples was measured in a nitrogen gas environment; and the temperature ranged from 300 K to above 700 K. The maximum applied field was 0.1 T. During the measurement, the temperature was raised at a rate estimated to be about 5 K.

Results and discussions

Crystal structure analysis

X-ray diffraction normally is one of the most useful routes to examine the crystal structure and to determine lattice parameters of materials. When replacing a part of Ni by Si in LaNi_5 alloy, crystal characteristics of the alloy remains unchanged compared to the LaNi_5 original material, although the Si atoms are smaller than the Ni atoms, this result is shown in Fig. 1. Fig. 2 shows the $\text{LaNi}_{4.5}\text{Si}_{0.5}$ alloy before and after cycling 10 cycles; the alloys remains as a CaCu_5 structure. Characteristics of the sample crystal decreased, which was

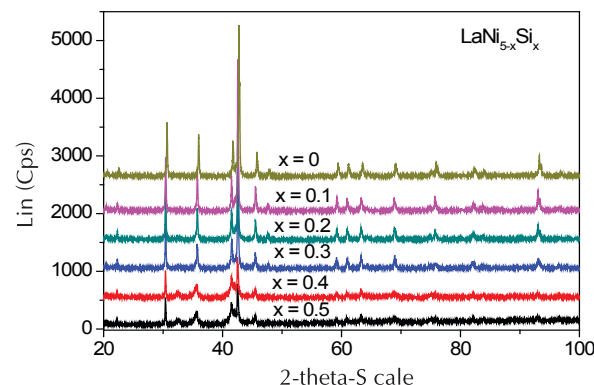


Fig. 1. The X-ray diffraction spectrum of the LaNi_5 and $\text{LaNi}_{5-x}\text{Si}_x$ ($x \leq 0.5$) alloys.

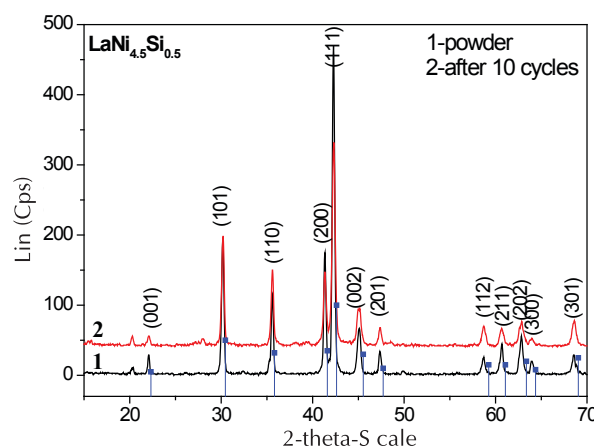


Fig. 2. The X-ray diffraction spectrum of the alloy $\text{LaNi}_{4.5}\text{Si}_{0.5}$ after 10 cycles.

presented for the expanded diffraction peaks and decreased in diffraction peaks intensity. This is because the hydrogen absorption process can cause stretch lattice, but not by much. The volume expansion on hydriding, $\Delta V/V$, decreases almost linearly with increasing silicon content, the decrease being almost proportional to the decrease in hydrogen content of the hydrides.

The magnetic properties

Measurements are carried out on the bulk samples, the powder samples after ground (not to absorb hydrogen) and powder samples were taken after charge-discharge at 10 cycles for the whole system. The magnetisation curves of the bulk samples and the powder samples after grinding are shown in Fig. 3. The magnetisation curve of bulk LaNi_5 and $\text{LaNi}_{5-x}\text{Si}_x$ samples is a straight line passing through the origin of this is characteristic of Pauli paramagnetic with magnetic susceptibility are listed in Table 1.

From the table above, we have clearly seen that the value of magnetic susceptibility of the compound $\text{LaNi}_{5-x}\text{Si}_x$ reduced when the mass or the Si composition increased. It is understandable that the elemental Si is not magnetic, while an increase in the element Si, decrease the magnetic element Ni, and it leads to a reduction of magnetic susceptibility.

Table 1. Magnetic susceptibility of the sample LaNi_5 and $\text{LaNi}_{5-x}\text{Si}_x$.

Order number	Sample	$\chi (10^{-6})$
1	LaNi_5	3.700
2	$\text{LaNi}_{4.9}\text{Si}_{0.1}$	2.813
3	$\text{LaNi}_{4.8}\text{Si}_{0.2}$	2.571
4	$\text{LaNi}_{4.7}\text{Si}_{0.3}$	2.326
5	$\text{LaNi}_{4.6}\text{Si}_{0.4}$	2.157
6	$\text{LaNi}_{4.5}\text{Si}_{0.5}$	1.531

Figure 3 also shows magnetisation curves of $\text{LaNi}_{5-x}\text{Si}_x$ powder samples after milling to a grain size of about 50 μm . The figure we see for all $\text{LaNi}_{5-x}\text{Si}_x$ powder samples shows super - magnetism. After milling, the magnetism of the samples increased, which was represented in the increasing moments of the sample since the magnetic field was tested. The reason is that the Ni particles are disabsorbed out from the surface of the particles due to La oxidation by oxygen and water vapour in the air during charging-discharging. The concentration of Ni particles on the surface of the material increases, which Ni is ferromagnetic elements thereby changing the magnetic state of the material.

Magnetisation curves of $\text{LaNi}_{5-x}\text{Si}_x$ samples taken after charge-discharge 10 cycles are shown in Fig. 4. We see that the magnetic moment of the samples increases remarkably. The reason for that is that the nanometer-scale Ni particles are disabsorbed out the surface of the particles due to La oxidation by oxygen and water vapour in the air during charging-discharging. The concentration of Ni particles on the

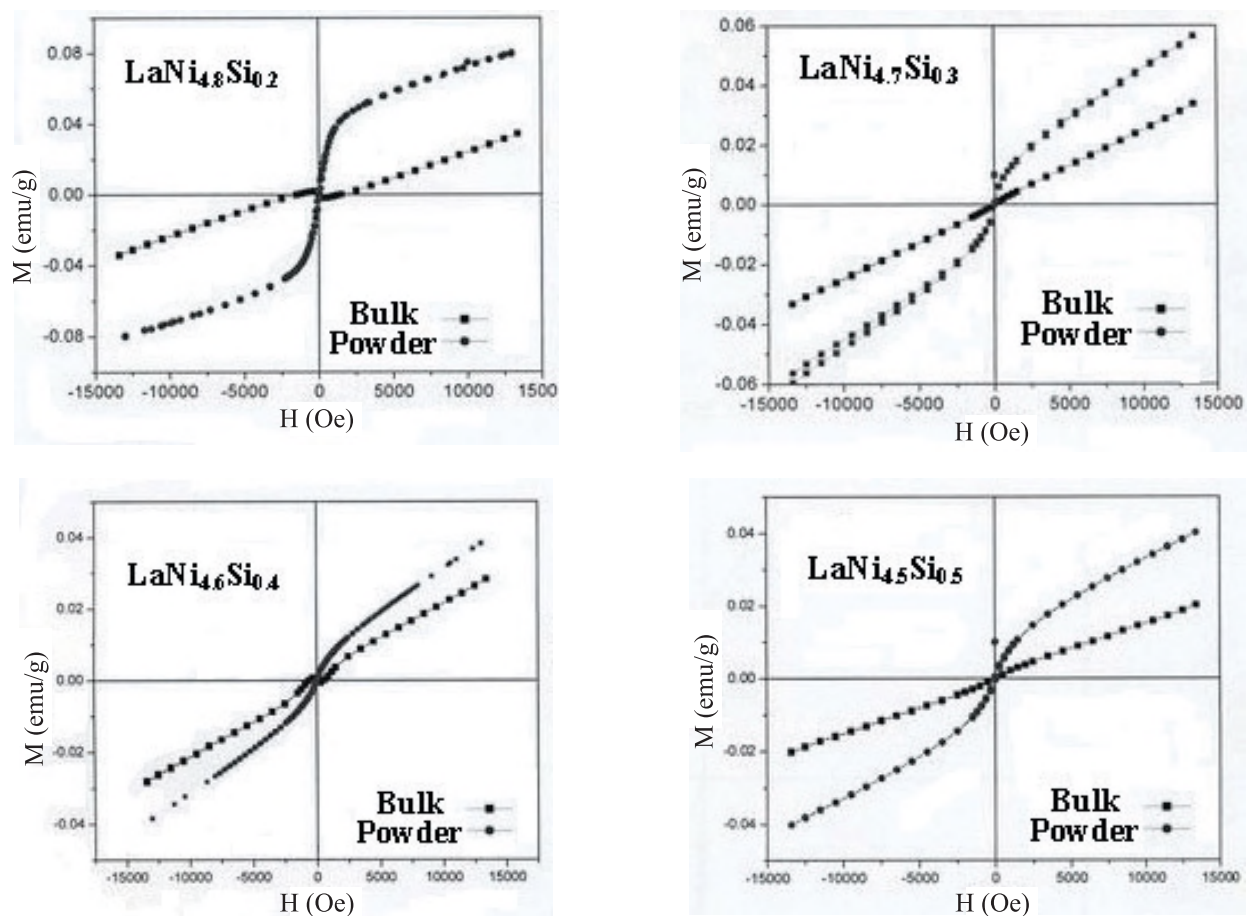


Fig. 3. Magnetisation curves of the powder samples and the bulk samples of $\text{LaNi}_{5-x}\text{Si}_x$.

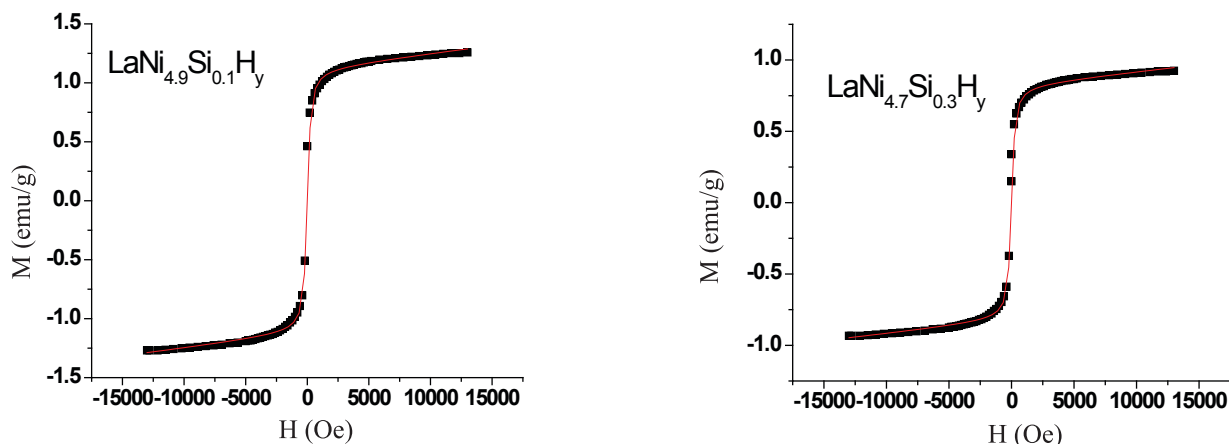


Fig. 4. Magnetisation curve of the sample $\text{LaNi}_{4.9}\text{Si}_{0.1}\text{H}_y$ and $\text{LaNi}_{4.7}\text{Si}_{0.3}\text{H}_y$ after charge-discharge 10 cycles.

surface of the material increases, which Ni is ferromagnetic elements thereby changing the magnetic state of the material. However, if the Ni atoms release too much, it will lead to the destruction of the material structure.

Changes to the characteristic phenomenon before and after hydrogenation by Ni atoms are releasing surface material during the hydrogenation was K. Suzuki analysed via the use of X-ray diffraction diagrams on two LaNi_5 and $\text{LaNi}_{4.7}\text{Al}_{0.3}$ materials systems [25]. The X-ray diffraction spectrum of the LaNi_5 and $\text{LaNi}_{4.7}\text{Al}_{0.3}$ alloys showed after 2000 charge-discharge cycles crystal characteristic reduced, reflected in the expansion and reduce the intensity of the diffraction peaks, have been shown by an appearance of the characteristic peak for the element Ni, which is the element causing a change of state of magnetic materials.

The results are quite consistent with previous studies on the electrochemical properties of the negative electrode materials used in battery recharging of Ni-MH [26]. When the charge-discharge process occurs in a negative electrode, Ni atoms released to constitute clusters of particles on the surface of the material. Therefore, increases the magnetic properties of materials. Through an examination of the nature of the material, the method for fast results and high accuracy, we can evaluate electrochemical process occurs at the negative electrode.

Conclusions

We have investigated the crystallographic and the magnetic characteristics of the intermetallic $\text{LaNi}_{5-x}\text{Si}_x$ ($x \leq 0.5$). The XRD patterns provide evidence of the single phase and crystallising in the hexagonal CaCu_5 -type structure in all

samples. The lattice parameters calculated by the Rietveld method, both a and c , remained mostly unchanged by the replacement of Ni with Si. The bulk sample is characteristic of Pauli paramagnetic. The value of magnetic susceptibility of the compound $\text{LaNi}_{5-x}\text{Si}_x$ reduces when the mass or the Si composition increases. For powder and after charge-discharge 10 cycles samples, the material transfer from the paramagnetic to superparamagnetic state was studied because Ni elements release surface material during charging-discharging. The magnetic susceptibility of the material increases as the number of charge-discharge cycles increase. The analysis showed that although measurements of magnetic characteristics are relatively simple but can give us quantitative information about the microscopic processes occurring in the negative electrode. Such magnetic characteristics measurements can be seen from the analytical method have high sensitivity used to evaluate the quality of the electrode through survey and comparison with standard samples.

ACKNOWLEDGMENTS

This work is partly supported by the Key Project DTKHCN/001/16 of the Ministry of Industry and Trade.

REFERENCES

- [1] T. Sakai, H. Miyamura, N. Kuriyama, H. Ishikawa, I. Uehara (1993), "Rare-earth-based hydrogen storage alloys for rechargeable nickel-metal hydride batteries", *J. Alloys and Compd.*, **192(1-2)**, pp.155-157.
- [2] S. Longchamp, F. Goubard (2004), " AB_5 -type intermetallic compound as a substrate for nickel hexacyanoferrate modified electrodes", *Sensors and Actuators B: Chem.*, **99(2-3)**, pp.516-524.

- [3] J. Yang, C. Tai, G. Marasinghe, et al. (2000), "Structural, electronic, and magnetic properties of $\text{LaNi}_{5-x}\text{T}_x$ ($\text{T} = \text{Fe}, \text{Mn}$) compounds", *Phys. Rev. B*, **63**(1), pp.14407.
- [4] L.T. Tai, B.T. Hang, N.P. Thuy, T.D. Hien (2003), "Magnetic properties of LaNi_5 -based compounds", *J. Magn. Magn. Mater.*, **262**(3), pp.485-489.
- [5] K.H. Buschow, H. Van Mai, A. Miedema (1975), "Hydrogen absorption in intermetallic compounds of thorium", *J. Less Common Met.*, **42**(2), pp.163-178.
- [6] C.K. Witham, R.C. Bowman, B.V. Ratnakumar, B. Fultz, S. Surampudi (1996), " AB_5 metal hydride alloys for alkaline rechargeable cells", in *Proceeding of the 11th Annu. Batter. Conf. Appl. Adv.*, pp.129-134.
- [7] G. Sandrock (2010), "Hydride Databases Introduction", *Hydrogen Storage Materials*, **1**, pp.1-10.
- [8] A. Dra (2008), "Crystal Structure and Hydrogen Sorption Properties of the $\text{YNi}_{5-x}\text{Ga}_x$ Alloys", *J. Acta Chim. Slov.*, **55**(4), pp.869-873.
- [9] R. Nakamura, K. Asano, K. Yoshimi, Y. Iijima (2006), "Thermal analysis of lattice defects in LaNi_5 ", *J. Alloys Compd.*, **413**(1-2), pp.211-213.
- [10] X.H. An, Y.B. Pan, Q. Luo, X. Zhang, J.Y. Zhang, Q. Li (2010), "Application of a new kinetic model for the hydriding kinetics of $\text{LaNi}_{5-x}\text{Al}_x$ ($0 \leq x \leq 1.0$) alloys", *J. Alloys Compd.*, **506**(1), pp.63-69.
- [11] K. Asano, Y. Hashimoto, T. Iida, M. Kondo, Y. Iijima (2005), "Hydrogen diffusion by substitution of aluminum and cobalt for a part of nickel in $\text{LaNi}_5\text{-H}$ alloy", *J. Alloys Compd.*, **395**(1-2), pp.201-208.
- [12] F. Stucki, L. Schlapbach (1980), "Magnetic properties of LaNi_5 , FeTi , Mg_2Ni and their hydrides", *J. Less Common Met.*, **74**, pp.143-151.
- [13] P. Millet, P. Dantzer (1997), "A new approach to the kinetics of $\text{LaNi}_5\text{-H}_2$ (g) systems based on impedance spectroscopy analysis", *J. Alloys Compd.*, **Vol.253-254**, pp.542-546.
- [14] S. Ino, M. Hosono, M. Sato, S. Nakajima, K. Yamashita, T. Izumi (2009), "A soft metal hydride actuator using LaNi_5 alloy and a laminate film bellows", in *Proceeding of the 2009 IEEE Int. Conf. Ind. Technol.*, pp.1-6.
- [15] E. Burzo (2007), "Crystal structures and magnetic properties of nickel-based compounds", *J. Optoelectronics and Advanced Materials*, **9**(4), pp.1113-1119.
- [16] A. Drašner, Z. Blažina (2004), "The effect of substitution of Ga for Ni on the hydrogen sorption properties of NdNi_5 ", *J. Alloys Compd.*, **381**(1-2), pp.188-191.
- [17] W. Li, B. Zhang, J. Yuan, Y. Yan, Y. Wu (2017), "Effect of Mo content on the microstructures and electrochemical performances of $\text{La}_{0.75}\text{Mg}_{0.25}\text{Ni}_{3.2-x}\text{Co}_{0.2}\text{Al}_{0.1}\text{Mo}_x$ ($x = 0, 0.10, 0.15, 0.20$) hydrogen storage alloys", *J. Alloys Compd.*, **692**, pp.817-824.
- [18] N. Biliškov, G.I. Miletić, A. Drašner, K. Prezelj (2015), "Structural and hydrogen sorption properties of $\text{SmNi}_{5-x}\text{Al}_x$ system - An experimental and theoretical study", *Int. J. Hydrogen Energy*, **40**(27), pp.8548-8561.
- [19] Z. Ma, W. Zhou, C. Wu, et al. (2016), "Effects of size of nickel powder additive on the low-temperature electrochemical performances and kinetics parameters of AB_5 -type hydrogen storage alloy for negative electrode in Ni/MH battery", *J. Alloys Compd.*, **660**, pp.289-296.
- [20] T. Erika, C. Sebastian, Z. Fernando, D. Verónica (2015), "Temperature performance of AB_5 hydrogen storage alloy for Ni-MH batteries", *Int. J. Hydrogen Energy*, **1**, pp.2-8.
- [21] X. Tian, G. Yun, H. Wang, et al. (2014), "Preparation and electrochemical properties of La-Mg-Ni-based $\text{La}_{0.75}\text{Mg}_{0.25}\text{Ni}_{3.3}\text{Co}_{0.5}$ multiphase hydrogen storage alloy as negative material of Ni/MH battery", *Int. J. Hydrogen Energy*, **39**(16), pp.8474-8481.
- [22] E.R. Pinatel, M. Palumbo, F. Massimino, P. Rizzi, M. Baricco (2015), "Hydrogen sorption in the $\text{LaNi}_{5-x}\text{Al}_x\text{-H}$ system ($0 \leq x \leq 1$)", *Intermetallics*, **62**, pp.7-16.
- [23] E.M. Borzone, M.V. Blanco, A. Baruj, G.O. Meyer (2014), "Stability of $\text{LaNi}_{5-x}\text{Sn}_x$ cycled in hydrogen", *Int. J. Hydrogen Energy*, **39**(16), pp.8791-8796.
- [24] J. Prigent, J.M. Joubert, M. Gupta (2012), "Modification of the hydrogenation properties of LaNi_5 upon Ni substitution by Rh, Ir, Pt or Au", *J. Alloys Compd.*, **511**(1), pp.95-100.
- [25] K. Suzuki, Kazuhiro Ishikawa, Kiyoshi Aoki (2000), "Degradation of LaNi_5 and $\text{LaNi}_{4.7}\text{Al}_{0.3}$ Hydrogen-Absorbing Alloys by Cycling", *Mater. Trans., JIM*, **41**(5), pp.581-584.
- [26] D.N. Ba, L.T. Tai, N.P. Duong, C.V. Tuan, T.Q. Huy (2013), "Electrochemical Properties of $\text{LaNi}_{5-x}\text{Ga}_x$ Alloys Used as the Negative Electrodes of Ni-MH Batteries", *Anal. Lett.*, **46**(12), pp.1897-1909.

A model for thermal conductivity of carbon nanotubes with ethylene glycol/water based nanofluids

Trong Tam Nguyen¹, Hung Thang Bui^{2*}, Ngoc Minh Phan^{1,2,3}

¹Graduate University of Science and Technology (GUST), Vietnam Academy of Science and Technology (VAST)

²Institute of Materials Science (IMS), Vietnam Academy of Science and Technology (VAST)

³Center for High Technology Development (HTD), Vietnam Academy of Science and Technology (VAST)

Received 25 April 2017; accepted 2 June 2017

Abstract:

Due to its unique thermal properties, carbon nanotubes (CNTs) have been used as additives in order to increase thermal conductivity and other mechanical properties of nanofluids. There have been many studies of thermal conductivity for single phase fluids containing CNTs; however, most commercial coolants are two-phase fluids, such as the mixture of ethylene glycol and water (E/W). Similarly, there are some models that can be used to predict thermal conductivity of single phase fluids containing CNTs but not yet as a model for thermal conductivity of the E/W solution containing CNTs. In this paper, we present a model to predict the thermal conductivity of CNTs nanofluids based on an E/W solution. The model is found to correctly predict trends observed in experimental data of V. Kumaresan, et al. with varying concentrations of CNTs in nanofluids.

Keywords: carbon nanotube, ethylene glycol, nanofluids, thermal conductivity, water.

Classification number: 2.1, 5.1

Introduction

Research into thermal dissipation materials of high power electronic devices has been receiving special interest from scientists and technologists. Besides finding new materials and technologies to increase component density and processing speed of electronic and optoelectronic devices, it is very important to find new materials and appropriate configuration to accelerate the thermal dissipation [1].

In recent years, there are many approaches that can improve the cooling system's performance; the most feasible one being to enhance the heat transfer (dissipation) performance through a working fluid without modifying either its mechanical designs or its key components. Researchers have recently shown a lot of interest in the issue of nanofluid thermal properties [2]. The heat transfer performance of nanofluids has been found to be enhanced by adding solid nanoparticles, including metals (Cu,

Au, Ag, Ni), metal oxides (Al₂O₃, CuO, Fe₂O₃, SiO₂, TiO₂), or ceramics (SiC, AlN, SiN) [3-6].

CNTs are one of the most valuable materials with high thermal conductivity (above 1,400 W/m.K compared to the thermal conductivity of Ag 419 W/m.K) [7-9]. Owing to their unique thermal properties, CNTs have been used as additives to increase the thermal conductivity and other mechanical properties of nanofluids [10-13].

So far, there have been many studies into the thermal conductivity of single phase fluids containing CNTs. However, most commercial coolants are two-phase fluids, such as the mixture of E/W. Similarly, there are some models used to predict the thermal conductivity of single phase fluids containing CNTs [14-31], but not yet a model for thermal conductivity of E/W solution containing CNTs.

In this work, we present a model for predicting the thermal conductivity of the CNT nanofluids based E/W solution, which takes into consideration the effects of size, volume fraction, and thermal conductivity of CNTs, as well as the properties of the base liquid. This model is found to correctly predict trends observed in the experimental data of V. Kumaresan, et al., with varying concentrations of CNTs in nanofluids.

The model

As we already know, CNT is a very good thermal conductor to be used in tubes, but also is a good insulator laterally for tube axis. On the other hand, CNT disperses nanofluids in all direction, randomly. Therefore, we need to replace the thermal conductivity property of CNT (k_{CNT}) with an effective thermal conductivity of CNT ($k_{eff-CNT}$) for all calculations. In the report [31], we calculated effective thermal conductivity of CNT ($k_{eff-CNT}$) as follows:

$$k_{eff-CNT} = \frac{1}{2} k_{CNT} \quad (1)$$

This model considers three paths for heat to flow in an E/W solution containing CNTs, one through which the E molecules allows one through the W molecules and the other through

*Corresponding author: Email: thangbh@ims.vast.vn

the CNTs. The total heat transfer through nanofluid can be expressed as:

$$q = q_E + q_W + q_{CNT} \quad (2)$$

$$q = -k_E A_E \left(\frac{dT}{dx} \right)_E - k_W A_W \left(\frac{dT}{dx} \right)_W - k_{eff-CNT} A_{CNT} \left(\frac{dT}{dx} \right)_{CNT} \quad (3)$$

Where A , k , and (dT/dx) denote the heat transfer area, thermal conductivity, and temperature gradient of the respective media. Subscripts “ E ”, “ W ” and “ CNT ” denote quantities corresponding to ethylene glycol, water and carbon nanotubes, respectively. The liquid medium and the CNTs are assumed to be in local thermal equilibrium at each location, which gives:

$$\left(\frac{dT}{dx} \right)_E = \left(\frac{dT}{dx} \right)_W = \left(\frac{dT}{dx} \right)_{CNT} = \left(\frac{dT}{dx} \right) \quad (4)$$

Thus, the equation (3) can be written as:

$$q = - \left(k_E A_E + k_W A_W + k_{eff-CNT} A_{CNT} \right) \left(\frac{dT}{dx} \right) \quad (5)$$

$$-k \left(A_E + A_W + A_{CNT} \right) \left(\frac{dT}{dx} \right) = - \left(k_E A_E + k_W A_W + k_{eff-CNT} A_{CNT} \right) \left(\frac{dT}{dx} \right) \quad (6)$$

$$k \left(A_E + A_W + A_{CNT} \right) = k_E A_E + k_W A_W + k_{eff-CNT} A_{CNT} \quad (7)$$

It is proposed that the ratio of heat transfer areas $A_E : A_W : A_{CNT}$ could be taken in proportion to the total surface areas of E molecules (S_E), W molecules (S_W), and nanotubes (S_{CNT}) per unit volume of the suspension. We take the E molecules, and W molecules to be spheres with radii of r_E , r_W , and the CNTs to be cylinders with radii r_{CNT} and length L , respectively. The surface area and volume of the individual liquid molecules can be respectively calculated as:

$$S_E = 4\pi r_E^2 \quad (8)$$

$$v_E = \frac{4}{3} \pi r_E^3 \quad (9)$$

$$S_W = 4\pi r_W^2 \quad (10)$$

$$v_W = \frac{4}{3} \pi r_W^3 \quad (11)$$

Note that the two ends of the CNTs are hemispherical, and therefore the surface area and volume of the individual CNTs can be respectively calculated as:

$$S_{CNT} = 4\pi r_{CNT}^2 + 2\pi r_{CNT} L \quad (12)$$

$$v_{CNT} = \frac{4}{3} \pi r_{CNT}^3 + \pi r_{CNT}^2 L \quad (13)$$

Total surface area can be calculated as the product of the number of particles and the surface area of those particles for each constituent. Denoting the fraction of the volume of the CNTs as ε_{CNT} , so that the volume fraction of the liquid is $(1 - \varepsilon_{CNT})$. Denoting the volume fraction of the E in based solution as ε_E , so the volume fraction of E in nanofluids as $(1 - \varepsilon_{CNT}) \cdot \varepsilon_E$ and the volume fraction of W in nanofluids as $(1 - \varepsilon_{CNT})(1 - \varepsilon_E)$. The number of particles for the three constituents can be calculated as, respectively:

$$n_E = \frac{(1 - \varepsilon_{CNT}) \varepsilon_E}{v_E} = \frac{(1 - \varepsilon_{CNT}) \varepsilon_E}{\frac{4}{3} \pi r_E^3} \quad (14)$$

$$n_W = \frac{(1 - \varepsilon_{CNT})(1 - \varepsilon_E)}{v_W} = \frac{(1 - \varepsilon_{CNT})(1 - \varepsilon_E)}{\frac{4}{3} \pi r_W^3} \quad (15)$$

$$n_{CNT} = \frac{\varepsilon_{CNT}}{v_{CNT}} = \frac{\varepsilon_{CNT}}{\frac{4}{3} \pi r_{CNT}^3 + \pi r_{CNT}^2 L} \quad (16)$$

The corresponding surface areas of the E molecules are given by:

$$S_E = n_E \cdot S_E = \frac{(1 - \varepsilon_{CNT}) \varepsilon_E}{\frac{4}{3} \pi r_E^3} \cdot 4\pi r_E^2 = 3 \frac{(1 - \varepsilon_{CNT}) \varepsilon_E}{r_E} \quad (17)$$

The corresponding surface areas of the W molecules are given by:

$$S_W = n_W \cdot S_W = \frac{(1 - \varepsilon_{CNT})(1 - \varepsilon_E)}{\frac{4}{3} \pi r_W^3} \cdot 4\pi r_W^2 = 3 \frac{(1 - \varepsilon_{CNT})(1 - \varepsilon_E)}{r_W} \quad (18)$$

The corresponding surface areas of the CNT phase are given by:

$$S_{CNT} = n_{CNT} \cdot S_{CNT} \quad (19)$$

$$S_{CNT} = 3\varepsilon_{CNT} \frac{4r_{CNT} + 2L}{4r_{CNT}^2 + 3r_{CNT}L} \quad (20)$$

$$S_{CNT} = 3\varepsilon_{CNT} \frac{4 \frac{r_{CNT}}{L} + 2}{\left(4 \frac{r_{CNT}}{L} + 3 \right) r_{CNT}} \quad (21)$$

Note that the CNT length is very large compared to the CNT radii, thus:

$$\frac{r_{CNT}}{L} \approx 0 \quad (22)$$

From (21) and (22), S_{CNT} is expressed as:

$$S_{CNT} = \frac{2\varepsilon_{CNT}}{r_{CNT}} \quad (23)$$

Taking $A_E : A_W : A_{CNT} = S_E : S_W : S_{CNT}$, we obtain:

$$k(S_E + S_W + S_{CNT}) = k_E S_E + k_W S_W + k_{eff-CNT} S_{CNT} \quad (24)$$

Substituting from equation (17), (18) and (23) into the expression for heat transfer rate in equation (24), we obtain:

$$k = \frac{k_E 3 \frac{(1-\varepsilon_{CNT})\varepsilon_E}{r_E} + k_W 3 \frac{(1-\varepsilon_{CNT})(1-\varepsilon_E)}{r_W} + k_{eff-CNT} \frac{2\varepsilon_{CNT}}{r_{CNT}}}{3 \frac{(1-\varepsilon_{CNT})\varepsilon_E}{r_E} + 3 \frac{(1-\varepsilon_{CNT})(1-\varepsilon_E)}{r_W} + \frac{2\varepsilon_{CNT}}{r_{CNT}}} \quad (25)$$

$$k = \frac{\frac{\varepsilon_E k_E}{r_E} + \frac{(1-\varepsilon_E)k_W}{r_W} + \frac{2\varepsilon_{CNT}k_{eff-CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}}{\frac{\varepsilon_E}{r_E} + \frac{(1-\varepsilon_E)}{r_W} + \frac{2\varepsilon_{CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}} \quad (26)$$

Note that $\varepsilon < 10\%$ in all experiments, $r_E \ll r_{CNT}$, $r_W \ll r_{CNT}$ from (26) we have:

$$k = \frac{\frac{\varepsilon_E k_E}{r_E} + \frac{(1-\varepsilon_E)k_W}{r_W} + \frac{2\varepsilon_{CNT}k_{eff-CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}}{\frac{\varepsilon_E}{r_E} + \frac{(1-\varepsilon_E)}{r_W}} \quad (27)$$

From (1) and (27), the effective thermal conductivity of CNT-nanofluids is expressed as:

$$k = \frac{\frac{\varepsilon_E k_E}{r_E} + \frac{(1-\varepsilon_E)k_W}{r_W} + \frac{\varepsilon_{CNT} k_{CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}}{\frac{\varepsilon_E}{r_E} + \frac{(1-\varepsilon_E)}{r_W}} \quad (28)$$

From (28), the enhancement of thermal conductivity of CNT-nanofluids is expressed as:

$$\Delta k = k - k_0 = \frac{\frac{\varepsilon_{CNT} k_{CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}}{\frac{\varepsilon_E}{r_E} + \frac{(1-\varepsilon_E)}{r_W}} \quad (29)$$

From (29), the percent enhancement of thermal conductivity of CNT-nanofluids is expressed as:

$$\% \Delta k = 100\% \cdot \frac{k - k_0}{k_0} = 100\% \cdot \frac{\frac{\varepsilon_{CNT} k_{CNT}}{3(1-\varepsilon_{CNT})r_{CNT}}}{\frac{\varepsilon_E k_E}{r_E} + \frac{(1-\varepsilon_E)k_W}{r_W}} \quad (30)$$

Validation of the model

In order to validate the model, we compared the experimental data of V. Kumaresan, et al. (2012) [32] with calculation data from our model. In the Kumaresan experiment, the average diameter of dispersed nanotubes is found to be 42.6 nm [32], therefore, the average radius of CNTs is $r_{CNT} = 21.3$ nm. In calculation from this, the radius of E molecule and W molecule are 0.12 nm and 0.1 nm, respectively; and the thermal conductivity of E and W are 0.26 W/mK and 0.6 W/mK, respectively [31].

In ref. [33], Li, et al. reported thermal conductivity of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are 2,400 W/mK and 1,400 W/mK, respectively, measured using the non-contact Raman spectra shift method. So we choose the thermal conductivity of CNTs in this calculation is $k_{CNT} = 1,400$ W/mK. Fig. 1 shows that the model has correctly predicted the trends observed in the experimental data of V. Kumaresan, et al. [32].

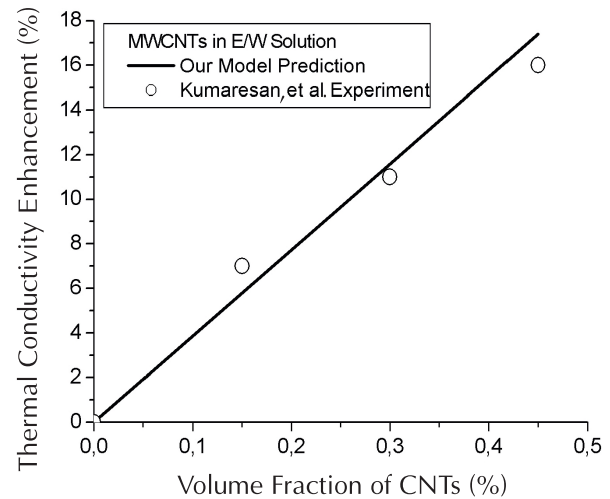


Fig. 1. The comparison between our model and the experimental data of V. Kumaresan, et al. in the case of dispersing MWCNTs in E/W solution.

Conclusions

We have developed a model for predicting the thermal conductivity of CNT nanofluids based E/W solution. Calculation results showed that the thermal conductivity of CNT nanofluids increased linearly with low volume concentration. The model was compared to experimental data of Kumaresan et al., and the result shows that the model correctly predicted the trends observed in experimental data. This model is close to the commercial coolant as well as has important implications for predicting the thermal conductivity of coolants based E/W containing CNTs for industrial applications.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from Vietnam Academy of Science and Technology (Project No. VAST.TD.QP.03/17-19) and the Vietnam National Foundation for Science and Technology Development (Project No. 103.99-2015.70).

REFERENCES

- [1] P.K. Schelling, L. Shi, K.E. Goodson (2005), "Managing heat for electronics", *Materials Today*, **8(6)**, pp.30-35.
- [2] Y.H. Hung, J.H. Chen, T.P. Teng (2013), "Feasibility Assessment of Thermal Management System for Green Power Sources Using Nanofluid", *Journal of Nanomaterials*, Article ID: 321261, 11 pages.
- [3] A.A. Avramenko, D.G. Blinov, I.V. Shevchuk, A.V. Kuznetsov (2012), "Symmetry analysis and self-similar forms of fluid flow and heat-mass transfer in turbulent boundary layer flow of a nanofluid", *Physics of Fluids*, **24(9)**, 092003.
- [4] A.A. Avramenko, D.G. Blinov, and I.V. Shevchuk (2011), "Self-similar analysis of fluid flow and heat-mass transfer of nanofluids in boundary layer", *Phys. Fluids*, **23(8)**, 082002.
- [5] R. Ni, S.Q. Zhou, and K.Q. Xia (2011), "An experimental investigation of turbulent thermal convection in water-based alumina nanofluid", *Phys. Fluids*, **23(2)**, 022005.
- [6] J. Kim, Y.T. Kang, and C.K. Choi (2004), "Analysis of convective instability and heat transfer characteristics of nanofluids", *Phys. Fluids*, **16(7)**, 2395.
- [7] CRC (2010), *Handbook of Chemistry and Physics*, 90th Edition, Internet Version 2010, Section 12, pp.198-199.
- [8] S. Berver, Y.K. Kwon, D. Tománek (2000), "Unusual high thermal conductivity of carbon nanotubes", *Phys. Rev. Lett.*, **84(20)**, p.4613.
- [9] P. Kim, L. Shi, A. Majumdar, and P. L. McEuen (2001), "Thermal transport measurements of individual multiwalled nanotubes", *Phys. Rev. Lett.*, **87(21)**, 215502.
- [10] N.M. Phan, H.T. Bui, M.H. Nguyen, H.K. Phan (2014), "Carbon-nanotube-based liquids: a new class of nanomaterials and their applications", *Adv. Nat. Sci.: Nanosci. Nanotechnol.*, **5(1)**, 015014 (5pp).
- [11] B.H. Thang, L.D. Quang, N.M. Hong, P.H. Khoi, P.N. Minh (2014), "Application of multi-walled carbon nanotube nanofluid for 450 W LED floodlight", *Journal of Nanomaterials*, Article ID 347909, 6 pages.
- [12] Y. Ding, H. Alias, D. Wen, R.A. Williams (2006), "Heat transfer of aqueous suspensions of carbon nanotubes (CNT nanofluids)", *International Journal of Heat and Mass Transfer*, **49(1-2)**, pp.240-250.
- [13] B.H. Thang, P.V. Trinh, L.D. Quang, N.T. Huong, P.H. Khoi, and P.N. Minh (2014), "Heat dissipation for the Intel Core i5 processor using multi-walled carbon-nanotube-based ethylene glycol", *Journal of the Korean Physical Society*, **65(3)**, pp.312-316.
- [14] D. Hemanth, H.E. Patel, V.R.R. Kumar, T. Sundararajan, T. Pradeep, S.K. Das (2004), "Model for heat conduction in nanofluids", *Physical Review Letters*, **93(14)**, 144301.
- [15] H.E. Patel, K.B. Anoop, T. Sundararajan, Sarit K. Das (2008), "Model for thermal conductivity of CNT-nanofluids", *Bull. Mater. Sci.*, **31(3)**, pp.387-390.
- [16] Y.J. Hwang, Y.C. Ahn, H.S. Shin, C.G. Lee, G.T. Kim, H.S. Park, J.K. Lee (2006), "Investigation on characteristics of thermal conductivity enhancement of nanofluids", *Current Applied Physics*, **6(6)**, pp.1068-1071.
- [17] L. Chen, H. Xie, Y. Li, W. Yu (2008), "Nanofluids containing carbon nanotubes treated by mechanochemical reaction", *Thermochimica Acta*, **477(1-2)**, pp.21-24.
- [18] G. Wu, J. Yang, S. Ge, Y. Wang, M. Chen, Y. Chen (2009), "Thermal conductivity measurement for carbon-nanotube suspensions with 3w method", *Advanced Materials Research*, **Vol.60-61**, pp.394-398.
- [19] J.C. Maxwell (1873), *A treatise on electricity and magnetism*, Clarendon Press.
- [20] R.L. Hamilton, O.K. Crosser (1962), "Thermal conductivity of heterogeneous two-component systems", *Ind. Eng. Chem. Fundam.*, **1(3)**, pp.187-191.
- [21] D.J. Jeffrey (1973), "Conduction through a random suspension of spheres", *Proc. Royal. Soc. London*, A335:355-367.
- [22] R.H. Davis (1983), "The effective thermal conductivity of a composite material with spherical inclusions", *Int. J. Thermophys.*, **7(3)**, pp.609-620.
- [23] R. Walvekar, I.A. Faris, M. Khalid (2012), "Thermal conductivity of carbon nanotube nanofluid-Experimental and theoretical study", *Heat Transfer-Asian Research*, **41(2)**, pp.145-163.
- [24] Y. Zheng and H. Hong (2007), "Modified model for effective thermal conductivity of nanofluids containing carbon nanotubes", *Journal of Thermophysics and Heat Transfer*, **21(3)**, pp.658-660.
- [25] W. Yu and S.U.S. Choi (2004), "The role of interfacial layers in the enhanced thermal conductivity of nanofluids: A renovated Hamilton-Crosser model", *Journal of Nanoparticle Research*, **6(4)**, pp.355-361.
- [26] S. M. Hosseini, A. Moghadassi, D. Henneke (2011), "Modeling of the effective thermal conductivity of carbon nanotube nanofluids based on dimensionless groups", *The Canadian Journal of Chemical Engineering*, **89(6)**, pp.183-186.
- [27] N.N. Venkata Sastry, A. Bhunia, T. Sundararajan, S.K. Das (2008), "Predicting the effective thermal conductivity of carbon nanotube based nanofluids", *Nanotechnology*, **19(5)**, 055704.
- [28] O.M. Wilson, X. Hu, D.G. Cahill, P.V. Braun (2002), "Colloidal metal particles as probes of nanoscale thermal transport in fluids", *Physical Review B*, **66(22)**, 224301.
- [29] Q.Z. Xue (2006), "Model for the effective thermal conductivity of carbon nanotube composites", *Nanotechnology*, **17(6)**, pp.1655-1660.
- [30] V.U. Unnikrishnan, D. Banerjee, J.N. Reddy (2008), "Atomistic-mesoscale interfacial resistance based thermal analysis of carbon nanotube systems", *International Journal of Thermal Sciences*, **47**, pp.1602-1609.
- [31] B.H. Thang, P.H. Khoi, P.N. Minh (2015), "A modified model for thermal conductivity of carbon nanotube-nanofluids", *Physics of Fluids*, **27**, 032002.
- [32] V. Kumaresan, R. Velraj (2012), "Experimental investigation of the thermo-physical properties of water-ethylene glycol mixture based CNT nanofluids", *Thermochimica Acta*, **545**, pp.180-186.
- [33] Q. Li, C. Liu, X. Wang, S. Fan (2009), "Measuring the thermal conductivity of individual carbon nanotubes by the Raman shift method", *Nanotechnology*, **20(14)**, 145702.

Classification and identification of Vietnamese honey using chemometrics based on ^1H -NMR data

The Anh Nguyen, Truong Giang Vu, Thi Lan Nguyen, Quang Trung Pham, Thi Thao Ta^{1*}

Faculty of Chemistry, University of Science, Vietnam National University

Received 12 April 2017; accepted 2 June 2017

Abstract:

Honey is a natural, sweet, and syrupy fluid which has been used in Vietnam in a variety of ways; as a food supplement, beauty product, and natural drug. However, quality control and characterization of honey are blind problems. Consumers, and even market management committees, must believe in the producer's quality standards without using any special techniques to evaluate the botanical origins of honey in the Vietnamese market. The chemical composition and physical properties of natural honey vary per plant species of which the honey bees scrounged. Longan flower-honey has a high price and is commercially produced in Yen Bai, Bac Giang, and the more well-known Hung Yen Province in Vietnam, and now is being confused with original flower honey. In this work, a total of 57 honey samples (longan and non-longan) from different geographic and botanical origins have been analysed in terms of ^1H -NMR spectroscopy, coupled also with multivariate statistical analysis methods. Principal component analysis followed by icoshift algorithm analysis comes about as a proficient device in recognising ^1H -NMR spectra of longan honey samples.

Keywords: botanical origin, chemometrics, classification, ^1H -NMR, identification, Vietnamese honey.

Classification number: 2.2

Introduction

As stated in the Codex Alimentarius Commission, honey is defined as a characteristic substance created by honey bees and is comprised of water and sugars, primarily fructose and glucose [1]. Other minor compounds include proteins, amino acids, flavours and aromatic molecules, pigments, vitamins, and numerous unpredictable parts establishing nutritious and organoleptic qualities. Honey is a global product due to its promptly accessible

source of vitality, and its antibacterial and antioxidant capacities [2, 3]. Bees are considered to produce honey to serve as their main source of food during times of scarcity or harsh weather conditions. Bees transform pollen from flowers and trees of various kinds to produce honey, including both in-house trees and forest trees.

Currently, Vietnam ranks sixth in the world in regards to honey export. According to the Vietnamese Beekeepers Association, in 2013, the total domestic

production of honey was more than 48,000 tonnes, with 37,000 tonnes exported. Recently, the honey export growth rate has steadily increased at a high rate (14%) [4]. Due to high market demand for forest honey, which often demands a much higher price, local producers often mix honey from various original and botanical sources, including lychee, coffee, *Melaleuca leucadendron* L., and especially longan; they have been known to also mix money with sugar.

Longan (*Dimocarpus longan*) is an evergreen fruit crop grown in tropical and subtropical climates and is considered as a traditional fruit of Vietnam, having its main production areas in the south: Tien Giang, Ben Tre, Dong Thap, Vinh Long, Can Tho, and Ba Ria-Vung Tau; and in the north: Bac Giang, Lao Cai, Yen Bai, Thai Nguyen, Phu Tho, Son La, Hung Yen, and Thanh Hoa. In 1997, the total area planted with longan was 60,000 ha, and grew to reach to 75,200 ha in 2002 [5]. Because of a higher price of longan honey than synthesised honey, it is necessary to control the honey quality and authenticity in order to preserve the production areas, to develop quality standards, and to protect consumers from commercial speculation. Vietnamese officials are encouraging the development of new analytical methods to control and verify quality specification for honey with different botanical origins, quality controls, and original trademarks.

As of late, numerous different studies have been published to develop new

^{*}Corresponding author: Email: thaott73@gmail.com

methodologies and diverse analytical techniques to evaluate either different or equal botanical and geographical origin of honey [6, 7]. Among the greater analytical methods applied in food characterization, nuclear magnetic resonance (NMR) is accepted as a powerful and trusted method [6-8] due to its non-destructible aspects, high reproducibility, and sensitivity as shown across a large range of utilizations. In contrast with chromatography, NMR requires a small amount of sample and simple sample preparation, so it can be used to perform metabolite characterization of honey for either geographical assessment or botanical assessment [9, 10].

The ^1H -NMR spectra of poly floral and honey samples were recorded and geographically characterised [11]. The classification of Brazilian honey's botanical origin by Principal Component Analysis and Hierarchical Cluster Analysis also using NMR analysis were investigated [12]. Independent component analysis had been also used to discriminate manuka honey from other floral honey types [13]. Factor analysis and general discriminant analysis was successfully applied to detect Honey Adulteration by Sugar Syrups [14]. The icoshift algorithm is based on the shift of spectral intervals, and is employed across all spectra simultaneously. The icoshift program is an open source and highly efficient program designed to solve signal alignment problems in metabolomic analysis [15]; however, it has not yet been applied to the ^1H -NMR spectra of honey.

In this work, the ^1H -NMR spectra of honey in water solvent was applied using a pulse sequence NOESYPR1D to saturate the signal of the solvent. The advantage of this method is that it has low cost and easy usage to prepare. A total of 57 honey samples coming from Vietnamese longan and other botanical origins were studied. By using Principal

components analysis (PCA) combined with mean-centering calculation and icoshift tool, ^1H -NMR spectra have been used for building a model and identifying longan honey among different honey samples.

Materials and methods

Materials and sample collections

A total of 57 honey samples were collected on the trading market. The original and botanical information of the samples was recognised based on its packaging and onsite information. Among the samples, there were 18 longan honey samples, 10 non-longan honey samples (coming from other fruits) and 29 test samples recognised as non-identified samples.

NMR analysis

An NMR solvent was prepared from double distilled deionized water and deuterated water (9:1 in volume). A 0.1 ml of the sample was dissolved in 0.3 ml of the $\text{H}_2\text{O}/\text{D}_2\text{O}$ solvent. The ^1H -NMR spectra were recorded at 300 K using a Bruker Advance 500 MHz (Bruker Biospin, Germany) operating at 11,7 T with a 5 mm BBFO probe. Solvent suppression was achieved by applying a presaturation scheme with low-power radiofrequency irradiation. The number of data points was 32 K, acquisition time was 2.04 s, the number of scans was 8 and spectral width was 8,012,820 Hz. An exponential function of LB 0.3 was applied before Fourier transformation, and the phase and baseline were automatically corrected using Topspin 3.2 (Bruker Biospin, Germany).

Statistical methods

NMR data was aligned, changed over into Excel 2016 (Microsoft) then transported into Matlab R2016a (The MathWorks, USA) for statistical analysis. Principal component analysis (PCA) was performed with mean-centering as a data pretreatment.

PCA is a chemometric standout

method amongst unsupervised ones used in analysing NMR data. It is an essential statistical tool for introductory examinations of extensive data sets to investigate likely patterns, classifications and identification of outliers. The goal of the principal component analysis is to explain the maximum amount of variance with the fewest number of principal components [11]. This method includes a dimensional reduction of the data set using a smaller number of axes. These components (PCs) are shown graphically as a score plot, which is a summary of the relationship among the observations. Coefficients, by which the original variables are multiplied to obtain the PCs, are represented in loading plots that summarise the variables (chemical shift data points) - which is a means to interpret the patterns seen in the score plot [16]. Samples (or observations) that were similar, or highly correlated with one another, were closed in the same group, whereas samples that were dissimilar, or uncorrelated, were clustered in different groups. The higher eigenvalues, the more information of PCs contains the original data matrix [17].

One of the most common normalising methods is mean-centering, which calculates the mean of each column and subtracts this from the column itself. Another way of interpreting mean-centered data is that each row of the mean-centered data includes only the differences of each row from the average sample in the original data matrix. In other words, mean-centering involves the subtraction of the variable averages from the data.

Icoshift toolbox for Matlab is an open source tool provided by the University of Copenhagen. The icoshift algorithm represents a powerful and versatile tool used for dealing with all kinds of signal alignment problems. It allows the researcher to choose among a large variety of options, from fully automated corrections of the whole NMR spectrum

to supervised and targeted interventions covering only selected spectral regions [15].

Results and discussions

Chemical characterization of honey samples

Fig. 1 represents a complete spectrum of a longan honey sample from 0 to 9 ppm in chemical shift. The spectra of 57 honey samples were combined in Fig. 2. It can be seen that, the main compounds show their dominant resonances at

regions of 1-2 ppm and from 3 to 5.5 ppm. The spectra were exported as text files from Topspin software into Matlab software to study the characteristics, identification and classification.

To specify compounds that characterise each part of the spectra, the whole spectra was divided into three main regions: 1-3 ppm, 3-5.5 ppm, and 6-8 ppm as shown in Fig. 3. The first region, 1-3 ppm, shows the appearance of two main peaks: lactic acid (1 ppm) and acetic acid (2.1 ppm). The

region 3-5.5 ppm shows the percent of carbohydrates, and dominant resonances of main monosaccharides, like: (α - and β - glucopyranose, β - fructopyranose, α - and β - fructofuranose). For instance, α and β - anomeric hydrogen of glucopyranose could be recognised at 5.2 and 4.6 ppm [17]. The last region, 6-8 ppm, represents formic acid and some the aromatic amino acids including tyrosine, phenylalanine; in here, almost peaks have the too small intensity and are not convergent.

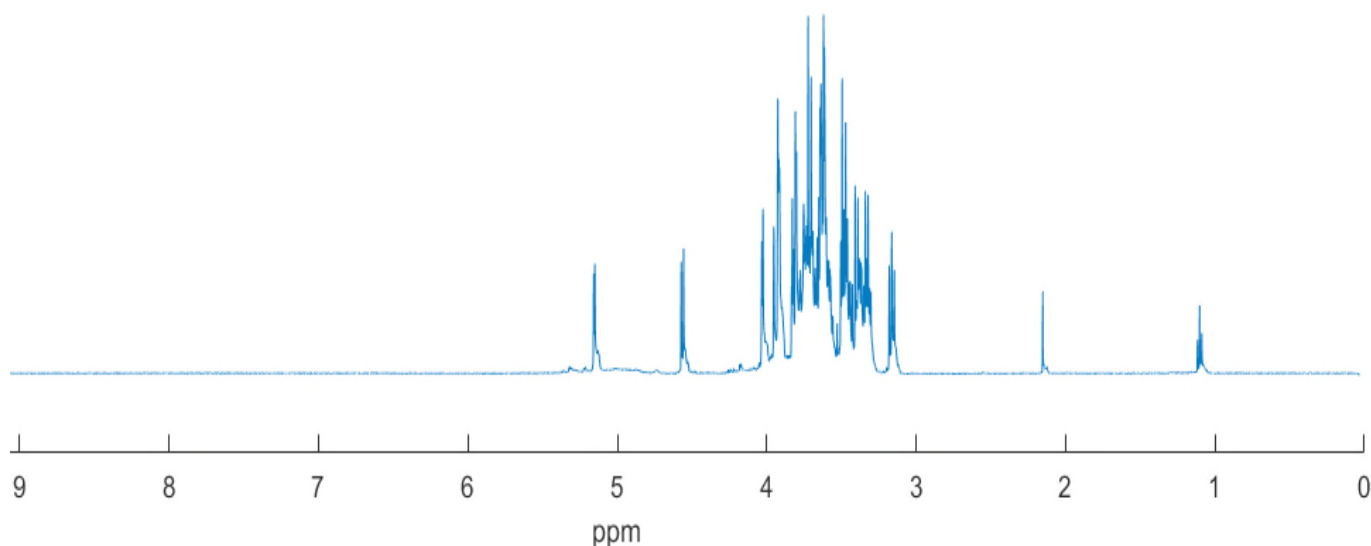


Fig. 1. A complete NMR spectrum of a longan honey sample.

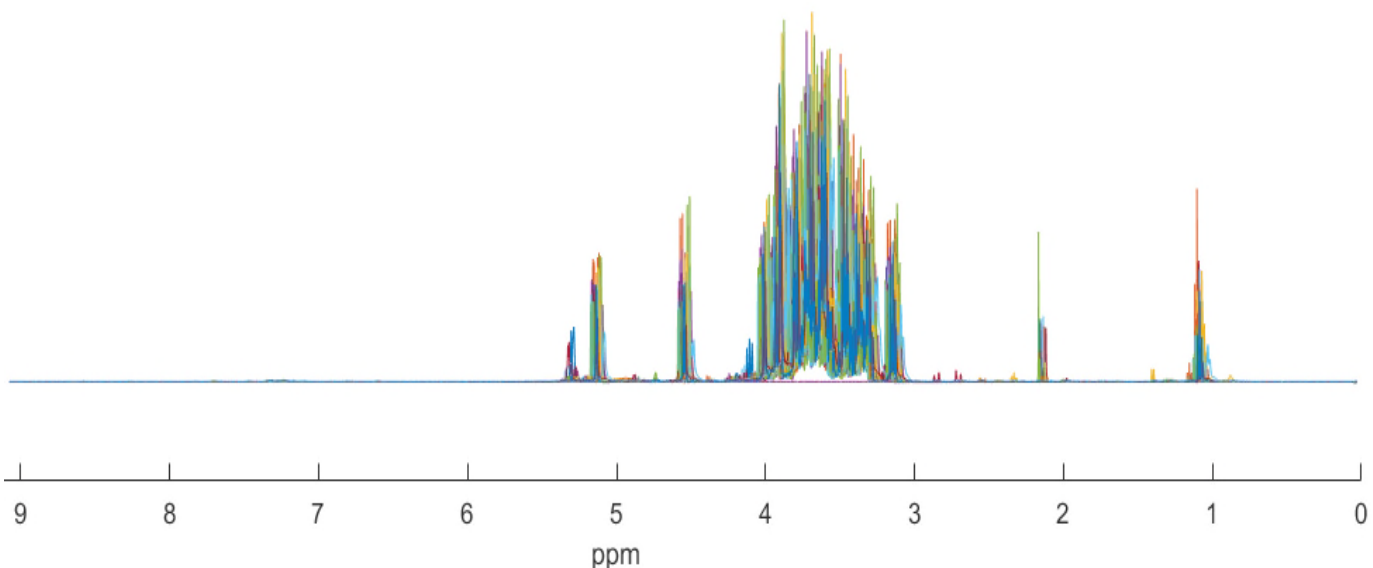


Fig. 2. The NMR combined spectra of 57 honey samples.

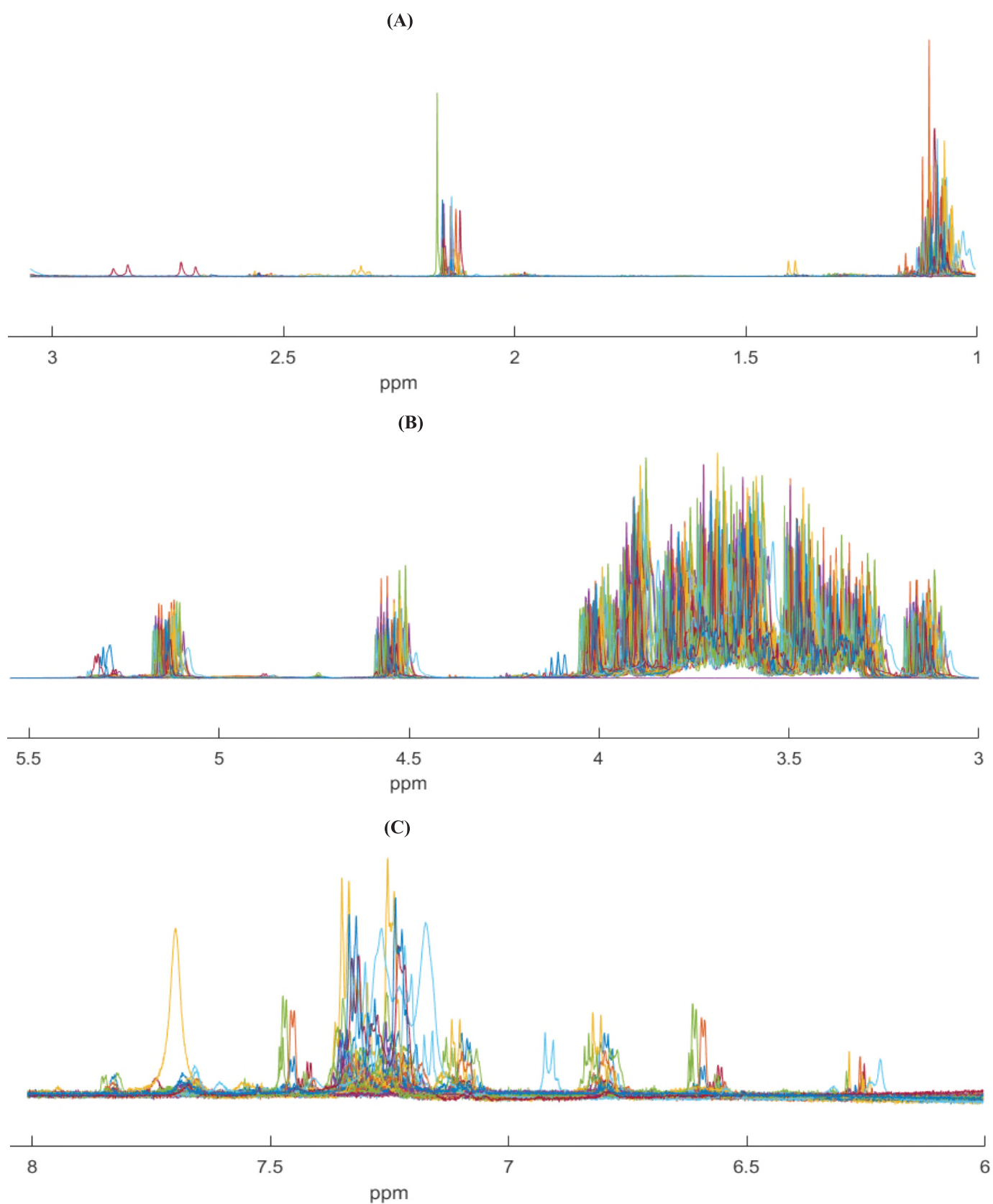


Fig. 3. ^1H -NMR spectra of honey samples in different regions corresponding to each group. (A) Acetic and lactic region; (B) Carbohydrate region; (C) Aromatic region.

It can be seen in the above figures, each honey sample gives us the differences in the concentrations of carbohydrate compounds, amino acids, lactic acids, and acetic acids. From this point of view, it can be proposed that the PCA method can be employed to discriminate longan flower honey and non-longan flower honey.

Chemometric application

Spectra transforming:

Figure. 2 shows the complexity and considerable deviation of each spectrum compared to others; it leads to the use of normalisation and mean-centering to standardise the data as well as subtract the variable averages.

Figure. 4A and 4B are the PCA score plots of the 1-3 ppm and 6-8 ppm regions, respectively. In these plots, it was impossible to distinguish the longan honey samples from others, due to the group-less distribution of them [18]. These results indicated that the concentrations of amino acids could not be used to discriminate honey samples with different origins because of their low quantities. Also, lactic and acetic acids are not able to distinguish the origins of honey because the amounts of these compounds vary due to the unprofessional collection, extracting and preserving techniques of farmers. However, lactic and acetic acids may consist of the information and the preservation time and conditions [19].

Data pretreatment method:

Figure. 5A represents the PCA score plot of the 3-5.5 ppm region. It can be seen that PC1 describes 26.23%, while PC2 describes 19.09% of the total variability; the samples are grouped into three clearly distinct clusters, and one of the figures has all 18 longan honey samples. It is highly possible that honey samples were botanically classified by their difference in carbohydrates ratio so that the glucose and fructose concentration can lead to a longan origin of honey. Compared to the PCA score plot without using data treatment by mean-centering (Fig. 4B), the increasing of the eigenvalue is almost two-fold.

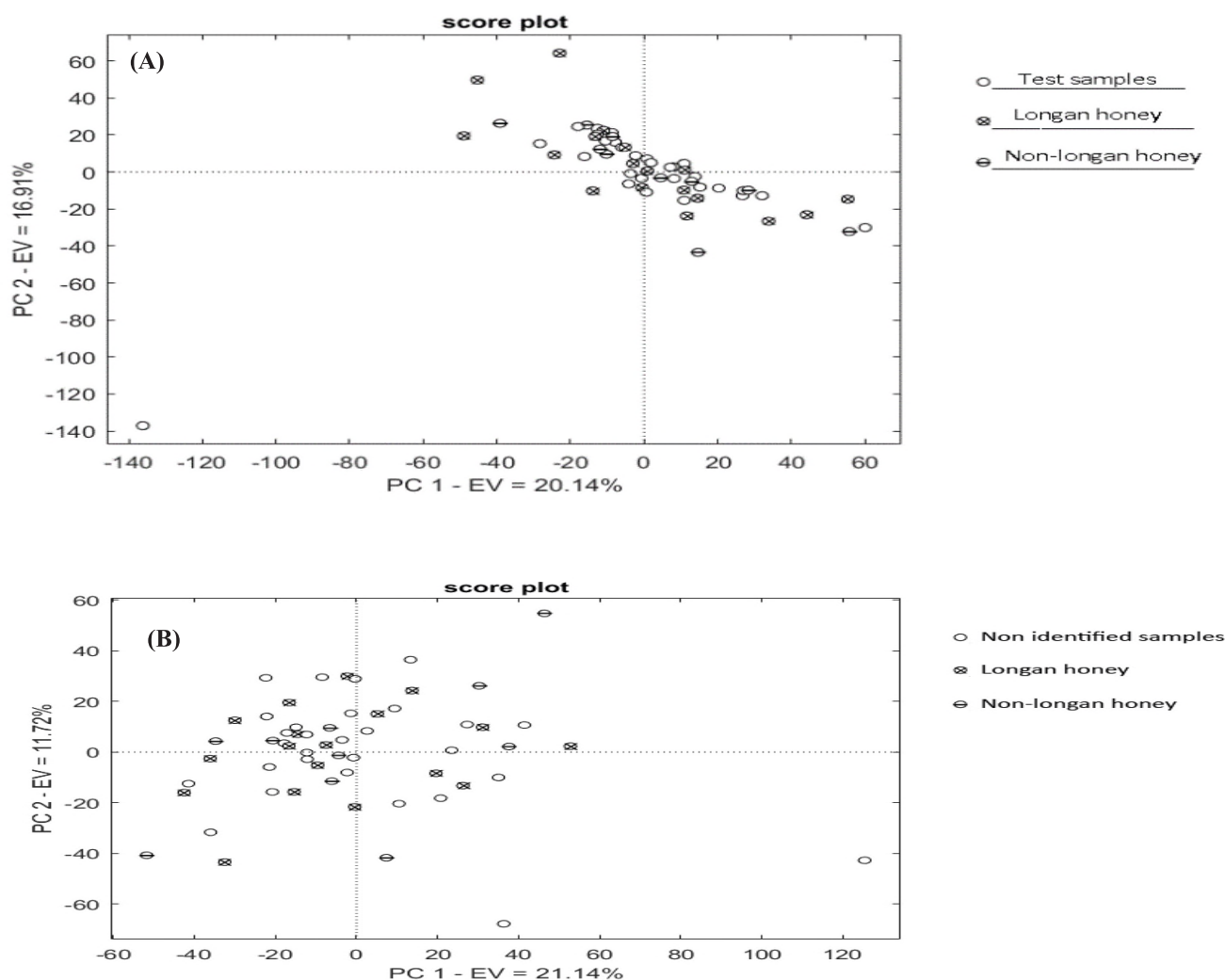


Fig. 4. PCA score plots of the ¹H-NMR spectra range: (A) 1-3 ppm and (B) 6-8 ppm regions.

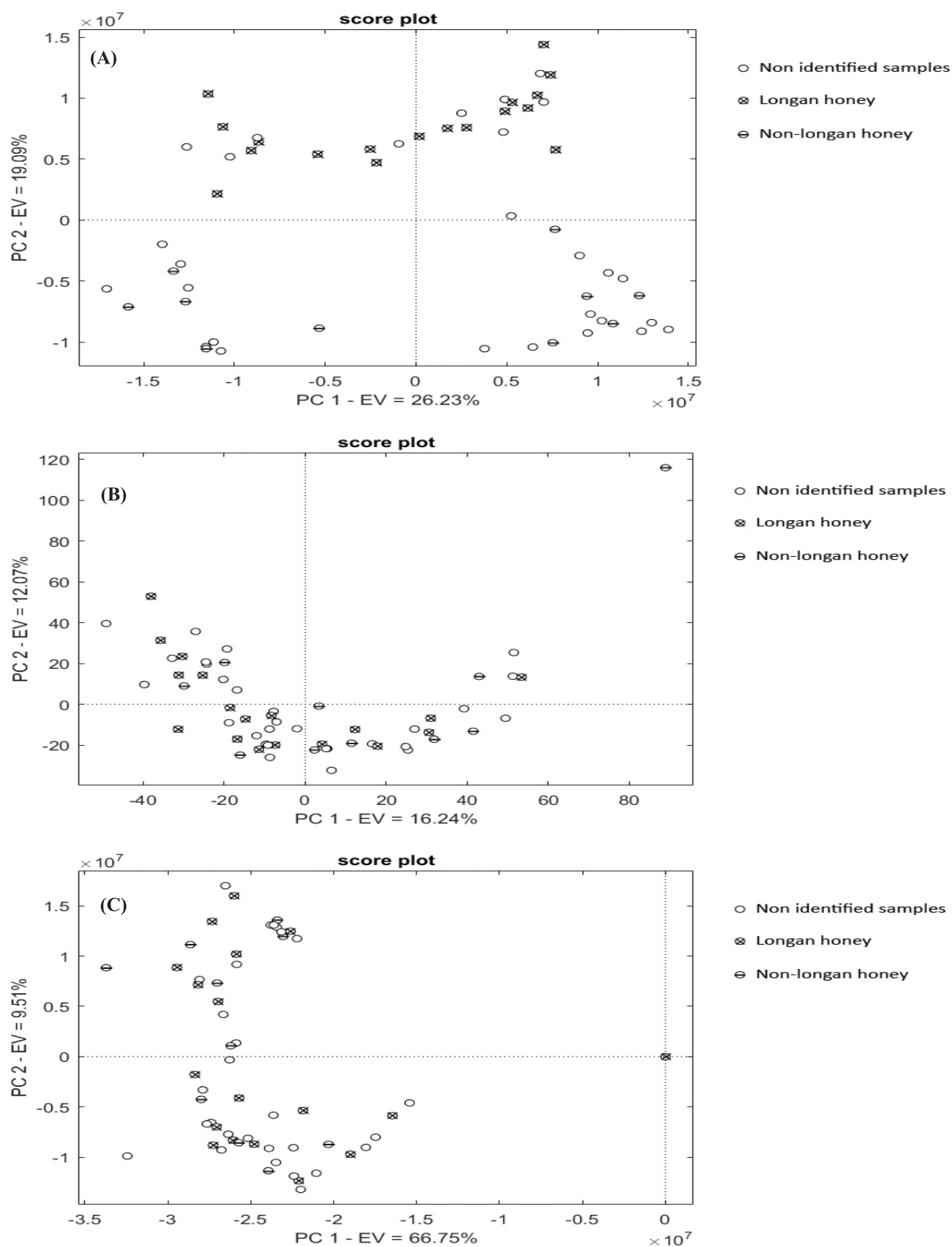


Fig. 5. PCA score plots of 3-5.5 ppm regions, with data pretreatment by: (A) mean-centering; (B) not treating and (C) range scaling.

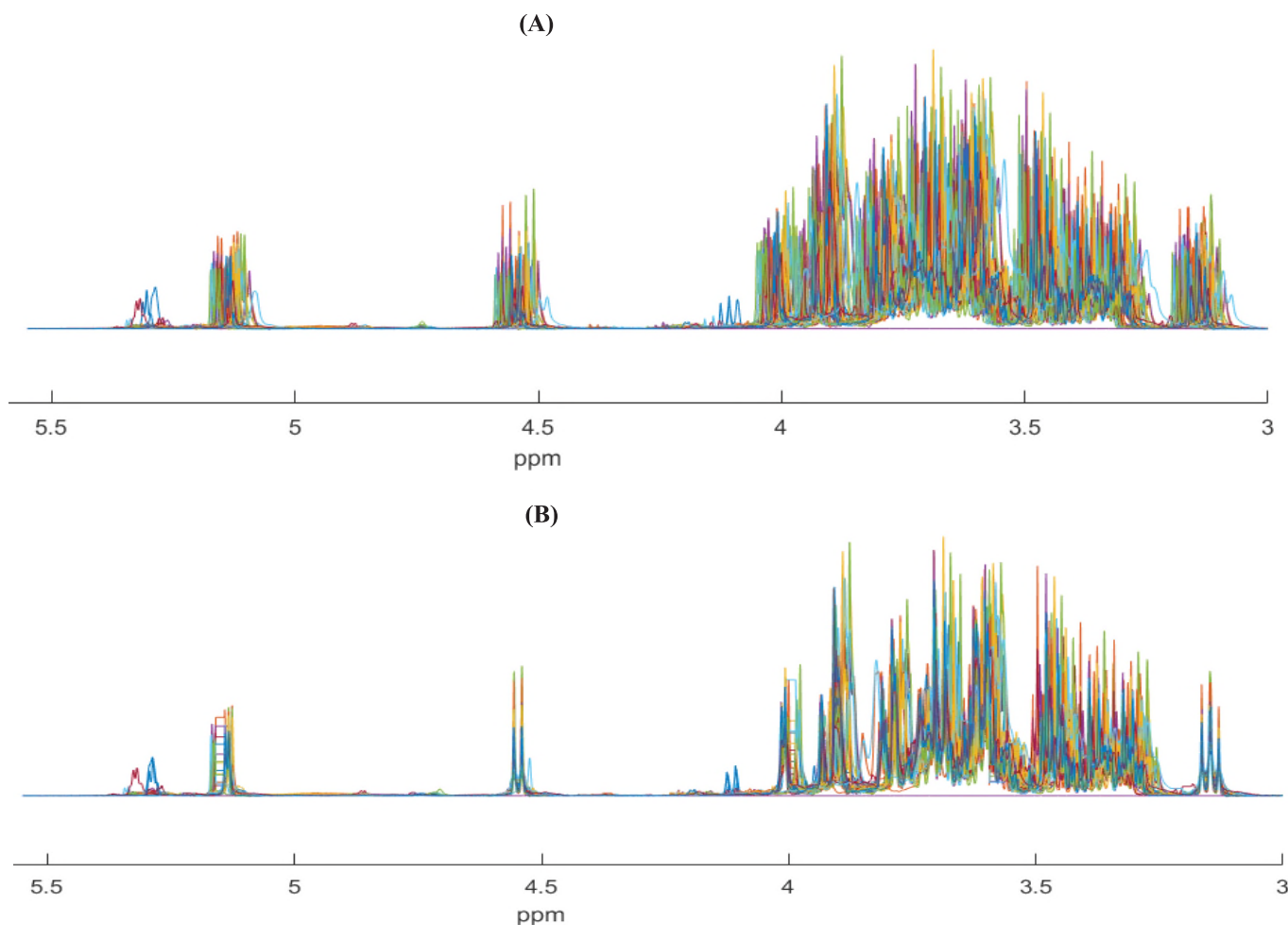


Fig. 6. ^1H -NMR spectra of 57 honey samples obtained with: (A) before using Icoshift algorithm; (B) after using Icoshift algorithm.

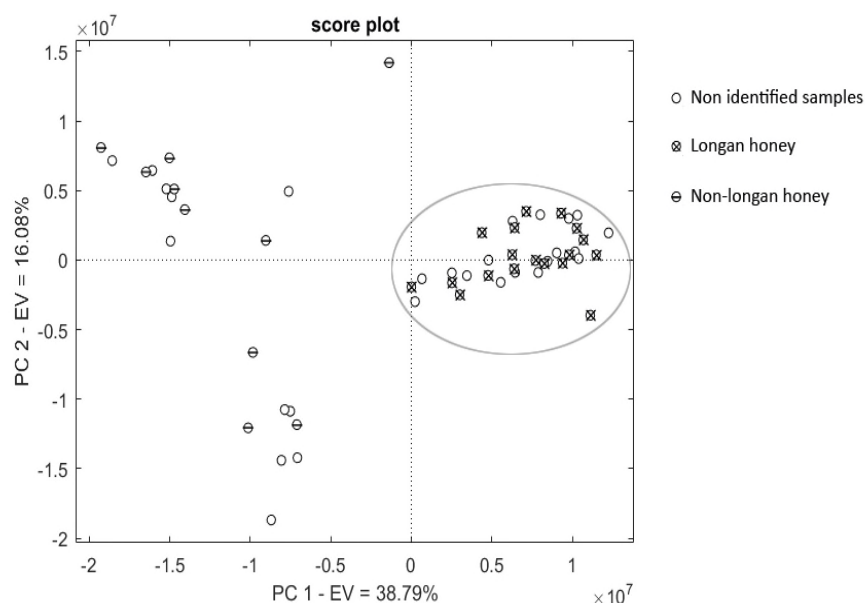


Fig. 7. PCA score plot of sample's spectra in the range of 3-5.5 ppm after Icoshift.

Icoshift application:

The results obtained by using Icoshift toolbox to align the spectral data sets of all 57 honey samples were displayed in Fig. 6B. The results obtained by using PCA showed a better score plot with higher eigenvalue and clearer grouped samples.

The eigenvalues of the two first PCs were estimated at about 54%, which is an acceptable number. In the circled group, the predicted samples included 18 longan honey and 16 non-identified honey samples being present. Therefore, it was reasonable that this group contained longan honey.

It can also be recognised that all the longan honey samples belonged in the circle, whereas non-longan honey samples belonged on the outside (Fig. 7). It suggests

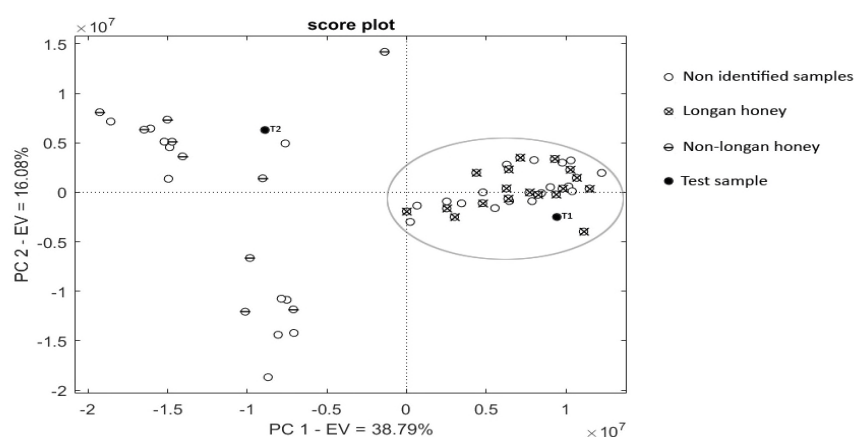


Fig. 8. PCA score plot of initial 57 honey samples and 2 test samples (T1 and T2).

that unknown samples' sources can be clearly identified based on their locations on the score plot.

Identification of unknown samples

For two unknown samples (T1 - a longan honey and T2 - coffee honey), the botanical sources can be classified as follows:

- Collect the data of ^1H -NMR spectrum of a honey sample in the range of 3-5.5 ppm.

- Extract the data into a spectrum of the data matrix and add the spectra (intensity vs. ppm) to the original data together with the 57 studied samples.

- Run the data pretreatment and PCA in the Matlab software.

The score plot obtained in Fig. 8 suggests that sample T1 is longan honey whereas T2 is not.

Conclusions

The ^1H -NMR spectra of honey samples in water solvent has been successfully applied for the classification of botanical origin of honey (longan flower honey or non-longan one). The ^1H -NMR data was pretreated by using the mean-centering algorithm. The PCA application was followed by icoshift algorithm which suggests good results in the classification of original longan honey based on the reference data of 57 honey samples in the range of carbohydrate 3-5.5 ppm. The longan honey (test sample) was grouped in

its cluster showing suitable results to identify if an unknown sample is longan honey or not. The application of the data of 57 honey samples and PCA showed the appropriate results in the recognition of two test samples belonged to longan honey or non-longan honey. It can be seen that ^1H -NMR spectroscopy coupled with multivariate methods followed icoshift algorithm is a useful method of classifying the botanical of honey in the Vietnamese market. Therefore, it will be necessary to look after more reliable samples to develop a complete, quick and simple method for commercial application.

REFERENCES

- [1] Codex Alimentarius Commission (2002), "Codex standard 12 Revised Codex Standard for Honey", *Stan. Methods*, **11**, pp.1-8.
- [2] S. Bogdanov (1997), "Nature and origin of the antibacterial substances in honey", *Lebensm.-Wiss. u.- Technol.*, **30**(7), pp.748-753.
- [3] E. Perez, A.J. Rodriguez - Malaver, P. Vit (2006), "Antioxidant capacity of Venezuelan honey in wistar rat homogenates", *J. Med. Food*, **9**(4), pp.510-516.
- [4] Cục Xúc tiến thương mại (2014), "Vietnam's honey export-opportunities and potentials", *VIETRADE*.
- [5] Southern Horticultural Research Institute-SOFRI (2014), *Proceeding of the Workshop on Increasing Production and Market Access for Tropical Fruit in Southeast Asia*, pp.13-17.
- [6] R. Karoui, J. De Baerdemaeker (2007), "A review of the analytical methods coupled with chemometrics tools for the determination of the quality and identity of dairy products", *Food Chem.*, **102**(3), pp.621-640.
- [7] J.F.D. Kelly, G. Downey, V. Fouratier (2004), "Initial study of honey adulteration by sugar solutions using mid-infrared (MID) spectroscopy and chemometrics", *Journal of Agricultural and Food Chemistry*, **52**(1), pp.33-39.
- [8] T.D. Alam, M.K. Alam (2004), "Chemometric analysis of NMR spectroscopy data: A review", *Annu. Rep. NMR Spectrosc.*, **54**(1), pp.41-80.
- [9] R.A. Prestes, L.A. Colnago, L.A. Forato, L. Vizzotto, E.H. Novotny, E. Carrilho (2007), "A rapid and automated low resolution NMR method to analyze oil quality in intact oilseeds", *Analytica Chimica Acta*, **596**(2), pp.325-329.
- [10] S. Bogdanov & M. Gfeller (2006), "Classification of honeydew and blossom honeys by discriminant analysis", *ALP Science*, **500**, pp.1-9.
- [11] J. Riedl, S. Esslinger, C. Faulh - Hassek (2015), "Review of validation and reporting of non-targeted fingerprinting approaches for food authentication", *Analytica Chimica Acta*, **885**, pp.17-32.
- [12] R. Consonni, and L. Xagliani (2008), "Geographical Characterization of Polyfloral and Acacia Honeys by Nuclear Magnetic Resonance and Chemometrics", *Agricultural and Food Chemistry*, **56**(16), pp.6873-6880.
- [13] F. Elisangela - Boffo, A. Leila - Tavares, C.T. Antonio - Tobias, M.C. Márcia - Ferreira, G. Antonio - Ferreira (2012), "Identification of components of Brazilian honey by ^1H -NMR and classification of its botanical origin by chemometric methods", *LWT - Food Science and Technology*, **49**(1), pp.55-63.
- [14] M. Spiteri, K.M. Roger, E. Jamin, F. Thomas, S. Guyader, M. Lees, D.N. Rutledge (2017), "Combination of ^1H -NMR and chemometrics to discriminate manuk honey from other floral honey types from Oceania", *Food Chemistry*, **217**, pp.766-772.
- [15] D. Bertelli, M. Lolli, G. Papotti, L. Bortolotti, J. Serra (2010), "Detection of Honey Adulteration by Sugar Syrups Using One-Dimensional and Two-Dimensional High - Resolution Nuclear Magnetic Resonance", *Agricultural and Food Chemistry*, **58**(15), pp.8495-8501.
- [16] F. Savorani, G. Tomasi, S.B. Engelsen (2010), "icoshift: A versatile tool for the rapid alignment of 1D NMR spectra", *Journal of Magnetic Resonance*, **202**(2), pp.190-202.
- [17] M. Spiteri, E. Dubin, J. Cotton, M. Poirer, B. Corman, E. Eric Jamin, M.L. Rutledge (2016), "Data fusion between high resolution ^1H -NMR and mass spectrometry: a synergetic approach to honey botanical origin characterization", *Analytical Bioanalytical Chemistry*, **408**(16), pp.4389-401.
- [18] J. Devillers, M. Morlot, M.H. Pham-Delegue, J.C. Dore (2004), "Classification of monofloral honeys based on their quality control data", *Food Chemistry*, **86**(2), pp.305-312.
- [19] R. Consonni, L.R. Cagliani, C. Cogliati (2012), "NMR characterization of saccharides in Italian honeys of different floral sources", *Agricultural and Food Chemistry*, **60**(18), pp.4526-34.

Scientific and technological achievements of the oil and gas industry of Vietnam and strategies to address future challenges

Thu Hien Nguyen, Tang Son Nguyen, Thanh Tung Dang*

Science & Strategy Division, Vietnam Petroleum Institute

Received 10 January 2017; accepted 4 May 2017

Abstract:

Over more than 40 years of the Vietnam National Oil and Gas Group (PVN) development, Science and Technology has been played a very important role and significantly contribute to the success of PVN in all core business areas. This paper reviews the achievements of Science & Technology activities of PVN so far as well as point outs key strategies to address future challenges of oil & gas industry in Vietnam in order to improve productivity, quality, production, business efficiency, and to achieve sustainable development.

Keywords: oil and gas industry, oil exploration and production, refining and petrochemical, science and technology strategy.

Classification number: 2.3

Introduction

Energy security is becoming an enormous global challenge. For the oil and gas sectors, the general context is the increasingly fierce competition from non-renewable resources. While demand for energy is increasing, supply is limited, and regions with large oil and gas potential are getting more and more difficult to access.

As a strategy to develop Vietnam's Oil and Gas industry, scientific and technological solutions are being used to improve productivity, quality, production, business efficiency, and in-depth sustainable development.

Over the years, PVN has achieved remarkable successes in all fields of

scientific and technological research and the application of resulting activities, contributing significantly to the sustainable development of PVN [1].

In addition to PVN's average revenue growth of 18.2% per year between 2006 and 2015, Vietnam's oil and gas industry has established synchronized activities for the exploration, production, processing, and distribution of petroleum products and services. This is an opportunity for scientific research activities across all fields to serve the whole chain of production and business activities for PVN. Accompanying each stage of development, research helps to solve problems in science and technology according to the actual requirements of production and

business, services, and applied directly to the operating activities and business of PVN. Petroleum scientific and technological advancements have made important contributions to a powerful Petro-Vietnam [2].

To date, PVN has been transferring, applying, and mastering many of the most modern technologies in the world to enhance PVN's operational activities.

In upstream petroleum exploration and production sectors, Vietnam's Oil and Gas industry has been using various modern technologies, including the latest applications of information technology, such as geophysical data interpretation and processing software; reservoir modeling and simulation; production design; and production technology to extract oil from basement rock. A range of the world's most advanced and specialized software solutions have been put to use, including the seismic data processing software [1, 3].

ProMax; the seismic interpretation from Landmark and Geoquest; the reservoir simulation from Geoquest (Eclipse); CMG software (IMEX, GEM, STARS); the drilling software Drilling Office; software for geophysics data interpretation of oil wells, Elan Plus; Finder databases; and others. PVN has studied technologies for drilling into

*Corresponding author: Email: tungdt01@vpi.pvn.vn

deep-sea regions, drilling into geological objects with high temperature and high pressure, horizontal drilling technology, and drilling technology for narrow oil wells. PVN also developed a summative assessment study of the construction of drilling, well completion work, and studies on development of appropriate drilling fluids for different geological conditions of Vietnam. Automatic control technology in drilling and extraction is also widely applied, with a special focus on horizontal drilling technology, drilling technology for narrow oil wells, automatic extraction wells on lightweight rig operation, and sub-sea well operations. Biological and chemical technologies have been applied to enhance the oil recovery efforts in operating oil wells, including at Bach Ho, Rang Dong, Su Tu Den, and Su Tu Vang. Information technology has been used to increase the accuracy of analytical results and to save analysis time for many kinds of samples: paleontology, petrography, geochemical studies, and rock mechanical studies.

In downstream sectors, scientific studies have focused on the analysis of crude oil and gas to establish a database of oil and gas fields in Vietnam. Analytical results are mainly used to build databases, and for reference and guidance plans to select the right processing methods and technologies, and to choose the appropriate type of crude oil [1].

Regarding the gas sector, PVN has implemented research on the gas market, gas pricing, gas price scenarios calculating influence from imported gas from abroad, gas market forecast, and a gas pricing mechanism for Ca Voi Xanh. In-depth studies on electricity have also been deployed, for example on the efficiency of PVN's investment

into gas and coal thermal power; and the operation and maintenance of power plants. Furthermore, studies into the feasibility of building power plants from household waste, as well as wind power potential, and on connecting wind power to the independent grid, alongside with studies of coal thermal power, waste handling and processing (ash, fly ash, separation of CO₂ from flue gas) [1, 2].

Regarding HSE in the oil and gas industry, research projects have achieved excellent results and have been applied to production, including establishing a system of advanced testing methods to control chemicals used in oil and gas harvesting, which assists environmental management agencies to assess the level of danger and manage risk for the use and disposal of toxic chemicals. PVN's research has helped to establish an environmentally sensitive map, oil drift models, and a responsive plan for oil spills. These have been applied to assess the environmental damage caused by oil spills which occurred in the Ganh Rai, Vung Tau Province in 2001; conduct quantitative studies assessing the risk of Vietnam - Russia Petroleum Joint Venture (VSP) marine works, and gas pipeline system of PVGAS for safety management; do research into working environments to provide management guidelines and improve working conditions; and establish a database of waste and environmental monitoring, and create a VMEGIS system, which helps to manage environmental issues relating to oil and gas operations and the rescue plan for oil spills when incident occurs [1, 2].

Highlights of specific scientific and technological achievements

Until now, four studies of PVN have been awarded the prestigious Ho Chi

Minh and State Prizes for Science and Technology. In Series 5 of the Ho Chi Minh and State Prizes for Science and Technology (2017), PVN was honored to be awarded three prizes of total 16 winning works. Earlier, in Series 4 (2012), PVN was awarded the prize for its work relating to oil extraction technology from basement rock. All the award-winning work are of great practical impact and has contributed significantly to the economic and social development of Vietnam [1].

The VSP group of works won the Ho Chi Minh Prize (2012) for the following study: "Effective exploration, discovery, and production of oil successfully conducted in the Pre-Tertiary granitoid basement rock of Cuu Long Basin, on the Vietnam continental shelf"

Before oil was discovered in the basement rock of Bach Ho, many well-known oil and gas companies, including Deminex, Bon Valley, and Agip, carried out exploration of oil and gas within the continental shelf of Vietnam. However, due to the use of traditional oil and gas exploration methods, those exploration missions did not come to any marketable discoveries. In terms of geology, this is an unusual supply of oil, as conventional oil is normally found in terrigenous sediment, but the oil at Cuu Long Basin is contained in the basement rock of Pre-Tertiary granitoid. This unusual existence of oil requires unconventional extraction technologies, and a rethinking of the classic rules of the oil and gas industry. So far there is still a lot of debate about permeability, and porosity, so this technology is progressively improving to determine the ultimate scientific truth. Vietnam is now known all over the world for mastering technology to exploit such a particular type of oil. Many other countries have asked VSP

for co-operation to research in order to help them to develop technology for similar oil supplies, including former CIS countries, Sudan, Venezuela, and Uzbekistan, to name a few.

Vietsovpetro not only has built a methodology for petroleum extraction from fractured basement rock, but has also contributed to research methodologies and reservoir models, technological solutions for fractured basement drilling, production with maintained reservoir pressure, optimized oil recovery factors at a high tempo, created software to calculate reservoir parameters, and mined constructing organization. Based on these developments, foreign oil and gas service companies have made technological improvements in research, drilling and extraction of oil from basement rock, and contributed to an increase in oil production in Vietnam. Currently in the Cuu Long Basin, oil and gas fields have already been discovered in basement rock and operations conducted to extract oil from areas including Bach Ho, Rong from Vietsovpetro, Nam Rong-Doi Moi from joint Vietnam-Russia-Japan; Su Tu Den, Su Tu Vang, Su Tu Nau from Cuu Long JOC; Rang Dong from JVPC; Ruby from Petronas; Ca Ngu Vang from Hoang Long - Hoan Vu JOC, and there are other discoveries including jade, diamonds, and pearls in Hai Su Den, Thang Long, Ho Xam South, which are soon going into operation. Granitoid fractured basement rock becomes objects of interest when conducting exploration into other sedimentary basins. Apart from the Cuu Long Basin, oil and gas have continued to be discovered in basement granitoid in the Nam Con Son Basin including Dai Hung, and Gau Chua Oil Fields. The scientific and technological achievements in

Vietnam's oil and gas industry possess practical value, not only for Cuu Long Basin, but also for other oil reservoirs on the continental shelf of Vietnam and the rest of the region. These achievements are the scientific contributions of science and technology for oil and gas industry in Vietnam and the world.

The work of VSP won the Ho Chi Minh Prize (2017) for the following study: "Technological research, development, and completion used to gather, process, and transport crude oil in the conditions of Vietsovpetro oil fields and their linked oil fields on the continental shelf of Vietnam"

This group of research works is systematic and comprehensive to cover the development of systems used to gather, process, and transport crude oil off the coast of Vietsovpetro, summing up all of the difficulties and challenges with transporting crude oil from Bach Ho and Rong via offshore underground pipe, collecting the length of the project; and is a comprehensive body of research into the rheological properties and processing solutions, transporting crude oil at Vietsovpetro; and works in research and development and innovative treatment technologies and transportation of crude oil offshore in line with the actual conditions of Vietsovpetro oilfields different than traditional technologies. This technology consists of a combination of technological solutions with varying degrees of flexibility, depending on the region and the exploitation period, and has been proven to be highly effective. The use of innovative technologies to transport gas-saturated oil via air preliminary separation and subsequent transportation of oil below its freezing temperature has contributed significantly and made fundamental changes to Vietsovpetro

oilfield development guidelines, from the initial fix-rigged model MSP fixed rigs to a simple rig model BK/RC. The application of transportation technology from Vietsovpetro has helped to connect many nearby small fields.

The result of the research conducted is a prerequisite for the development of future overall schemes connecting all oil and gas fields on Vietnam's continental shelf, and create a chance to connect small fields, marginal fields, enhance efficiency in the utilization and maximize resources for the country.

The research works of PV Shipyard won the Ho Chi Minh Prize (2017) for the following study: "A study of the detailed design and application of technology to fabricate assemble and launch self-elevated rig in water depth of 90 m and suitable for the conditions of Vietnam"

During the period of 2009-2012, with support from the Ministry of Science and Technology, PVN ordered PV Shipyard and the National Research Institute of Mechanical Engineering to carry out a national research project to "study the detailed design and application of technology to fabricate, assemble, and launch a jack-up rig in a water depth of 90 m, and suitable for conditions of Vietnam". This project covers 11 scientific research and technological development topics aimed at enhancing self-reliance, increasing local content, training staff in the field of design and manufacture for oil rigs and aimed at self-reliance of design and fabrication of oil and gas drilling rigs; contributing to the construction and development of key mechanical design and fabrication of oil rigs and mechanical equipment gauge serve the cause of rapid and sustainable development of PVN.



Fig. 1. The 90 m water depth Tam Dao 03 rig marked a breakthrough in the science and technology understanding of the PV Shipyard, and laid a foundation for the design and manufacture of drilling rigs for the industry in Vietnam.
Source: pvn.vn.

In 2011, a 90 m depth self-elevating rig was set up at Tam Dao 3 (Fig. 1). This was the first domestic rig ever constructed by PetroVietnam, and was successfully launched and put into use two months before schedule. This event marked a maturation and the mastering of high-tech for Vietnam's oil and gas industry. The project added Vietnam to the list of countries capable of designing and manufacturing oil and gas rigs. The 90 m water rig has a weight of nearly 12 thousand tons, is 145 feet in length, and operates in water depths of up to 90 meters, with a capability to drill to depths of 6.1 km. The rig can withstand hurricane winds equivalent to and above level 12, and withstand extreme weather conditions. Tam Dao 03 is the first 90 m depth self-elevating rig built by PV Shipyard, and was completed two months before the deadline, and

was registered into the USA Maritime Registry with ABS certification, which shows the rig to meet international standards. The rig is registered to be used by the joint venture Vietnamese - Russia Vietsovpetro, with the stable and effective operation since June 2012.

This scientific research project is a key factor determining the success of the project which is to build the Tam Dao 03 rig and afterwards, have a major role to build the Tam Dao 05 drilling rig across all phases, from design, procurement of materials, and construction equipment manufacturing. For the Tam Dao 05 rig project, PV Shipyard was completely proactive in the implementation of projects, increasing the localization rate, reduce dependence on foreign technologies less than the rig Tam Dao 03 project.

The Tam Dao 03 rig includes the following applications: 1. It applied advanced process design methods for modern drilling rigs; 2. It applied advanced design software; 3. It applied the work rig developed to the highest quality possible. The project increased the rate of localization of the project to build the Tam Dao 05 rig at 39% local engineering compared with the three other projects at Tam Dao at 34.7%. At the same time, there was a reduction in foreign expert work time from around 43,000 hours (Tam Dao 03) to 11,000 hours (Tam Dao 05). Besides, the project construction period for Tam Dao 05 was reduced to 32 months, although the mass fabrication 1.5 times compared with the volume of projects built in Tam Dao 03.

Rig design technology had a key role in the development of the PV Shipyard.

Until now, the PV Shipyard has both basic and advanced technological foundations. The PV Shipyard workforce is also professionally trained on scientific research projects.

The work of VSP won the State Prize for Science and Technology (2017) for the following study: “A study of the optimal plan to build, launch, and install super-sized tripods in water depths of over 100 m to be suitable for the conditions of Vietnam”

This study analyzed and selected methods to manufacture tripods using a combination of large blocks. In this project, researchers calculated the base plan by launching a scheme of sliding (Skidding loadouts). This study calculated methods by laying the base for a self-releasing (Launching) rig, and worked on onshore fabrication, and construction works were completed to the calculations, procedures, and plans for combination blocks “panel” so adults can stand up to 20,000 T, which is the length up to 150 meters base. This project also created a panel rotary volume flip code by combining multiple cranes that can be up to 1,000 tons. This innovation is the embodiment of a flip turn lifting large panel blocks using multiple cranes (6 cranes) combined hydraulic lift system and “tie back”. Regarding the launch and installation at sea, construction work was

completed through a fully standardized process, solving the problem of launching and installing gauge bases by means of self-discharge lines with a condition of equipment in Vietnam. The outstanding creative element here was it was the first time that we were able to perform the stand by a reporter dedicated domestic barge, and was converted and used successfully and safely. The barges used exclusively launched the first base and the only one in Vietnam (VSP-05) and has been optimized to apply barges VSP-05 conversion and marine cranes with lifting capacity is limited (Paracel 1,200 tons).

Science and technology development strategy of PVN during 2016-2025 period and guideline to 2035

In the future, the energy sector in general, and in particular the oil and gas industries, faces a number of problems, such as the depletion of fossil fuel resources, and a high demand for energy, fuel and material requirements for the production to be increasingly strict (Fig. 2). Global climate change, environmental protection requirements lead to increasingly strict standards on waste, improvements to the efficiency of use of resources and energy saving, development of new energy sources to replace traditional ones, the energy demand shifted towards reducing the share of coal and oil, increasing the

proportion of electricity and gas.

The trend of scientific and technological applications to improve the efficiency of resource use and energy will contribute to significantly reduce demand for fossil fuels and reducing environmental pollution. Sources of clean and renewable energy will replace fossil energy sources, which are likely the causes of environmental pollution. Research and application of new scientific and technical solutions is a key engine for this energy transformation [4].

In the context of dynamic and unpredictable development in both economic and social aspects, the role of science and technology in production is increasingly appreciated. The issue of innovation, technological application and transfer, operational optimization and energy savings to improve the efficiency of production operations is the core issue throughout the whole industry. The biggest challenge for the development of the science and technology knowledge of PVN now is to rapidly improve the quality of human resources to develop scientific and technological improvements to meet the quality evenly research, not only extensively but also intensively, soon to reach the advanced level for the region across all areas.

The development of science and

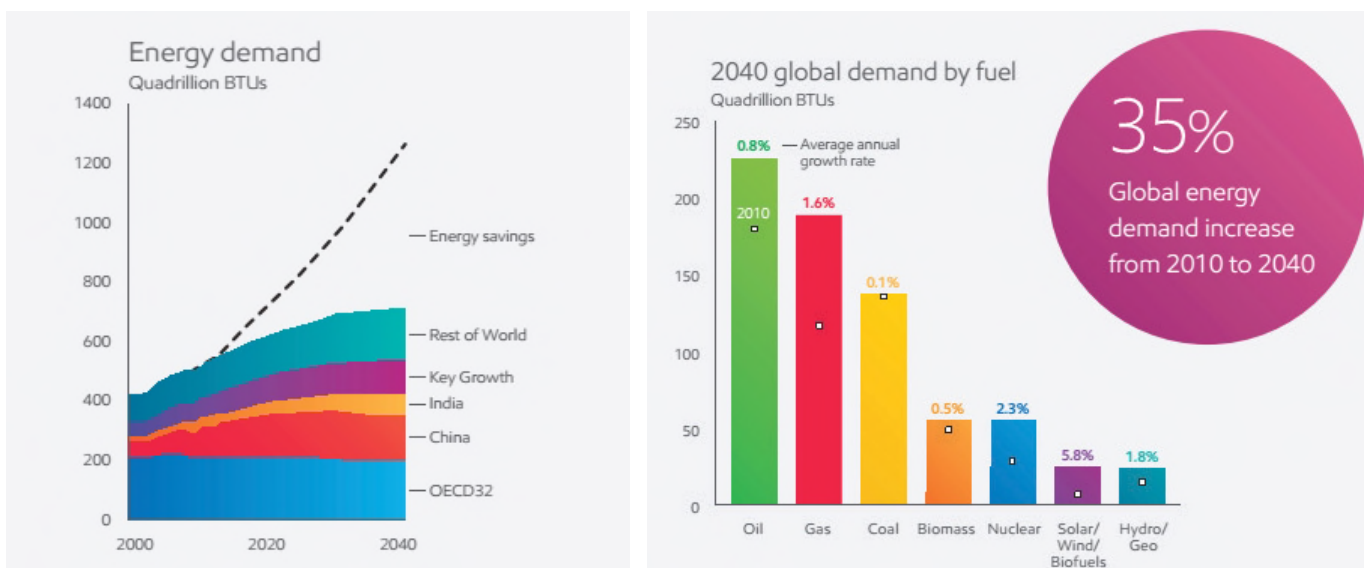


Fig. 2. World's demand of energy and proportions of each type.

technology in Vietnam's oil and gas industry is under the general trend of the world oil and gas industry, in particular by sector is as follows [1, 3]:

Exploration sector

Seismic exploration: Development has been made towards the application of advances in physics and information technology in order to improve reception quality, improve the resolution of objects, improve the exploratory study of small sizes, and at great depths, do better in the complex geological environment. The development of hardware and software that allows direct analysis object images in 3D, 4D is being progressed.

Well-logging: This development is based on the latest progresses in physical science, engineering technology, electronics, and software, and is used to obtain information directly and more reliably, reducing downtime for measuring drill well logging, increasing curve and maximum parameters in one measurement, diverse audience services, and environmental conditions. Intelligent solutions integrate multiple curves, different materials using artificial intelligence network (ANN), and improve reliability prediction calculations physical properties of reservoirs.

Oil and gas production sector

Drilling technology: Aiming at offshore and deep water drilling to expand the scope of exploration using mining and drilling technologies applied to achieve the following objectives: reduce the costs of drilling, drill deep water areas, and integrate with other technologies. The drilling technology trends being developed are as follows:

- Casing drilling technology;
- Single-diameter well drilling technology;
- Slim hole, microphone hole drilling technology;
- Seismic imaging while drilling, real-time drilling technology;
- Drilling fluids suitable for areas with high temperature, high pressure;

- Drilling under balancing pressure;
- Drilling of wells with multi-diameter;
- Horizontal drilling;
- Drilling technology in deep water offshore areas well requires great drilling depths.

Production technology:

- The technology group to enhance water injection efficiency, improving primary oil recovery (IOR);
- Technology for low and ultra-low permeability wells such as tight oil, shale gas, etc., requires exploitation by hydraulic seam cracks;
- Technology object extraction carbonate objects;
- Technology in the area of offshore deep water (groundwater well head, etc.);
- Technology to strengthen the management and supervision of the mining process (automatic measurement systems, management and exploitation online, etc.);
- Technology non-traditional mining: tight oil, gas shale, tar sand, mines permafrost zone, etc;

- Advanced Technologies, including tertiary (or enhanced) oil recovery (EOR) technology mix (such as hydrocarbon, CO₂, N₂); chemical, thermal, physical and chemical micro (MEOR) technologies; water alternating gas (WAG). These methods are most widely developed as chemical methods (surfactant, polymer, alkane, alkali-polymer (AP), alkaline-surfactant-polymer (ASP), alkaline-surfactant-polymer (SAP), and nano-materials). Technological products aimed to match the characteristics of reservoir rock, including fluidity and heat resistance to increase the scope, efficiency, and environmental friendliness, and biodiversity.

Platform Technology:

- Lightweight construction platform: Lightweight and minimal construction platform is often used to exploit critical oil and gas fields;

- Common platform: The gantry concrete or steel platform is used at less than 200 m deep. Concrete structures are capable of being built with fixed steel structures which are widely used in the North Sea since water depths are less than 300 m. This type of rig is also used for both critical and large fields;

- Elastic platform: SPAR type suitable rigs with a depth of over 1,500 m and for geologically weak foundation;

- Tension Leg Platform-TLP: Theoretically, TLP rigs can work in unlimited water depths;

- Submersible platform is used to exploit deep water fields. The current floating facilities are used for economically-technically critical fields (marginal fields and fields with high depth);

- The floating facilities: Popularly used in deep water areas. Systems of this type have emerged synthesis: Extraction - processing - contains - oil (FSO/FPSO);

- Special rigs used for permafrost conditions: Mainly suitable for northern waters of Russia and Europe.

Gas sector

Development of technologies to effectively collect gas from small and offshore gas fields using pipe-free methods such as FLNG, FCNG, FGTL, and Micro-GTL:

CNG production technology: Onshore CNG production technology has been commercialized and used for years. However, the offshore counterpart, referred to as FCNG (Floating compressed natural gas) has not yet been commercialized. The two CNG manufacturing technologies whether onshore or offshore have the same principles (implementation process).

Among the unconventional technologies for gas collection, FCNG is the simplest. The core of the technology is the process of compressing and storing gas at high pressures, so it is likely to be widely commercialized in the next few years. The gas fields in Vietnam mainly consist of small ones with reserves

<< 1 tcf and scattered and thus FCNG technology is a promising and suitable technology.

Offshore LNG production technology:

The production process of LNG offshore (floating LNG production - FLNG) is not different from the onshore LNG production; however, the FLNG vessel itself integrates with many components, including gas collection equipment, gas treatment equipment for air separation and liquefaction, a storage tank system, and product transfer systems for the LNG tankers.

Floating GTL, micro-GTL technology:

Floating GTL Technology (GTL Floating - FGTL) or Micro-GTL process is not essentially different from GTL - Gas to liquid process. The ultimate aim of both processes is transforming natural gas into liquid products of high economic value. However, the technology aims FGTL transformation toward efficient natural gas resources in the small gas fields, offshore products into a high-value liquid medium capable of transporting both by traditional methods as vessels and pipes.

- The development of advanced technologies to control and improve the efficiency of operation of the air: Technology for risk mitigation;

- Research and development technology for collecting, processing, and using gas and gas products in order to increase processing efficiency, expand markets, and ensure the required product quality and the environment:

- + CO₂, H₂S treatment technology using membrane, sea water, etc.;

- + Technology for transformation of natural gas into high-value products;

- + Fuel conversion technology: LNG, CNG, GTL, etc.;

- The development of advanced technologies to improve gas efficiency: Saving energy, improving the efficiency of combustion.

Petrochemical and refining sector

Refining technology:

Reduced requirements of feedstock and improved product quality: One popular global trend in crude oil exploitation is the movement away from the traditional crude oils (reserves declining) to crude oils with high density, which contain more sulfur and impurities. Moreover, the strict requirement of product quality, especially environmental standards, is becoming increasingly challenging. Therefore, in terms of technology, the current major trends in the field of oil refining is improved, upgraded oil filtration technology to processing sulfur and impurities-contained heavy crude oil is used, and concentrations of chemicals in products which have an adverse effect on the environment (sulfur impurities, olefin, aromatics) are reduced.

Technologies which are expected to grow in the near future to meet the requirements of both hydro-related raw materials and products technologies: Hydro-treating, hydrocracking, and hydrogen production technology.

Improving the processing capacity:

To increase profits, oil refineries, at present and in the future, are likely to increase their processing capacity. Thus the size of a maximum capacity line and equipment in for the oil refining industry will also increase because of the construction of a chain and large equipment, which will reduce overall investment performance and operating costs over two or more lines or small appliances.

Integration with Petrochemistry:

Because conventional petrochemical profits remain stable at higher profiting oil refineries, done to extend the value-added chain, reduce risk, and increase profits, they tend to build refineries to currently and in future maximize integration with petrochemicals from refined petroleum products. The integrated oil refinery can follow the following directions:

- Using naphtha from oil refining (light naphtha, heavy naphtha) for use

as raw materials for steam cracking workshops to produce Olefin (ethylene, propylene, and butadiene) from which produces resins, solvents, and chemicals, and is the lead manufacture of ethylene, propylene, and butadiene;

- Using naphtha from oil refining (heavy naphtha, FCC naphtha) for use as raw materials for workshops reforming and BTX extraction to the production of aromatic compounds (benzene, toluene, and xylenes with p-Xylene in particular) which produces all kinds of plastic, solvents, fibers, and chemicals as BTX derivatives;

- Combining the two directions above.

Petrochemical Technology:

Production of platform chemicals from methane is becoming more popular because natural gas reserves in the world are more plentiful than oil, and the main ingredient in natural gas is methane (C1). New technological trends are transforming methane into the middle of petrochemical products, saving valuable time with olefin and aromatic factors and thereby the production of petrochemical products. Converting methane into the intermediate petrochemical products of high value O₂ is oriented in the following directions:

- Indirectly through Methanol: MTO technology (methane to olefins), MTP (Methane to Propylene), and MTA (Methane to Aromatics);

- Directly through OCM Technology (oxidative Coupling of Methane) for Ethylene production.

Production of petrochemical products from petrochemical raw materials of biological origin:

With the need to reduce the effects of total emissions on the environment, the use of biological materials, such as ethanol, to produce the intermediate petrochemical products (ethylene) and other petrochemical products, such as PE biological chain; and the use of bio-PET is also developing as a trend. Also, the demand for biodegradable plastic products after being discharged

to the environment is also on the rise. Consequently, technology must be able to process raw petrochemical materials of biological origin, which are usually not as pure as petrochemical raw materials from petroleum. More specifically, the following petrochemical technologies will be able to use all kinds of biological materials with biodegradable materials derived from petroleum hardly decomposed from which to form the plastic medium whose properties facilitate the manufacture and use of such petroleum derivative plastics that are likely to decompose when discharged into the environment.

Safety, health, environment sectors

Cleaner production technologies: To meet the increasingly strict environmental protection requirements and use of resources in a most efficient, clean trend in production in the oil industry, which includes an optimization of the operation and application of new technologies of energy savings, oil and gas resources must be efficiently used to reduce emissions.

CO₂ storage and sequestration technologies: Aimed at reducing the greenhouse gas emissions causing climate change, the current separation technologies of stored CO₂ (CO₂ capture and storage) are being developed and applied in the petroleum sector in which CO₂ is separated from emissions of power plants, refineries, petrochemical processing or from natural gas. CO₂ is separated after being buried in exploited gas fields or aquifers which have suitable geological structures.

Power sector

- Continue to develop and improve technology configuration for the power plant in order to improve capacity, reduce emissions, and diversify resources. Over time the following technologies have been developed:

+ For gas-powered plants, gas turbines combine the cycle generation F last life (F4, F5) or Generation H;

+ For coal-fired power plants, boiler parameters supercritical and ultra-

supercritical;

- Development, clean energy and technology improvements, renewable energy such as hydro, wind, and solar, in order to improve performance, the capacity and the share of renewable energy in the energy structure measure;

- O₂.

Conclusions

Through 55 years of operation, PVN has evolved from an “apprentice” and “employee” for foreign oil companies to the one who can master difficult tasks in the exploration, exploitation, storage, transportation, and processing of oil and gas. Success has been enjoyed via the construction of Tam Dao 03 and Tam Dao 05 platforms; construction and operation of the project such as the Bien Dong 01; operating and ensuring safety for the Dung Quat Refinery; and the construction and safe operation of gas-fired power plants, and fertilizer production plants. It is a testament to the determination and continuous efforts of several generations of PVN leaders and employees towards scientific and technological research and application activities, and has contributed significantly to the sustainable development of the organization. Technological solutions and scientific content have contributed more and more to production and business activities. To date, PVN has managed five basic areas: exploration and exploitation, gas industry, power industry, processing industry, and high-tech services. Scientific and technological activities have been continuing to bring high economic efficiency and contribute greatly to the success of PVN.

To implement the “Strategy for development of PVN by 2025 and guidelines to 2035” with an aim of making PVN the core unit of the oil and gas industry; to have dynamic, strong financial resources, and science and technology backing with high competitiveness in domestic and international markets, an important contribution in the process of building, developing, and protecting the country.

On the basis of an assessment of the status and level of scientific and technological capacity, the Group’s scientific and technological development perspective is: “Science and Technology are the essential tools to improve competitiveness and to ensure the successful implementation of the development strategy of PVN. The scientific and technological development aims to raise the level of science and technology for the petroleum industry and contribute to the development of world’s science and technology, in which the focus is the applied research and basic research in service-oriented applications for operating business activities and petroleum science. The petroleum scientific and technological activities should be implemented on the basis of internal resources, incorporated with the cooperation at both domestic and international scales, and accompanied by research - training - production to create synergy strength and enhance competitiveness”.

In the future, to overcome the challenges of domestic and global oil and gas industry changes, the PVN will determine scientific and technological developmental strategies for the organization based on an overall objective to raise scientific and technological resources; improve research capacity, mastery, innovation, creativity, and consulting; and to develop technological options to reach the advanced levels of the area in 2025. This will reach an advancement level with the rest of the world in a number of core areas for after 2035; contributing to improving the competitiveness and the successful implementation of the development strategy of PVN.

REFERENCES

- [1] Vietnam Petroleum Institute (2016), “Science and Technology Strategy of PetroVietnam in the period of 2016-2025, vision to 2035”.
- [2] Vietnam Petroleum Institute (2016), “Oil & gas industry master plan to 2025, vision to 2035”.
- [3] Truong Minh (2013), *Collection of Scientific research in oil & gas industry*, Science and Engineering Publishing, Hanoi.
- [4] Exxon Mobil report “The Outlook for Energy: A view to 2040”.

New rice varieties adapted to climate change in the Mekong River Delta of Vietnam

Chi Buu Bui^{1*}, Thi Lang Nguyen²

¹Institute of Agricultural Sciences for Southern Vietnam (IAS), Vietnam

²Cuu Long Delta Rice Research Institute (CLRRRI), Vietnam

Received 1 August 2016; accepted 21 October 2016

Abstract:

Although Vietnam is the second largest rice exporter in the world, the country has only recently met the demand for sustainable food supply at a national level, and not yet at a household one. In the early 1970s, the average rice yield in the Mekong River Delta (MRD) was 1.9 tons/ha and the annual production weighed 4 million tons. The current rice productivity and production has gained over 6 tons/ha and 24 million tons/year, respectively. This is a substantial increase. This progress in the MRD is in brief, due mostly to (1) Government policy, (2) Water management, and (3) Technological innovation and application with an emphasis on varietal improvement. As an impact of the ongoing El Nino Southern Oscillation (ENSO) phenomenon, severe droughts and salinity intrusion has been occurring in Vietnam's MRD and has caused varying degrees of damage to agriculture, fisheries and the livelihoods of people in the region. New rice genotypes have been released via both conventional breeding and marker-assisted selection. The introgression of target genes from wild species viz. *Oryza officinalis*, *O. australiensis*, *O. rufipogon* into cultivars has been conducted successfully. Commercial rice varieties, which are currently growing in the MRD for export, are listed as Jasmine 85, OM3536, OM4900, IR64, OM6162, ST3, ST5...

Keywords: climate change, El Nino, gene introgression, marker-assisted selection, wild rice.

Classification number: 3.1

Introduction

The two primary rice bowls of Vietnam are the MRD, accounting for 54% of Vietnamese rice production, and the Red River Delta (RRD) contributing 17%. Both delta regions are comprised of large areas of fertile low laying land which is highly suitable for rice production. Both are blessed with abundant water from rain and from two of the largest rivers in the country. Affected by the monsoonal climate, rain, and the flows of those two rivers, the region

experiences strong seasonal variations, suffering through floods in the rainy season, and water scarcity and salinity intrusion in the dry season. In addition, about half of the MRD is covered with acid sulphate soil. Water resource management helps alleviate the above production constraints and contributes to very high productivity in the delta regions, with rice crop intensity > 2 and yields of more than 5.4 tons/ha/crop over the delta regions.

Although Vietnam is the second largest

rice exporter, it has only recently met the demand for food security at the national level, however, not yet at the household level. Many Vietnamese people do not have access to sufficient food. People who have an insufficient food supply account for 6.7% of the overall Vietnamese population, and 8.7% in rural areas. Approximately one million people in mountainous regions use maize and cassava instead of rice because of their severely restricted access to food sources due to low incomes and poor infrastructure in rural areas [1].

In Vietnam's sustainable development strategy, food security is always a top priority, seen as an ends to gain livelihood, political and social stability. After the financial crisis of 2009, one could observe a noticeable growth in the appreciation of the essential role of agriculture by the global society. Vietnam has recently been identified as an outstanding example of a country that has successfully overcome economic crisis from food security issues arising from agriculture challenges.

Intensive cultivation of rice in the Mekong Delta has led to an increased threat of brown plant hopper populations and continuously adapting agriculture virus strains (e.g. blast), which transmit ragged stunt and yellowing diseases that further risk food security in the region.

Vietnam needs to deal with its decline in agricultural land for rice as well as future climate shocks including sea-level rise. Based on *status quo*, by 2030, projected decline in yields of rice in Vietnam in North Delta could be -2.2% and in South Delta could be -5.6%. This trend continues till 2050 where an overall Vietnam could experience yield loss by 20%. Subsequently, yield loss could be 32.6% in North Delta and 7.8 to 8.6% in

*Corresponding author: Email: buu.bc@iasvn.org

South Delta. By 2080, the picture continues to be negative. Predictions suggest that North Delta may experience loss of 16.5% annually. However, yield changes may vary between the seasons: -17.5% (summer/autumn), +11% (autumn/winter) in the South Delta; -23.5% (summer/autumn), +17.2% (autumn/winter) in the Ca Mau region of the South Delta, Vietnam [2].

Climate changes caused by El Nino have created severe drought and salinity intrusion into the MRD. On March 15, 2016, the Vietnamese government and the United Nations Development Program (UNDP) organized a meeting with donors, international organizations, and other partners to discuss joint efforts for drought response and recovery in the region.

In response to this urgent call, the CGIAR (Consultative Group of International Agriculture Research) Centers operating in Vietnam, in collaboration with MARD (Ministry of Agriculture and Rural Development), organized a joint field assessment in the MRD to first-hand observe and assess the effects of the drought and salinity intrusion currently experienced by the region.

The CGIAR will contribute to solving the food security issue by studying global data and analyses from Vietnamese scenarios, and calculate forecasts, outline plans, and recommend CSR (Climate Smart Agriculture) options for integration into current and future donor-development intervention programs. These findings and plans can also be used to identify opportunities for research and development of programs that ensure future food security preparedness.

The current challenges for Vietnamese agriculture could be identified as follows: climate change due to extremely harmful weather, reduced arable land, water shortage, food and energy crisis, and population blooming.

Vietnam must deal with rising sea water levels to 15-90 cm by 2070, and a rapidly (21% rate) increasing level of rain fall by the end of century 21 as compared to the period of 1980-1999.

Varietal improvement for exporting and climate change

In the past, Vietnam had suffered a

series of long and terrible wars during which rice productivity could not meet demand. In the case of the MRD, during those wartimes rice fields yield less than 2.0 tons/ha due to deepwater rice at 1.7 million ha including 0.5 million ha of floating rice which had a low yield. Rice production in 1975 was calculated at 4.0 million tons/year, but steadily rose to current yield records of 21 million tons in 2010. This change is substantial. Vietnam imported rice and wheat during those war time at roughly 2 million tons/year until 1989.

Recent agricultural progress in Vietnam is mostly due to (1) Government policy, (2) Water management, and (3) Technological innovation and application with an emphasis on varietal improvement. Vietnam has become a rice exporter (Table 1).

Table 1. Global rice market situation and Vietnam role (million tons/year).

Country	2009	2010	2011	2012*
India	2.15	2.23	4.64	8.00
Vietnam	5.95	6.73	7.00	7.00
Thailand	8.57	9.05	10.65	6.50
Pakistan	3.19	4.00	3.41	3.75
USA	3.02	3.87	3.21	3.45
Others	6.52	5.67	7.24	6.75
Global	29.40	31.55	36.15	35.45

*Estimated.

The most recent objectives of rice breeding has been to develop new varieties of rice suitable to match food security levels and maximize rice cultivator profit.

From 1991 until 2000, focus was on water management strategies to promote irrigation to rice fields, augment rice production, prevent salt intrusion, and improve sulfate toxicity in the Plain of Reeds (Dong Thap Muoi) of MRD. Rice growing areas yield as much as 5.7 million ha in 1986 rising to 7.66 million ha in 2000. That means that 2 million ha were augmented over 15 years (34%).

Rice breeding is now considered to be the most important focus in order to raise crop yield productivity. Throughout human history, food crises have often been solved by new inventions; these so called green revolutions have helped peoples deal with hunger. Due to IRRI Cooperation's

rice improvement programs in Vietnam which installed IRRI elite lines throughout various regions, prebreeding derivatives have been introduced that allowed for rice crop cross breeding, which enriches the gene pool of wild rice species (*O. officinalis*, *O. australiensis*, *O. rufipogon*).

The new rice breed IR8 is called Than Nong 8 and Nong Nghiep 8 in South and North Vietnam respectively. The breed provides a much higher yield compared to other leading local breeds. Subsequently, new varieties had been engineered and released as Than Nong 73-2, IR36, IR42, IR19660, IR48, IR4570, IR8423, IR64 in the South. However, due to Vietnam's history with war, the country's yield potential has not been fully utilized partially due to the lack of intensification. Since 1975, new technologies have not been fully adopted due to the lack of appropriate agriculture policies, which are needed to motivate rice farmers to use those new technologies. The lack of adequate policies is one of the key reasons why Vietnam imported rice during the period of 1962 until 1987, during which times includes a period of four continuous years that Vietnam had to import over one million tons/year from international sources. In 1967, 1.25 million ton was imported; in 1968, 1.23 million tons was imported; in 1969, 1.03 million tons was imported and in 1970 rice imports peaked at 1.26 million tons imported.

Vietnam became a rice exporter in 1989, two years after the new policies from Doi Moi were implemented. At that time, the leading rice varieties were IR64, IR17494, IR50404, VND95-20, OM1490, OM576. These new varieties have helped average grain yield continuously increase at 0.11 tons/ha/year in the most recent 20 years.

These changes are due to breeding and seed programs, and certified rice seeds have been used from 2% up to 28-30% during the 10 most recent years in the MRD [3]. With a 10% grain yield resulting from the use of certified seeds, a gain of at least 500 thousand tons is estimated annually. This success becomes a prerequisite to develop the new program "One Must, Five Reductions" by MARD used to support sustainable rice production in the MRD (the program requires the use of certified seeds, a reduced seeding rate, N fertilizer application, pesticides, postharvest loss,

and water consumption).

In the beginning, rice farmers had a very negative opinion of certified seeds; 53% of the farmers understood less than 40% about certified seeds. However, after participating in community-based farmer group demonstrations, trainings and field visits, 87% of the farmers understood 70% about certified seeds [4]. Nearly half of the farmers had low opinions about the seeds (47%), and approximately 1/3 of them had high a high opinion of the seeds (40%) with three reductions and three gains before participation in community-based farmer groups showing the benefits of certified seeds, training farmers on seed use, and observing field demonstrations. However, after the demonstrations, more than 80% of them agreed to accept the new varieties of rice seeds that offer short duration genotypes resistant to brown plant hopper, blast and a higher export quality (Table 2).

Table 2. Farmers' perceptions of certified seeds and three reductions and three gains (% farmers) (n = 230).

Level of perception	Participation in community-based farmer groups, training and visiting field demonstration		χ^2
	<i>Before</i>	<i>After</i>	
<i>Certified seeds</i>			
Low	53	4	11,224**
Medium	10	9	
High	37	87	
Total	100	100	
<i>Three reductions three gains</i>			
Low	47	1	12,865**
Medium	13	1	
High	40	98	
Total	100	100	

**Significant at 1%.

Level of perception: Low: $\leq 40\%$ right answers toward perception on technology; medium: $> 40-70\%$ right answers toward perception on technology; high: $> 70\%$ right answers toward perception on technology.

New rice varieties adapted to farmers' demand under climate change

Commercial rice varieties: Currently,

rice varieties grown in the MRD for export are Jasmine 85, OM3536, OM4900, IR64, OMCS2000, OM6162 (drought tolerance), ST3, ST5, VND95-20.

Promising rice varieties: Recently released by MARD are rice varieties incorporating HYV (High Yielding Variety) and good grain quality properties as OM5756, AG1 (OM6377), HG2 (OM6161), OM6162, OM6677, OM6976, OM7347 (aroma and drought tolerance). Most of these rices have a long slender grain shape and are good export quality.

MAS (Marker-Assisted Selection) for grain quality properties

AC (Amylose Content): AC is one of the important properties of grain quality. A polymorphic microsatellite sequence closely linked to the *Wx* gene has been reported. Analysis of chromosome 6 with 20 SSRs, markers RG42 and Waxy were used to select the promising lines.

Three new varieties with low AC such as: OM4900, Hau Giang 2, OM7347 were released due to MAS [5].

Aroma: The genomic clone RG28, which is tightly linked to the *frg* gene in rice, provides a performance MAS in the rice breeding program. This study was conducted to identify the target gene by using SSR (RM223) and STS designed

from RG28 on Chromosome 8 (RG28FL-RB) linked to *fgr* gene in rice to obtain salt tolerance genotype plus aroma such as OM4900 (3 dS/m at seedling stage).

Major biotic and abiotic stresses under climate change

To sustain current yield levels, rice improvement has been focused on releasing new genotypes with stable tolerance at the target stresses under climate change. Brown plant hopper blooms up beside blast and bacterial leaf blight damage.

The favorable soil for rice cultures in the two largest granaries in Vietnam is alluvial soil at 1.18 million ha (30.1%) in MRD. The unfavorable areas with various soil types is presented in Table 3.

Table 3. Problem soils in rice culture areas in MRD.

Soil type	MRD (%)
Acid sulfate	40.8
Salinity	18.9
Peat soils	20.0
Grey soils	3.4

In acid sulfate soils, low pH, aluminum toxicity, iron toxicity, and low phosphorous are considered as the primary limiting factors for rice growth. Currently, water management and agronomic practices have been recommended. Some improved genotypes have been identified to tolerate drought, salinity, and acid sulfate, but it is not stable due to climate change and unfavorable weather patterns.

For biotic stresses, brown plant hopper is often considered as rice production's most harmful pest. Due to increases in population and changing biotypes, current rice genotypes are not able to resist this pest for long periods. Introgression of target genes from wild rice species into cultivars is being implemented.

Blast disease is another important biotic stress. Many isolates have been collected throughout Vietnam, and gene *Pi-2* is considered to control most virulent strains in Vietnam beside other genes.

Bacterial leaf blight is the most significant problem in Northern Vietnam during the two main seasons, and in southern Vietnam during the monsoon season. Genotypes with *xa-5*, *xa-13*, *Xa-*

7, *Xa-4*, *Xa-21* can control the virulent bacteria in Vietnam [3, 6].

Varietal improvement has provided farmers with the best materials available from pure line selection, introduction, and local hybridization.

Approximately 5,000 types of local rice and hundreds of populations of four wild rice species: *O. rufipogon*, *O. nivara*, *O. officinalis*, *O. granulata* have been collected, catalogued and evaluated. The resulting materials have provided donors for biotic and abiotic stresses [7]. Rice germplasm evaluation assisted by DNA markers has been conducted at some institutions in Vietnam to supply reliable information to rice breeders to assist with selecting appropriate materials.

The use of a gene pool from wild rice species fully introduces a true introgression of desirable traits into HYVs such as AS996 (IR64/*Oryza rufipogon*), the first derivative which is tolerant to some major biotic and abiotic stresses, short duration, high yield, and wide adaptability, in particular Dong Thap Muoi acid sulfate soil area.

Focus from biotechnology applications of rice have been on: (1) The functional genomics to improve the traits in which the conventional breeding is not available, (2) MAS in rice breeding, and (3) Genetic diversity assessment. Rice biotechnology has significant potential to help the field of agriculture to contribute to the goal of country-wide sustainable development, which is to adapt to climate change with an emphasis on salinity, submergence, and drought and heat tolerance; to increase the nutritional and market values of food; and to enhance the stability and sustainability of the agro-ecosystem.

Several wild species with strong degrees of resistance to pests have been identified. *O. rufipogon* is a wild rice breed strongly tolerant to acid sulfate soil which occurs in Dong Thap Muoi, Vietnam, and has been identified to have an appropriate population for QTL mapping of aluminum toxicity with its derivative as the AS996 genotype [8].

The use of MAS will increase the efficiency of selecting a rice breeds most desirable traits. Supported by the Rockefeller Foundation (RF), one can identify necessary facilities for this PCR-

based markers' application. Teams use RAPD, AFLP, SSR, STS to detect target genes that control the resistance of major biotic and abiotic stresses. Drought, salinity, and acid sulfate are the key challenges beside major biotic stresses in a rice breeding program used for food security.

Drought tolerance was assessed by both phenotyping and genotyping (RM201 on Chromosome 9) to develop OM7759, OM7930 and OM7935. Genotype OM6162 is growing in large scale areas. However, the stress of drought is still the biggest challenge, in particular at flowering stage.

Salinity tolerance was conducted under field conditions and MAS (Chromosome 1 and 8). The promising rice lines at seedling stage with survival days of 15-20 days, were noticed as: EC = 7 dS/m: OM5629, OM8108; EC = 5 dS/m: OM6600, OM8104 (+ drought tol), OM6328; EC = 4 dS/m: OM6677, OM6377, OM2395 and MNR4; EC = 3 dS/m: OM4900, OM6162 (+ drought tol); and the rice genotype did not express its salt stress in reproductive stages yet.

Heat tolerant rice genotypes can be recognized through multilocal yield testing and MAS (Chromosome 3 and 4) as followed: OM8108, HTL1, HTL2, HTL3 and HTL4. Heat shock protein HSP90 was notice to express in rice plant via Western blotting under temperature of over 36°C daily and 27°C nightly, in Ninh Thuan province.

Phosphorus-deficient soils is another major yield-limiting factor in the Mekong Delta, especially in acid sulfate soils. A major QTL for P uptake had been mapped on Chromosome 12 (RIL6 population of AS996/OM2395). OM4498 and AS996 were selected and developed successfully in MRD.

Sub1, a major QTL on Chromosome 9, provides protection for 10-18 days of complete submergence. IR64 *Sub1* has been exploited in rice breeding in MRD to obtain submergence tolerance rice varitey OM1490 - *Sub1*.

Looking to the future

Looking to the future, one must (1) Broaden the genetic background of rice varieties from both landraces and wild rice

species; (2) Break the yield ceiling and stabilize productivity; (3) Improve grain quality and nutrition value of rice; and (4) Meet the demand of climate changing and water deficit to have drought tolerant genotypes and others tolerant to abiotic stress.

REFERENCES

- [1] Nguyen Van Bo, Bui Chi Buu (2010), "Rice Production in Vietnam: Achievements, Opportunities and Challenges", *Vietnam Fifty Years of Rice Research and Development*, Agric Publisher, pp.19-36.
- [2] P. Teng, Mely Caballero-Anthony, Jonatan Anderias Lassa (2016), "The Future of Rice Security Under Climate Change", *NTS Report*, 4 (S. Rajaratnam School of International Studies).
- [3] Bui Chi Buu (2009), "Rice Production in Vietnam", in *Proc. of The First Vietnam Rice Festival in Hau Giang*, VNA Publishing House, pp.17-32.
- [4] Truong Thi Ngoc Chi, Tran Quang Tuyen, Tran Thi Ngoc Mai, Nguyen Van Khang, Le Van Tinh, Le Thi Ngoc Anh (2011), "Effect of Community-Based Farmer Groups with Same Preference on Adoption of Technology", *OmonRice*, 18, pp.157-166.
- [5] Nguyen Thi Lang, Bui Chi Buu, Nguyen Van Viet, A.M. Ismail (2010), "Strategies for Improving and Stabilizing Rice Productivity in The Coastal Zones of The Mekong Delta, Vietnam", *Tropical Deltas and Coastal Zones: Food Production, Communities and Environment at the Land and Water Interface*, pp.209-222.
- [6] Bui Chi Buu, Nguyen Thi Lang (1998), "Rice Germplasm Evaluation Assisted by DNA Marker for Bacterial Leaf Blight", *Agricultural Biotechnology: Laboratory, Field, and Market*, CISRO, Darwin, Australia, pp.69-70.
- [7] Bui Chi Buu, D.W. Puckridge (2001), "Impact of Agricultural Research and Training on The Productivity and Farming Systems in The Mekong Delta", *The Impact of Agricultural Research for Development in Southeast Asia*, Proc. of an Int. Conf. Held at The Cambodian Agric. Res. and Develop. Inst., Phnom Penh, Cambodia, pp.123-135.
- [8] Bay D. Nguyen, D.S. Brar, B.C. Buu, N.V. Tao, P.N. Luong, H.T. Nguyen (2003), "Identification and Mapping of The QTL for Aluminum Tolerance Introgressed from The New Source, *O. rufipogon* Griff., Into Indica Rice (*Oryza sativa*L.)", *Theor. Appl. Genet.*, 106(4), pp.583-593.

Sustainable rice cultivation in the deep flooded zones of the Vietnamese Mekong Delta

Alexander Chapman¹, Stephen Darby¹, Emma Tompkins¹,
Christopher Hackney¹, Julian Leyland¹, Pham Dang Tri Van²,
Thanh Vu Pham^{2*}, Daniel Parsons³, Rolf Aalto⁴, Andrew Nicholas⁵

¹Geography and Environment, University of Southampton, United Kingdom

²College of Environment and Natural Resources, Can Tho University, Vietnam

³Geography and Environment, University of Southampton, United Kingdom

⁴Department of Geography, University of Hull, United Kingdom

⁵Department of Geography, University of Exeter, United Kingdom

Received 8 April 2017; accepted 5 June 2017

Abstract:

This paper explains how the management of the full-dyke system in the deep flooded zones of the Vietnamese Mekong Delta affects rice cultivation, and outlines how alternative dyke management strategies could offer more sustainable adaptations in the face of future environmental threats. The current management of the ‘full-dyke’ network has been successful in promoting triple-cropping rice cultivation, but this practice has prevented sediment deposition on the land surface. River-borne sediments deposited on the delta land surface have high economic value because they are (i) rich in nutrients (potentially 26 million USD/yr of free fertiliser to An Giang Province) and can (ii) help to maintain the Mekong Delta land above sea-level. Without a continuing supply of sediment to the delta, triple-cropping paddies may not continue to be sustainable or profitable for the majority of rice farmers over the next 10 to 20 years. The economic value of sediment as a free fertiliser is particularly important to poor farmers, as without sediment, they run a significant risk of debt due to fluctuations in rice, fertiliser, and other input prices. With incoming loads now declining, sediment must be managed carefully as a resource. Our projections show that the best use of the remaining sediment resource can be achieved by allowing full paddy flooding only in years of high sediment potential, and this would greatly increase the sustainability of rice agriculture in the face of future environmental change. This recommended policy is an option with few regrets, in that its other benefits include maximising groundwater replenishment, ensuring freshwater availability during drought periods (including countering salt water intrusion), cleansing rice paddies of pests and disease, and tempering downstream flooding. If triple-rice-cropping continues to have priority, financial support will particularly be needed to provide help to poorer farmers coping with increases in artificial fertiliser prices.

Keywords: *deep flooded, dyke, Mekong Delta, rice, rice cultivation, sediment, sustainability.*

Classification number: 3.1

Introduction

The Vietnamese Mekong Delta (VMD), one of the most productive agricultural regions on Earth, is vital to global food security, the Vietnamese economy, and sustaining the livelihoods of its 18 million residents [1]. The VMD is vulnerable to environmental changes, such as with the size and timing of floods and droughts, sea-level rise, and salt-water intrusion, which come as a result of climate change and human modifications to the Mekong River’s basin upstream [2]. These environmental changes, combined with local development pressures, represent significant threats to the Delta, such that the VMD is now facing a major sustainability challenge [3]. Among the most serious of the expected impacts is the ongoing decline of the Mekong River’s sediment load. Our work has shown that in the last 30 years, there have been substantial declines in sediment load as a result of climate change and extraction of sand from the river bed [4]. We, and other scientists, also project this decline to continue in the future, by between 50-90% over the next 20-30 years, due to a combination of ongoing climate change and trapping behind upstream dams [4, 5]. However, at present a significant sediment resource is still available (Hung et al. estimate 6.83 kg/m²/year) [6]. The deposition of nutrient-rich sediments is important both in replenishing agricultural soils and building land height across the Delta, but it is especially significant in the ‘deep flooded zones’ in the north (Fig. 1).

*Corresponding author: Email: ptvu@ctu.edu.vn

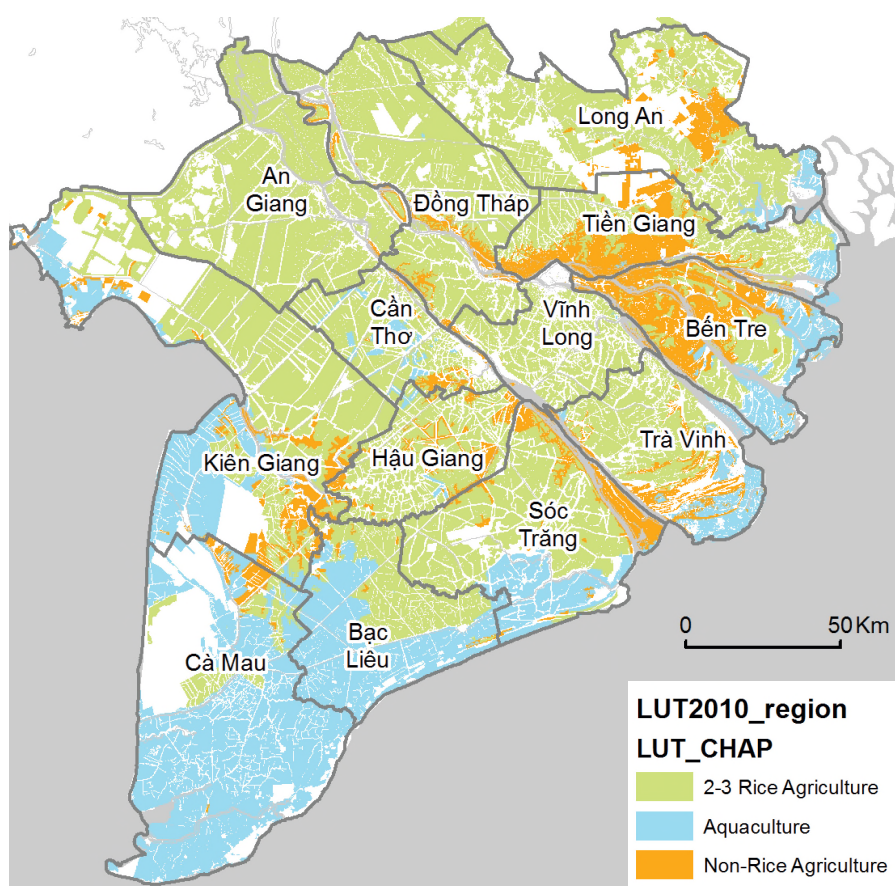


Fig. 1. Map and agro-ecological zones of the Vietnamese Mekong Delta.

Methods

Data collection

Strategic decisions on the VMD's hydraulic operations are guided by targets set at the national level, but specific decisions on the system's management are made and controlled at the provincial level of governance. We conducted a semi-structured focus group with four high-ranking provincial officials to clarify the context within which decisions on hydraulic operations are made.

Our objective was to examine the impacts of high dykes, triple-cropping, and sediment exclusion from a socioeconomic perspective. Floodplain sedimentation can be highly spatially and temporally variable and hence difficult to measure; data is sparsely available on local rates in the VMD. However, farmers of the VMD are aware of the fertilising effects of fluvial sediment, and most typically work any sediment left by inundation

into an even spread around their paddies. While previous research has shown that perceptions may differ from physical measurements, the strong local knowledge of this phenomenon meant that we posited that farmer perceptions could provide a meaningful estimation. Asking farmers to make quantitative estimates of the depth of the sediment (if any) left behind by the monsoon was a new approach to analysing the deltaic environment. To help this process, farmers were presented with visual aids (a scale showing different depths, and a diagram). Some simple validation checks of the farmers' reported sediment values and greater detail on the data collection and analysis can be found in the supplementary information.

Framework

The cause and effect relationship framework are used to structure our methods, analysis, and discussion. The method is a simple framing tool, pioneered

by the OECD [7]. The framework encourages the presentation of a problem's cause and impacts in a format that is clear and easily translated into policy. Studies used the expert approach, Participatory Rural Appraisal (PRA), and local staff consultation. Data analysis used regression models and calculations of the economic value of the sediment.

Results and discussions

Threats to the Vietnamese Mekong Delta

The deep flooded zone is home to around a third of the Delta's population, and is the most intensive rice-growing region in Vietnam. In the deep flooded zones, and particularly in An Giang Province, an extensive network of high dykes (the 'full-dyke' system, see Abbreviations) has proven very effective in regulating and controlling the annual flood, leading to favourable conditions for intensive rice agriculture, with yields improving on average by around 2-3% year-on-year since 2000 [8]. This yield growth has largely been achieved by introducing triple-cropping to new areas. However, just as they are barriers to flood waters, full-dykes also exclude sediment from rice growing compartments. As a result of this, the deep flooded zones now face the multiple challenges of a sinking floodplain, declining soil fertility, and increasing agricultural input costs at a time when a sediment resource is still available.

Stakeholders (Fig. 2) have highlighted both the potentially positive and negative impacts of the 'full-dyke' system. It is clear that the local stakeholders (provincial and district officials, especially those from Department of Agricultural and Rural Development (DARD) and Department of Natural Resources and Environment (DONRE), as well as members of the Farmers Union) [9] value the positive socioeconomic impacts derived from the way in which the full-dyke system is currently managed; albeit stakeholders also have concerns about valuable environmental resources being degraded. Despite these concerns, MARD made new commitments to large-scale expansion

of triple-rice-cropping and supporting infrastructure as recently as 2015, with the deep flooded zones to take the largest share (Decision No. 101/QĐ-BNN-TT). Our research provides an early warning that livelihoods, which depend to a large extent on the natural resources excluded by triple-cropping, face long-term threats from declining productivity, inequality, and the sinking of the Delta below sea-level. Our work, therefore, challenges some of the assumptions made by local stakeholders and decision makers, but also identifies potential and attainable adaptations that could help to protect natural capital in the VMD for future generations.

Focus on key sediment-related impacts of the current system

Our research challenges some of the assumptions (Fig. 2) made by local stakeholders about the full-dyke system, and highlights how triple-rice-cropping inside the full-dyke rings threatens the

ability to meet policy objectives set out by the national and provincial authorities. Specifically, we have shown that the consequence of a full-dyke system is to reduce the depth of nutrient-rich sediment deposited on rice paddies by a factor of five [10].

The socio-economic implications of this decline are serious. Our data highlights the high short-term costs caused by the loss of the fertilising nutrients within the deposited sediments. We estimate sediment is currently providing around 11 million USD/yr of free fertiliser in An Giang Province's remaining flooded areas, and that is around 15 million USD/yr which is being excluded and lost due to the full-dyke system. This figure is less than the gross profit (c. 80-150 million USD/yr) currently being derived from the third rice crop, but the loss of free fertilisation is critical to the economic wellbeing of poorer farmers and is, therefore, in tension

with the government's objectives that target greater equality, poverty alleviation, and sustainability in the delta. Moreover, additional long-term costs (due to damages incurred by rising sea-levels as a result of the reduced natural rate of land building) associated with the exclusion of sediment from paddy compartments are not currently considered in cost-benefit analyses of the triple-cropping policy.

Farmer concerns:

- Free fertilisation from nutrient-rich sediment is of particular value to poorer farmers. On average, natural sediment delivered from floods improves agricultural efficiency by 2% and increases the annual profits of an average size paddy by around \$190 per year. The benefit of this free input is critical to poor farmers who operate within narrow profit margins; without it, their vulnerability to volatility in fertiliser price increases. Our data suggests approximately 48% of farmers are operating paddies of 1.5 ha or less in size (and 32% below 1 ha) while only 12% operate what we consider to be 'large' paddies (greater than 5 ha).

- Triple-cropping increases inequality and debt in poorer households. Triple-cropping places a much higher reliance on artificial fertilisation. Our work shows that after around 10-15 years of triple-cropping, farmers apply almost twice as much fertiliser per year as they do under double-cropping [11]. Poorer farmers who lack the technical capacity to apply fertiliser efficiently incur higher costs and an intense workload.

Government concerns:

- Sediment deposition is the only way to offset the sinking of the delta region into the sea. The most recent research shows that the deep-flooded zones of the Delta are sinking at around 1cm per year due to natural processes and subsidence linked to groundwater extraction [12]. Without sediment to raise the ground-level, the economic costs of infrastructure development to prevent land loss will grow and the viability of livelihoods in the long-term future will be threatened. While sediment loads are declining, full-

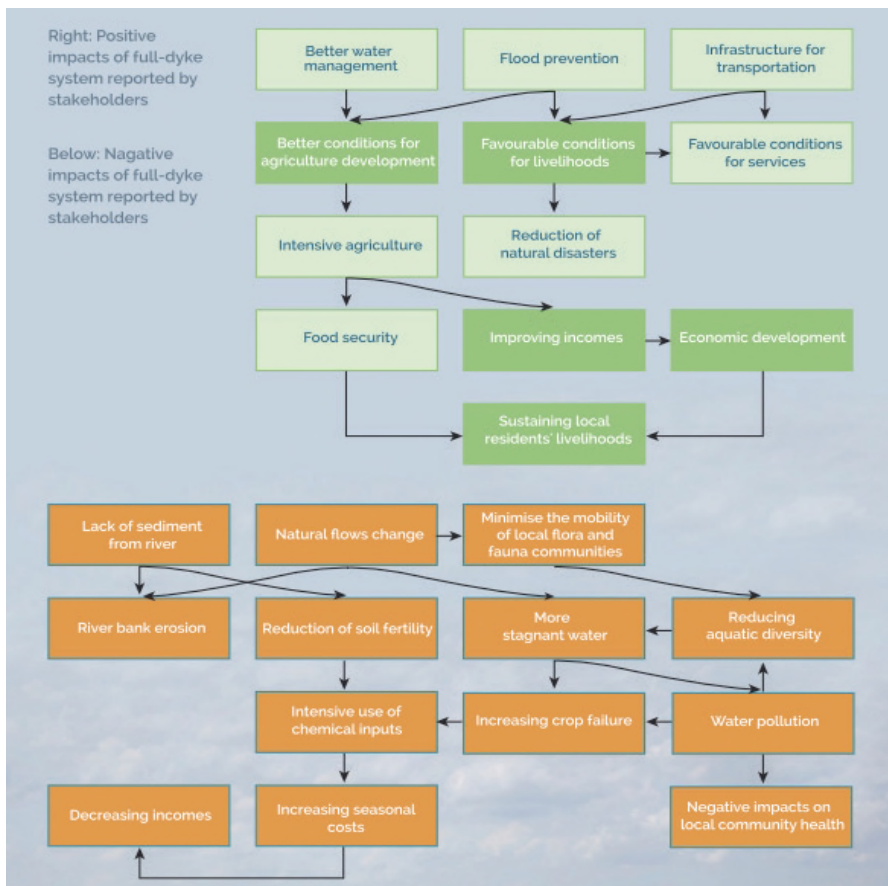


Fig. 2. Focus group output comprising reported positive and negative impacts of the full-dyke system.

dykes are excluding sediment from paddy compartments at a time when considerable sediment resource still remains available.

- Our projections (Fig. 3) show that high productivity levels currently associated with triple-cropping are not sustainable in the long-term. Triple-cropping depletes the nutrient buffer provided by sediment [13]. In the future, the total annual yield per hectare inside the full-dykes will fall lower than that which could be achievable inside low dyke rings (also termed August dyke rings) which allow monsoon season inundation. This decline represents a long-term threat to government revenues from export taxes.

- The triple-cropping system increases the vulnerability of farmers to debt and contributes to social issues. As a growing number of poor farmers are forced to sell their land to pay debts, unemployment and migration levels will grow. There is already evidence of net migration out of the deep-flooded zones of the delta at far higher rates than other delta provinces and indeed other provinces around Vietnam. The current high rates of net migration will be a result of multiple factors, but the triple-cropping strategy is also a contributing factor.

The impacts of different dyke management policy options

Our model projections compared outcomes from a range of alternative cropping policies against the current dominant strategy of uninterrupted triple-cropping [10]. These alternate policies (Fig. 4) represent potential adaptations of current practice in response to different future scenarios of sediment decline.

Double-cropping inside full-dyke systems with paddy inundation annually through sluice gate operation: Over the short-term (5-10 years), this adaptation provides farmers access to natural free-sediment bound nutrients and allows farmers to seek alternative sources of income during the wet season, offering farmers greater resilience to economic shocks. However, the effectiveness of this policy over the long-term is threatened by the construction of multiple hydropower dams upstream in the Mekong Basin, which may significantly reduce the overall supply of sediment. In addition, flooding paddies only through sluice gates may reduce the quantity of sediment reaching the floodplain when compared with traditional flooding via dyke-overflow.

The 3-3-2 cropping rotation: This policy (recommended by An Giang

Provincial Government) marginally increases average sediment deposition, but our evidence shows that it does not significantly improve socio-economic outcomes. Challenges, particularly with debt, are presented when sluice gates are opened in low-flood years that only achieve low sediment deposition.

Opening of sluice gates in years of high sediment potential: This adaptation allows floods with high sediment potential to be released onto paddies to ensure maximum sediment deposition is achieved during breaks from rice-cropping, and also has the benefit of reducing flooding intensity in downstream regions. Effective implementation of this policy relies on accurate information about the conditions of the Mekong River upstream and effective communication with all levels of agricultural administration to ensure farmers are warned in advance that flooding of the dyke rings will take place. As flood predictions are imperfect, there may be instances where flooding disrupts farmers who began sowing their fields with rice-. In these cases, steps will need to be taken to support farmers, and information and training may need to be disseminated to minimise risks and costs.

Conclusions and recommendations

Support to farmers

Our projections suggest that any policy which involves triple-cropping will need to be supported by the provision of greater and increasing financial assistance to farmers, especially the poorest farmers. Productivity levels are rapidly declining, fertiliser prices are rising, and the need to fertilise crops to maintain yields is rising. Opening sluice gates to allow sediment deposition and flooding to take place, may, on average, be advantageous to the majority of farmers; however, due to the unpredictable nature of floods, farmers may suffer economic losses during the period when sluice gates are opened and rice cultivation is stopped. As such, we recommend local governments to identify ways to assist farmers financially, both during the period of flooding and also in the early weeks of the following season. Our model suggests an effective mechanism

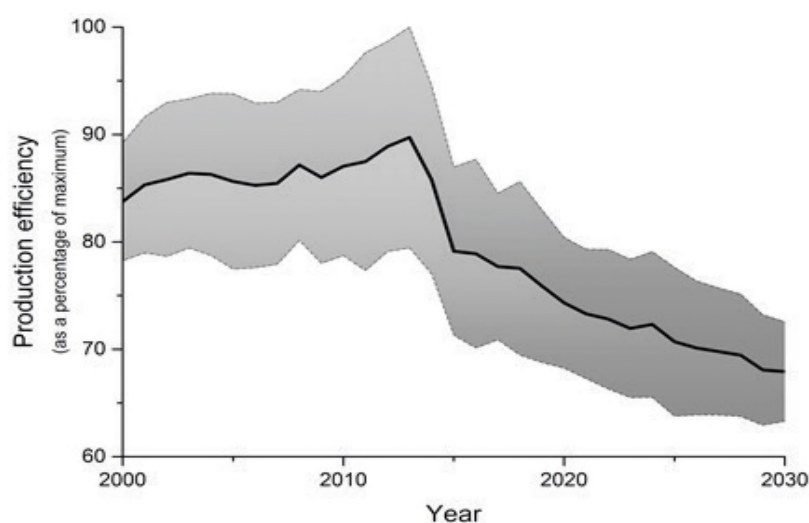


Fig. 3. Model projections of agricultural input efficiency (yield achieved against fertiliser applied) between 2000-2030 assuming the continuation of uninterrupted triple-cropping.

for assisting farmers is to subsidise, or guarantee farmers a fertiliser or rice price. This gives farmers greater income security during an uncertain period. The quantity of financial assistance required might be reduced through greater and wider provision of training to improve farmers' technical capacities to apply inputs.

scenarios.

Mechanisms for providing financial support to the poorest farmers when sluice gates are opened and when fertiliser prices are high (or rice prices low) should be implemented.

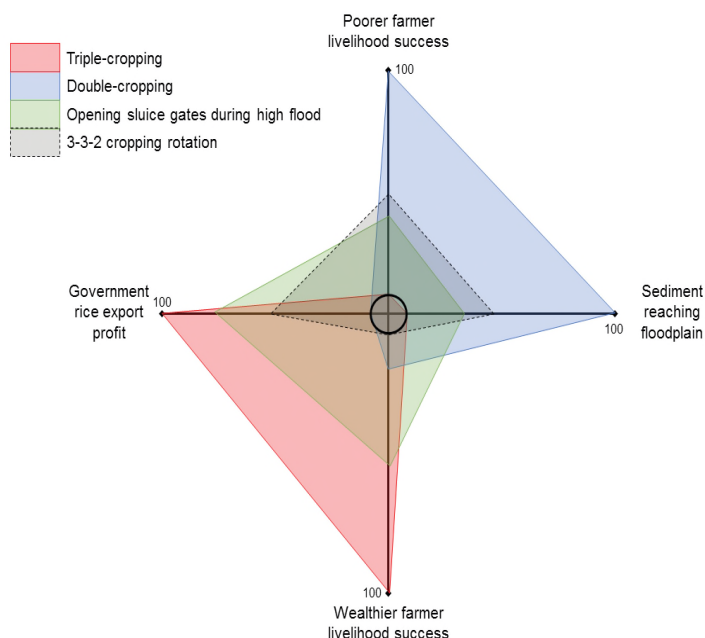


Fig. 4. Model projected trade-offs between four policies, showing relative outcomes against four indicators.

Recommendations for provincial dyke and agricultural management

Our projections suggest that option 3 (opening sluice gates during years of high sediment potential) represents the strongest middle-ground between different stakeholders' interests and environmental services, and for achieving long-term sustainability.

Careful planning, with reference to the most up-to-date research [8] is required alongside any of the three policies described above, to ensure sluice gate operation encourages maximum sediment deposition.

We believe that investment into improving the agricultural input practices of the poorest farmers would be an effective complement to all of our tested

In Fig. 4, policy outcomes are defined on a normalised comparative scale, and are based on a mid-range estimate of sediment decline (51% decline over the next 20 years). For further information on methods behind this figure, please refer to Chapman and Darby (2016) and further details are available in Chapman (2016) [14].

ACKNOWLEDGEMENTS

Research funded by the UK Natural Environment Research Council (awards NE/JO21970/1, NE/JO21571/1 and NE/JO21881/1 to the Universities of Southampton, Hull and Exeter) with support from a UK Economic and Social Science Research Council Impact Accelerator Account award and with advice from Public Policy @ Southampton.

REFERENCES

- [1] Data from the General Statistics Office of Vietnam, available at: <https://www.gso.gov.vn>.
- [2] F. Renaud & C. Künzer (2012), "The Mekong Delta System: Interdisciplinary analyses of a River Delta", *Springer Environmental Science and Engineering*, <http://link.springer.com/book/10.1007/978-94-007-3962-8>.
- [3] E. Anthony, G. Brunier, M. Besset, M. Goichot, P. Dussouillez, V. Nguyen (2015) "Linking rapid erosion of the Mekong River Delta to human activities", *Scientific Reports*, **5**, p.14745.
- [4] S.E. Darby, C.R. Hackney, J. Leyland, M. Kumm, H. Lauri, D.R. Parsons, J.L. Best, A.P. Nicholas, R. Aalto (2016), "Fluvial sediment supply to a mega-delta reduced by shifting tropical-cyclone activity", *Nature*, **539**, pp.276-279.
- [5] G. Kondolf, Z. Rubin, J. Minear (2014), "Dams on the Mekong: Cumulative sediment starvation", *Water Resources Research*, **50**, pp.5158-5169.
- [6] N. Hung, J. Delgado, A. Günter, B. Merz, A. Bardossy, H. Apel (2014), "Sedimentation in the floodplains of the Mekong Delta, Vietnam Part II: Deposition and erosion", *Hydrological Processes*, **28**, pp.3145-3160.
- [7] OECD (2003), *OECD indicators: development, measurement and use*, Paris.
- [8] An Giang Statistics Office (2013), *An Giang Statistical Yearbook*, Long Xuyen.
- [9] A focus group involving 30 An Giang stakeholders was held on the 8th April 2016 and was comprised of members of DARD at province level (9), DARD at district level (7), DONRE at province level (4), DONRE at district level (3), Farmers Union at province level (1), An Giang University (1), District People's Committees (1), DPI (1), DST (1), DOF (1), DOC(1).
- [10] A. Chapman, S. Darby, H. Hong, E. Tompkins, T. Van (2016), "Adaptation and development trade-offs: Fluvial sediment deposition and the sustainability of rice-cropping in An Giang Province, Mekong Delta", *Climatic Change*, In press, <http://link.springer.com/article/10.1007/s10584-016-1684-3>.
- [11] A. Chapman, S. Darby (2016), "Evaluating sustainable adaptation strategies for vulnerable mega-deltas using system dynamics modelling: Rice agriculture in the Mekong Delta's An Giang Province, Vietnam", *Science of the Total Environment*, **559**, pp.326-338.
- [12] L. Erban, S. Gorelick, H. Zebker (2014), "Groundwater extraction, land subsidence, and sea-level rise in the Mekong Delta, Vietnam", *Environmental Research Letters*, **9**, p.084010.
- [13] N. Hoa, B. Janssen, O. Oenema, A. Dobermann (2006), "Comparison of partial and complete soil K budgets under intensive rice cropping in the Mekong Delta, Vietnam", *Agriculture, Ecosystems and Environment*, **116**, pp.121-131.
- [14] A. Chapman (2016), *Evaluating an adaptation: Rice-sediment trade-offs in the Vietnamese Mekong Delta*, PhD thesis, University of Southampton.

Evaluation of saline tolerance at the seedling stage of various maize cross combinations

Huu Hung Nguyen^{1*}, Van Vang Luong,
Thi Minh Tam Ngo, Hoai Son Vu, Xuan Thang Nguyen

Maize Research Institute of Vietnam (MRI)

Received 28 December 2016; accepted 9 March 2017

Abstract:

This study was conducted at the Maize Research Institute of Vietnam using 24 maize hybrids, identified as STM1 to STM24, at five different salt concentrations, which were 0, 50, 100, 150, and 200 mM NaCl. After seven days of germination at room temperature, seedlings were transplanted to nutrient-rich solutions salinized with salt. 17 day-old seedlings were then harvested to calculate their growth parameters of the day to seedlings fully wilt and degradation, root and shoot length, seedling dry matter production, and content. As the study results show, salt stress caused the reduction of plant growth in all hybrids, however, the STM10 and STM21 varieties showed the best salt tolerance, while STM17 and STM18 presented most salt-sensitive. The analyzed results for Na⁺ and K⁺ showed that among the hybrids, STM21 had lower Na⁺ and higher K⁺ contents in both roots and shoots of the seedlings than others.

Keywords: maize hybrid, salt tolerance, seedling.

Classification number: 3.1

Introduction

Salinization is the accumulation of salt in the soil, which strongly influences the agricultural production, the health of the environment, and the economy of the country [1]. According to Wild (2003), about 15% of the land in the world has been eroded, and the physical and chemical characteristics of the Earth's soil have been modified by various factors, including soil salinization [2].

In Vietnam, saline soils are formed primarily from salt or salt-water moving in from the sea or from underground movements of salt sources up to the soil surface. Another reason for soil salinity is saline water used to water fields because of a lack of fresh water. Evaporation during crop farming also causes soil surface salinization. There are about three million hectares of land with high salinization and acidification distributed mainly

across the provinces of the Mekong River Delta, including in the areas of: Bac Lieu, Ca Mau, Kien Giang, Tra Vinh, and Ben Tre; and the provinces of the Red River Delta, including: Quang Ninh, Hai Phong, Thai Binh, Nam Dinh, Ninh Binh, and Thanh Hoa [3].

Most crops tolerate salinity to a threshold level, above which salt levels will begin to decrease crop productivity [4]. Maize is a pollinated crop and is able to tolerate salinity [5]. Research into maize genotypes is needed to determine materials for maize breeding programs to produce high yield varieties with salt tolerance. Studies into the salt tolerances of maize have been published by scientists [6-10], and have given a scientific basis for salinity tolerance in maize. In Vietnam, salt tolerance in maize has not been studied yet. Facing the evolution of adverse climate change and the increase of the salinization of agricultural land, research of breeding salt-tolerant crops in general, and maize in particular, is an urgent matter to aid in the development of agriculture. Studies of the saline tolerance of maize in fields are very difficult because of the heterogeneity of soil's physical and chemical properties, and fluctuations in seasonal rainfall. Therefore, we conducted the "Evaluation of salt tolerance ability on maize hybrids at the seedling stage" using an objective assessment of maize hybrids for salt tolerance.

Materials and methods

Materials

The research was conducted at MRI (Dan Phuong, Hanoi) using materials from 24 maize crosses: STM1, STM2, STM3... STM24.

Methods

The maize seeds were soaked in distilled water and germinated in sand medium in individual lines for five to seven days at room temperature, and then transplanted to a Yoshida nutritional solution, which was salinized with salt (NaCl) at four concentrations: 50, 100, 150, and 200 mM, including a control of 0 mM. Additional salt was applied in the small amount at 50 mM every five days after transplanting, and the remaining salt was provided after seven days. The nutritional solution was changed twice per week. The experiment was arranged in a randomized complete block design (RCBD) with three replications. After 17 days, the plants were harvested separately in line and treated. Harvested plants were washed in tap water and two times with distilled water, and the following observation parameters were recorded:

Survival date and salt tolerance ability: The times from when the plants were transplanted until their deaths in the saline solution.

Shoot length: Five plants were taken for plants measurement; the length between the collar and the tip of the longest leaf were measured in cm, and the mean value was reported as the shoot length.

Root length: The plants were measured for shoot length and

*Corresponding author: Email: hungnmri@gmail.com

were used for root length measurements. The length between the collar and the tip of the longest root was measured in cm, and mean value was reported as the root length.

Plant dry weight: Five plants were used for the shoot and root length measurement and were first dried under shaded conditions for some time, and then dried in a hot oven maintained at $70^{\circ}\text{C} \pm 2$ for 48 hours, and then cooled and weighed. The plant dry weight was expressed in gram/plant.

Salt Tolerant Index (STI):

$$\text{STI (\%)} = \frac{\text{Total plant dry weight at treatment } S_x}{\text{Total plant dry weight at treatment } S_0} \times 100$$

S_x : Treatment at salt concentration x; S_0 : Treatment at salt concentration 0.

Ion Na^+ , K^+ accumulation: Ion Na^+ , K^+ accumulation was measured using a flame photometer.

Statistical analysis: Data showing the variances of factorial design was analyzed using three replications using IRRISTAT statistical software.

Results and discussions

Survival date and salt tolerance ability

The observations showed that all crosses reduced growth and wilt after transplanting into the saline solution; this presented very differently between the hybrids. In the S4 treatment, the plants yielded the most degradation without the death plants until 17 days of culture in a nutritional solution including salt. Among them, STM21 showed the highest tolerance in comparison to others (Fig. 1).



Fig. 1. The effect of salinity on different maize crosses after 17 days of transplanting in 200 mM NaCl (S4).

Shoot length

The data in Table 1 shows that the shoot lengths of all crosses reduced when grown in saline solution. In the comparison between S4 (200 mM NaCl) and S0 (0 mM NaCl), STM17 presented the highest reduction of shoot length percentage (45.78%) followed by STM18 (43.98%), whereas the decrease that was the lowest was in STM21 with only 19.72%. The average shoot length of all hybrids decreased by 36.05% when grown in nutritional solution with 200 mM NaCl. It

demonstrated that the salinity of the soil made a great impact on the growth of plants. This was also founded by Carlos, et al. (2007) and André, et al. (2004), when they researched into salt tolerance in maize [6, 11].

Table 1. The effect of salinity on shoot length of different maize crosses at the seedling stage.

No.	Crosses	Shoot length (cm)					Mean	Reduced* (%)
		Treatments						
		S0 (0 mM NaCl)	S1 (50 mM NaCl)	S2 (100 mM NaCl)	S3 (150 mM NaCl)	S4 (200 mM NaCl)		
1	STM1	31.57	31.03	30.03	25.73	20.13	27.70	36.22
2	STM2	35.67	33.73	32.18	27.48	24.47	30.71	31.40
3	STM3	33.17	32.87	31.87	27.57	21.63	29.42	34.77
4	STM4	34.02	32.62	32.72	27.30	20.88	29.51	38.61
5	STM5	31.97	31.67	30.67	25.97	19.37	27.93	39.42
6	STM6	30.55	29.47	28.47	23.77	17.17	25.88	43.81
7	STM7	32.60	32.30	31.30	26.70	20.10	28.60	38.34
8	STM8	33.80	33.50	31.33	27.97	21.37	29.59	36.78
9	STM9	33.80	32.67	31.33	26.87	21.70	29.27	35.80
10	STM10	33.48	33.18	34.37	28.47	25.10	30.92	25.04
11	STM11	34.40	32.53	31.53	27.67	22.80	29.79	33.72
12	STM12	32.93	32.63	31.63	27.33	21.33	29.17	35.22
13	STM13	33.73	33.43	33.53	28.00	25.02	30.74	25.84
14	STM14	29.87	29.57	28.57	23.87	17.27	25.83	42.19
15	STM15	31.53	31.23	30.23	25.53	18.93	27.49	39.96
16	STM16	34.50	32.67	33.20	25.93	21.90	29.64	36.52
17	STM17	33.20	31.40	28.90	22.33	18.00	26.77	45.78
18	STM18	31.13	29.90	28.90	23.00	17.60	26.11	43.98
19	STM19	32.07	31.77	30.77	26.07	20.63	28.26	35.66
20	STM20	34.20	31.23	30.23	25.40	20.93	28.40	38.79
21	STM21	33.13	33.90	32.90	28.80	26.60	31.07	19.72
22	STM22	32.27	31.97	30.97	25.13	22.30	28.53	30.89
23	STM23	32.68	32.38	31.38	23.63	21.80	28.38	33.30
24	STM24	27.13	26.83	25.83	21.13	15.20	23.23	43.47
Mean		32.64	31.85	30.95	25.90	20.93	28.46	36.05
		LSD _{0.05} : 3.49					CV%: 17.6%	

*Reduced shoot length in the comparison between S4 to S0.

Root length

Root length also was affected by salinity, shown by increasing salt concentrations that reduced root growth (Table 2) and which positively decreased when increasing amounts of salt concentration. However, the response of maize hybrids to salinity was different. Results showed that the average root length of STM18 was the shortest (9.53 cm), while STM10 was at 16.93 cm. However, reductions of root length between the S0 (0 mM NaCl) and S4 (200 mM NaCl) treatments were seen, and the STM21 was reduced the lowest at 18.15%. Studies of maize in salt stress by Khan and Mcneilly (2005) also showed that maize can be grown in saline conditions, but root length decreases rapidly with increasing salt concentration [12].

Table 2. The effect of salinity on root length of different maize crosses at the seedling stage.

		Root length (cm)						
No.	Crosses	Treatments					Mean	Reduced* (%)
		S0 (0 mM NaCl)	S1 (50 mM NaCl)	S2 (100 mM NaCl)	S3 (150 mM NaCl)	S4 (200 mM NaCl)		
1	STM1	17.87	17.67	16.67	14.87	12.87	15.99	27.99
2	STM2	16.07	15.87	14.87	13.07	11.07	14.19	31.12
3	STM3	16.80	16.60	15.60	13.80	11.73	14.91	30.16
4	STM4	17.82	17.62	16.62	15.15	14.22	16.28	20.21
5	STM5	19.10	18.90	16.83	14.63	13.90	16.67	27.23
6	STM6	16.20	16.00	15.00	13.20	11.00	14.28	32.10
7	STM7	18.67	18.47	16.23	15.93	13.73	16.61	26.43
8	STM8	15.93	19.60	14.73	13.47	11.33	15.01	28.87
9	STM9	18.60	18.40	16.80	14.60	13.67	16.41	26.52
10	STM10	19.80	15.73	18.33	16.27	14.53	16.93	26.60
11	STM11	16.20	16.00	15.00	13.40	11.53	14.43	28.81
12	STM12	17.93	17.73	16.73	15.07	13.07	16.11	27.14
13	STM13	18.25	18.05	17.05	16.00	14.38	16.75	21.19
14	STM14	17.23	17.03	16.03	14.23	12.03	15.31	30.17
15	STM15	17.05	16.85	15.85	14.05	11.85	15.13	30.50
16	STM16	16.73	16.53	15.53	13.73	11.53	14.81	31.08
17	STM17	16.75	16.55	14.82	12.62	10.48	14.24	37.41
18	STM18	17.20	17.00	14.47	12.20	9.53	14.08	44.57
19	STM19	16.07	15.87	14.87	13.07	11.80	14.33	26.56
20	STM20	18.00	17.80	15.87	15.20	13.33	16.04	25.93
21	STM21	18.00	17.80	18.20	16.33	14.73	17.01	18.15
22	STM22	17.87	17.67	16.33	15.27	13.87	16.20	22.39
23	STM23	16.23	16.03	15.03	13.23	12.07	14.52	25.67
24	STM24	16.58	16.38	15.38	13.58	11.38	14.66	31.36
Mean		17.37	17.17	15.95	14.29	12.49	15.45	28.26
LSD _{0.05} : 3.42		CV%: 13.80%						

*Reduced root length in the comparison between S4 to S0.

Plant dry weight

As seen with shoot length and root length, the dry weight of the seedlings decreased with increasing salt concentrations. The average plant dry weight of all crosses at S0 treatment was 0.535 gram/plant, however, it decreased to 0.448 gram/plant at S4 concentration. The dry weight of the seedlings in saline conditions obtained was very different between crosses; STM21 showed the highest dry weight at 0.521 gram/plant, while the lowest observed was in STM17 (0.404 gram/plant). The crosses which presented high plant dry weights in salt concentrations were seen to have high salt tolerant indexes. The results showed that STM21, STM10, and STM13 were

Table 3. The effect of salinity on plant dry weight of different maize crosses at the seedling stage.

No.	Crosses	Plant dry weight (gram/plant)					Mean	Salt tolerant index (%)
		Treatments						
		S0 (0 mM NaCl)	S1 (50 mM NaCl)	S2 (100 mM NaCl)	S3 (150 mM NaCl)	S4 (200 mM NaCl)		
1	STM1	0.502	0.491	0.451	0.425	0.371	0.448	73.79
2	STM2	0.547	0.508	0.469	0.441	0.387	0.470	70.81
3	STM3	0.515	0.476	0.427	0.396	0.341	0.431	66.19
4	STM4	0.503	0.465	0.419	0.424	0.317	0.425	63.00
5	STM5	0.490	0.499	0.440	0.392	0.362	0.437	73.93
6	STM6	0.569	0.529	0.468	0.422	0.349	0.467	61.34
7	STM7	0.528	0.490	0.441	0.405	0.339	0.441	64.20
8	STM8	0.501	0.460	0.414	0.380	0.312	0.413	62.28
9	STM9	0.487	0.513	0.468	0.373	0.300	0.428	61.58
10	STM10	0.577	0.536	0.509	0.491	0.450	0.513	77.94
11	STM11	0.528	0.490	0.439	0.405	0.344	0.441	65.15
12	STM12	0.548	0.510	0.460	0.419	0.358	0.459	65.27
13	STM13	0.560	0.525	0.498	0.487	0.442	0.503	78.99
14	STM14	0.515	0.469	0.408	0.362	0.317	0.414	61.53
15	STM15	0.523	0.482	0.424	0.374	0.301	0.421	57.62
16	STM16	0.516	0.476	0.419	0.369	0.293	0.415	56.78
17	STM17	0.541	0.448	0.395	0.350	0.289	0.404	53.51
18	STM18	0.554	0.449	0.403	0.357	0.276	0.408	49.73
19	STM19	0.547	0.508	0.460	0.433	0.367	0.463	67.05
20	STM20	0.569	0.511	0.460	0.416	0.354	0.462	62.21
21	STM21	0.575	0.541	0.522	0.502	0.465	0.521	80.81
22	STM22	0.538	0.495	0.460	0.436	0.405	0.467	75.34
23	STM23	0.549	0.510	0.454	0.417	0.357	0.457	65.07
24	STM24	0.550	0.511	0.451	0.404	0.327	0.449	59.36
Mean		0.535	0.496	0.448	0.412	0.351	0.448	65.63
LSD _{0.05}		0.067				CV%: 9.30%		

more salt tolerant than other crosses (Table 3). Research on salt tolerance in maize from Muhammad, et al. (2010) found that salt tolerances in maize varieties are very different, at which have high dry matter production and the ability for better growth and development in salt stress [10].

Ion Na⁺, K⁺ accumulation

As observed through experiments, the crosses of tolerant and saline sensitive seedlings were selected for measuring ion Na⁺ and K⁺ accumulation. The results in Table 4 show the levels of Na⁺ accumulation in plants that increased from increasing the salt concentration, and the average Na⁺ content in S0 was 0.375%, increased to 4.951% in S4 treatment. Among the crosses, STM18 accumulated the highest Na⁺ at 5.552%, while STM21 showed the lowest (4.392%) in the same salt concentration of 200 mM NaCl.

Table 4. The effect of salinity on ion Na⁺ accumulation of different maize crosses.

No.	Crosses	Na ⁺ content (% dry weight)					Mean
		Treatments					
		S0 (0 mM NaCl)	S1 (50 mM NaCl)	S2 (100 mM NaCl)	S3 (150 mM NaCl)	S4 (200 mM NaCl)	
1	STM10	0.363	0.795	1.733	3.355	4.509	2.151
2	STM13	0.373	0.848	1.786	3.408	4.988	2.281
3	STM17	0.390	1.022	2.579	4.352	5.472	2.763
4	STM18	0.363	1.102	2.659	4.432	5.552	2.822
5	STM21	0.380	0.702	1.639	3.262	4.392	2.075
6	STM22	0.380	0.943	2.043	3.665	4.795	2.365
Mean		0.375	0.902	2.073	3.746	4.951	2.409
LSD _{0.05}		0.146				CV%: 13.40%	

For potassium, the accumulation of K⁺ in all crosses as shown to decrease with increasing salt concentrations (Table 5). The average ion K⁺ accumulated in the crosses of S0 (0 mM NaCl) at 3.016%, which reduced to 1.931% in the S4 concentration (200 mM NaCl). The STM21 maintained K⁺ absorption at the highest of all treatments, followed by STM10. The STM18 was recognized as the cross which showed the lowest K⁺ uptake.

Table 5. The effect of salinity on ion K⁺ accumulation of different maize crosses.

No.	Crosses	K ⁺ content (% of dry weight)					Mean
		Treatments					
		S0 (0 mM NaCl)	S1 (50 mM NaCl)	S2 (100 mM NaCl)	S3 (150 mM NaCl)	S4 (200 mM NaCl)	
1	STM10	3.037	2.927	2.787	2.473	2.183	2.681
2	STM13	3.027	2.933	2.807	2.517	2.227	2.702
3	STM17	3.003	2.730	2.220	1.780	1.480	2.243
4	STM18	2.977	2.733	2.223	1.693	1.393	2.204
5	STM21	3.023	3.080	2.860	2.570	2.280	2.763
6	STM22	3.030	2.937	2.587	2.312	2.022	2.577
Mean		3.016	2.890	2.581	2.224	1.931	2.528
LSD _{0.05} : 0.113		CV%: 12.10%					

Conclusions

Based on the results of these studies of saline tolerant maize cross combinations at the seedling stage, the conclusions are as follows:

1) Shoot length, root length, and plant dry weight of all crosses were reduced as salt concentration increased. The STM17, STM18 reduced the highest, while the STM21 presented the lowest reduction of both shoot length and root length. The STM21 produced the best salt tolerant index, followed by STM13 and STM10.

2) The accumulation of ion K⁺ decreased, and the

absorption of Na⁺ increased in the presence of salt stress. The STM21 showed the best accumulation of K⁺ and elimination of Na⁺.

3) The effect of salinity on the crosses was very different, especially among them identified as STM21 and STM10, which had the highest salt tolerant index.

REFERENCES

- [1] P. Rengasamy (2006), "World salinization with an emphasis on Australia", *J. Exp. Bot.*, **57**(5), pp.1017-1023.
- [2] Wild (2003), *Soil, Land, and Food: Managing the Land during the Twenty first Century*, Cambridge University Press, UK.
- [3] Ho Quang Duc, et al. (2010), *Saline soil and acid soils in Vietnam*, Soils and Fertilizers Research Institute (SFRI).
- [4] M.A. Khan, M.U. Shirazi, M. Ali, S. Mumtaz, A. Sherin, M.Y. Ashraf (2006), "Comparative performance of some wheat genotypes growing under saline water", *Pak. J. Bot.*, **38**(5), pp.1633-1639.
- [5] E. Paterniani (1990), "Maize breeding in tropics", *Cri. Rev. Plant Sci.*, **9**(2), pp.125-154.
- [6] Carlos Daniel Giaveno, Rafael Vasconcelos Ribeiro, Gustavo Maia Souza, and Ricardo Ferraz de Oliveira (2007), "Screening of tropical maize for salt stress tolerance", *Crop Breeding and Applied Biotechnology*, **7**, pp.304-313.
- [7] M. Akaram, M. Asghar Malik, M. Yasin Ashraf, M. Farrukh Saleem, M. Hussain (2007), "Competitive seedling growth and K⁺/Na⁺ ratio in different maize (*Zea Mays* L.) hybrid under salinity stress", *Pak. J. Bot.*, **39**(7), pp.2553-2563.
- [8] J.L. Karmoker, Shamin Farhana, Parveen Rashid (2008), "Effects of salinity on ion accumulation in maize, (*Zea mays* L. cv. Bari-7)", *Bangladesh J. Bot.*, **37**(2), pp.203-205.
- [9] S. Schubert, A. Neubert, A. Schierholt, A. Sumer, C. Zorb (2009), "Development of salt-resistant maize hybrids: The combination of physiological strategies using conventional breeding methods", *Plant Sci.*, **177**(3), pp.196-202.
- [10] Muhammad Akram, Muhammad Yasin Ashraf, Rashid Ahmad, Ejaz Ahmed Waraich, Javed Iqbal, Muhammad Mohsan (2010), "Screening for salt tolerance in maize (*Zea mays* L.) hybrid at an early seedling stage", *Pak. J. Bot.*, **42**(1), pp.141-154.
- [11] André Dias de Azevedo Neto, José Tarquinio Prisco, Joaquim Enéas-Filho, Claudivan Feitosa de Lacerda, José Vieira Silva, Paulo Henrique Alves da Costa, Enéas Gomes-Filho (2004), "Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes", *Brazilian Journal of Plant Physiology*, **16**(1), pp.31-38.
- [12] A.A. Khan, T. Mcneilly (2005), "Triple test cross analysis for salinity tolerance based upon seedling root length in maize (*Zea mays* L.)", *Breeding Science*, **55**(3), pp.321-325.

Identification of the fungus *Erysiphe diffusa* causing powdery mildew disease on soybeans in Vinh Phuc, Vietnam

Thi Thanh Tam Le^{1*}, Van Liem Nguyen¹, Van Truc Pham², Yukio Sato³

¹Plant Protection Research Institute (PPRI), Vietnam Academy of Agricultural Sciences (VAAS)

²Department of Plant Protection in Vinh Phuc

³Toyama Prefecture University (TPU), Japan

Received 16 November 2016; accepted 3 March 2017

Abstract:

Fungus *Erysiphe diffusa* Cooke & Peck 2000, which causes powdery mildew disease on soybeans (*Glycine max* (L) Merr) cultivated in Vinh Phuc Province, has already been identified using a combination of molecular biotechnology and an updated standard morphological classification for the confirmation of a pathogenicity test to fulfill Koch's postulate. Six sequences of the ITS rRNA region of this fungus on soybeans have already been registered in GenBank with accession numbers including KM260706 and KM260708 - KM260712.

Keywords: *Erysiphe diffusa*, pathogenicity test, powdery mildew, soybean.

Classification number: 3.1

Introduction

Vietnam is a tropical country with a monsoon climate characterized by heavy rainfall, high humidity, and hot temperatures. These are favourable conditions for the development and outbreak of various diseases on plants. Powdery mildew is one of the most severe diseases of these, often damaging and being found on many kinds of plant species belonging to various botanic families.

Around the world, powdery mildew disease is damaging areas used for cultivating soybeans, including Asia (China, Japan, Korea...) and America (USA, Argentina...). The disease became an epidemic in Korea, reducing approximately 65-70 percentage of the crop yield in many intensively cultivated areas [1]. In Oita Province, Kyushu Island, Japan, powdery mildew disease outbreak on soybean to epidemic-

extremes and caused a 35-40% reduction in yield [2].

The severe outbreak of powdery mildew disease is caused by a fungus belonging to the genus *Erysiphe*, and has been reportedly found on several soybean samples in parts of East Asia, including in Vietnam, since 1998 [3]. The formation of the sexual period (chasmothecia) is an important key to classify powdery mildew fungi. However, the taxonomic and phylogenetic positions of this fungus have not been recognized due to a lack of a sexual stage. In fact, it has been difficult to find out the formation of chasmothecia in tropical countries including Vietnam. Therefore, studies and the application of morphological classification based on the sexual stage of powdery mildew fungus have hardly been developed in tropical areas.

According to the statistics from the

General Statistics Office of Vietnam and Ministry of Agriculture and Rural Development, although the country of Vietnam is a great consumer market of soybeans, the acreage in Vietnam has had a decreasing trend from 181,000 to 120,000 ha, with an average yield of 14.3 ta/ha, which is in comparison with that of the world at 25 ta/ha. At present, soybeans are the main winter crops in regions with two annual rice seasons, like in Vinh Phuc Province. In 2014, Yen Lac was the district with the greatest soybean cultivation area in Vinh Phuc (1,128 ha), in which Lien Chau was the commune of 196 ha, concentrating on an area with a dyke. DT96, DT84 are popular soybean varieties cultivated with other local ones. Soybean cultivation has not only increased the income for farmers but also spread-out regions flush with intensively commercial productions, step by step and opening in the direction of changing crop structures sustainable for the winter season in Yen Lac District in particularly and Vinh Phuc Province in general. However, powdery mildew fungus has been reported as one of the major agents limiting acreage and productivity of soybeans in this locality. The disease has already damaged new varieties that have high yield such as DT26, DT84... [4].

To study the fundamentals of controlling powdery mildew disease effectively, the Ministry of Science and Technology of

*Corresponding author: Email: thanhtamle10_2012@yahoo.co.uk

Vietnam has approved a Vietnam-Japan international co-operation project, titled the “Research and application of biotechnology for the classification and control of powdery mildew diseases of some crops in Vietnam” for the 2013-2015 period. This article outlines the latest study results for taxonomy of powdery mildew fungus on soybeans grown in Vinh Phuc, Vietnam ranging from the species level, based on a combination of molecular biotechnology and a standard morphological classification, which has been updated and implemented creatively during 2014 by the Plant Protection Research Institute (PPRI) [5].

Materials and methods

The place for gathering powdery mildew fungus samples: The Tam Duong Commune of Tam Dao District, the Yen Lap Commune of Vinh Tuong District, and the Lien Chau Commune of Yen Lac District in Vinh Phuc Province.

The preparation of the fungus source: Powdery mildew fungus was isolated from disease samples found on soybeans characterized by symptoms on its medium leaves gathered in Vinh Phuc Province.

The observation of morphology of powdery mildew fungus: Conidiophore and conidia were extracted from powdery mildew disease infected soybean samples, which are still fresh, have been separated using transparent adhesive tape and then put onto lamen, and the number of conidia was counted and noted for its characteristics of conidiophore, foot cell, appressorium..., which was observed using a composed microscope at objective lens set at 40 X. In each sample, 100 conidia and 30-foot cells were measured for length and width.

The observation of the germination of conidia powdery mildew fungi was carried out according to the method of Hirata (1942) [6].

Artificial inoculation: In order to define plant pathogens, artificial inoculation was carried out under glass house conditions on the DT84 soybean variety, which was two weeks old and had one or two true leaves via press disease infected leaf to the fresh young one of 30 soybean plants. Meanwhile, 30 other freshly non-infected soybean plants were considered as negative controls.

DNA extraction: The total DNA genome of powdery mildew fungi was extracted from the conidia of six disease samples on soybeans of the Coc Nong local variety and DT84 popular ones which were gathered from Tam Duong Commune located in Tam Dao District, Vinh Phuc Province via a DNA Easy Blood and Tissue Kit (QIAGEN).

Polymerase Chain Reaction (PCR): In order to clone ITS rDNA regions, universal primer pairs HF1/HR4 were used according to the method of Tam, et al. (2015) [7]. The resulting product of the first PCR was used as the only template for the second PCR. The first PCR was carried out in a 0.5 ml tube with total 20 ml reaction volume, in which 0.5 µl was put into each primer (forward and reverse) at a preparation of 20 µM concentration, 10 µl Taq Polymerase Master Mix (TaKaRa, Tokyo), 4.0 µl dd H₂O, and 5.0 µl gDNA of powdery mildew fungi. The reaction were run in a PCR thermal cycler SP (Takara, Kyoto, Japan) under thermal cycles containing the following steps: Denature at 94°C for 3 mins; followed by 30 small cycles, each including denature at 94°C for 30 secs, annealing at 58°C for 30 secs and extended at 72°C for 48 secs. The reaction was then finished at 72°C for 10 mins.

The second PCR was carried out in a total 30 µl reaction volume containing 20 µl Taq PCR Master Mix, 1.0 µl of each primer (forward and reversed at a concentration of 20 µM), 5.0 µl dd H₂O, and 3.0 µl of the first PCR product at

gDNA template.

The PCR product was then isolated using 2% agarose gel electrophoresis with a 1% TAE buffer, presented as expected at band after submerging in 1% TAE containing Ethidium Bromide or Green Safe for 30 mins and then taking a photograph using UV sol gel machine. The band gel of the PCR product was cut and purified using QIAquick Gel ExtractionKit (QIAGEN) as described according to the manufacturer’s instructions.

Sequencing and analysis: Both directions of the DNA fragment were sequenced in order to analyze its ITS rDNA using the universal primer pairs HF1/HR4 mentioned above. The purified DNA fragment from the second PCR was then sent for sequencing at the First Base Co. (Malaysia/Singapore) or at Macrogen Co. (Korea).

Sequences at two directions were assembled using DNA star Lasergene 11 Core Suite software (<http://www.dnastar.com/t-allproducts.aspx>). Using BLAST, NCBI for searching sequences with the high identity of 98-100%. Then, using ClustalX Package [8] to align nucleotide sequences of powdery mildew fungi ITS rDNA was obtained in this study and compared to others in the DDBJ Database. After that, the alignment was seen and cut, or selected in MEGA5.2 [9].

Phylogenic trees obtained from analyzed data was found using method maximum parsimony (MP). MP analysis ran in PAUP 4.0 b10 [10]. Brands with bootstrap (BS) values were checked using BS analysis [11], and with 1,000 replications using optional conditions of stepwise addition previously ordered.

Results and discussions

Morphological characteristics of powdery mildew fungi damaging on soybeans in Vinh Phuc Province

Powdery mildew fungi mainly

damaged medium and old leaves of soybeans. The first symptom that was present was white spots on both sides of the leaves, appearing after 1-2 weeks. These disease spots spread widely and merged together so that the whole leaf looks like it was covered by powdered lime. As observed under a composed microscope, the hypha of fungi mycelium had a sinuous form, and sometimes was present with big patches. Appressoria on hypha often had lobed or opposite lobed shapes, or multi-lobed shapes. Conidiophore often grew straight to its hypha. Conidiophore produced conidia singly with 1-2 dividing cells. Conidiophore had a length size measuring $(43.3\text{--}48.3\text{--}66.5\text{--}71.5) \mu\text{m}$. Footcell had a cylindrical form with a lengthxwidth size measuring $(25\text{--}27.5\text{--}35\text{--}37.5) \times 6.3\text{--}7.5\text{--}(8.8) \mu\text{m}$. Conidia had a lengthxwidth size measuring $(27.5\text{--}30\text{--}40\text{--}45) \times (15\text{--}17.5\text{--}20\text{--}22.5) \mu\text{m}$ with lengthxwidth ratio calculated at $(1.6\text{--}1.8\text{--}2.0)$. Conidia had various kinds of shapes including cylindrical, egg, or oval shapes, but all without fibrosin. Conidia had a germination type of *Pseudoidium*. Chasmothecium had not yet been found. According to the morphological characteristics of anamorph stage (or unsexual stage) in comparison with the important classification keys which had been updated by Braun and Cook (2012) [5], powdery mildew fungus damaging on soybeans in Vinh Phuc, Vietnam belongs to *Erysiphe* genus (Fig. 1).

Artificial inoculation

Pathogenicity of powdery mildew fungi was defined using an artificial inoculation experiment and by pressing soybean leaves that were infected by powdery mildew onto 1-2 fresh young leaves of two-weeks old soybean plants of the DT84 variety, which is popularly cultured for production in real fields. Inoculation of powdery mildew fungi was performed on 30 fresh young

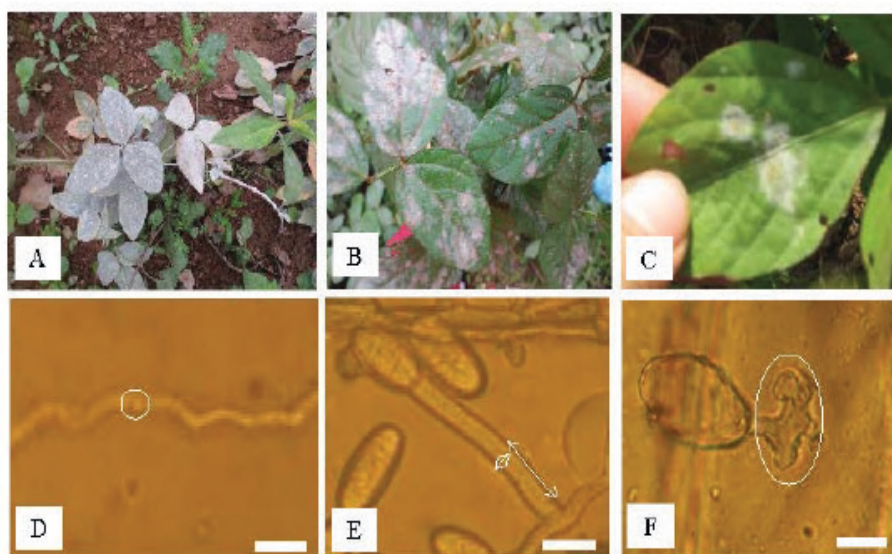


Fig. 1. Several key morphological characteristics of powdery mildew fungi damage on soybeans in Vinh Phuc, Vietnam.

(A, B, C) The symptoms of powdery mildew fungi were found on leaves of soybeans in glass house conditions in PPRI and on the fields of Vinh Phuc; (D) Hypha with a big patch shown highlighted by a white circle; (E) Conidiophore with length and width of foot-cell shown highlighted using white arrows; (F) Conidia germinated with *Pseudoidium* type shown highlighted by a white circle. The length of the bars is equal to (D, E) $30 \mu\text{m}$ and (F) $15 \mu\text{m}$ respectively.



Fig. 2. Several photos illustrating experiments of powdery mildew fungi on soybeans in glass house conditions in PPRI.

(A) An experiment of artificial inoculation; (B, C, D) Experiments into the sensitivity of powdery mildew fungi on soybean varieties (PPRI, 2014).

soybean plants. Meanwhile, 30 fresh others were used as a negative control (Fig. 2).

The plants were maintained in glass house conditions in PPRI from 26 to 28°C . Inoculated leaves developed typical symptoms after 10 days, while control plants didn't present any symptoms. The fungus presented on inoculated leaves had similar morphological characteristics to the one in the original source of inoculation.

Moreover, its sequence ITS rDNA from PCR product was 100% identical to the one in the original source of inoculation, fulfilling the demands of Koch postulate.

PCR for cloning ITS rDNA of powdery mildew fungi on soybean leaves

PCR results in order to define powdery mildew fungi on soybeans to species level are illustrated in Fig. 3 below.

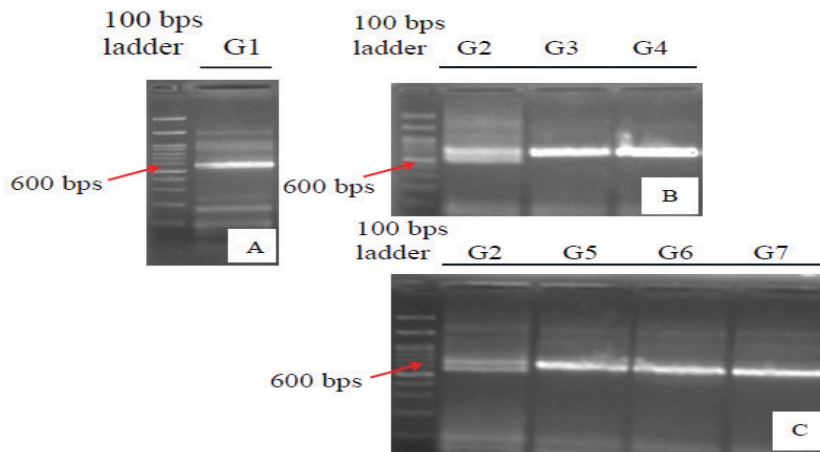


Fig. 3. Illustration containing PCR results for cloning ITS rDNA of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam (PPRI, 2014).

Material sources of powdery mildew fungi using for analysis phylogenic tree based on ITS rDNA

Sequences of the ITS rDNA of powdery mildew fungi from six soybean samples has been registered with the GenBank Accession (GA.) from G1 and G3-G7, while others extracted are from the DNA Database.

The establishment and analysis of the phylogenic tree MP based on the sequences of the ITS rDNA of powdery mildew fungi which damage soybeans in Vietnam by PAUP* 4.0

Six sequences of the ITS rDNA region of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam were aligned with 17 sequences of the same genes of powdery mildew fungi belonging to the *Erysiphe* genus causing damage on other host plants obtained from the DNA Databases. Sequences of the ITS rDNA region of powdery mildew fungi *E. glycines* on *D. oxyphyllum*, GenBank Accession (GA.) AB015927, and *A. edgeworthii*, GA. AB015934 in Japan were used as an outgroup. The alignment sequences containing 23 taxa and 579 characters were observed to have 24 characters (equal to 4.15%) variable and 86 characters (equal to 14.85%) informative for phylogenic analysis. The analysis of the MP used PAUP, running

Heuristic searches and calculating the likelihoods of the results obtained, including transition/transversion = 2 (kappa = 4.0437887). Nucleotide frequencies were hypothesized as A = 0.19871, C = 0.27734, G = 0.27874, T = 0.24521. In 8,016 rearrangements, the

number of satisfied trees was two, and the score of the best tree was 133. The Kishino-Hasegawa test was used to find out the best tree; tree No. 1, which had the parameters of a tree length at 133, a consistency index (CI) at 0.9323, a retention index (RI) at 0.9692, a rescaled consistency index (RC) at 0.9036, and a homoplasy index (HI) at 0.0849. A heuristic search using a branch-swapping algorithm and Tree-Bisection-Reconnection (TBR) was carried out with 1,000 replications to establish a phylogenetic tree, in which its clusters would have bootstrap values similar to those represented in Fig. 4.

Analysis of the phylogenic tree based on the sequences of its ITS rDNA regions of powdery mildew fungi on soybeans (*G. max*) in Vinh Phuc, Vietnam, and around the world, as well as powdery mildew fungi of *Erysiphe* genus on other

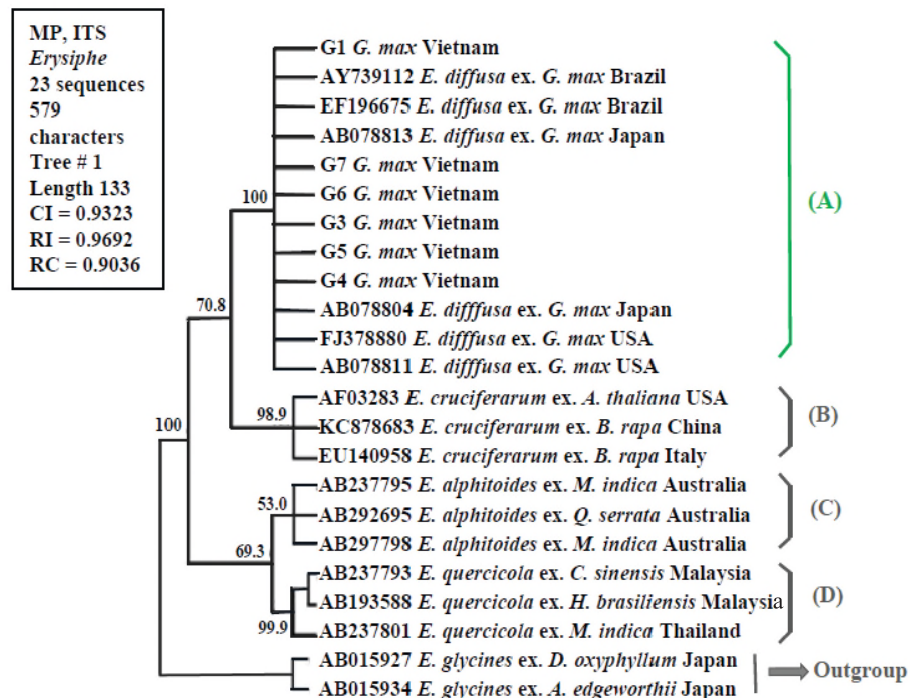


Fig. 4. Phylogenetic tree MP, PAUP 4.0 based on analysis sequences of the ITS rDNA region of powdery mildew fungi on soybeans in Vinh Phuc, Vietnam and powdery mildew fungi on other host plants in the world.

Clusters (A) = *E. diffusa*; (B) = *E. cruciferarum*; (C) = *E. alphetoides*; (D) = *E. quercicola*; Outgroup = *E. glycines*. Bootstrap values calculated with 1,000 replications using optimal conditions of previously ordered stepwise addition were observed only presented at bootstrap values > 50%.

host plants, had already been shown that powdery mildew fungus was on soybean samples from Vinh Phuc, Vietnam; G1 and G3-7 along with more found on samples from Brazil, GA. AY739112, EF196675; 2 ones from America, GA. FJ378880, AB078811; and two more from Japan, GA. AB078813, AB078804, all belonging to cluster A, *Erysiphe diffusa* with 100 bootstrap value support.

From previous studies, *Erysiphe diffusa* Cooke & Peck, 2000 is synonymous with *Microsphaera diffusa* Cooke & Peck, 1872, and *Trichocladia diffusa* Cooke & Peck, 1927. This powdery mildew fungus has a host range including soybeans (*Glycine max*), French beans (*Phaseolus vulgaris*), and other plants with flowers from Fabaceae (*Alysicarpus longifolius*, *Apios americana*, *Crotalaria brevidens*, *Lespedeza bicolor*, *Lupinus perennis*, *Oxytropis campestris*...) and distributes in North America (Canada, Mexico, America), Middle and South America, Galapagos Archipelago, Asia (China, Korea, Japan, East USSR). *E. diffusa* could be divided into two lower levels of species based on morphological characteristics of appressorium on chasmothecium of teleomorph or sexual stage.

(1) *E. diffusa* var. *diffusa* has the length of appressoria 1.5-2.5 times longer than the diameter of chasmothecia. Its appressoria are shorter and rather stiff. This species often distributes on host plant genres including *Apios*, *Desmodium*, *Dolichos*, *Glycine*, *Glycyrrhiza*, *Lespedeza*, *Phaseolus*, *Senna*, and *Strophostyles*.

(2) *E. diffusa* var. *elongata* (Braun, comb. Et stat.nov.) MycoBank, No. 561423 has sinuous appressoria with their length of 2-4.5 times longer than the diameter of chasmothecia. This species often distributes onto host plant genres including: *Desmodium*, *Psoralea*, *Robinia*, and *Ruprechtia*.

However, reports of *E. diffusa* from Asia were uncertain of species found. The definition in the Asian reports

of *Lespedeza* was unclear. *E. diffusa* happened on a wide range of crops belonging to Fabaceae, and sometimes represented as a complex of several closely affiliated species, having allied taxa which were not separated yet [5].

For example, the molecular biological analysis of samples of powdery mildew fungi on soybeans gathered from Japan, Korea, Vietnam, and America showed that damage caused by the outbreak of the disease was a result of the combination of two species of *Erysiphe*. One species found at the sexual stage was the *Erysiphe glycines*. The other in Hanoi, Vietnam was uncertain because its sexual stage still had not been found yet, but it was suggested that it was likely an *E. diffusa*, the causative agent for powdery mildew fungi on soybeans in America considered so due to the identity of its ITS sequences. However, authors have not affirmed that it was exactly *E. diffusa* because there was an absence of its chasmothecia and, therefore, it was continuously asked to find out its sexual stage for accurate classification to species level [3]. Nowadays, the powdery mildew fungi can be fully classified at a species level, even without the observation of sexual stages. This is done using updated morphological characteristic keys of anamorph in combination with application biotechnology to sequence its ITS rDNA [5].

In this study, powdery mildew fungus in Vinh Phuc has morphological characteristics belonging to the *Erysiphe* genus, and has ITS rDNA identical to *E. diffusa* pulled from soybeans in Brazil, Japan, and America with a 100 bootstrap value supported in its phylogenetic tree MP which is being analyzed using specific gene software, PAUP* 4.0.

Therefore, this paper does not need a present sexual stage, as it has already announced the first result in the definition of the powdery mildew fungus on soybeans in Vinh Phuc, Vietnam at the species level, *Erysiphe diffusa* Cooke & Peck, 2000 [12].

Conclusions

The powdery mildew fungus which damages soybeans in Vinh Phuc, Vietnam has been defined as *Erysiphe diffusa* Cooke & Peck 2000.

REFERENCES

- [1] H.D. Shin (2000), "*Erysiphaceae* of Korea", Nat. Inst. Agric. Sci. Tech., Suwon, Korea.
- [2] W. Hasama, T. Kato (2000), "New records in Japan for powdery mildew on soybeans by *Oidium* sp. of *Erysiphe polygoni* type (in Japanese)", *Kyushu Pl. Prot. Res.*, **46**, pp.18-21.
- [3] S. Takamatsu, Hyeon-Dong shin, Uralak Paksiri, Saranya Limkaisang, U. Taguchi, Nguyen Thi Binh, Y. Sato (2002), "Two *Erysiphe* species associated with a recent outbreak of soybean powdery mildew: results of molecular phylogenetic analysis based on nuclear rDNA sequences", *Mycoscience*, **43**(4), pp.333-341.
- [4] Vu Trieu Man (2007), *Plant Pathology Book*, Agricultural Publish House.
- [5] U. Braun, R.T.A. Cook (2012), "Taxonomic Manual of the Erysiphales (Powdery Mildews)", *CBS Biodiversity*, **Series No.11**, CBS, Utrecht.
- [6] K. Hirata (1942), "On the shape of germ tubes of *Erysipheae*", *Bull. Chiba. Coll. Hortic.*, **5**, pp.34-49.
- [7] L.T.T. Tam, P.N. Dung, N.V. Liem (2016), "First report of powdery mildew caused by *Erysiphe cruciferarum* on *Brassica juncea* in Vietnam", *Plant Disease* (APS Journals), **100**(4), p.856, <http://dx.doi.org/10.1094/PDIS-06-15-0678-PDN>.
- [8] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins (1997), "The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools", *Nucleic Acids Research*, **25**(24), pp.4876-4882.
- [9] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar (2011), "MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods", *Molecular Biology and Evolution*, **28**(10), pp.2731-2739.
- [10] D.L. Swofford (2001), *PAUP: Phylogenetic analysis using parsimony and other methods, Version 4.0 beta 5*, Sinauer Associates, Sunderland, MA.
- [11] J. Felsenstein (1985), "Confidence limits on phylogenies: An approach using the bootstrap", *Evolution*, **39**(4), pp.783-791.
- [12] D.F. Farr, A.Y. Rossman (2014), *Fungal Databases. Syst. Mycol. Microbiol. Lab*, Online publication, ARS, USDA, Retrieved 6 November 2014, <http://nt.ars-grin.gov/fungaldb/index.cfm>.

Evaluating the initial result of transanal and transvaginal NOTES for colorectal cancer

Nhu Hiep Pham*, Huu Thien Ho, Anh Vu Pham, Hai Thanh Phan, Thanh Xuan Nguyen, Xuan Dong Pham, Tien Nhan Van, Nghiem Trung Tran, Trung Vy Pham, Si Doan Diem Tran, Trung Hieu Mai, Le Minh Chau Dao

Hue Central Hospital, Vietnam

Received 15 October 2016; accepted 10 January 2017

Abstract:

Objective: Natural Orifice Transluminal Endoscopic Surgery (NOTES) is an important evolution in minimally invasive surgery (MIS) nowadays. This paper presents the techniques and early results of the pure transanal and transvaginal laparoscopies (NOTES) used for the treatment of colorectal cancer. **Material and method:** Prospective studies were conducted at Hue Central Hospital, Vietnam. **Patients:** From December 2013 to September 2015, 22 colorectal cancer patients (18 rectum, 3 sigmoid tumors and 1 descending colon), adenocarcinoma, $T \leq T_3N_1M_0$. **Methods:** The patients were placed in lithotomy and Trendelenburg positions, and the lone-star retractor was placed in the anus (rectum cancer) or vagina (sigmoid cancer). The surgical cavity was then inflated with CO₂ and set at 12 mm/Hg. Dissection was continued until inside of the abdominal cavity (transanal technique). After that, the rectum was pushed into the abdominal cavity. The IMA and IMV were divided (TME included) in both techniques. After finishing dissection, the specimens were pulled out through the anus or vagina to prepare anastomosis. Coloanal and colorectal anastomosis were either hand-sewn (6 cases) or sealed with EEA staplers (16 cases). **Results:** 2 patients needed one more 5 mm umbilical port in RLQ, 2 patients needed two 5 mm trocars (post radiation hemorrhage, and urethral perforation). One patient converted to open and 1 patient converted to the HYBRID-NOTES procedure. The operation time was 258±40 (190-300) minutes. All patients required minimal analgesia. Bowel movement returned on the first day to 16 patients (average: two days, maximum: three days). The hospital stay was 7±2.8 (4-14) days. Kirwan classification (sphincter function) was very good (stage I: 18). **Conclusions:** Pure transanal and transvaginal laparoscopies for the treatment of colorectal cancer are feasible and safe. We believe that this is the first pure transvaginal laparoscopy (NOTES) for human in the world. A multicentric study in a large numbers of patients and a long follow-up is necessary.

Keywords: colorectal cancer, Hue Central Hospital, Natural Orifice Transluminal Endoscopic Surgery.

Classification number: 3.2

Introduction

From the first transgastric liver biopsy of Kallo, appendectomy of Rao in 2004, and first transvaginal cholecystectomy of Jacques Marescaux in 2007, Natural Orifice Transluminal Endoscopic Surgery (NOTES) is seen as the newest technique in minimally invasive surgery methods [1]. At many centers around the world, laparoscopic surgery conducted through natural orifices NOTES was tested on bodies from body snatchers, bodies of animals, and after that, it was applied on people to positive results. However, the report of NOTES use for patients with colorectal cancer is very limited [2]. In Vietnam, there are only a few cases of colorectal cut by Hybrid NOTES or a few cases of transvaginal cholecystectomy were reported, and no reports of NOTES for patients with colorectal cancer.

Objective: To introduce our first experiences in research and application of NOTES for colorectal cancer.

Materials and methods

We prospectively studied 22 patients who suffered from descending colons, sigmoid, or rectal cancers from 12/2013 to 9/2015. Patient's consents were obtained. All patients underwent elective surgery using the technique: Transanal or transvaginal endoscopic surgery.

Patient selection criteria included ASA 1-3, Body Mass Index (BMI) < 30 kg/m², tumor size < 5 cm and tumor stage (Dukes classification) ≤ T3. Patients were not in situations of

*Corresponding author: Email: nhuhieppham@yahoo.com.vn



Fig. 1. Operation team.

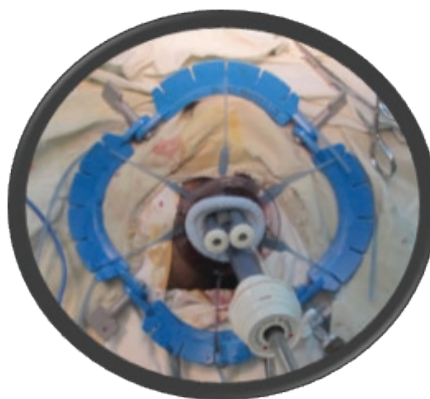
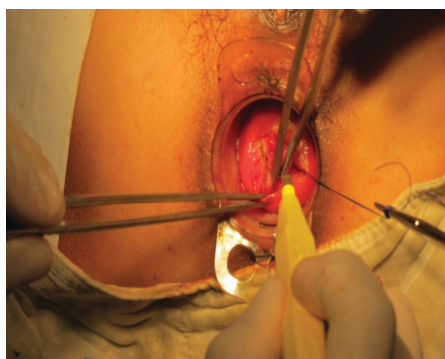


Fig. 2. Mucosal dissection and SILS port placement in anus.

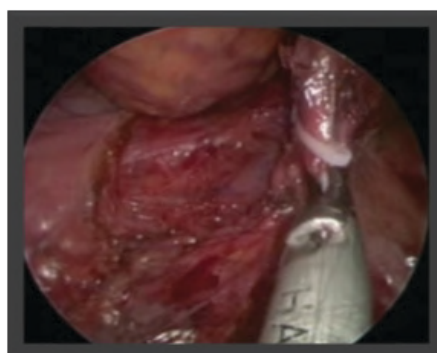


Fig. 3. Place SILS port in vagina and clip IMA by hemolock.

intestinal occlusion or sub-occlusion. Female patients with sigmoid cancer which could be operated by transvaginal endoscopic surgery had menopause and didn't have inflamed or infected vaginas. Exclusion criteria included pregnancy or distant metastasis.

Surgical technique: Pre-operative preparation for patients was similar to conventional laparoscopic colorectal resection. Under general anesthesia, patients were placed in the lithotomy position with a bladder catheter. The surgeon and first assistant stood between the patient's two legs. The laparoscopic

system was placed on the patient's left side. (Fig. 1). For instruments, we used a single access port (Covidien), Optic 30°, 5.5 mm, 50 cm and standard laparoscopic grasper with different lengths.

Transanal endoscopic surgery was used for rectal tumors and transvaginal for sigmoid and descending colon tumors.

In the transanal approach, lone-star retractors (for lower rectal cancer) or anal dilators from the Covidien hemorrhoidectomy set (for intermediate and high rectal tumors) were placed in the anus. Rectal lumen with purse-strings closed 1 cm below the inferior margin of the tumor by Prolene 2.0 and mucosal dissection started at 1 cm below the point of entry by the monopolar scalpel to go through the rectal wall (Fig. 2).

The dissection was from posterior and then around the rectum. When the space created enough for the SIL port of Covidien, it was placed (Fig. 3).

CO₂ inflation was done with a pressure of 12 mm/Hg. TME was continued around the rectum with either a harmonic scalpel or monopolar hook. A peritoneal fold was opened anteriorly and then around the rest of the way. The rectum then was pushed into the abdominal cavity. Mesocolon vessels were divided whether by hemolock or by endo GIA. Told fascia was then freed.

The length of the colon was checked to see if it was enough for a pull-through. The tumor and colon were then pulled out through the anus and resection was done 6 cm proximal to the tumor. Then, anastomose was performed via hand-sewn or EEA device.

For the transvaginal approach, posterior fornix was opened about 2.5 cm between two retraction sutures and SIL port device (covidien) was placed. After determination of tumor position, sigmoid was then divided under the tumor at 2 m through a mesentery window created next to sigmoid wall. The vessels were divided by hemolock or by endo-GIA. After the dissection finished, the tumor was pulled out through the vagina and the colon was resected 6 cm proximal to the tumor and prepare for anastomosis. Anastomosis was performed by EEA.

In difficult cases or intra-operative complication situations, we placed additional port 5 mm in order of priority: trans-umbilical, right lower quadrant and left lower quadrant.

Data collected consisted of age, gender, BMI, tumor position, intraoperative complications, conversion rate to conventional laparoscopy, additional ports, post-operative complications, post-operative pain, specimen length, Quirk's assessment for TME, postoperative TNM staging, sphincter function (Kirwan) and follow-up time and actions.

Chemo-radiation: Adjuvant and neoadjuvant followed Hue Oncology Center protocol.

Results

From 12/2013 to 9/2015, 22 colorectal resections were performed by NOTES, in which there were 18 Transanal and four transvaginal. Male/female: 11/11. Mean age: 51,6±12,1 (30-96) years old. BMI 21,2±2,5 (17,3-27,3).

Lesions (Table 1, 2)

Table 1. Tumor location (18 rectum, three sigmoid, one descending colon).

Distance from anal margin	<5 cm	5-10 cm	>10-15 cm	>15 cm
N	6	6	6	4

Table 2. Classification.

I	II	III	
T2N0M0	T3N0M0	T2N1M0*	T3N1M0*
12	4	2	4

*: 1≤N1≤3 nodes (+).

Techniques (Table 3)

Table 3. Techniques of resection.

Anterior resection	Low-anterior resection	Pull-through	Transvaginal
6	6	6	4

Method to perform anastomosis:

Hand-sew: 6 coloanal,

EEA stapler: 16 coloanal

Operative duration: 258±40 (190-300) minutes.

Complications/Conversions to conventional laparoscopy (Table 4, 5)

The mean length of each specimen was 29.6±4.5 cm. No residual tumor cells at proximal resection margins were found in any patients. Quirk's assessment for TME was good in 18 cases of rectal cancer.

Evaluation of sphincter function following Kirwan was Kirwan I in all

Table 4. Causes of additional trocars (4 patients).

Reasons	NOTES	Number of trocar	N
Post-radiation/Hemorrhage	Anus	2	1
Limited working space/instruments	Anus	1	1
Disorientation/Loss of control	Anus	1	1
Fat/BMI: 27.3	Vaginal	2	1

Table 5. Causes of conversion techniques.

Reasons	Location of tumor	Convert technique	N
Hemorrhage due to injury of iliac artery	Low rectal cancer	Laparotomy	1
Location of tumor was lower posterior fornix, so surgeon didn't dissect transvaginal	Sigmoid tumor	Hybrid-NOTES	1

Mean bowel movement return was 2±0.5 days. Mean hospital stay was 7±2.8 (4-14) days. Mean VAS on the first post-operative day was 3.4±0.5 points.

There was one patient with post-operative complication recorded, and experienced leakage anastomosis coloanal on the 4th day, which showed redo-anastomosis and ileostomy.

patients at three months. No mortality and local recurrence at the end of this study was recognized with a median follow-up time of 12 months.

Discussions

Laparoscopic surgery has become increasingly popular in surgical practice and in the treatment of colorectal cancer. Although conventional laparoscopic

surgery has already significantly reduced the invasiveness of the procedure, many researchers [3, 4] are currently investigating the matter to maximize the advantages of minimal invasiveness by reducing the number of working ports (single port surgery), the size of instruments (mini-laparoscopy), and performing surgery via natural orifices (hybrid NOTES, or pure NOTES) [5-8].

At Hue Central Hospital, we have been performing laparoscopic natural orifice specimen extraction for ultra-low rectal cancers since 2007 with results presented at several domestic and international conferences [9, 10]. It could be considered an intermediate step toward NOTES for colorectal cancer at our hospitals. On the other hand, we also have had experiences with the transanal Soave procedure in pediatric patients with Hirschsprung's disease [11], considered as NOTES for benign disease. On these platforms, we decided to perform NOTES on patients suffered colorectal cancer.

Our study consists of 22 cases of operation by transanal and transvaginal NOTES, in which 16 cases were considered as pure NOTES. The results showed that NOTES was feasible and safe with a mean operative time of less than four hours and low complication rates.

Regardless of the type of procedure, surgeons always have to ensure the surgical and oncologic safety of patients. Therefore, there are three important issues that need to be considered when performing NOTES: proper indication, technical competency, and good outcome (short-term as well as long-term). In our study, we chose patients with tumors $\leq$ T3 and without ganglion invasion. Patients with obesity were also a contra-indication in our study. Related to the position of tumors, most published reports focus on rectal cancers in which Transanal NOTES

can be applied [12-16]. However, in our study, for rectal cancers, we performed Transanal NOTES for tumors in all three parts of the rectum using two different procedures (lower anterior and intersphincteric) which were feasible and safe. Our remarks correspond to the opinions of Isha Ann Emhoff [17] in a review of NOTES for colorectal cancer. For sigmoid cancer, we chose to perform transvaginal NOTES. In transvaginal NOTES, the position of posterior vaginal fornix corresponded to the recto-sigmoid junction, so we determined the tumor position that way. We first resected the sigmoid under the tumor, 2 cm through the mesenteric window, which was created next to sigmoid wall. The division of mesenteric vessels was then conducted. There was one study published that mentioned this technique, but used for benign diseases in human [18]. Therefore, we consider this to be the first publication in the world of pure transvaginal resection for sigmoid cancer.

There were four patients in our study needing additional ports (Table 4). The reasons were loss of control, limited working space, thick mesocolon, and hemorrhaging. Regarding disorientation, in the first cases, we intended to go far back, but when we needed to be familiar with the surgical field, the disorientation was managed. To solve the problem of limited working space, we used long optic 5 mm instruments with different lengths and a harmonic scalpel. In addition to that, we noted that in order to have a good working space, we needed to open the peritoneal folder when the rectum was totally freed. If not, the CO₂ would go into the abdominal cavity and resulting in reduced perirectal space. Concerning the thick mesocolon, we determined that it is best if the elected patients have a BMI under 25 kg/m². In review of Emhoff, most patients operated on using NOTES had a BMI under 25 kg/m² [17]. One intra-operative hemorrhage

occurred in our study. The patient had a T3 and ganglion invasion, and suffered a short-course of radiotherapy four weeks before the operation; this situation led to challenges with coagulation of the middle rectal artery; however, with two trocars from the abdomen, we controlled the bleeding.

Following Antonio [19] and new research to the present, the dissection from anal of TME (down-to-up) had many advantages in terms of oncology than the traditional dissection, the only difficulty that required experienced surgeons and TME dissection at the beginning of the surgery through the anus. One patient who was converted to open surgery is one of the first patients of the study group and the surgeon had "go too far" off TME.

In regard to anastomosis, in our study, the hand-sewn technique was performed in six low rectal cancers. In the other 16 patients anastomosis was performed using staplers. Both Leroy [15] and Zhang [16] performed coloanal anastomosis by hand-sewn interrupted sutures. We saw that in some situations, when the anastomose was high enough, performing the anastomosis by stapler was easier.

One problem encountered relating to NOTES, was intra-abdominal cavity infection due to a colon being pulled out through an "infection source" anus or vagina. However, some reports of NOSE or NOTES supported the safety of these techniques [5, 12-16, 20]. Our study did not recognized any intra-abdominal cavity infections.

Until now, most NOTES articles published have been case reports. Therefore, long-term oncologic results are not available. However, a study of 20 patients from S. Atallah, et al. [2] investigates 20 colorectal cancer patients treated with hybrid NOTES, as well as our study about NOSE

techniques used for the treatment of colo-rectal cancer showed no local recurrence after a 6-month follow-up [9, 10]. Similar results were found in our study with the follow-up duration of 12 months. In addition, Pathologic findings of our specimens showed good quality in TME following Quirk assessments and no residual cancer tissues in proximal resection margins in any patients. We believe that this indicates the effectiveness of this technique.

Rapid postoperative recovery and less pain in this study were advantages of this technique. Sphincter function in 18 patients was good (Kirwan I) after three months. So, we considered that long duration of retraction didn't affect anal sphincter.

Conclusions

Transanal and transvaginal NOTES for rectal cancer is feasible, safe and effective. Pure transvaginal NOTES for colon cancer at Hue Central Hospital could be considered as the first report conducted in the world. However, multi-centric studies with larger series and longer follow-up to evaluate the surgical as well as oncologic outcomes are necessary.

REFERENCES

- [1] J. Marescaux, B. Dallemagne, S. Perretta, et al. (2007), "Surgery without scars: report of transluminal cholecystectomy in a human being", *Arch. Surg.*, **142**(9), pp.823-826, discussion 826-827.
- [2] S. Atallah, B. Martin-Perez, M. Albert, et al. (2014), "Transanal minimally invasive surgery for total mesorectal excision (TAMIS-TME): results and experience with the first 20 patients undergoing curative-intent rectal cancer surgery at a single institution", *Tech. Coloproctol.*, doi: 10.1007/s10151-013-1095-7.
- [3] I.A. Emhoff, Grace Clara Lee, Patricia Sylla (2013), "Transanal colorectal resection using natural orifice transluminal endoscopic surgery (NOTES)", *Digestive Endoscopy*, **26** Suppl. 1, pp.29-42, doi: 10.1111/den.12157.
- [4] Dang Tam, Le Quang Nhan, Pham Cong Khanh (2010), "Evaluate feasibility and effectiveness with some technical modifies of NOTES transvaginal cholecystectomy combine with minilaparoscopy", *Journal of Surgery*, **14**(1), pp.161-165.
- [5] A.M. Wolhuis, A. de Buck van Overstraeten, S. Fieuws, K. Boon, A. D'Hoore (2015), "Standardized laparoscopic NOSE-colectomy is feasible with low morbidity", *Surg. Endosc.*, **29**(5), pp.1167-1173, doi: 10.1007/s00464-014-3784-3.
- [6] S.J. Park, K.Y. Lee, B.M. Kang, S.I. Choi, S.H. Lee (2013), "Initial experience of single-port laparoscopic surgery for sigmoid colon cancer", *World J. Surg.*, **37**(3), pp.652-656, doi: 10.1007/s00268-012-1882-8.
- [7] Say-June Kim, Byung-Jo Choi, Sang Chul Lee (2015), "A novel single-port laparoscopic operation for colorectal cancer with transanal specimen extraction: a comparative study", *BMC Surg.*, **15**(1), p.10, doi: 10.1186/1471-2482-15-10.
- [8] S. Bona S.M., M. Montorsi (2012), "Minilaparoscopic Colorectal Resections: Technical Note", *Minimally Invasive Surgery*, **Vol.2012**, doi: 10.1155/2012/482079.
- [9] Pham Nhu Hiep, Pham Anh Vu, Ho Huu Thien (2010), "Laparoscopic surgery in rectal cancer: a retrospective analysis", *International Journal of Colorectal Disease*, **24**(7); pp.1465-1469.
- [10] Tran Ngoc Thong, Dang Ngoc Hung, Hoang Trong Nhat Phuong, Le Manh Ha, Pham Anh Vu, Pham Nhu Hiep, Le Loc (2014), "Initial results of laparoscopic low anterior resection with specimen retraction via natural orifices (Hybrid NOTES) for upper and middle rectal cancer", *Journal of clinical medicine/Hue Central Hospital*, ISSN:1859-3895, **22**, pp.161-166.
- [11] Pham Anh Vu, Pham Nhu Hiep, Ho Huu Thien (2010), "Transanal one-stage endorectal Pull-through for Hirschprung disease: Experiences with 51 newborn patients", *Pediatric Surgery International*, **Vol.26**, pp.589-592.
- [12] P. Sylla, D.W. Rattner, S. Delgado, A.M. Lacy (2010), "NOTES transanal rectal cancer resection using transanal endoscopic microsurgery and laparoscopic assistance", *Surg. Endosc.*, **24**(5), pp.1205-1210, doi: 10.1007/s00464-010-0965-6.
- [13] R. Zorron, H.N. Phillips, D. Coelho, L. Flach, F. Lemos, R. Vassallo (2012), "Perirectal NOTES access: 'down-to-up' total mesorectal excision for rectal cancer", *Surgical Innovation*, **19**(1), pp.11-19.
- [14] J.J. Tuech, V. Bridoux, B. Kianifard, et al. (2011), "Natural orifice total mesorectal excision using transanal port and laparoscopic assistance", *European Journal of Surgical Oncology*, **37**(4), pp.334-335.
- [15] J. Leroy, Brian Donncha Barry, Armando Melani, et al. (2013), "No-Scar Transanal Total Mesorectal Excision-The Last Step to Pure NOTES for Colorectal Surgery", *JAMA Surg.*, **148**(3), pp.226-230.
- [16] H. Zhang, Y.-S. Zhang, X.-W. Jin, et al. (2013), "Transanal single-port laparoscopic total mesorectal excision in the treatment of rectal cancer", *Tech. Coloproctol.*, **17**, pp.117-123.
- [17] Isha Ann Emhoff, Grace Clara Lee, Patricia Sylla (2013), "Transanal colorectal resection using natural orifice transluminal endoscopic surgery (NOTES)", *Digestive Endoscopy*, **26**, doi: 10.1111/den.12157.
- [18] M. D'Hondt, D. Devriendt, F. Van Rooy, F. Vansteenkiste, E. Dozois (2014), "Transvaginal pure NOTES sigmoid resection using a single port device", *Tech. Coloproctol.*, **18**(1), pp.77-80, doi: 10.1007/s10151-013-1005-z.
- [19] Antonio M. de Lacy, David W. Rattner, Cedric Adelsdorfer, et al. (2013), "Transanal natural orifice transluminal endoscopic surgery (NOTES) rectal resection: 'down-to-up' total mesorectal excision (TME)-short-term outcomes in the first 20 cases", *Surg. Endosc.*, **27**, pp.3165-3172.
- [20] Nguyen Minh Hai, Ho Cao Vu (2010), "Laparoscopic colorectal surgery with specimen extraction via natural orifices (HYBRID N.O.T.E.S)", *Journal of Medicine of Ho Chi Minh city*, **Vol.14**-Supplement of No.2, pp.147-150.

Effects of elicitors on the growth and active compounds of *Panax vietnamensis* cells in the bioreactor

Van Men Chu*, Tuan Anh Vu

Vietnam Military Medical University

Received 16 October 2016; accepted 6 January 2017

Abstract:

In this study, the effects of elicitors on the growth and bioactive ingredients of *Panax vietnamensis* cell mass are evaluated. The medium composition was fixed with a MS liquid medium supplemented with 6.42 mg/ml NAA, kinetin of 0.11 mg/l, saccharose of 34.3 g/l, a pH of 5.6-5.8, temperature of 24-26°C, conducted at 50 rpm, and culture time of 14 days. The results showed that the best elicitor is jasmonic acid, at a concentration of 100 μ mol/l, and for an optimal exposure of the bioactive ingredients to jasmonic acid for 12 days and 14 days after starting the culture. Under select conditions, the cell mass yield was 20.01 g/l and contents of ginsenoside Rg1, Rb1, and Rd were 0.288, 0.302, and 0.146%, respectively.

Keywords: biomass, MS, *Panax vietnamensis*, saponin.

Classification number: 3.3, 3.5

Introduction

Ngoc Linh Ginseng (*Panax vietnamensis* Ha et Grushv.) belongs to the Araliaceae family, and is a unique and valuable herb found on Ngoc Linh Mountain. Currently, this species has been included in the Red Book of Vietnam. Natural Ngoc Linh Ginseng only grows at altitudes above 1,500 meters, mainly concentrating in the Ngoc Linh Mountain area of the Dak To (Kon Tum) and Tra My (Quang Nam) Districts. Ngoc Linh Ginseng has many biological uses including nutritious, tonic, antioxidant, anti-aging, and hepatoprotective effects. Ngoc Linh Ginseng is a rare and valuable medicinal plant, which has been exhaustively exploited [1, 2].

Today, plant cell biomass technology has been widely applied in many fields of pharmaceuticals, cosmetics and functional foods. Vietnam Military Medical University (VMMU), together with Ajou University in South Korea, has been successfully carrying out collaborative projects, namely: *Research collaboration on the development of the production procedure for Vietnamese ginseng (Panax vietnamensis) biomass as a raw material for health products to serve publics* (2007) [3]. This collaborative project has been highly appreciated by the scientific committee and has been recommended for further progress to develop a complete biomass production procedure of Ngoc Linh Ginseng cells. Based on the results of the research project, VMMU have been granted a patent for the methodology for a Ngoc Linh Ginseng biomass production procedure by the Intellectual Property Department of the Vietnamese Ministry of Science and Technology (2009) [4]. As a result of the above mentioned success, scientists from VMMU continue to carry out this ministry-level project, now titled the *Study on the Vinantonic preparations from Vietnamese ginseng cellmass*, this product has been



150-year-old roots of *Panax vietnamensis*.

*Corresponding author: Email: hvqy@vmmu.edu.vn

approved by the Ministry of Health and is now circulated nationwide [5].

Based on the results of these successful projects, the Ministry of Science and Technology has agreed to allow VMMU to carry out a national-level project: “*Ngoc Linh Ginseng cellmass production process optimization and Vinatonic preparation*” [6]. To implement this project, a study of conditions, especially oxygen concentration for optimized cell biomass grown in 100 liter bioreactors, is important. In this article, we evaluate the effects of oxygen concentration on the development and content of active ingredients in Vietnamese ginseng cell biomass in 100 liter bioreactors.

Experimentals

Instrument, reagents

- Instrument: Incubator 2 NU-440-400E, Orbital shaker, Incubator ISF4-W, 100 liter Bioreactor Biostat C200 (Germany).

- Reagents: Myo-inositol, Nicotinic acid, Glycine, Pyridoxine hydrochloride, Thiamine hydrochloride, Kinetin, Glucose, Sucrose, Acid 2.4 - dichloro phenoxy acetic, Kali nitrate, Amoni nitrate, Magnesium sulfate, Kali dihydro phosphate, and other reagents that meet culture standards.

Material and methods

Material: *Panax vietnamensis* cellmass (provided by the Biomedical and Pharmaceutical Applied Research Center at VMMU).

Methods: Cultured *Panax vietnamensis* cellmass was studied in an optimal medium, including: (MS: Medium supplemented with 6.42 mg/l of NAA; 0.11 mg/l of kinetin; 34.3 gram of saccharose in 1 liter of culture medium). Different elicitors were used to increase the content of bioactive compounds in the ginseng cellmass. After each culture batch, the ginseng cell was filtered. Then the dry cellmass and quantify ginsenoside in the cellmass were weighted. After analyzing the results, the best elicitor for cellmass and bioactive compounds was selected.

- Select suitable elicitors: Different elicitors were used including jasmonic acid, ferulic acid, caffeic acid, methyl jasmonate, and yeast extract. These elicitors were added on the 12th day of the culture cycle. After finishing the culture cycle, the cellmass was filtered, collected, and quantified as the bioactive compounds in dry cellmass. From

Table1. Results of elicitor selection.

Elicitor	Ginsenoside (%)			Dried cells weight (g/l)
	<i>Rg1</i>	<i>Rb1</i>	<i>Rd</i>	
Without elicitor	0.2521±0.010	0.2722±0.012	0.1430±0.005	20.26±2.05
Jasmonic acid	0.3432±0.013	0.3847±0.019	0.1711±0.007	18.58±1.88
Methyl jasmonate	0.3164±0.013	0.3441±0.014	0.1597±0.005	18.40±1.88
Ferulic acid	0.2583±0.009	0.2529±0.010	0.1259±0.004	19.19±2.14
Caffeic acid	0.2469±0.010	0.2878±0.014	0.1315±0.005	19.60±2.26
Yeast extract	0.3096±0.012	0.3536±0.014	0.1838±0.007	18.60±2.28

the results, the best elicitor was selected.

- Select optimal elicitor concentration: After 12 days of the culture cycle, ginseng cells were exposed to jasmonic acid at different concentrations: 50, 100, 150, 200, 250 and 300 µmol/l; and allowed to continue to culture for two more days. The cellmass and quantitated active compounds in cellmass were then collected. From the results, the best concentration of jasmonic acid was selected.

- Select the suitable time of adding the elicitor: Jasmonic acid was added at a concentration of 100 µmol/l to the culture medium on the 1st, 3rd, 5th, 7th, 9th, 11th, 12th, 13th and 14th day of the culture cycle. Two days after adding jasmonic acid, the ginseng cells were wilted quantitated ginsenoside content in the dry cellmass. From the results, the best time to add an elicitor was identified.

- Select the optimal exposure time for the elicitor: On the 12th day of culturing the ginseng cells, the cells were exposed to jasmonic acid at a concentration of 100 µmol/l, over 12, 24, 36, 48, 60 and 72 hours after exposing the cells to the elicitor. The cells were then filtered, dried and quantified to measure the content of ginsenoside. From the results, the optimal exposed time to the elicitor was selected.

Results and discussions

Selecting elicitor types

In order to culture the ginseng cellmass in optimal MS medium, on the 12th day of culturing, the cells were exposed to jasmonic acid at a concentration of 100 µmol/l; methyl jasmonate (100 µmol/l), ferulic acid (100 µmol/l), caffeic acid (100 µmol/l), and yeast extract (100 µg/g fresh weight). 48 hours after exposing the cells to the elicitor, the cells were filtered, dried to constant weight, and

quantified as to the content of ginsenoside. Results are presented in Table 1.

When elicitors were used, the content of ginsenoside *Rb1*, *Rg1*, *Rd* was enhanced compared to those of the control. Among elicitors used, jasmonic acid, methyl jasmonate, and yeast extract showed the best results.

Experimental results showed that, jasmonic acid, methyl jasmonate, and yeast extract can be used for the cultivation of Vietnamese ginseng roots cellmass as elicitors in order to increase the synthesis of bioactive compounds to enhance the saponin content of the final products. For further studies, jasmonic acid was used.

Table 1 shows that when an elicitor was used, the dried cellmass was lower, compared to that of the control group; thus elicitors stimulated the synthesis of active compounds, at the same time reducing the ginseng cellmass growth. Our results were similar to other published results of plant cell biomass, and this was explained by the elicitors as well as strange agents, and pathogens, when exposed to cells, inhibited the growth of ginseng cells. In response to that observation, the cells have a defense mechanism to stimulate the synthesis of phytoalexin (almost all active compounds are phytoalexin).

The effects of elicitor concentrations on the content of bioactive compounds in Vietnamese ginseng cellmass in 100 bioreactor liter showed the culture of the ginseng cellmass in optimal MS medium on the 12th day of culturing, the cells were exposed to jasmonic acid at different concentrations at 50 µmol/l, 100 µmol/l, 150 µmol/l, 200 µmol/l, 250 µmol/l and 300 µmol/l. The contents of ginsenosides in dry cellmass are shown in Table 2.

Table 2. Results for selection of elicitor concentrations.

Concentration ($\mu\text{mol/l}$)	Ginsenoside (%)			Dry cell weight (g/l)
	<i>Rg1</i>	<i>Rb1</i>	<i>Rd</i>	
0	0.2512 $\pm$ 0.012	0.2709 $\pm$ 0.011	0.1421 $\pm$ 0.005	20.22 $\pm$ 2.33
50	0.3032 $\pm$ 0.010	0.3308 $\pm$ 0.013	0.1544 $\pm$ 0.007	19.26 $\pm$ 2.36
100	0.3494 $\pm$ 0.013	0.3852 $\pm$ 0.016	0.1705 $\pm$ 0.006	18.63 $\pm$ 2.25
150	0.3470 $\pm$ 0.015	0.3890 $\pm$ 0.014	0.1728 $\pm$ 0.006	18.15 $\pm$ 2.26
200	0.3311 $\pm$ 0.012	0.3283 $\pm$ 0.014	0.1614 $\pm$ 0.007	17.87 $\pm$ 1.89
250	0.3290 $\pm$ 0.011	0.2761 $\pm$ 0.009	0.1559 $\pm$ 0.008	17.06 $\pm$ 1.73

Table 3. Influences of adding-time of an elicitor on cellmass and ginsenoside.

Days	Ginsenoside (%)			Dry cell weight (g/l)
	<i>Rg1</i>	<i>Rb1</i>	<i>Rd</i>	
1	0.0892 $\pm$ 0.004	0.1032 $\pm$ 0.004	*	12.74 $\pm$ 1.42
4	0.1201 $\pm$ 0.004	0.1181 $\pm$ 0.005	*	12.64 $\pm$ 1.45
6	0.1702 $\pm$ 0.006	0.1296 $\pm$ 0.006	0.0518 $\pm$ 0.002	13.02 $\pm$ 1.59
8	0.2015 $\pm$ 0.008	0.1795 $\pm$ 0.006	0.0540 $\pm$ 0.002	15.39 $\pm$ 1.86
10	0.2864 $\pm$ 0.010	0.3086 $\pm$ 0.013	0.1281 $\pm$ 0.006	17.94 $\pm$ 2.23
12	0.3408 $\pm$ 0.014	0.3624 $\pm$ 0.013	0.1479 $\pm$ 0.005	18.25 $\pm$ 1.93
14	0.3495 $\pm$ 0.017	0.3838 $\pm$ 0.016	0.1714 $\pm$ 0.007	18.58 $\pm$ 1.92
16	0.3290 $\pm$ 0.012	0.3686 $\pm$ 0.012	0.1629 $\pm$ 0.005	17.66 $\pm$ 1.79
20	0.2506 $\pm$ 0.010	0.2707 $\pm$ 0.009	0.1430 $\pm$ 0.005	16.95 $\pm$ 1.74

*Undetected.

Table 4. Effects of exposed time to an elicitor on the content of ginsenoside and cellmass.

Exposed time (hours)	Ginsenoside (%)			Dry cell weight (g/l)
	<i>Rg1</i>	<i>Rb1</i>	<i>Rd</i>	
12	0.288 $\pm$ 0.010	0.302 $\pm$ 0.011	0.146 $\pm$ 0.005	20.01 $\pm$ 0.66
24	0.306 $\pm$ 0.011	0.308 $\pm$ 0.013	0.148 $\pm$ 0.006	19.66 $\pm$ 0.68
36	0.325 $\pm$ 0.013	0.341 $\pm$ 0.011	0.160 $\pm$ 0.008	19.21 $\pm$ 0.76
48	0.351 $\pm$ 0.017	0.381 $\pm$ 0.013	0.174 $\pm$ 0.006	18.52 $\pm$ 0.64
60	0.316 $\pm$ 0.011	0.331 $\pm$ 0.014	0.152 $\pm$ 0.006	17.30 $\pm$ 0.69
72	0.275 $\pm$ 0.009	0.290 $\pm$ 0.010	0.136 $\pm$ 0.005	15.31 $\pm$ 0.74

Results in Table 2 show that, when jasmonic acid was used at the concentration of 100 $\mu\text{mol/l}$, the content of ginsenoside in dry cellmass was the highest, so this concentration was selected as a suitable concentration for culturing the Vietnamese ginseng.

Selecting of the suitable time and duration to add an elicitor

Culturing ginseng cells in the optimal MS medium supplemented with jasmonic acid at a concentration of 100 $\mu\text{mol/l}$ on the 1st, 3rd, 5th, 7th, 9th, 11th, 12th, 13th and 14th day of the culture cycle, is optimized after two days of the addition of jasmonic acid. The cells were filtered and dried to determine the weight of the cellmass and content of ginsenosides in the cellmass. Results are shown in Table 3.

Selecting the optimal exposed time to an elicitor

On the 12th day of culturing ginseng cells, the cells were exposed to jasmonic acid at a concentration of 100 $\mu\text{mol/L}$, from 12, 24, 36, 48, 60 and 72 hours after exposure to an elicitor, the cells were filtered, dried and quantified the content of ginsenoside. Results are shown in Table 4.

Results show that the optimal exposure time to jasmonic acid was 48 hours (two days), when the content of ginsenosides in the cellmass was the highest.

Conclusions

In this study, the effects of elicitors on the growth and bioactive ingredients of *Panax vietnamensis* cellmass were evaluated. The best elicitor was jasmonic acid, the best concentration of jasmonic acid was 100 $\mu\text{mol/l}$, and the optimal time of exposure to jasmonic acid was 12 days and 14 days after starting the culture. With select conditions, the cellmass yield was 20.01 g/l and the contents of ginsenoside *Rg1*, *Rb1* and *Rd* were 0.288, 0.302 and 0.146 %, respectively.

ACKNOWLEDGEMENTS

This study is part of the national project, code number KC10.DA02/11-15, belonging to the National Key Sci-Tech Program KC10/11-15.

REFERENCES

- [1] Do Huy Bich, et al. (2003), *Medicinal Plants and animals do drugs in Vietnam*, Vol.1-2, Science and Technics Publishing House.
- [2] Do Tat Loi (2005), *Medicinal Plants and Drugs from Vietnam*, Medical Publishing House Company Limited, pp.808-810.
- [3] Military Medical Academy (2007), *Research collab oration on the development of the production procedure for Vietnamese ginseng (Panax vietnamensis) biomass as a raw material for health products to serve publics*.
- [4] Le Bach Quang, et al. (2009), *Method for Ngoc Linh ginseng biomass production procedure of Ngoc Linh ginseng cells*, Patent by the Intellectual Property Department, Vietnam Ministry of Science and Technology.
- [5] Nguyen Van Long, et al. (2010), *Study on the Vinatonic preparations from Vietnamese ginseng cellmass*, the project of Ministry of Defense.
- [6] Vu Tuan Anh, et al. (2013), *The national level project namely Ngoc Linh ginseng cellmass production process optimization and Vinatonic preparation*.

Trophic structure of free-living nematodes in the Saigon River, Vietnam

Thi My Yen Nguyen, Xuan Quang Ngo*

The Department of Environmental Management and Technology,
Institute of Tropical Biology, Vietnam Academy of Science and Technology

Received 20 April 2017; accepted 30 May 2017

Abstract:

Sub-tidal free-living nematodes in 12 stations along the Saigon River were investigated in both dry and rainy seasons during 2014 and 2015. In total, 157 nematode genera were found to belong to 59 families, and 11 orders of two classes of Enoplea and Chromadorea were identified. The trophic structure of free-living nematode communities was analysed spanning the overall seasons to see that deposit-feeders developed dominantly in the Saigon River indicating their association with food types including particles, bacteria, diatom, etc.. The dominance of deposit-feeders was contributed to a high abundance of genera in each station, including *Theristus*, *Teschellingia*, *Monhystera*, *Thalassomonhystera*, *Paraplectonema*, *Daptonema*, *Aphanonchus*, *Sphaerotheristus*, and *Geomonhystera*. Epistrate-feeder types were found as the second most abundant group. The remaining feeding types, such as chewers and suction-feeders, were in rather low proportion at most stations.

Keywords: *free-living nematodes, Saigon River, Trophic structure, Vietnam.*

Classification number: 3.4

Introduction

The trophic structures and ecological functioning of free-living nematodes in aquatic environment have been well investigated and documented [1-4]. Nematodes have been found to feed on a diversity of food webs, including bacteria, microalgae, fungi, detritus, suspended organic matter, plants, and animal organisms. The first study of nematode feeding types was conducted by Wieser (1953) [4], and was based on the morphological structure and armature of the buccal cavity. In this study, Wieser differentiated four feeding groups based on lateral optical section of the buccal cavity of the fixed specimens of marine nematodes: 1A) selective deposit-feeders with minute-small stoma without teeth, 1B) Non-selective deposit-feeders with somewhat unarmed stoma, 2A) Epistrate-feeders with medium-sized stoma and small teeth, and 2B) Predators/omnivores with large stoma and large teeth/mandibles. This classification has been widely used in analyses on the trophic structure of free-living nematode [5].

Later some modification and development of Wieser's feeding type classification were proposed by Jensen (1987) [1] and Moens & Vincx (1997) [3], and these were mostly according to nematode cultures and food sources. Specifically, Jensen (1987) [1] distinguished four feeding types of free-living aquatic nematodes: 1) deposit-feeders, 2) epistrate-feeders, 3) scavengers, and 4) predators. However, this scheme does not refer to the nematode with stylet as feeding apparatus. While Moens and Vincx (1997) [3] classified the Westerschelde estuarine nematodes into six major feeding guilds: 1) Microvores, 2) ciliated feeders, 3) deposit-feeders, 4) epigrowth-feeders, 5) facultative predators, and 6) predators.

Additionally, Yeates, et al. (1993) [6], described eight trophic groups based on its source of food, such as 1) plant feeders, 2) hyphal feeders, 3) bacterial feeders, 4) substrate ingesters, 5) predators of animals, 6) unicellular eukaryote feeders, 7) dispersal or infective stage of parasites, and 8) omnivores. This classification was suitable to apply to research on nematode trophic structure and feeding habits in soil ecology. Moens, et al. (2004) [7], also differentiated nematode feeding type structure into eight trophic groups: 1) plant feeders, 2) hyphal feeders, 3) bacterial feeders, 4) substrate ingestion, 5) carnivores, 6) unicellular eukaryote feeders, 7) animal parasite, and 8) omnivores. This trophic structure classification was updated for Yeates' system. Some nematode groups may feed on one or more of a type of food source, depending on their

*Corresponding author: Email: ngoxuanq@gmail.com

feeding habits.

Particularly, in order to facilitate the functional role of nematodes in oligotrophic lake sediment and based on morphological characteristics of the buccal cavity in combination with available food, Traunspurger (1997) [8] grouped free-living nematodes into four feeding types: 1) deposit-feeders: without teeth in the buccal cavity, swallowing food and feeding on bacteria and unicellular eukaryotes; 2) epistrate-feeders: possessing small teeth in the buccal cavity, they tear and swallow the food composed of bacteria, unicellular eukaryotes, diatoms and other algae; 3) chewers: with a voluminous, sclerotised buccal cavity with one or more teeth and denticles which feed on predators on protozoa, other nematodes, rotifers, tardigrades and other small animals; and 4) suction-feeders/omnivores: are characterised by the presence of a stylet and are supposedly omnivorous, they pierce different kinds of food (algae, vascular plants, epidermal cells and root hairs, fungi and animals) by their stylet.

Traunspurger's 1997 [8] feeding type classification grouped selective and non-selective deposit-feeders into deposit-feeder similar to Jensen (1987) [1], and subdivided the 2B group of Wieser (1953) [4] into chewers and suction-feeders. Applying this feeding type classification is not only quite simple but also provides full information of nematode ecological role in the benthic food webs.

Therefore, the classification from Traunspurger (1997) [8] was selected to apply for studying on the trophic structure of free-living nematodes in the Saigon River. Objectives of this work are to understand how free-living nematode feeding structures and their feeding diversity in the Saigon River of Vietnam.

Materials and methods

Sampling stations

Sampling field trips were conducted during the dry and rainy seasons of 2014 and 2015 (March 2014, September 2014, and March 2015, September 2015, respectively) at 11 ports along the Saigon River and one reference location in Cu Chi District. These were coded as SG (Saigon River), from SG1 to SG12 respectively: SG1 (Cu Chi District), SG2 (Tan Cang Port), SG3 (Ba Son Shipyard), SG4 (Saigon Port), SG5 (Tan Thuan Dong Port), SG6 (Ben Nghe Port), SG7 (Joint Company of Logistic Development No.1 Port - VICT), SG8 (Saigon New Port), SG9 (Bien Dong Port), SG10 (Saigon Shipbuilding Port), SG11 (Lotus Port), and SG12 (Navioil Port) (Fig. 1).

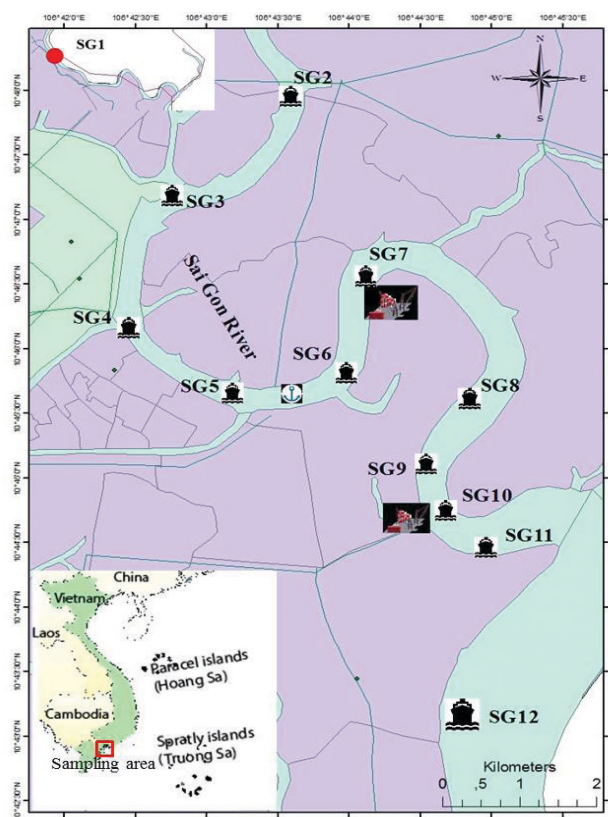


Fig. 1. Sampling stations of free-living nematode communities in study areas.

Sampling collection and laboratory experiments

In each station, sediment samples were collected using a boat with ponar grab, and plastic cores of 3.5 cm diameter (10 cm² surface area). The cores were pushed down into the sediment for up to 10 cm deep. At each station, triple samples were taken and put into 150 ml plastic bottles. All samples were then transferred to a laboratory for processing and analysis. In the laboratory sediment samples, the samples were fixed by 7% formalin solution at 60°C temperature, and gently stirred before decanting, extracting, mounting, and making slides according to Smol (2007) [9]. Nematode specimens were identified to a genus level based on classification keys of Warwick, et al. (2005) [10], Zullini (2005) [11], V.T Nguyen (2007) [12], and the NEMYS database of the Ghent University in Belgium [13].

The nematode trophic structure was identified according to Traunspurger (1997) [8], which included four feeding type groups: deposit-feeders, epistrate-feeders, chewers, and suction-feeders based on the buccal cavity structure, and food source, and calculated by percentage. Characteristics of these groups are clearly described in Table 1. This function-based

approach offers several advantages: (i) in order to assign a feeding category, it is not necessary to identify specimens to species level, and (ii) it focuses on the ecological role of nematode communities [14].

Table 1. Classification of nematode feeding types according to Traunsperger (1997) [8].

Feeding type	Shape of buccal cavity	Food sources
deposit-feeders (swallowers)	no teeth in buccal cavity	bacteria, unicellular eukaryotes
epistrate-feeders (tear and swallowers)	small teeth present in buccal cavity	bacteria, unicellular eukaryotes, diatoms, other microalgae
chewers	voluminous, sclerotised buccal cavity with one or more teeth and denticles	predators of protozoa, nematodes, rotifers, enchytraeids and tardigrades
suction-feeder	stylet present	omnivorous, algae, plant, fungi and animal

Trophic diversity of Heip, et al. (1985) [15] was applied in order to discover how their diversity:

Trophic index = $\sum \theta^2$ (total square percentage of feeding types).

Data analysis

Nematode data was processed using Microsoft Excel 2007. Nematode densities were all converted in order to calculate their abundance per 10 cm². The significant difference of nematode variables between stations was detected using two-way PERMANOVA analysis. The software PRIMER 6.0 adds on PERMANOVA and STATISTICA 7.0 were applied for significant different test with the number of permutations 9999.

Results

General characteristic of free-living nematode communities

A total of 157 free living nematodes genera were identified over 12 stations within four seasons along the Saigon River. They belonged to 59 families of 11 orders and two classes of Enoplea and Chromadorea. Of these, the highest diversity of nematode genera composition was 61.78% of total belonging to the class Chromadorea. Although the number of taxa in this area was lower in comparison with the adjacent river, the Mekong estuarine system, where 230 nematode genera, 58 families of two classes of Enoplea and Chromadorea were found [16], and were quite higher than the Cua Luc Estuary in North Vietnam [17] with 66 species, 52 genera and 17 families. In a study of Italian contaminated harbours, only 72 genera, and 26 families were reported [18].

In our study, the genera *Parodontophora*, *Terschellingia*, and *Rhabdolaimus* were the most dominant in the river over the seasons. However, some genera showed high densities in only one season: *Daptonema*, *Geomonhystera*, and *Sphaerolaimus* in the 2014 dry season; *Thalassomonhystera* in the 2014 rainy season; *Achromadora*, *Mononchulus* in the 2015 dry season; and *Mesodorylaimus* in the rainy season of 2015. Several genera were abundant in two or the three seasons including *Dorylaimus*, *Diplolaimelloides*, *Ironus*, *Monhystera*, *Mylonchulus*, *Paraplectonema*, *Punctodora*, *Sphaerotheristus*, *Aphanonchus*, and *Theristus*. This was completely different from the Mekong estuaries where *Halalaimus*, *Rhynchonema*, *Parodontophora*, *Terschellingia*, *Onyx*, *Leptolaimoides*, *Oncholaimellus*, *Omicronema*, *Rhinema*, *Haliplectus*, and *Desmodora* were found to be dominant [5]. According to Heip, et al. (1985) [15], dominant nematode genera in European estuarine rivers such as in Germany, the United Kingdom, Belgium, Finland, South American, the Netherlands, and France were *Adoncholaimus*, *Anoplostoma*, *Axonolaimus*, *Daptonema*, *Leptolaimus*, *Microlaimus*, *Monhystera*, *Metachromadora*, *Ptycholaimellus*, *Sabatieria*, *Theristus*, *Tripyloides*, and *Viscosia*. The difference in the composition and the dominant genera of nematode communities could be explained by different environmental conditions, especially sediment characteristics [16].

Densities of nematode communities ranged from 13.33±2.89 (inds/10 cm²) at SG4 in the dry season of 2014 to 5863±2396.46 (inds/10 cm²) at SG8 in the rainy season of 2015. The results indicated that SG8 was occupied the highest densities in 2014 and the rainy season of 2015. The nematode communities at SG8 station during the rainy season of 2015 indicated overall higher densities compared to the other stations (Fig. 2).

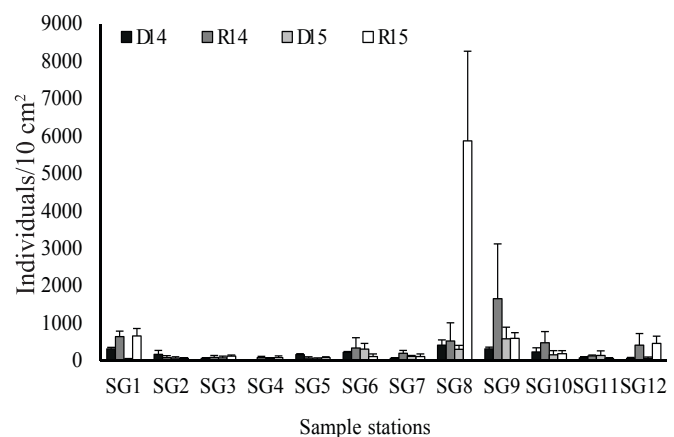


Fig. 2. Densities of nematode communities in the Saigon River.

D: Dry season, R: Rainy season.

The trophic structure of free-living nematode communities

A total of 157 free living nematode genera were identified in Saigon River harbours, and they were classified into four main feeding types according to Trautspurger (1997) [8], which are: deposit-feeders, epistrate-feeders, chewers, and suction-feeders. In which, deposit-feeders prevail in whole communities, with 50 genera (31.85% total), and they also occupied dominantly in all seasons. Particularly, this group prevailed high percentage from 35.56% (SG4) to 95.93% (SG8) in total individuals during the dry season of 2014; from 46.03% (SG1) to 90.94% (SG8) in the rainy season of 2014; from 36.96% (SG2) to 79.95% (SG8) in the dry season of 2015; and from 45.33% (SG4) to 92.67% (SG12) in the rainy season of 2015. Specifically, they were highly dominant over stations during the rainy season of 2014 (46.03% at SG5-90.94% at SG8) (Fig. 3). The highest percentage of deposit-feeders nematode group overall stations indicates that particles, bacteria, diatom, etc., was copious in the sediment [8]. The results of a two-way PERMANOVA analysis for deposit-feeders group showed a significant difference between stations ($p = 0.0007$), but there were no indication in seasonal effect ($p = 0.062$) as well as interaction of seasons and stations ($p = 0.34$).

The dominant trophic groups in the Saigon River related to its genera richness. This was demonstrated by a contributory rate of the dominant genera. During the rainy season of 2014, *Theristus* (occupied 31.7-84.8% total individuals in the stations from SG2 to SG12), *Terschellingia* (42.49% at SG1), and more *Monhystera*, *Thalssomnhystra*, and *Paraplectonema* were the abundant genera. The genera of *Daptonema* (70.38% in total of number individuals at SG6, 88.09% at SG8, and 97.35% at SG7), *Monhystera* (62.98%, 38.66%, 34.99%, and 23.67% correspond to SG12, SG9, SG10, and SG3, respectively), *Terschellingia* (80.55% at SG11, 23.365% at SG9, and 20.45% at SG1), *Aphanonchus* and *Sphaerotheristus* (both equal to 23.9% at SG4), *Geomonhystera* (17.8% at SG2) were found to be high in the dry season of 2014. *Theristus* (at SG1, SG2, and from SG6 to SG12 with 10.55-68.71%), *Terschellingia* (10.24-28.65% at SG4, SG7, SG8, SG11, and SG12), *Sphaerotheristus* (21.16-26.87% at SG3, SG4, and SG10), *Paraplectonema* (20% at SG1), *Aphanonchus* (10.24-22.51% at SG1, SG2, and SG4) were more present in the dry season of 2015. *Theristus* (from SG2 to SG12 with 21.2-87.37%), *Terschellingia* (78.3% at SG1, and 66.65% at SG12), *Aphanonchus* (at SG2 with 12.22%) were dominant in the wet season of 2015. These genera belong to the deposit-feeders group. The high percentage of genera richness lead to the dominated extremely of deposit-feeders group which are without teeth in the buccal cavity, swallows the foods, feed on bacteria, and unicellular eukaryotes.

The second high percentage of feeding type was epistrate-feeders with 47 genera (29.94%). They also presented quite high percentages in each station. For instance, the station SG5 (in the dry season 2014 with 35.81%, the dry season 2015 with 39.32% and 36.50% in the rainy season 2015), SG1 (40.29% in the dry 2015), and SG7 (32.78% in the rainy season 2015) had the numerous presence of the genera *Parodontophora*, *Rhabdolaimus*, *Punctodora*, *Achromadora*, *Udonchus*, and *Simanema*. The epistrate-feeding nematodes had a small tooth in the buccal cavity, they tear and swallow the foods, feed on feed on bacteria, unicellular eukaryotes, diatoms and other algae. There was a significant difference between stations for feeding epistrate-feeders (Two-way PERMANOVA of $p = 0.48$). Nevertheless, it was not found in season ($p = 0.501$) and interaction between seasons and stations ($p = 0.7$).

For chewers and suction-feeders, they contributed a very low percentage to the communities (Fig. 3). However, the results of the two-way PERMANOVA analysis showed some interesting features in these groups. The relative abundance of chewers were found significant differences in both stations ($p = 0.0034$) and seasons ($p = 0.0003$). In contrast, only suction-feeders were found to be significantly different between seasons ($p = 0.0036$), but not in stations ($p = 0.073$) and two factors interaction ($p = 0.078$).

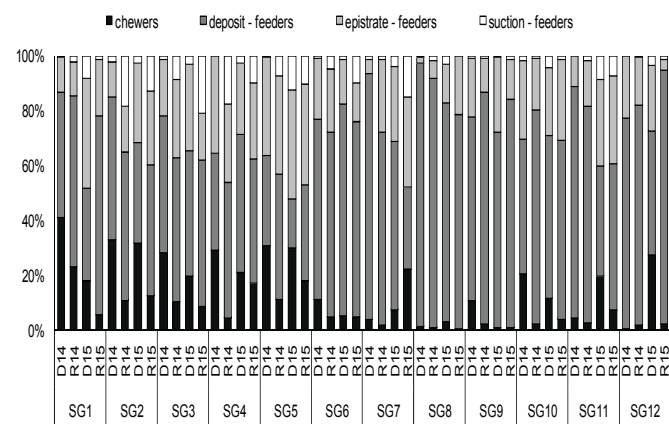


Fig. 3. A percentage of free-living nematode communities in the Saigon River in dry (D) and rainy (R) season from 2014-2015.

The trophic index of free-living nematode communities in the Saigon River during four sampling time ranged from 0.32 ± 0.03 (lowest in SG5) to 0.77 ± 0.13 (highest in SG8) (Fig. 4). From station SG1 to SG5, trophic value index was quite low and showed high from station SG6 to SG12. The diversity of nematode communities in the Saigon River from Ben Nghe Port (SG6) to downstream present a tendency higher in comparison to the upper part.

The two-way PERMANOVA analysis for the trophic index showed a significant difference between stations along the river ($p = 0.0003$), and between dry and rainy season ($p = 0.006$), but there was no difference in interaction effect ($p = 0.387$).

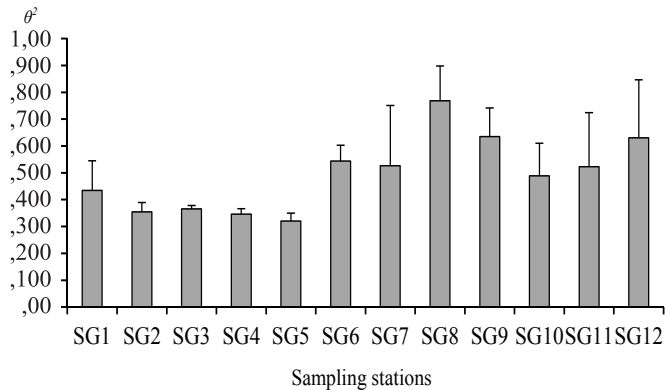


Fig. 4. The average and standard deviation of trophic index of nematode communities in four sampling times.

Discussion

The nematode community's density in our study was shown with a larger deviation in comparison to other research. Most specifically, from 168.7 to 1602.6 inds/10 cm² in harbor stations in Italy [18]; 454.0±289.9 - 3137.7±337.1 inds/10 cm² in the Mekong estuaries [5]; and 67-1666 inds/10 cm² in the Westerschelde [19]; 317-1002 inds/10 cm² in Shin River, Kasuga River, and Tsumeta River in Takamatsu, Japan [20]. However, nematode densities in the Saigon River were lower than that in the Oosterschelde [21] with values of 100-7100 inds/10 cm² or five European estuaries [22] with values from 130-14500 inds/10 cm².

Analysed results of the trophic structure of free-living nematode communities in this study were also found in the same line with the study of Gheskiere, et al. (2004) [23], in a tourists impacted sandy beach of the De Panne, Belgium. The authors reported that non-selective deposit feeders dominated all of the zones, except in the drift line, where epistratum feeders were dominant on sandy beaches. According to Moreno, et al. (2008) [18], the dominant trophic group of nematode communities at Marina Degli Aregai Port in Italy was represented by the non-selective deposit-feeders (1B: 61%), and followed by the epistrate-feeders (2A: 23%). In addition, at Station O of the Genoa-Voltri Port, the trophic groups had a high presence of deposit-feeders (59%) when we incorporate selective deposit-feeders (1A: 31%), and non-selective deposit-feeders (1B: 28%) together, followed by epistrate-feeders (2A: 38%).

Contrasting deposit-feeders to epistrate-feeders, suction-feeders, and chewers had a lower presence across almost all

stations over the seasons. They contributed from 19.75% and 18.47% in total (with 31 and 29 genera), respectively. In all probability, the genera belong to these groups were a small percentage of the nematode communities. Whereas Nicholas, et al. (1992) [24], recorded that the predaceous nematode species were highest in number (>86%) in the sediments of the shore of Lake Alexandrina, Bogut & Vidakovic (2002) [25] reported that chewers were the major group of nematofauna at the eulittoral of Lake Sakadas: 68.28% of total nematofauna at submerged site A, 70.13% at site B (at the land-water interface), and 54.16% at emerged site C. Suction-feeders were the next important nematofauna group: 19.98% at site A, 23.60% at site B, and 36.97% at the site C, followed by deposit-feeders (5.98-11.78%). They also supported irregularities in the water level and the quantity of food available, and had a major influence on the changes in the distribution of nematode feeding-types. The chewers group had voluminous, sclerotised buccal cavities with one or more teeth and denticles, and feed on predators including protozoa, other nematodes, rotifers, tardigrades, and other small animals. While suction-feeders, which are omnivores, are characterised by the presence of a stylet and are supposedly omnivorous, they piercing different kinds of food (algae, vascular plants, epidermal cells and root hairs, fungi and animals) by using their stylets.

Furthermore, it was interesting that the trophic structure of free-living nematode communities also can indicate the environmental situation. Moreno, et al. (2008) [18] found the nematode trophic structure in three stations with different environmental conditions in the Genoa-Voltri Ports: highly polluted station M, polluted station I, and less polluted in station O. The trophic structure of nematode communities was characterised by a dominance of epistrate-feeders (2A: 33% at station M, 38% at station O and 46% at station I), following by selective deposit-feeders (1A: 33% at station I), and non-selective deposit-feeders (1B: 31% at station M). At Portosole Port, the dominant trophic group was found to be epistrate-feeders (2A: 59%), followed by non-selective deposit-feeders (1B: 24%). This was in line with results from the Saigon River where found mainly deposit-feeders and epistrate-feeders meanwhile very few chewers and suction-feeders which feed protozoa, nematodes, rotifers, enchytraeids, and tardigrades as well as omnivorous, algae, plant, fungi, and small animals.

In addition, Dražina, et al. (2014) [14] informed the community that specific trophic nematode groups occupied differently depending on freshwater substrate and available food sources, different parts of river beds and lakes basin. For instance, in sandy mud sediment, deposit-feeders prevail. In the periphyton habitats harbour, where was rich and diverse nematode communities, epistrate-feeding nematodes

dominated. These authors established the domination of suction-feeding nematodes in stream bryophytes; a high percentage dorylaimid nematodes in terrestrial mosses across Europe and in bryophytes and in the deeper layers of the tufa substrates.

Conclusions

Feeding type structures of free-living nematode communities in the Saigon River were characterised by a dominance of nematode feeding type deposit-feeders and epistrate-feeders. The other feeding types, such as chewers and suction-feeders, were rather low proportion in this river. Dominant feeding types were influenced by high abundant genera occupied in the habitat and food available.

ACKNOWLEDGEMENTS

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.06-2013.66.

REFERENCES

- [1] P. Jensen (1987), "Feeding ecology of free-living aquatic nematodes", *Marine Ecology Progress Series*, **35**, pp.187-196.
- [2] T. Moens, M. Bergtold, W. Traunspurger (2006), "Feeding ecology of free-living benthic nematodes", In: *Freshwater nematode: Ecology and Taxonomy*, editor by Eyalew-Abebe with Traunspurger W. and Andr  ssy I.", The CABI Publishing, pp.105-131.
- [3] T. Moens, M. Vincx (1997), "Observations on the feeding ecology of estuarine nematodes", *Journal of Marine Biological Association of the United Kingdom*, **77**(1), pp.211-227.
- [4] W. Wieser (1953), "Die Beziehung zwischen Mundh  hlengestalt, Ern  hrungsweise und Vorkommen bei freilebenden marinen Nematoden", *Ark. Zool.*, **4**, pp.439-484.
- [5] X.Q. Ngo, A. Vanreusel, N. Smol, N.C. Nguyen (2010), "Meiobenthos Assemblages in the Me Kong estuarine system with special focus on free-living marine Nematodes", *Ocean Science Journal*, **45**(4), pp.213-224.
- [6] G.W. Yeates, T. Bongers, R.G.M. De Goede, D.W. Freckman, and S.S. Georgieva (1993), "Feeding habits in soil nematode families and genera-an outline for soil ecologists", *Journal of Nematology*, **25**(3), pp.315-331.
- [7] T. Moens, G.W. Yeates, P. De Ley (2004), "Use of carbon and energy sources by nematodes", *Nematology Monographs & Perspectives*, **2**, pp.529-545.
- [8] W. Traunspurger (1997), "Bathymetric, seasonal and vertical distribution of feeding-types of nematodes in an oligotrophic lake", *Vie Et Milieu*, **47**(1), pp.1-7.
- [9] N. Smol (2007), "General Techniques of the ICP-Nematology", *Lecture syllabus MSc Nematology Ghent University*, 38p.
- [10] R.M. Warwick, H.M. Platt, P.J. Somerfield (2005), *Free-living marine nematodes. Part III. Monhysterids*, The Linnean Society of London and the Estuarine and Coastal Sciences Association, London, 296p.
- [11] A. Zullini (2005), "The Identification manual for freshwater nematode genera", *Lecture syllabus MSc Nematology Ghent University*, 210p.
- [12] V.T. Nguyen (2007), *Fauna of Vietnam: Free-living nematodes (orders Monhysterida, Araeolaimida, Chromadorida, Rhabditida, Enoplida, Mononchida, and Dorylaimida)*, Science and Technics Publishing House, Hanoi, **22**, 445p.
- [13] K. Guilini, T.N. Bezerra, T. Deprez, G. Fonseca, O. Holovachov, D. Leduc, D. Miljutin, T. Moens, J. Sharma, N. Smol, A. Tchesunov, V. Mokievsky, J. Vanaverbeke, A. Vanreusel, M. Vincx (2016), *NeMys: World Database of Free-Living Marine Nematodes*, accessed at www.nemys.ugent.be.
- [14] T. Dra  ina, M.   poljar, B. Primc, I. Habdija (2014), "Nematode feeding types in a tufa-depositing environment (Plitvice Lakes, Croatia)", *Natura Croatica*, **23**(1), pp.89-99.
- [15] C. Heip, M. Vincx, G. Vranken (1985), "The ecology of marine nematodes", *Oceanography and Marine Biology: An Annual Review*, **23**, pp.399-489.
- [16] X.Q. Ngo, N.C. Nguyen, N. Smol, L. Prozorova, A. Vanreusel (2016), "The strong link of intertidal nematode communities with sediment features in the Mekong estuaries provides a useful tool for biomonitoring", *Environmental Monitoring and Assessment*, **188**(2), pp.1-16.
- [17] O. Pavlyuk, T. Yulia, V.T. Nguyen, D.T. Nguyen (2008), "Meiobenthos in Estuary Part of Ha Long Bay (Gulf of Tonkin, South China Sea, Vietnam)", *Ocean Science Journal*, **43**(3), pp.153-160.
- [18] M. Moreno, L. Vezzulli, V. Marin, P. Laconi, G. Albertelli, M. Fabiano, (2008), "The use of meiofauna diversity as an indicator of pollution in harbours", *Journal of Marine Science*, **65**(8), pp.1428-1435.
- [19] K. Soetaert, M. Vincx, J. Wittoeck, M. Tulkens, D. Van Gansbeke (1994), "Spatial patterns of Westerschelde meiobenthos", *Estuarine, Coastal and Shelf Science*, **39**(4), pp.367-388.
- [20] Y. Supaporn, T. Kuninao, M. Shigeru (2006), "Temporal change of the environmental conditions of the sediment and abundance of the nematode community in the subtidal sediment near a river mouth with tidal flats", *Plankton Benthos Research*, **1**(2), pp.109-116.
- [21] N. Smol, K.A. Willems, J.C. Govaere, A.J.J. Sandee (1994), "Composition, distribution and biomass of meiobenthos in the Oosterschelde estuary (SW Netherlands)", *Hydrobiologia*, **282-283**, pp.197-217.
- [22] K. Soetaert, M. Vincx, J. Wittoeck, M. Tulkens (1995), "Meiobenthic distribution and nematode community structure in five European estuaries", *Hydrobiologia*, **311**(1-3), pp.185-206.
- [23] T. Gheskiere, H. Eveline, V. Jan, V. Magda, D. Steven (2004), "Horizontal zonation patterns and feeding structure of marine nematode assemblages on a macrotidal, ultra-dissipative sandy beach (De Panne, Belgium)", *Journal of Sea Research*, **52**(3), pp.211-226.
- [24] W.L. Nicholas, A.F. Bird, T.A. Beech, A.C. Stewart (1992), "The nematode fauna of the Murray River Estuary, South Australia. The effects of the barrages across its mouth", *Hydrobiologia*, **234**(2), pp.87-101.
- [25] I. Bogut, J. Vidakovic (2002), "Nematode feeding-types at the eulittoral of Lake Sakadas (Kopacki rit Nature Park, Croatia)", *Natura Croatica*, **11**(3), pp.321-340.

Review on molecular markers for identification of Orchids

Thi Huyen Trang Vu^{1*}, Thi Ly Le², Truong Khoa Nguyen³, Duy Duong Tran³,
Hoang Dung Tran⁴

¹School of Agricultural Science and Biotechnology, Nguyen Tat Thanh University

²School of Biotechnology, Ho Chi Minh International University

³Genetic Engineering Division, Institute of Agricultural Genetics

⁴Nguyen Tat Thanh University

Received 1 March 2017; accepted 2 June 2017

Abstract:

Orchidaceae is one of the most valuable plant groups all over the world, and is also an impressively large and complex family of flowering plants. Effective molecular tools used for the identification of orchid species should be developed to support traditional morphological approaches. This study reviews most of the DNA fragments that have been used as taxon identifiers in researches conducted on Orchidaceae in order to assess potential molecular markers and metric measurements for the identification of orchid species.

Keywords: DNA barcode Orchids, DNA fingerprinting, molecular identification Orchidaceae, molecular markers, molecular phylogeny.

Classification number: 3.5

Introduction

Orchidaceae is one of the largest and most complex families of flowering plants, comprising of approximately 22,500 species belong to 736 currently recognised genera [1]. Orchids have many values ranging from the beauty of their flowers to therapeutic properties in some species. However, taxons of Orchidaceae are endangered, this is mainly because of over-collection and habitat destruction, and all species are included in Conventions on International Trade of Endangered Species of Fauna and Flora (CITES) I and II [2]. Illegal trade and imitations have also become increasing problems. Unfortunately, laws banning these issues and their enforcement have met obstacles mostly due to the imperceptible difference of species' morphology. So, it is very difficult to identify orchid species and their inter-species hybrids using

traditional classification; even those with fertile parts. Besides this, the species can be transported in a vegetative state, as seeds or as fragments [3]. Accurate authentication of orchid species is critical for biodiversity conservation and effective utilisation of orchids as plant resources [4].

Many researchers have, therefore, tended to develop genetic tests that can cheaply and easily determine the present species. "DNA barcodes" tools are promising options in providing a practical, standardised, species-level identification approach that can be used for biodiversity assessment, life history, ecological studies, and forensic analysis [5]. DNA barcode refers to the use of a single segment of DNA to identify specific coding information that offers discriminating ability of the living taxa, even if only a small fragment of the organism at any stage of development is available [6]. The potential DNA

regions used as barcodes should match some key criteria: i) The universality of amplification and sequencing; ii) The pattern of intraspecific vs. interspecific variation; and iii) The power to identify species [7].

The selection of a barcode locus is complicated due to the trade-off that arises between the need for universal application in a wide range of taxa and sequence substitution saturation [5]. The single region 5' end of cytochrome c-oxidase I (COI) from the mitochondrial genome is quite successfully used for the identification of animals [8, 9]. However searching for DNA barcode in plants is far more challenging than in animals. Mitochondrial genes, including COI, in plants have low rates of synonymous substitution [10, 11], a large structural rearrangement in the genome, and import of sequences from nucleus and chloroplast [12]. Because of these problems, they are not recommended to use for DNA barcodes for plants. So, the nuclear and chloroplast genomes are focused to look for identifying markers for plants. Until now, no single sequences can be sufficient to identify all plant species. Even the use of a combination of multi-locus barcodes also gives different levels of discrimination in different groups of plants (Table 1).

The aim of this paper is to assess potential molecular markers for the identification of orchids. We review most of the DNA fragments that have been used as taxon identifiers in researches on Orchidaceae and other land plants, including orchids. The capability of taxon discrimination is often evaluated along with the construction of a phylogenetic tree. So we also use phylogenetic articles as literature sources for seeking suitable sequences. Highly evaluated markers proposed by previous authors will be deeply discussed to summarise a database of molecular candidates for orchid authentication.

*Corresponding author: Email: vutihuyentrang.vu@gmail.com

Table 1. Summary of advantages and disadvantages of molecular loci in plants.

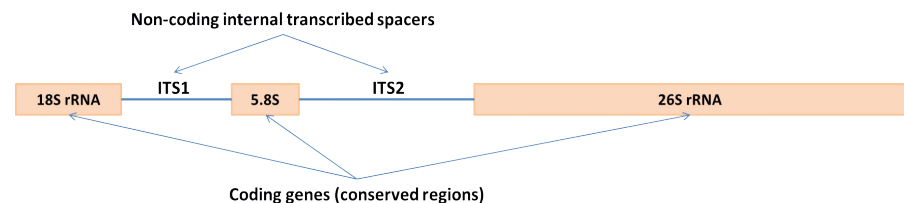
Loci	Advantages	Disadvantages
Nuclear regions (such as ITS)	- High variability - High number copies in cell - Biparental inheritance → more information	- Universal across different groups of organisms → unexpected contamination - Intra-genomic variability, divergent paralogous copies (multiple functional copies), pseudogenes → poor-quality sequences
Chloroplast gene	- High copy number - Maternal inheritance information → no reflection of complexity - Some genes are highly variable - The high variable regions → used for low-level identification (species, under-species) - The lower variable regions → used for high-level identification (genus, family, tribe...)	- Low evolutionary rate → few choices
Mitochondrial genes		- Low rate of sequence change - Genome structure of mitochondrial in plant rapidly change
Intergenic spacer and intron	- High variability (do not encode any products → faster evolve than coding regions)	- Difficult to amplify, sequence and align - Too variable, even intra-species
Low-copy nuclear genes (such as <i>Xdh</i>)	- High variability	- Low copy number - The present and absent of introns, the size of introns, the substitution rate are greatly variable and poorly studied → no universal

Studied barcodes for Orchid taxa

Single locus barcodes

The ITS region: In plants, nuclear genes (particularly introns) and spacers exhibit the highest variability [13]. The internal transcribed spacers (ITS) of nuclear ribosomal regions were proposed as a variable molecular marker for detecting genetic variation among genera, species, and within species. The two internal transcribed spacers (ITS1 and ITS2) do not encode any product but permit it to evolve at a faster rate than the ribosomal coding regions. For example, the ITS length of the aligned sequence in *Holcoglossum* (Orchidaceae) was 567 bp and it contained 26 informative sites and 27 variable sites [14]. The ITS exhibits high resolution at the species level [5]. The ITS has been shown to have unparalleled species resolutions compared with candidate barcodes proposed thus far [15]. Besides that, they exist in cells with high numbers of copies [5, 9]. The flanked regions 5.8S at the middle and 18S, 26S at the two ends of the ITS fragment, are conserved sequences which are useful to develop primers [9] (Fig. 1). The high retrieval rate of amplicons of the ITS [5, 16] may be due to these characteristics. The ITS regions were evaluated and found to have high-quality bidirectional sequences [5].

In addition, the ITS with nuclear genes can provide more complex information which relates to biparental inheritance in comparison with plastid markers [17]. The length of the ITS is about 600 bp [18-20], which can satisfy the length requirements of barcoding.

**Fig. 1. Coding genes.**

The ITS spacer, although often highly variable, also reached a number of limits for DNA barcoding [5]. Using the ITS as a barcode still has its challenges [21]. The high variability among intra-genomic systems, on the contrary, was a disadvantage of species discrimination [22]. The CBOL Plant Working Group did not recognize the ITS as a suitable locus for DNA barcoding because there were many factors that affected the quality of sequences from direct sequencing of PCR products, including such reasons as the presence of intra-genomic variability, the divergent paralogous copies (multiple functional copies) within individuals and

pseudogenes [22]. The coexistence of variation orthologous copies of the ITS in the hybrid genomes of *Paphiopedilum* leads to poor-quality sequences which consisted of multiple peaks. Thus, the ITS was not found to be suitable neither for species resolution nor for getting an insight into the parentage of the hybrids (50% species resolution for eight natural species) [6]. Another disadvantage of the ITS was that it could not be amplified from some barium samples because the ITS is too variable to guarantee reliable alignments and contains variable indels (insertion/deletions) at the species level [23].

However, the internal transcribed spacer region (ITS) of ribosomal DNA has proved to be an effective marking barcoding progress. At the genus level, the ITS clearly distinguishes between the two genera *Paphiopedilum* and *Phragmipedium*, and also the *Cypripedioid* genera [24]. At the species level, Kress, et al. (2005) [5] has evaluated that the ITS has a much higher divergence value than any of

the plastid regions studied and has an amplification success rate of 88%. Therefore this region was proposed as a potentially usable DNA region for the application of barcoding to flowering plants using the optional supplementary marker *trnH-psbA*. The ITS was also evaluated to be an effective candidate DNA barcode for *Orchidaceae* [16, 18, 25]. The PCR success rate of the ITS was high [16], 100% in 158 wild orchid samples [18], and in *Holcoglossum* [6, 14]. Although the combination of the ITS with another sequence showed a greater ability to identify species, the ITS sequence alone was still an effective barcode among proposed loci

[*rbcL*, *matK*, *atpF-atpH*, *rpoB*, *rpoC1*, *trnH-psbA*, *trnL-F*, and ITS] [25]. The ITS was employed successfully in *Dendrobium* (Orchidaceae). Specifically, the phylogenetic relationship and the differentiation of 11 medicinal *Dendrobium* spp. from one another and from two adulterant species *Pholidotaarticulata* and *Flickingeria comate* could be analysed using this locus [20]. The specific nucleotide sequences of the ITS is used for the identification and phylogeny of 20 *Dendrobium* species, in which ITS1 and ITS2 regions exhibit more variation than the 5.8S rDNA [19]. The single ITS barcode revealed to be the best DNA barcode affording 100% species resolution based on 129 congeneric species of *Dendrobium*, and 93% based on sets of sequences from both the experiment and the GenBank. This resolution value of the single ITS barcode was higher than other single barcodes *matK*, *rbcL*, *rpoB*, *rpoC1* and even combined barcodes [*matK+rbcL*] and [*matK+rpoB+rpoC1*] (33, 20, 18, 17, 80.77 and 92.31% respectively) in the study [16]. Using nuclear ribosomal ITS sequence data, genetic units in *Grammatophyllum speciosum* complex (Orchidaceae) were totally recognised at species level to be *G. speciosum* Blume, *G. wallisii* Rchb.f., *G. kinabaluense* Ames and C. Schweinf., *G. pantherinum* Rchb.f., and *G. cominsii* Rolfe [26].

In orchid phylogenetic research, there have been many different markers used, among them is ITS, which has usually been a favourite choice of researchers [27-31]. The ITS tree of 16 *Paphiopedilum* species and two varieties found in Vietnam, received strong Jack-knife support for phylogenetics analysis [32]. However as discussed in the barcoding field, not any single or multiple-locus markers could totally identify the species, and so the combination of regions in phylogenetic studies could not get the strong support in all clades of phylogenetic trees [33-35]. The ITS was used and combined with other regions in most of the phylogenetic studies thus far [3, 36-40]. The combined data matrices often

get better results than individual ones [41-43]. With a higher taxonomic level (section, genus, subgenus, tribe, subfamily), ITS, *matK*, *trnL* and *rbcL* were most often used [44-49], e.g. three subgenera of *Cymbidium* genus could be distinguished clearly from among ten species by its ITS (ITS1+5.8S+ITS2) [50].

In general, the ITS is worth its use for barcoding projects; and in order to increase resolution effects, ITS2 can be an alternative solution, or supplementary markers maybe accompanied as discussed in the combination barcodes latter.

ITS2: Located between ribosomal genes 5.8S and 28S of the ITS regions, recently ITS2 has been highly noticed as a valuable barcode for many plants. The ITS2 spacer provides structural elements necessary for correct pre-rRNA processing and probably has a function in the regulation of the transcription of active ribosomal subunits [51]. As an identifying marker, this sort of ITS fragment does not only receive benefits from the long ITS possess but also overcomes the limits of a full-length region.

In ITS2, the success rates of both PCR and sequencing were very high [3], mostly 100% in *Dendrobium* [51] or 93.8% in a wide range of plants instead of 42.3% for full ITS [52]. This is because the length of ITS2 is quite short at about 248 bp in *Dendrobium* [51], and the design of the universal primers for this sequence is easy due to the availability of flanked conserved regions (5.8S and 26S rRNA). This characteristic was also ideal for barcoding, which should be short enough to recover amplicons from degraded DNA [23, 53] and can overcome the trouble of the universal ITS from contaminated microorganism [53].

The rate of successful identification with the ITS2 is high at 92.7% at the species level and 99.8% at the genus level [52]. Early from 2001, Lau, et al. (2001) [53] discovered the significant

ability of ITS2 for differentiating medicinal *Dendrobium* species from one another and also from non-orchids and adulterants. In 2010, the 50790 ITS2 sequences of plants were downloaded from GenBank and were evaluated according to their sequence lengths, GC content, intra- and inter-specific divergence, and efficiency of identification. The study proposed that the ITS2 locus shows significant sequence variability at the species level or lower, and should be used as a universal DNA barcode for identifying plant species. Among them all, the success rates for using the ITS2 region to identify *Orchidaceae* taxa at the species level (%) were variable in different genera *Scaphyglottis* 100.0, *Satyrium* 98.3, *Dendrobium* 91.9, *Dichaea* 81.8, *Disa* 79.7, *Masdevallia* 79.6, *Paphiopedilum* 76.6, *Telipogon* 76.1, *Cymbidium* 74.1, *Dendrochilum* 71.2, *Cyrtorchilum* 69.3, *Phalaenopsis* 65.9, *Oncidium* 65.1, *Maxillaria* 62.9, *Gomesa* 49.1, *Diuris* 31.1, and *Ophrys* 22.7 [54]. In the study on 43 samples of *Dendrobium*, the ITS2 analyses showed a significant divergence between the inter- and intra-specific genetic distances, and the presence of a barcoding gap was obvious [51]. The variability of ITS2 was sufficient enough to distinguish even closely related species. The phylogenetic analysis of ITS2 regions of 64 *Dendrobium* species also showed good results when the cluster analysis mainly supported a relationship between the species of *Dendrobium* established by traditional morphological methods and many previous molecular analyses [51] (Table 2).

***trnH-psbA*:** *trnH-psbA* is a non-coding intergenic spacer which is a rapidly evolving region. *trnH-psbA* was early on highly evaluated by Kress, et al. (2005) [5] because of its high interspecific variation, high length variation, good priming sites, and it was proposed to be the most viable candidate for a single-locus barcode for land plants identification [55]. Actually, *trnH-psbA* was quite easily amplified with a success rate that either might reach 100% in

large numbers of land plants [5, 56] or at least more than 90% in a wide range of plants [3, 7, 52, 56-58]. In orchids particularly, PCR rates were also very high, up to 100% in *Dendrobium* [59, 60], *Oncidium* [61], *Holcoglossum* [14], and *Cymbidium* [62] (Fig. 2).

trnH-psbA was popularly known to have a high sequence divergence [5, 55, 56] due to its large number of insertions and deletions (indels). The species resolution rates of this region in plants were the highest in many research studies, for examples (82.6%) out of the nine other loci ITS, *rbcL*, *ndhJ*, *matK*, *rpoB2*, *rpoC1*, *ycf5*, *accD* [55] and (59%) out of the nine other loci *cox1*, 23S rDNA, *rpoB*, *rpoC1*, *rbcL*, *matK*, *atpF-atpH*, *psbK-psbI* [58]. In the big DNA barcoding project of the CBOL Plant Working Group, *trnH-psbA* also showed the highest species discrimination (69%) in comparison with another six loci (*atpF-atpH*, *matK*, *rbcL*, *rpoB*, *rpoC1*, *psbK-psbI*) from 259 samples of 95 species of 34 genera seed plants [7]. Species resolution rate of *trnH-psbA* in other land plant researches was 67.6%, second high among five screening markers *psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, ITS [52].

In *Orchidaceae*, species resolution of this sequence varied from different genera and different studies. Among six markers (*trnH-psbA*, *accD*, *rpoC1*, *rpoB*, *matK* and *ndhJ*), only *trnH-psbA* could resolve 8 of 11 Mesoamerican orchid species (72.7%), which was just lower than *matK* (10/11). Proportion of monophyletic species recovered with UPGMA of *trnH-psbA* were 90.6%, the same as *matK*, and the two highest among eight loci *accD*, *ndhJ*, *matK*, *trnH-psbA*, *rbcL*, *rpoB*, *rpoC1*, and *ycf5* for 172 individuals of 86 species (including 71 individuals of 48 orchid species + other angiosperm species) [57]. On *Dendrobium*, in testing the unique marker *trnH-psbA*, Yao, et al. (2009) calculated the intergenic spacers of all species (0.3 to 2.3%) and the intraspecific variation (0 to 0.1%) [59]. This research's result meant that the

barcoding gap was obvious, and so the locus was evaluated as an effective spacer for barcoding *Dendrobium* species and for differentiating *Dendrobium* species from other adulterating species. But in 2011, the species resolution of *trnH-psbA* on *Dendrobium* was 79.3% over 504 samples (lower than *matK* 88.8%, *atpF-atpH* 82.4%, and *rbcL* 79.8% [63], or 8.14%, which is lower than the ITS, ITS2, and *matK* [60]. On *Holcoglossum* (*Orchidaceae*), 52 individuals belong 12 species were analysed for barcode with six markers *rbcL*, *matK*, *atpF-atpH*, *psbK-psbI*, *trnH-psbA* and ITS. Species resolution of *trnH-psbA* was 5/12 species (using Neighbor-Joining algorithm), equal to ITS 5/12 and lower than *matK* 6/12 [14].

In general, *trnH-psbA* was highly evaluated early-on, but less favourable at a later time due to some obvious problems. The most common complaint was about *trnH-psbA*. That it was generally too difficult to align in land plants [5, 55] as well as in *Orchidaceae*. *trnH-psbA* possesses many indels in their sequences [5, 55, 56]. The mononucleotide (A/T) repeats (or known as homopolymers) and/or small tandem repeats (AT) were frequently noted in this intergenic spacer [3, 7, 55, 56]. The existence of homopolymers (AAAA/TTTTTT) for most non-coding regions, including *trnH-psbA*, interrupted the sequence runs and caused problems with obtaining high-quality bidirectional sequences [3, 7, 58, 64], especially with the forward primer, as in *Dendrobium* [16]. The results were both lower and overlapped between bidirectional reads or only allowed partial sequences to be obtained. This is also another major limitation for this locus. In orchids and some other monocots, beside of these similar problems of indels and repeats [16], the genomic rearrangement of the non-homologous inverted repeat has been also found [15, 55], and especially the insertion of well-conserved exon-copies of *rpl12* and *rps19* (known as pseudogene) [57, 59, 65]. The indels, repeats and inverted repeats likely in *trnH-psbA* sequences made this region

not only significantly complex, but also significantly different in length, and those were the reasons for the difficulty of alignment for analysis. This problem either led to the failure of sequence alignment or the requirement of manual editing [7].

The length of *trnH-psbA* in a wide range of plants is quite short at about 400 bp, to satisfy the criteria of the barcode, which should be short enough for easy amplification [5, 7]. On the other hand, there was research that showed that the short length of *trnH-psbA* makes a lack of information for barcode and phylogenetic analysis [56]. In orchids, the length of *trnH-psbA* are 850 bp in *Dendrobium* (included entire regions *psbA-trnH* 722-785 bp plus regions of *rpl12* - 279 bp and *rps19* - 19 bp) [59], 739 bp in *Holcoglossum* [14]. However, the containing of *rpl12* and *rps19* in orchids and some monocots caused much longer in size of this region up to > 1000 bp and meets the problem of PCR and sequencing [7, 65]. The high difference in length caused multiple bands in few samples of *Dendrobium* that hard to recognise which one is correct *trnH-psbA* segment, and the bad quality sequence made this locus excluded from the analysis [16].

In another point of view, the difficulty of alignment was not a major obstacle [5] compared to the benefit provided by their sequence information. Indels are useful pieces of information for species discrimination, i.e. they could help to distinguish three species of genus *Solidago*, which cannot be separated due to low sequence divergence, and Kress, et al. (2005) hoped for an improvement of DNA barcoding tools for which to utilize the indel information [5]. Thus, this locus is still valuable for many research projects, to be used as potential barcodes, especially with a combination of supplement barcodes, which will be discussed later.

matK: *matK* is the gene coding for the maturase K protein. This is also a rapidly evolving gene [7] that potential as an identification molecular marker

that can be used in many barcoding and phylogenetic studies. This region was evaluated at much higher levels of sequence variations for species discrimination [7, 65]. Lahaye, et al. (2008b) [66] suggested that *matK* was the preferred universal barcode for flowering plants, including orchids and re-affirmed best potential of this region in Lahaye. The results of DNA barcode library for 20 endangered *Orchidaceae* species distributed in Mexico using the barcodes *matK* and *rbcL* showed that single *matK* allowed for the identification of the most orchid species [67]. The species resolution of *matK* was 100% for *Paphiopedilum* [6], 5/5 species for five medicinal *Dendrobium* species [68] and 6/12 for *Holcoglossum* [14] in comparing with other studied single regions.

matK has also been proved to have the same problem as *trnH-psbA* with homopolymer runs of mononucleotide repeats for some taxonomic groups and led to low-quality bidirectional sequences [3, 58, 68]. However this rate was not significant, and only a few samples of *matK* amplification in *Dendrobium* gave multiple bands [16]. But *matK* suffered most by a low amplification success rate. From 96 individuals, at 96 species of 48 genera of land plants, the PCR rate of *matK* was just 39.3%, far lower than other screening loci (*trnH-psbA*, *rbcL*, ITS1, *ndhJ*, *rpoB2*, *rpoC1*, *ycf5*, *accD*) [55]. Fazekas, et al. (2008) used 10 primer pairs for sequencing reactions of *matK*, but the success rate could still not covered all the samples (88%) [58]. The poor PCR recovery might due to the nonuniversal primers.

This problem could be overcome through design improvements or by improving the specific primers [7, 65, 68]. Using the specific primer pairs 390F and 1326R from Cuénoud, et al. [69], it could get 100% amplification of *matK* [57]. But particularly in orchids, the amplification rates of *matK* are quite good. These rate could be up to 100% ([61] - *Oncidiinae*, [62] - Thailand *Cymbidium*, [60] - *Dendrobium*) or at

very high range 95.23% ([6] - Indian *Paphiopedilum*), 92.31% ([14] - *Holcoglossum*), and 99.32% ([16] - *Dendrobium*). Then, *matK* is still a good choice for the barcoding of orchids, especially if good primers could be developed.

rbcL: *rbcL* is the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene in the plastid genome. The single region of *rbcL* was not high favoured because it is too long (1428 bp [5]) and contains highly conserved regions [61]. As with high universality, the amplification rates of *rbcL* were rather good in most of the studies, from 90 to 100% in a broad range of plants [3, 5, 15, 55, 57, 63, 64], and also in orchids [6, 14, 16, 60]. Although *rbcL* could get high-quality bidirectional sequences and well universality [7, 63], this poor variable region could discriminate well at the genus level, but could not show adequate resolution at the species level in plants [3, 5, 64, 70] and in orchids particularly [57]. In contrast with PCR success rates, resolution rates were very low [69.8% - [55], 75% - [64], 79.8% - [63], 58.02% - [15], 26.4% - [3] and not suitable for molecular identification in many *Orchidaceae* species (*Oncidiinae*, *Paphiopedilum*, *Holcoglossum* and *Dendrobium*) [6, 14-16, 25, 61]. However, *rbcL* was highly considered in many combined barcode, as discuss later.

ycf1: Another locus that is “more variable than *matK*” [71] or “any existing plastid candidate barcodes and can serve as a barcode for land plants”, that was *ycf1*, proposed as a barcode by Dong, et al. (2015) [15]. Within the plastid genome, *ycf1* spans the small single copy (SSC) and the inverted repeat (IR) regions. The section of *ycf1* in the IR region is short (less than one kilobase long) and conserved. In contrast, the section of *ycf1* in the SSC region has high sequence variability in seed plants [15]. *ycf1* was known to be absent from some genera, but exists in orchids, including *ycf1a* and *ycf1b*. 357 of 420 tree species could be distinguished using

ycf1b (85%), which was better than any of the *matK*, *rbcL*, and *trnH-psbA* and even slightly better than the core barcode *matK+rbcL* (71.31%) [15]. The reason that this valuable region received little attention for DNA barcoding or molecular systematic purposes at low taxonomic that is *ycf1* is too long (5709 bp in *Nicotiana tabacum*) and is too variable to permit the design of universal primers. However, the high variability of *ycf1* indicates its potential value in DNA barcoding of land plants. Phylogenetic application of *ycf1* had been found for *Orchidaceae* [30, 49, 71, 72] and was evaluated as the most variable and parsimony-informative among five other chloroplast genes (*matK*, *rbcL*, *rpoC1*, *rpoC2* and *ycf2*) [71, 73]. *ycf1* should be more tested in molecular identification of orchids.

atpF-atpH: Recently, Kim, et al. (2015) [74] suggested the intergenic spacer *atpF-atpH* as a barcoding marker after concluding a 100% species discrimination ability of this region on 28 individuals of four species of Korean *Cypripedium-Orchidaceae*. The results obtained from such a set of observations were found, such as sequence variations, species-specific SNPs, indels differences, length variations, and the use of species-specific primers (ARMS method for amplification refractory mutation system). The authors also suggested identification using electrophoresis based on length variations of sequences. *atpF-atpH* has significant length variations among species and was used for molecular identification and phylogenetic study of low taxonomic level plant species, although it does not function independently [15, 21]. However, this region was not suitable for *Holcoglossum* orchid DNA barcoding study [25] and fell for recovery of high-quality bidirectional sequences [7].

rps16-trnQ: Jhong-Yi Lin and his group have explored that the *rps16-trnQ* marker showed the best discrimination power and was considered to be the best DNA barcode in the study. Another 15 of 27 studied cpDNA markers were

also recognised as highly variable among moth orchids, with polymorphic information contents of 8.0 and were suggested to combine with *rps16-trnQ* [75].

***trnL-F*:** *Orchidaceae*, *trnL-F* regions were evaluated to be effective rabbit DNA barcode marker gene [25]. *trnL-F* was also used in many phylogenetic studies of orchid taxa such as *Orphrys*, *Angraecinae*, *Epidendroideae*, *Arethuseae*, *Vandae*, *Bulbophyllum*, *Coryciinae*, *Cypripedium*, *Tangtsinia*, and *Orchiade* [2, 29, 40-42, 76-81].

***psbK-psbI*:** Like *trnH-psbA*, the intergenic spacer *psbK-psbI* showed

good discriminatory power but had the lowest sequencing success in these trials, and substantial problems generating bidirectional reads [7]. The *Orchidaceae*, *psbK-psbI* showed the highest mean to be interspecific at a K2P distance (0.1192), followed by *matK* (0.0803), *atpF-atpH* IGS (0.0648), *trnH-psbA* IGS (0.0460) and *rbcL* (0.0248) [44]. By overcoming the obstacles and difficulties of the intergenic spacer as discussed in *trnH-psbA*, this region can be used as a potential molecular marker for orchids.

Multi-locus barcodes

In an effort to find universal barcodes

for a wide range of plants as well as orchids, it was clear that no single locus could be sufficient in this role for both universality and resolvability, and multi-locus barcodes seem to be more robust and effective. As Kress, et al. (2005) has suggested, it may need more than one locus for species-level discrimination [5].

In 2007, the two-locus barcode *trnH-psbA+rbcL* was first proposed by Kress and Erickson [55] with the species resolution increased to 85% for angiosperms comparing to the highest 82.6% of single locus *trnH-psbA*. Actually, the combination of *trnH-psbA+rpoB* or *trnH-psbA+rpoC1* was 85% too, but the PCR success rate of *rbcL* in the study was higher than *rpoB* and *rpoC1*, and then the totally result of *trnH-psbA+rbcL* was better.

In the same year 2007, Chase, et al. first suggested the three-locus barcodes either of *matK+rpoC1+rpoB* or *matK+rpoC1+trnH-psbA* [65]. The combination of two or three loci of *matK*, *rpoC1*, *rpoB* and *trnH-psbA* was also recommended for 11 Mesoamerican orchid species in this year and the resolution results showed that *matK+rpoC1+rpoB* 100% (discriminate 11/11 species), *matK+rpoC1+trnH-psbA* and *matK+rpoB+trnH-psbA* 90.9% (10/11 species), and the single *matK* 90.9% (10/11 species). In the study of Singh, et al. (2012) on *Dendrobium* (*Orchidaceae*), the combination *matK+rpoC1+rpoB* gave the highest resolution (94.44%) among other three-locus barcodes, just lower than the ITS (100%). From this result, they suggested that “barcodes, if based on the single or limited locus, would be specific taxa” [16]. On the subject *Cymbidium* (*Orchidaceae*), both the two options 1) The *matK+rpoC1+trnH-psbA*, and 2) The *matK+rpoB+trnH-psbA* have succeeded 100% species resolution of 19 *Cymbidium* in Thailand [62]. However, without *rpoC1* or *rpoB*, the two-locus *matK+trnH-psbA* barcode achieved only moderate improvement (90.9%) in comparing with the single *matK*

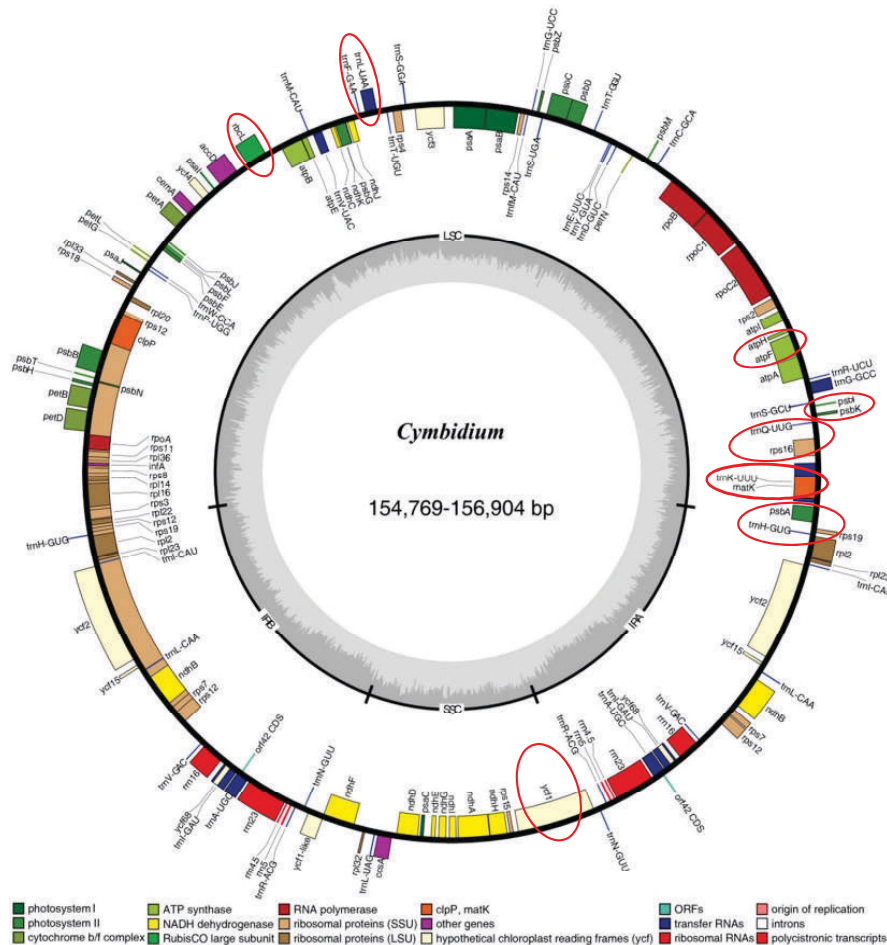


Fig. 2. Gene map of *Cymbidium* chloroplast genome [82] with notations for potential loci for barcoding (With Inverted repeat regions (IRa and IRb); small single copy (SSC) and large single copy (LSC) regions. Genes on the outside of the map are transcribed clockwise and genes on the inside of the map are transcribed counterclockwise. Loci in rectangle shapes are potential markers focused in this review).

resolution (90.6% of 48 orchid species plus 38 angiosperm species) [57].

In 2008, Fazekas, et al. selected a more loci combinations for barcoding 92 species of land plants. A multilocus plant barcoding region should have multiple regions chosen from among three of the coding (*rbcL*, *rpoB*, *matK*) and two of the non-coding regions (*trnH-psbA*, *atpF-atpH*) (61-69%). As all combinations assessed using four to seven regions had only marginally different success rates (69-71%); values that were approached by several two- and three-region combinations (61-69%) [58]. This meant that no single combination clearly outperformed all others. This situation was also proved in the study of Hollingsworth, et al. (2009) [21] with some three-locus combination of *rbcL*, *rpoC1*, *matK*, and *trnH-psbA*.

In 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group first recommended the two-locus combination of *rbcL+matK* as a plant barcode. This combination represented a practical solution to a complex trade-off between universality, sequence quality, discrimination, and cost [7]. Now it is generally agreed that a plant barcode will combine more than one locus (5-7), including a phylogenetically conservative coding locus (*rbcL*) with one or more rapidly evolving regions (partial *matK* gene and the intergenic spacer *trnH-psbA*). Thus Kress, et al.

(2009) tested on three loci *rbcL*, *matK* and *trnH-psbA* whether the use of multilocus supermatrices to generate phylogenetic hypotheses at the species-level would improve the resolution power. The results showed that the core-combining barcode proposed by CBOL *rbcL+matK* just discriminated 92%, while *trnH-psbA+rbcL* did 95%. The three-locus combination *rbcL+matK+trnH-psbA* discriminated 98% [64]. *rbcL+matK* was 93.1% species resolution and *rbcL+matK+trnH-psbA* was 95.3% [63]. Fazekas, et al. (2012) also suggested the combination of *rbcL+matK* as the core barcode with another supplement barcode (ITS or *trnH-psbA*) [83].

In 2010, ITS2 and *psbA-trnH* sequences were highly evaluated, with 93.8% and 23.8% PCR success rates in 1,433 species of 551 genera in 135 families from four phyla (Angiosperms, Gymnosperms, Ferns and Mosses), respectively, while its ITS fragments were only successfully amplified in 42.3% of the experiments; and the identification rate of the *psbA-trnH* region was 96.5% at the genus level using the nearest distance method; 72.8%, at the species level. ITS2+*psbA-trnH* was strongly recommended as a core and complementary barcode for a broad series of plant taxa [52].

On the contrast with CBOL (2009) [7], in the BOL project in 2011 on 1,757 species of seed plants in China,

the core barcode *rbcL+matK* gave the lowest resolution (49.7%) among other two-locus barcodes, the highest discrimination rate of the three-locus barcode was 81.8% by *matK+trnH-psbA*+ITS. *rbcL+matK* has high species identification power at the species level in just some taxonomic groups (e.g. *Orchidaceae*). The project proposed that the ITS or ITS2 should be incorporated into the core barcode (*rbcL*, *matK*) for seed plants [3]. The combination of *matK*+ITS showed a greater ability to identify species than *matK* or the ITS alone in *Holcoglossum* [14], in *Dendrobium* and *Paphiopedilum* [60].

On the subject *Oncidium* genus, a combination of *trnH-psbA+trnF-ndhJ* was proposed as a potential barcode by correct phylogenetic placement of 13/15 *Oncidiinae* hybrid varieties [61]. As intergenic spacers were recognized to be high variable, Kim, et al. (2014) [44] also suggested the combination of three intergenic spacers *atpF-atpH+psbK-psbI+trnH-psbA* as the best option for barcoding of the Korean orchid species, the resolution up to 98.8%, among 26 possible combinations of the five regions *rbcL*, *matK*, *atpF-atpH*, *psbK-psbI* and *trnH-psbA*.

In general, the use of combined barcode could give better resolution in most but not all cases depending on taxon specification (Table 2).

Table 2. Summary of studies comparing DNA barcoding regions in plants.

Study	Studied regions	Samples	Amplification (% success)	Sequence separation (%)	Molecular markers recommendation
Kress, et al. (2005) [5]	ITS, <i>trnH-psbA</i> , <i>atpB-rbcL</i> , <i>psbM-trnD</i> , <i>trnC-ycf6</i> , <i>trnL-F</i> , <i>trnK-rps16</i> , <i>trnV-atpE</i> , <i>rpl36-rps8</i> , <i>ycf6-psbM</i>	Set 1: 19 species/8 genera/7 families of angiosperm	<i>trnH-psbA</i> , <i>rpl136-rpf8</i> , <i>trnL-F</i> } 100% <i>trnC-ycf6</i> , <i>ycf6-psbM</i> } 90% Other regions = 73-80%	Sequence divergence: - ITS (2.81%) - <i>trnH-psbA</i> (1.24%) - <i>rpl136-rpf8</i> , <i>trnL-F</i> (0.44%) - <i>atpB-rbcL</i> (0.63%) - <i>trnC-ycf6</i> (0.55%)	<i>trnH-psbA</i> , ITS
	ITS, <i>rbcL</i> , <i>trnH-psbA</i>	Set 2: 83 individuals/83 species/72 genera/50 families of angiosperm	<i>trnH-psbA</i> = 100%, <i>rbcL</i> = 95%, ITS ≤ 88%		<i>trnH-psbA</i> >> <i>rbcL</i>

Taberlet, et al. (2006) [84]	<i>trnL</i> , P6 loop	more than 100 plant species		<i>trnL</i> 67.3% P6 loop 19.5%	
Kress and Erickson (2007) [55]	<i>trnH-psbA</i> , <i>rbcL</i> , ITS1, <i>ndhJ</i> , <i>matK</i> , <i>rpoB2</i> , <i>rpoC1</i> , <i>ycf5</i> , <i>accD</i> of angiosperms, gymnosperms, ferns, mosses, and liverworts	96 individuals/ 96 species/48 genera/43 families of land plants	<i>trnH-psbA</i> = 95.8% <i>rbcL</i> = 92.7% <i>rpoC1</i> = 83.3% <i>accD</i> , <i>rpoB</i> ≈ 80% <i>ndhJ</i> = 70%, ITS1 = 60.4% <i>ycf5</i> = 50% <i>matK</i> = 39.3%	<i>trnH-psbA</i> (82.6%) ITS (81.5%) <i>rbcL</i> (69.8%) Other loci (≤ 70%) <i>trnH-psbA+rbcL</i> , <i>trnH-psbA+rpoB2</i> , <i>trnH-psbA+rpoC1</i> } (85%) Other pairs of two loci (≤ 82.5%)	Two-locus barcode: <i>trnH-psbA+rbcL</i>
Chase, et al. (2007) [65]					Three-locus barcode: <i>rpoC1+rpoB+matK</i> <i>rpoC1+matK+trnH-psbA</i>
Lahaye, et al. (2008a) [57]	<i>accD</i> , <i>ndhJ</i> , <i>matK</i> , <i>rbcL</i> , <i>trnH-psbA</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>ycf5</i>	172 individuals/86 species (48 orchid species +38 species from 13 angiosperm families)	All other regions = 95-100% (except <i>ycf5</i> and <i>ndhJ</i>)	<i>trnH-psbA</i> , } 90.6% <i>matK</i> <i>matK+trnH-psbA</i> (90.9%) Other loci (≤ 87.5%) All barcodes combine (93.1%)	<i>matK</i> <i>matK+trnH-psbA</i>
Lahaye, et al. (2008b) [66]	<i>accD</i> , <i>ndhJ</i> , <i>matK</i> , <i>rbcL</i> , <i>trnH-psbA</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>ycf5</i> , <i>atpF-atpH</i> , <i>psbK-psbI</i>	101 individuals/38 species			<i>matK</i>
Fazekas, et al. (2008) [58]	<i>cox I</i> , <i>matK</i> , 23S rDNA, <i>rpoB</i> , <i>rpoC1</i> , <i>rbcL</i> , <i>trnH-psbA</i> , <i>atpF-atpH</i> , <i>psbK-psbI</i>	251 individuals/92 species/32 genera of land plants	% sequencing success 23S rDNA, <i>rbcL</i> = 100% (2 primer pairs used) <i>trnH-psbA</i> = 99% <i>rpoC1</i> = 95% (3 primer pairs used) <i>rpoB</i> = 92% (5 primer pairs used) <i>matK</i> = 88% (10 primer pairs used) <i>psbK-psbI</i> = 85% <i>cox I</i> = 72% <i>atpF-atpH</i> = 65%	<i>trnH-psbA</i> (59%) <i>matK</i> (56%) <i>atpF-atpH</i> , <i>psbK-psbI</i> (45%) <i>rbcL</i> , <i>rpoB</i> (42-48%) <i>cox I</i> (10%) 23S rDNA (7%) <i>rpoB+rpoC1</i> (50%) <i>matK+atpF-atpH+psbK-psbI</i> (69%) <i>rbcL+trnH-psbA</i> , <i>matK+atpF-atpH</i> } 64% <i>rpoB+rpoC1+matK</i> (61%)	Combinations of 3-4 loci from: <i>rbcL</i> , <i>rpoB</i> , <i>matK</i> , <i>trnH-psbA</i> , <i>atpF-atpH</i>
CBOL (2009) [7]	<i>atpF-atpH</i> , <i>matK</i> , <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>psbK-psbI</i> , <i>trnH-psbA</i>	907 samples from 550 species genera seed plants	<i>psbK-psbI</i> = 77% all others = 90-98%	<i>rpoC1</i> (38%), <i>rpoB</i> (40%), <i>atpF-atpH</i> (50%), <i>matK</i> (57%), <i>rbcL</i> (58%), <i>trnH-psbA</i> (58%) <i>psbK-psbI</i> (64%) 2-locus combinations (59-75%) 3-locus combinations (65-76%) All 7 loci combination (73%) <i>rbcL+matK</i> (72%)	<i>rbcL+matK</i>
Hbllingsworth, et al. (2009) [21]	<i>atpF-atpH</i> , <i>matK</i> , <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>psbK-psbI</i> , <i>trnH-psbA</i>				some combination of <i>rbcL</i> , <i>rpoC1</i> , <i>matK</i> , <i>trnH-psbA</i> .

Kress, et al. (2009) [64]	<i>rbcL</i> , <i>matK</i> , <i>trnH-psbA</i>	1,035 samples/296 species/181 genera of plants	<i>rbcL</i> = 93% <i>trnH-psbA</i> = 94%, but problems sequencing <i>matK</i> = 69%	<i>matK</i> (99%) <i>trnH-psbA</i> (95%) <i>rbcL</i> (75%) <i>matK+rbcL</i> (92%) <i>trnH-psbA+rbcL</i> (95%) <i>rbcL+matK+trnH-psbA</i> (98%) When both sequences recovery and correct assignment were taken into account: Of the 286 species <i>trnH-psbA</i> (90%) <i>rbcL</i> (70%) <i>matK</i> (69%)	<i>rbcL+matK+trnH-psbA</i>
Yao, et al. (2010) [54]	ITS2	50,790 plant and 12,221 animal ITS2 sequences GenBank		Dicotyledons (76.1%) monocotyledons (74.2%) gymnosperms (67.1%) ferns (88.1%) mosses (77.4%) animals (91.7%)	ITS2
Chen, et al. (2010) [52]	<i>psbA-trnH</i> , <i>matK</i> , <i>rbcL</i> , <i>rpoCl</i> , <i>ycf5</i> , ITS2, ITS	5,905 species/1,010 genera/219 families/7 phyla (Angiosperms, Gymnosperms, Ferns, Mosses, Liver-worts, Algae and Fungi)	<i>psbA-trnH</i> = 92.8% ITS2 = 93.8% ITS = 42.3%	At specie level: ITS2 (92.7%) <i>psbA-trnH</i> (67.6%) At genus level: ITS2 (99.8%) <i>psbA-trnH</i> (> 95%)	ITS2 or ITS2+ <i>psbA-trnH</i>
BOL (2011) [3]	<i>rbcL</i> , <i>matK</i> , <i>trnH-psbA</i> , ITS	1,757 species/141 genera/75 families/42 orders seed plants	<i>rbcL</i> = 94.5%, <i>matK</i> = 91.0%, <i>trnH-psbA</i> = 90.2%, ITS = 88.0%	ITS (67.2%) ITS2 (54.6%) <i>rbcL</i> (26.4%) <i>trnH-psbA</i> +ITS (79.1%) <i>trnH-psbA</i> +ITS2 (69.7%) <i>matK</i> +ITS (75.3%) <i>matK</i> +ITS2 (66.1%) <i>rbcL</i> +ITS (69.9%) <i>rbcL</i> +ITS2 (58.5%) <i>rbcL+matK</i> (49.7%) <i>matK+trnH-psbA</i> +ITS (81.8%) <i>matK+trnH-psbA</i> +ITS2 (75.0%) <i>rbcL+matK</i> +ITS (77.4%) <i>rbcL+matK</i> +ITS2 (68.5%) <i>rbcL+matK+trnH-psbA</i> (62.0%) <i>rbcL+matK+trnH-psbA</i> +ITS 82.8% <i>rbcL+matK+trnH-psbA</i> + ITS2 (77.2%)	ITS/ITS2 supplement to core barodes <i>rbcL</i> , <i>matK</i>
Burgess, et al. (2011) [63]	<i>rbcL+matK</i> , <i>rpoCl</i> , <i>trnH-psbA</i> , <i>atpF-atpH</i>	2,130 sequences/436 species/269 genera of land plants	<i>rbcL</i> = 91.4% <i>rpoCl</i> = 74.5%	<i>matK</i> (88.8%) <i>atpF-atpH</i> (82.4%) <i>rbcL</i> (79.8%) <i>trnH-psbA</i> (79.3%) <i>rpoCl</i> (73.1%) <i>rbcL+matK</i> (93.1%) <i>rbcL+matK+trnH-psbA</i> (95.3%) Combination 5 loci (97.3%)	<i>rbcL+matK</i>
Fazekas, et al. (2012) [83]					Core [<i>matK+rbcL</i>] + supplements (ITS, <i>trnH-psbA</i>)
Dong, et al. (2012) [85]	23 loci present in at least three genera				<i>ycf1-a</i> , <i>trnK</i> , <i>rpl32-trnL</i> , <i>trnH-psbA</i> , followed by <i>trnSUGA-trnGUCC</i> , <i>petA-psbJ</i> , <i>rps16-trnQ</i> , <i>ndhC-trnV</i> , <i>ycf1-b</i> , <i>ndhF</i> ,

					<i>rpoB-trnC</i> , <i>psbE-petL</i> , and <i>rbcL-accD</i> at genus level
Han, et al. (2013) [23]	ITS, ITS2	Set 1: 91 species/5 orders dry medicinal product and herbarium specimens Set 2: 12861 ITS and ITS2 sequences/8,313 species/8,313 species from 1699 genera, GenBank	ITS2 = 91% ITS = 23%	At the species level: ITS (89.2%) ITS2 (79.2%) At the genus level: ITS (97.5%) ITS2 (93.8%)	ITS2
Dong, et al. (2015) [15]	<i>ycfI</i> (<i>ycfIa</i> , <i>cfIb</i>), <i>rbcL</i> , <i>matK</i> , <i>trnH-psbA</i>	1352 sequences of <i>matK</i> , <i>rbcL</i> and <i>ycfI</i> from 420 species/179 genera/76 plant families.	<i>rbcLb</i> = 99.18%, <i>matK</i> = 91.43%, <i>ycfIb</i> = 85.31%	<i>ycfIb</i> (73.97%) <i>rbcLb</i> (58.02%) <i>matK</i> (57.56%) <i>rbcLb+matK</i> (71.31%) <i>ycfIb+rbcLb</i> (81.39%) <i>ycfIb+matK</i> (79.83%) <i>ycfIb+rbcLb+matK</i> (86.33%)	<i>ycfI</i> (<i>ycfIa</i> , <i>ycfIb</i>)
		7 relatively well-sampled plant groups		<i>ycfIb</i> highest	
Gigot, et al. (2007) [86]	11 Mesoamerican orchid species	<i>trnH-psbA</i> , <i>accD</i> , <i>rpoC1</i> , <i>rpoB</i> , <i>matK</i> , <i>ndhJ</i>	All = 100%	<i>accD</i> 3/11 (27.3%) <i>matK</i> 10/11 (90.9%) <i>ndhJ</i> 1/11 (9.1%) <i>rpoB</i> 6/11 (54.5%) <i>rpoC1</i> 4/11 (36.4%) <i>trnH-psbA</i> 8/11 (72.7%) <i>rpoC1+rpoB+matK</i> 11/11 (100%) <i>rpoC1+matK+trnH-psbA</i> 10/11 (90.9%) <i>rpoB+matK+trnH-psbA</i> 10/11 (90.9%)	Combination 2 or 3 of <i>rpoC1</i> , <i>rpoB</i> , <i>matK</i> , <i>trnH-psbA</i>
Yao, et al. (2009) [59]	<i>psbA-trnH</i>	17 <i>Dendrobium</i> species, 1 adulterance	<i>psbA-trnH</i> = 100%	Intergenic variation of all species 0.3 to 2.3% Intraspecific variation 0 to 0.1%	<i>psbA-trnH</i>
Wu, et al. (2010) [61]	<i>trnH-psbA</i> , <i>matK</i> , <i>trnF-ndhJ</i> , <i>ycfI-trnR</i> , <i>accD</i> , <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i>	15 Oncidiinae hybrid varieties	All = 100%	Correct phylogenetic placement of 13/15 varieties	<i>trnH-psbA+trnF-ndhJ</i>
Parveen, et al. (2012) [6]	ITS, <i>matK</i> , <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i>	8 species + 3 hybrids <i>Paphiopedilum</i>	ITS, <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i> } 100% <i>matK</i> = 95.23%	<i>matK</i> (100%) ITS (50%) <i>rbcL</i> (25%) <i>rpoB</i> , <i>rpoC1</i> (12.5%)	<i>matK</i>
Xiang, et al. (2011) [14]	<i>rbcL</i> , <i>matK</i> , <i>atpF-atpH</i> , <i>psbK-psbI</i> , <i>trnH-psbA</i> , ITS of <i>Holcoglossum</i>	12 species	<i>rbcL</i> = 100% <i>matK</i> = 92.31% ITS = 100% <i>trnH-psbA</i> = 100% <i>atpF-atpH</i> (low) <i>psbK-psbI</i> (low)	<i>rbcL</i> lowest <i>matK</i> 6/12 ITS 5/12 <i>trnH-psbA</i> 5/12 <i>matK</i> +ITS 7/12 <i>matK+trnH-psbA</i> 6/12 ITS+ <i>trnH-psbA</i> 6/12 <i>matK</i> +ITS+ <i>trnH-psbA</i> 7/12	<i>matK</i> or <i>matK</i> +ITS/ITS2

Wu, et al. (2012) [20]	ITS	11 <i>Dendrobium</i> , 2 adulterant species	ITS = 100%	100%	ITS
Chiang, et al. (2012) [19]	ITS	20 <i>Dendrobium</i> species	ITS = 100%		ITS
Singh, et al. (2012) [16]	<i>matK</i> , <i>rbcL</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>trnH-psbA</i> , ITS	Set 1: 292 individuals/36 species <i>Dendrobium</i> Set 2: 52 species (36 studied species + Genbank)	<i>rpoC1</i> = 100% <i>matK</i> = 99.32% <i>rpoB</i> = 99.2% ITS = 98.97% <i>rbcL</i> = 96.91%	ITS (100%) <i>matK</i> (80.56%) <i>rpoB</i> (55.56%) <i>rbcL</i> (41.67%) <i>rpoC1</i> (38.89%) <i>matK+rpoB+rpoC1</i> (94.44%) <i>matK+rbcL</i> (86.11 %) ITS (100%) <i>matK</i> (76.92%) <i>rpoB</i> (51.2%) <i>rpoC1</i> (42.31%) <i>rbcL</i> (38.46%) <i>matK+rpoB+rpoC1</i> (92.31 %) <i>matK+rbcL</i> (80.77%)	ITS, <i>matK+rpoB+rpoC1</i>
Siripiyasin, et al. (2012) [62]	<i>matK</i> , <i>rpoB</i> , <i>rpoC1</i> , <i>trnH-psbA</i>	19 species <i>Cymbidium</i> Thailand	All = 100%	All 100% species resolution	<i>trnH-psbA+matK+rpoC1</i> <i>trnH-psbA+matK+rpoB</i>
Yukawa, et al. (2013) [26]	4 genetic units in <i>Grammatophyllumsp eciosum</i> complex	ITS	All = 100%	Discriminate 4 different species of <i>Grammatophyllum</i>	ITS
Kim, et al. (2014) [44]	<i>rbcL</i> , <i>matK</i> , <i>atpF-atpH</i> , <i>psbK-psbI</i> and <i>trnH-psbA</i>	89 species of Orchidaceae	All = 100%	<i>trnH-psbA</i> (83.5%) <i>rbcL</i> (60.5%) <i>atpF-atpH+psbK-psbI+trnH-psbA</i> (98.8%)	<i>atpF-atpH+psbK-psbI+trnH-psbA</i>
Feng, et al. (2015) [51]	ITS2	Set 1: 64 species from <i>Dendrobium</i>	ITS2 = 100%	85.9% (by BLAST1 method), 82.8% (by nearest genetic distance method)	ITS2
	<i>trnN-rpl32</i> , <i>petN-psbM</i> , <i>petA-psbJ</i> , <i>trnF-ndhJ</i> , <i>trnE-trnT</i> , <i>accD-psaI</i> , <i>rps15-ycf1</i> , <i>psbA-trnK</i> , <i>atpF</i>	2 species <i>Phalaenopsisaphrodite</i> subsp. <i>Formosana</i> and <i>P. amabilis</i>	All = 100%		<i>trnN-rpl32</i> , <i>petN-psbM</i> , <i>petA-psbJ</i> , <i>trnF-ndhJ</i> , <i>trnE-trnT</i> , <i>accD-psaI</i> , <i>rps15-ycf1</i> , <i>psbA-trnK</i> , <i>atpF</i>
Lin, et al. (2015) [75]	<i>petN-psbM</i> , <i>petA-psbJ</i> , <i>trnT-psbD</i> , <i>trnF-ndhJ</i> , <i>trnN-rpl32</i> , <i>rps16-trnQ</i> , <i>rps16</i>	19 moth orchids species	All = 100%	<i>petN-psbM</i> 16/19 <i>petA-psbJ</i> 16/19 <i>trnT-psbD</i> 16/19 <i>trnF-ndhJ</i> 16/19 <i>trnN-rpl32</i> 16/19 <i>rps16-trnQ</i> 19/19 <i>rps16</i> 15/19 <i>trnL</i> 18/19	<i>rps16-trnQ</i>
Kim, et al. (2015) [74]	<i>rpoC2</i> , <i>atpF-atpH</i>	4 species of <i>Cypripedium</i>	<i>rpoC2</i> = 100% <i>atpF-atpH</i> = 100%	100%	<i>atpF-atpH</i>
Xu, et al. (2015) [60]	ITS, ITS2, <i>matK</i> , <i>rbcL</i> , <i>trnH-psbA</i>	Set 1: 184 species <i>Dendrobium</i>	All = 100%	ITS (31.93%) ITS2 (22.29%) <i>matK</i> (10.48%) <i>trnH-psbA</i> (8.14%) <i>rbcL</i> (5.56%) ITS+ <i>matK</i> (76.92%) ITS+ <i>matK+trnH-psbA</i> (73.13%) ITS2+ <i>matK</i> (64.84%) <i>matK+rbcL</i> (24%)	ITS+ <i>matK</i>

Some measurements for evaluating effects of molecular markers

Different metrics to evaluate the molecular markers are usually discussed in reference studies. It often suggests that the sequence lengths should be short enough (400-800 bp) for DNA extraction, amplification, and sequencing, but certainly must be long enough to contain sufficient information for sequence divergence [14, 57]. The sequence should possess conserved flanking sites for developing universal PCR primers [55] but routinely retrievable with a single primer pair [7]. Easy alignment is also one of the considered criteria [57] although in the situation of *trnH-psbA* and some other intergenic spacers which are known as so variable that hard to align, the difficulty of alignment is not a major obstacle when comparing with their advantages of variation sites [5].

The most concerned factor to identifying loci is good discriminatory power [3, 14, 16, 62]. This power is either based on sequence divergence or variability [5, 55, 57]. The potential parsimony-informative characters or known as nucleotide substitutions are the ones that much contribute to the divergence between sequences [56, 74, 83]. The one with the most features used to measure significant species-level genetic variability and divergence is “DNA barcoding gap”, which is presented between intra- and inter-specific variations. High interspecific, but low intraspecific divergence, are expected to achieve maximal species discrimination sequencing [6, 14, 16, 20, 26, 51, 54, 57, 59, 60, 62, 86]. Indel fragments (insertions and deletions) also contain much useful information for identification work [20], e.g. it can help to distinguish three species of genus *Solidago* despite low sequence divergence [5]. Nucleotide substitutions account for about 70% and indels account for about 30% of all mutations in the chloroplast genome [56]. However, this information is still not used effectively for available bioinformatics tools. The relative amounts of indels need to be

further tested [56, 74]. Bioinformatics tools for barcoding should be developed to use indel information [5].

Some studies are also concerned about the GC content of the sequences [20, 54]. GC content is found to be variable with different organisms. DNA with high GC-content is more stable than DNA with low GC-content (Mega net/help). Sequence length variation is also a helpful feature in some cases [54, 74, 75]. Kim, et al. (2015) even suggests using electrophoresis to identify species based on length variation [74]. PCR-based (multiplex and ARMS) method which determine specific SNPs has been used in analyses of sequence taxonomy [19, 74]. The secondary structure of the ITS2 region could provide useful information for species identification and could be considered as a molecular morphological characteristic [54].

To achieve optimal effect for barcoding, the utilization of many different pieces information is necessarily, such as barcode setting gap, length variation, indel variation... DNA barcodes can be very effective in the context of a clearly circumscribed floristic sample or plant community, and that additional data, such as geography and morphology may be required to obtain higher rates of species identification in other contexts [64].

Conclusions

Since the last classification of *Orchidaceae* in 2003, there has been major progress in the determination of relationships, despite that almost all of the problematic placements recognised in the previous classification 11 years ago have now been resolved by molecular methods [9]. However barcoding for the identification of plants, as well as orchid species, still faces many problems and needs improvement. These improvements are now continued to achieve by different ways. New DNA regions more potential and suitable that can overcome the available limits are ongoing investigated. Complete sequenced DNA genomes are used as

references to screen for a new locus, from plastid genome, to mitochondrial genome and to nucleus genome.

The combinations of multi-locus barcodes are now highly considered as one of the improvement solutions to obtain the best resolution results. Many factors should be cared about such as how many and which ones would be combined. The final aim is to both maximise the loci to get the best efficiency, and minimise the loci to decrease cost and time (e.g. selection of a 2-locus barcode is based on costs and can prevent further delays in implementing a standard barcode for land plants) [7]. The selections of combining loci depend on the characteristics of each locus. No single locus has shown high levels of universality and resolvability [21], and no single barcoding region has an ability to resolve species to the same degree as nearly any of the multilocus barcoding methods [58]. The combination may include a phylogenetically conservative coding locus (*rbcL*) with one or more rapidly evolving regions (part of the *matK* gene and the intergenic spacer *trnH-psbA*). Chloroplast genome sequences contain regions that are highly variable, and this variability of chloroplast genes differs markedly among genera [75, 85]. However, primers designing for these intergenic regions are the challenges with barcodes which, if based on the single or limited locus, would be specific taxa. So Singh, et al. (2012) [16] have recommended the use of whole chloroplast genome as single locus barcode in future will help. Improve the effective of available potential markers which low amplification rate with specific primers was one of the choices, e.g. *matK* much higher levels of sequence variation and so possess high ability of species discrimination, but need improve PCR primers [57]; in some cases, single- or multiple-primer sets are necessary [3, 87].

To accurately determine the relationship between either the species or higher taxonomic level,

the molecular markers used should be able to clearly separate studied taxa at first. Then the barcoding markers are closely related to phylogenetic markers. Developing resolution of molecular for authentication of taxa means developing reliability of phylogenetic study; and conversely, many barcoding studies used the phylogenetic tree as one of the metrics to measure the discrimination ability of the molecular regions. As our ranking based on a small statistic from about 50 phylogenetic references in this research, the most used locus is the ITS (80%), following by *matK* (46%), *trnL-F* (28%), *rbcL* (24%), *trnL* (20%), *trnH-psbA* (14%), *ycf1* (8%), *Xdh*, *trnS-G*, *trnK*, *atpI-atpH* (6%), and some other regions (lower than 5%).

REFERENCES

- [1] M.W. Chase, K.M. Cameron, J.V. Freudenstein, A.M. Pridgeon, G. Salazar, C. Van Den Berg, A. Schuiteman (2015), "An updated classification of *Orchidaceae*", *Botanical Journal of the Linnean Society*, **177**(2), pp.151-174.
- [2] X.G. Xiang, D.Z. Li, W.T. Jin, H.L. Zhou, J.W. Li, et al. (2012), "Phylogenetic placement of the enigmatic orchid genera *Thaia* and *Tangtsinia*: Evidence from molecular and morphological characters", *Taxon*, **61**(1), pp.45-54.
- [3] BOL China Plant Group (2011), "Comparative analysis of a large dataset indicates that ITS should be incorporated into the core barcode for seed plants", *Proceedings of the National Academy of Sciences of the United States of America*, **108**, pp.19641-19646.
- [4] H.M. Kim, S.H. Oh, G.S. Bhandari, C.S. Kim, C.W. Park (2014), "DNA barcoding of *Orchidaceae* in Korea", *Mol. Ecol. Resour.*, **14**(3), pp.499-507.
- [5] W.J. Kress, K.J. Wurdack, E.A. Zimmer, L.A. Weigt, D.H. Janzen (2005), "Use of DNA barcodes to identify flowering plants", *Proceedings of the National Academy of Sciences of the United States of America*, **102**, pp.8369-8374.
- [6] I. Parveen, H.K. Singh, S. Raghuvanshi, U.C. Pradhan, S.B. Babbar (2012), "DNA barcoding of endangered Indian *Paphiopedilum* species", *Mol. Ecol. Resour.*, **12**(1), pp.82-90.
- [7] CBOL Plant Working Group (2009), "A DNA barcode for land plants", *Proc. Natl. Acad. Sci. U.S.A.*, **106**(31), pp.12794-12797.
- [8] P.D.N. Hebert, A. Cywinska, S.L. Ball, J.R. De Waard (2003), "Biological identifications through DNA barcodes", *Proc. Biol. Sci.*, **270**(1512), pp.313-321.
- [9] V.S. Shneyer (2009), "DNA barcoding is a new approach in comparative genomics of plants", *Russ. J. Genetics.*, **45**(11), pp.1267-1278.
- [10] K.H. Wolfe, W.H. Li, P.M. Sharp (1987), "Rates of Nucleotide Substitution Vary Greatly among Plant Mitochondrial, Chloroplast, and Nuclear DNAs", *Proc. Natl. Acad. Sci.*, **84**(24), pp.9054-9058.
- [11] G. Drouin, H. Daoud, J. Xia (2008), "Relative Rates of Synonymous Substitutions in the Mitochondrial, Chloroplast and Nuclear Genomes of Seed Plants", *Mol. Phylogenet. Evol.*, **49**(3), pp.827-831.
- [12] Y. Cho, Y.L. Qiu, P. Kuhlman, J.D. Palmer (1998), "Explosive Invasion of Plant Mitochondria by a Group I Intron", *Proc. Natl. Acad. Sci. USA*, **95**(24), pp.14244-14249.
- [13] M.E. Mort, J.K. Archibald, C.P. Randle, et al. (2007), "Inferring Phylogeny at Low Taxonomic Levels: Utility of Rapidly Evolving cpDNA and Nuclear ITS Loci", *Am. J. Bot.*, **94**(2), pp.173-183.
- [14] X.G. Xiang, H. Hu, W. Wang, X.H. Jin (2011), "DNA barcoding of the recently evolved genus *Holcoglossum* (*Orchidaceae*: *Aeridinae*): A test of DNA barcode candidates", *Molecular Ecology Resources*, **11**(6), pp.1012-1021.
- [15] W. Dong, C. Xu, C. Li, J. Sun, Y. Zuo, S. Shi, T. Cheng, J. Guo, S. Zhou (2015), "*ycf1*, the most promising plastid DNA barcode of land plants", *Scientific Reports*, **5**, p.8348.
- [16] H.K. Singh, I. Parveen, S. Raghuvanshi, S.B. Babbar (2012), "The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of *Dendrobium* species", *BMC Research Notes*, **5**, p.42.
- [17] M.W. Chase, M.F. Fay (2009), "Barcoding of plants and fungi", *Science*, **325**(5941), pp.682-683.
- [18] M.Z. Huang (2010), *DNA Barcoding of Hainan Island Orchid Species Based on matK and ITS Sequences*, <http://mt.china-papers.com/1/?p=185765>.
- [19] C.H. Chiang, T.A. Yu, S.F. Lo, C.L. Kuo, W. Peng, H.S. Tsay (2012), "Molecular authentication of *Dendrobium* species by multiplex polymerase chain reaction and amplification refractory mutation system analysis", *J. Am. Soc. Hortic. Sci.*, **137**(6), pp.438-444.
- [20] C.T. Wu, S.K. Gupta, A.Z.M. Wang, S.F. Lo, C.L. Kuo, Y.J. Ko, C.L. Chen, C.C. Hsien, H.S. Tsay (2012), "Internal transcribed spacer sequence based identification and phylogenetic relationship of herba *Dendrobii*", *J. Food Drug Anal.*, **20**(1), pp.143-151.
- [21] M.L. Hollingsworth, A. Clark, L.L. Foerrest, et al. (2009), "Selecting barcoding loci for plants: Evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants", *Molecular Ecology Resources*, **9**(2), pp.439-457.
- [22] I. Alvarez, J.F. Wendel (2003), "Ribosomal ITS sequences and plant phylogenetic inference", *Mol. Phylogenet. Evol.*, **29**(3), pp.417-434.
- [23] J.P. Han, Y. Zhu, X. Chen, et al. (2013), "The short ITS2 sequence serves as an efficient taxonomic sequence tag in comparison with the full-length ITS", *BioMed Research International*, pp.1-7.
- [24] C.L. Morrison, K. Hovatter, M. Eackles, A.P. Spidle, T.L. King (2005), "Molecular identification of Cypripedioid orchids in international trade", *Selbyana*, **26**(12), pp.196-216.
- [25] H. Hao (2010), *DNA Barcoding and Molecular Phylogeny of Holcoglossum Schltr. (Orchidaceae)*, <http://www.dissertationtopic.net/doc/364760>.
- [26] T. Yukawa, A. Kinoshita, N. Tanaka (2013), "Molecular identification resolves taxonomic confusion in *Grammatophyllum speciosum* complex (*Orchidaceae*)", *Bulletin of the National Museum of Nature and Science*, **39**(3), pp.137-145.
- [27] S. Bernados, M.A. Santos, D. Tyteca, F. Amich (2006), "Phylogenetic relationships of Mediterranean Neottieae and Orchideae (*Orchidaceae*) inferred from nuclear ribosomal ITS sequences", *Acta Botanica Gallica*, **153**(2), pp.153-165.
- [28] J.M. Burke, M.J. Bayly, P.B. Adams, P.Y. Ladiges (2008), "Molecular phylogenetic analysis of *Dendrobium* (*Orchidaceae*), with emphasis on the Australian section *Dendrocoryne*, and implications for generic classification", *Aust. Syst. Bot.*, **21**(1), pp.1-14.
- [29] D.L. Szlachetko, P. Tukałło, J. Mytnik-Ejsmont, E. Grochowska (2013), "Reclassification of the *Angraecum*-alliance (*Orchidaceae*, *Vandoidae*) based on molecular and morphological data", *Biodiv. Res. Conserv.*, **29**, pp.1-23.
- [30] G. Sramkó, V.A. Molnár, J.A. Hawkins, R.M. Bateman (2014), "Molecular phylogeny and evolutionary history of the Eurasiatic orchid genus *Himantoglossum* s.l. (*Orchidaceae*)", *Ann. Bot. (Oxford)*, **114**(8), pp.1609-1626.
- [31] K. Srikuhnath, S. Sawasdechai, T.K. Jantapanon, P. Pongtongkam, S. Peyachoknagul (2015), "Phylogenetic Relationship of *Dendrobium* Species in Thailand Inferred from Chloroplast *matK* Gene and Nuclear rDNA ITS Region", *The Horticulture Journal*, **84**(3), pp.243-252.
- [32] K.H. Trung, T.D. Khanh, L.H. Ham, T.D. Duong, N.T. Khoa (2013), "Molecular Phylogeny of the Endangered Vietnamese *Paphiopedilum* Species Based on the Internal Transcribed Spacer of the Nuclear Ribosomal DNA", *Advanced Studies in Biology*, **5**(7), pp.337-346.
- [33] J.A.N. Batista, K.S. Borges, M.W.F. De Faria, K. Proite, A.J. Ramalho, et al. (2013), "Molecular phylogenetics of the species-rich genus *Habenaria* (*Orchidaceae*) in the New World based on nuclear and plastid DNA sequences", *Molecular Phylogenetics and Evolution*, **67**(1), pp.95-109.
- [34] X.G. Xiang, A. Schuiteman, D.Z. Li, W.C. Huang, S.W. Chung, J.W. Li, H.L. Zhou, W.T. Jin, Y.J. Lai, Z.Y. Li, et al. (2013), "Molecular systematics of *Dendrobium* (*Orchidaceae*, *Dendrobieae*) from mainland Asia based on plastid and nuclear sequences", *Mol. Phylogenet. Evol.*, **69**(3), pp.950-960.
- [35] W.T. Jin, X.H. Jin, A. Schuiteman, D.Z. Li, X.G. Xiang, W.C. Huang, J.W. Li, L.Q. Huang (2014), "Molecular systematics of subtribe *Orchidinae* and Asian taxa of *Habenariinae* (*Orchideae*, *Orchidaceae*) based on plastid *matK*, *rbcL* and nuclear ITS", *Mol. Phylogenet. Evol.*, **77**, pp.41-53.
- [36] W.M. Whitten, M.A. Blanco, N.H. Williams, S. Koehler, G. Carnevali, R.B. Singer, L. Endara, K.M. Neubig (2007), "Molecular phylogenetics of *Maxillaria* and related genera (*Orchidaceae*: *Cymbidieae*) based on combined molecular data sets", *Amer. J. Bot.*, **94**(11), pp.1860-1889.
- [37] M. Novello, M. Beyer, E.A. Veasey, S. Koehler (2013), *Molecular phylogeny of the Brazilian Atlantic forest orchid genus *Brasiliorchis**, <https://atbc.confex.com/atbc/2013/webprogram/Paper2086>.
- [38] F. Martos, S.D. Johnson, C.I. Peter, B. Bytebier (2014), "A molecular phylogeny reveals paraphyly of the large genus *Eulophia* (*Orchidaceae*): A case for the reinstatement of *Orthochilus*", *International Association for Plant Taxonomy*, **63**(1), pp.9-23.
- [39] M.H. Li, G.Q. Zhang, Z.J. Liu, S.R. Lan (2014), "Revision of *Hygrochilus* (*Orchidaceae*: *Epidendroideae*: *Aeridinae*) and a molecular phylogenetic analysis", *Phytotaxa*, **159**(4), pp.256-268.
- [40] Y. Tang, T. Yukawa, R.M. Bateman, H. Jiang, H. Peng (2015), "Phylogeny and classification of the East Asian *Amitostigma* alliance (*Orchidaceae*: *Orchideae*) based on six DNA markers", *BMC Evolutionary Biology*, **15**, p.96.
- [41] M. Soliva, A. Kocyan, A. Widmer (2001), "Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (*Orchidaceae*) based on nuclear and chloroplast DNA sequences", *Mol. Phylogenet. Evol. In press*, **20**(1), pp.78-88.
- [42] G.A. Fischer, B. Gravendeel, A. Sieder, J. Andriantiana, P. Heiselmayr, P.J. Cribb, E.C. Smidt, R.

- Samuel, M. Kiehn (2007), "Evolution of resupination of Madagascar species of *Bulbophyllum* (Orchidaceae)", *Molecular Phylogenetics and Evolution*, **45**(1), pp.358-376.
- [43] A. Chochai, I.J. Leitch, M.J. Ingrouille, M.F. Fay (2012), "Molecular phylogenetics of *Paphiopedilum* (Cyrtipedioideae, Orchidaceae) based on nuclear ribosomal ITS and plastid sequences", *Botanical Journal of the Linnean Society*, **170**(2), pp.176-196.
- [44] H.M. Kim, S.H. Oh, G.S. Bhandari, C.S. Kim, C.W. Park (2014), "DNA barcoding of Orchidaceae in Korea", *Mol. Ecol. Resour.*, **14**(3), pp.499-507.
- [45] W.M. Whitten, N.H. Williams, M.W. Chase (2000), "Subtribal and generic relationship of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: Eombined molecular evidence", *American Journal of Botany*, **87**(12), pp.1842-1856.
- [46] H. Topik, P.H. Weston, T. Yukawa, M. Ito (2012), "Phylogeny of subtribe aeridiinae (Orchidaceae) inferred from DNA sequences data: Advanced analyses including Australasian genera", *Jurnal Teknologi (Sci. Eng.)*, **59**(1), pp.87-95.
- [47] M. Moudi, R. Go (2015), "Monophyly of four sections of genus *Dendrobium* (Orchidaceae): Evidence from nuclear ribosomal DNA internal transcribe spacer (ITS) sequences", *International Journal of Bioassays*, **4**(1), pp.3622-3626.
- [48] M. Moudi, C.S.Y. Yong, M.N. Saleh, J.O. Abdullah, R. Go (2013), "Phylogenetic analysis among four sections of genus *Dendrobium* Sw. (Orchidaceae) in Peninsular Malaysia using *rbcl* sequence data", *International Journal of Bioassays*, **2**(6), pp.932-937.
- [49] L. Li, D.P. Ye, M. Niu, H.F. Yan, T.L. Wen, S.J. Li (2015), "Thunioopsis: A New Orchid Genus and Phylogeny of the Tribe Arethuseae (Orchidaceae)", *PLoS ONE*, **10**(8), e0132777.
- [50] S.K. Sharma, J. Dkhar, S. Kumaria, P. Tandon, R.S. Rao (2012), "Assessment of phylogenetic inter-relationships in the genus *Cymbidium* (Orchidaceae) based on internal transcribed spacer region of Rdn", *Gene*, **495**(1), pp.10-15.
- [51] S. Feng, Y. Jiang, S. Wang, M. Jiang, Z. Chen, Q. Ying, H. Wang (2015), "Molecular Identification of *Dendrobium* Species (Orchidaceae) Based on the DNA Barcode ITS2 Region and Its Application for Phylogenetic Study", *Int. J. Mol. Sci.*, **16**(9), pp.21975-21988.
- [52] S.L. Chen, H. Yao, J.P. Han, et al. (2010), "Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species", *PLoS ONE*, **5**(1), e8613.
- [53] D.T. Lau, P.C. Shaw, J. Wang, P.P. But (2001), "Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA", *Planta Med.*, **67**(5), pp.456-460.
- [54] H. Yao, J. Song, C. Liu, et al. (2010), "Use of ITS2 region as the universal DNA barcode for plants and animals", *PLoS ONE*, **5**(10), e13102.
- [55] W.J. Kress, D.L. Erickson (2007), "A two-locus global DNA barcode for land plants: The coding *rbcl* gene complements the non-coding *trnH-psbA* spacer region", *PLoS One*, **2**(6), e508.
- [56] J. Shaw, E.B. Lickey, E.E. Schilling, R.L. Small (2007), "Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetics studies in angiosperms: The tortoise and the hare III", *American Journal of Botany*, **94**(3), pp.275-288.
- [57] R. Lahaye, M. Van Der Bank, D. Bogarin, et al. (2008a) "DNA barcoding the floras of biodiversity hotspots", *Proceedings of the National Academy of Sciences*, **105**(8), pp.2923-2928.
- [58] A.J. Fazekas, K.S. Burgess, P.R. Kesanakurti, et al. (2008), "Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well", *PLoS ONE*, **3**(7), e2802.
- [59] H. Yao, J.Y. Song, X.Y. Ma, et al. (2009), "Identification of *Dendrobium* species by a candidate DNA barcode sequence: The chloroplast *psbA-trnH* intergenic region", *Planta Medica*, **75**(6), pp.667-669.
- [60] S. Xu, D. Li, J. Li, X. Xiang, W. Jin, W. Huang, X. Jin, L. Huang (2015), "Evaluation of the DNA barcodes in *Dendrobium* (Orchidaceae) from mainland Asia", *PLoS One*, **10**(1), e0115168.
- [61] F.H. Wu, M.T. Chan, D.C. Liao, C.T. Hsu, Y.W. Lee, et al. (2010), "Complete chloroplast genome of *Oncidium* Gower Ramsey and evaluation of molecular markers for identification and breeding in Oncidiinae", *BMC Plant Biol.*, **10**, pp.68-80.
- [62] P. Siripiyasing, K. Kaenratana, P. Mokkamul, T. Tanee, R. Sudmoon, A. Chaveerach (2012), "DNA barcoding of *Cymbidium* species (Orchidaceae) in Thailand", *Afr. J. Agric. Res.*, **7**(3), pp.393-400.
- [63] K.S. Burgess, A.J. Fazekas, P.R. Kesanakurti, et al. (2011), "Discriminating plant species in a local temperate flora using the *rbcl*+ *matK* DNA barcode", *Methods in Ecology and Evolution*, **2**(4), pp.333-340.
- [64] W.J. Kress, D.L. Erickson, F.A. Jones, et al. (2009), "Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama", *Proceedings of the National Academy of Sciences*, **106**(44), pp.18621-18626.
- [65] M.W. Chase, R.S. Cowan, P.M. Hollingsworth, et al. (2007), "A proposal for a standardised protocol to barcode all land plants", *Taxon*, **56**(2), pp.295-299.
- [66] R. Lahaye, V. Savolainen, S. Duthoit, O. Maurin, M. Van Der Bank (2008b), "A test of *psbK-psbI* and *atpF-atpH* as potential plant DNA barcodes using the flora of the Kruger National Park (South Africa) as a model system", *Nature Proceedings*, pp.1-23.
- [67] V. Sosa, T. Mejia-Saules, M.A. Cuellar, A.P. Vovides (2013), "DNA Barcoding in Endangered Mesoamerican Groups of Plants", *Botanical Review*, **79**(4), pp.469-482.
- [68] H. Asahina, J. Shinozaki, K. Masuda, Y. Morimitsu, M. Satake (2010), "Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcl* sequences", *J. Nat. Med.*, **64**(2), pp.133-138.
- [69] P. Cuénoud, V. Savolainen, L.W. Chatrou, M. Powell, R.J. Grayer, M.W. Chase (2002), "Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid *rbcl*, *atpB* and *matK* DNA sequences", *Am. J. Bot.*, **89**(1), pp.132-144.
- [70] M. Von Crautlein, H. Korpelainen, M. Pietilainen, J. Rikkinen (2011), "DNA barcoding: A tool for improved taxon identification and detection of species diversity", *Biodiversity Conservation*, **20**(2), pp.373-380.
- [71] K.M. Neubig, W.M. Whitten, B.S. Carlsward, et al. (2009), "Phylogenetic utility of *ycf1* in orchids: a plastid gene more variable than *matK*", *Plant Systematics and Evolution*, **277**(1), pp.75-84.
- [72] R. Arévalo, K. Cameron (2013), "Molecular Phylogenetics of Mormolyca (Orchidaceae: Maxillariinae) based on Combined Molecular Data Sets", *Linkesteriana*, **13**(1-2), pp.1-11.
- [73] Y.Y. Guo, L.Q. Huang, Z.J. Liu, X.Q. Wang (2016), "Promise and challenge of DNA barcoding in Venus Slipper (*Paphiopedilum*)", *PLoS ONE*, **11**(1), e0146880.
- [74] J.S. Kim, H.T. Kim, S.W. Son, J.H. Kim (2015), "Molecular identification of endangered Korean lady's slipper orchids (*Cypripedium*, Orchidaceae) and related taxa", *Botany*, **93**(9), pp.603-610.
- [75] C.S. Lin, J.J.W. Chen, Y.T. Huang, M.T. Chan, H. Daniell, W.J. Chang, C.T. Hsu, D.C. Liao, F.H. Wu, S.Y. Lin, C.F. Liao, M.K. Deyholos, G.K.S. Wong, V.A. Albert, M.L. Chou, C.Y. Chen, M.C. Shih (2015), "The location and translocation of *ndh* genes of chloroplast origin in the Orchidaceae family", *Scientific Reports*, **5**, 10p, doi: 10.1038/srep09040.
- [76] B.S. Carlsward, W.M. Whitten, N.H. Williams (2003), "Molecular phylogenetics of Neotropical leafless Angraecinae (Orchidaceae): Reevaluation of generic concepts", *Int. J. Pl. Sci.*, **164**(1), pp.43-51.
- [77] C. Van den Berg, D.H. Goldman, J.V. Freudenstein, A.M. Pridgeon, K.M. Cameron, et al. (2005), "An overview of the phylogenetic relationships within Epidendroideae inferred from multiple DNA regions and recircumscription of Epidendreae and Arethuseae (Orchidaceae)", *American Journal of Botany*, **92**(4), pp.613-624.
- [78] B.S. Carlsward, W.M. Whitten, N.H. Williams, B. Bytebier (2006), "Molecular phylogenetics of Vandaeae (Orchidaceae) and the evolution of leaflessness", *Amer. J. Bot.*, **93**(5), pp.770-786.
- [79] C. Micheneau, B. Carlsward, M. Fay, B. Bytebier, T. Pailler, M. Chase (2008), "Phylogenetics and biogeography of Mascarene angraecoid orchids (Vandaeae, Orchidaceae)", *Molecular Phylogenetics and Evolution*, **46**(3), pp.908-922.
- [80] R.J. Waterman, A. Pauw, T.G. Barraclough, V. Savolainen (2009), "Pollinators underestimated: a molecular phylogeny reveals widespread floral convergence in oil-secreting orchids (sub-tribe Coryciinae) of the Cape of South Africa", *Mol. Phylogenet E.*, **51**(1), pp.100-110.
- [81] J.H. Li, Z.J. Liu, G.A. Salazar, P. Bernhardt, H. Perner, Y. Tomohisa, X.H. Jin, S.W. Chung, Y.B. Luo (2011), "Molecular phylogeny of *Cypripedium* (Orchidaceae: Cyrtipedioideae) inferred from multiple nuclear and chloroplast regions", *Mol. Phylogenet. E.*, **61**(2), pp.308-320.
- [82] J.B. Yang, M. Tang, H.T. Li, D.Z. Zhang (2013), "Complete chloroplast genome of the genus *Cymbidium*: Lights into the species identification, phylogenetic implications and population genetic analyses", *BMC Evol. Biol.*, **13**, pp.84-96.
- [83] A.J. Fazekas, M. Kuzmina, G. Newmaster Steven, P.M. Hollingsworth (2012), "DNA barcoding methods for land plants", *Methods in Molecular Biology*, **858**, pp.223-252.
- [84] P. Taberlet, E. Coissac, F. Pompanon, L. Gielly, C. Miquel, A. Valentini, T. Vermet, G. Corthier, C. Brochmann, E. Willerslev (2006), "Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding", *Nucleic Acids Res.*, **35**(3), e4.
- [85] W. Dong, J. Liu, J. Yu, L. Wang, S. Zhou (2012), "Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding", *PLoS ONE*, **7**(4), e35071.
- [86] G. Gigot, J. Van Alphen-Stahl, D. Bogarin, J. Warner, M.W. Chase, et al. (2007), "Finding a suitable DNA barcode for mesoamerican orchids", *Linkesteriana*, **7**(1-2), pp.200-203.
- [87] C.S. Ford, K.L. Ayres, N. Toomey, N. Haider, J. Van Alphen Stahl, L.J. Kelly, N. Wikstrom, P.M. Hollingsworth, R.J. Duff, S.B. Hoot, R.S. Cowan, M.W. Chase, M.J. Wilkinson (2009), "Selection of candidate coding DNA barcoding regions for use on land plants", *Botanical Journal of the Linnean Society*, **159**(1), pp.1-11.

Morphological and molecular data reveal a new species of *Alocasia* (Araceae) from Vietnam

Hong Thien Van¹, Phi Nga Nguyen², Ngoc Toan Tran³, Hong Truong Luu^{4*}

¹Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City

²Department of Ecology and Evolutionary Biology, University of Science - Vietnam National University HCMC

³GreenViet

⁴Southern Institute of Ecology, Vietnam Academy of Science and Technology

Received 20 February 2017; accepted 26 April 2017

Abstract:

Alocasia rivularis (Araceae) is proposed as a new species from central Vietnam. The new species is morphologically similar to those of the Cuprea Group of *Alocasia*, but according to results of the *trnL-trnF* IGS and *matK* sequences, and based on morphological differences, it is clearly different from the closest congeners. Phylogenetic analysis, descriptions, and illustrations are provided.

Keywords: *Alocasia rivularis*, Araceae, central Vietnam, new species, phylogeny.

Classification number: 3.5

Introduction

Alocasia was first used as a section of *Colocasia* (Araceae) by Schott (1832) [1] and was raised to generic rank by Don in Sweet (1839) [2]. It includes more than 100 species occurring primarily in the subtropical eastern Himalaya as throughout subtropical and tropical Asia into the tropical western Pacific and eastern Australia [3-5]. In Vietnam, seven species of *Alocasia* have been recorded, including *A. cucullata*, *A. evrardii*, *A. lecomtei*, *A. longiloba*, *A. macrorrhizos*, *A. Odora*, and *A. Vietnamensis*, plus another three species with doubtful distribution in Vietnam, namely *A. hainanica*, *A. Auminata*, and *A. navicularis* [6-8]. In general, Vietnam is one of the countries where the genus is least understood [9].

In November of 2015, we conducted a field trip to Nui Dau Mountain, Nghia Hanh District, in Quang Ngai Province, and encountered a population of an aroid species superficially resembling either *Alocasia* or *Colocasia* (Araceae), which have distinguished peltate leaf blades and fruiting spathes. A set of specimens were then collected (Luu 1110, SGN). Our subsequent surveys collected up until 2016 of the areas did not yield inflorescences. Our examination of the collected specimens determined that the fruit were 6-8 mm in diameter, had 1 to 2 mature seeds of 5-6 mm in diameter, and had 12 to 13 unfertilised and

reduced seeds with basal placentation. These facts would suggest placing the species (which is hereafter referred to as *A. rivularis*) in *Acolosia* rather than in *Colocasia* as the latter has < 3 mm fruits with many small seeds and ovaries with parietal placentation [5, 10]. *A. rivularis* seems to resemble those of the Cuprea Group (such as *A. beccarii* Engl., *A. cuprea* K.Koch, *A. perakensis* Hemsl., and *A. peltata* M.Hotta), which typically has nearly completely peltate adult leaves alternating with long cataphylls and the staminate zone of the spadix being mostly to completely within the lower spathe [4]. However, in our collections, no cataphylls alternating with adult leaves were found, and the position of the staminate zone within the spathe was not known. Besides that, the number of ovules per ovary, which is possibly 13-14 as drawn from the examined fruits, is a few more than the range of 6 to 10 found in known species of *Alocasia*, but lower than that (i.e. very numerous) found in those of *Colocasia* [3-5, 9, 11]. Due to the morphological divergence of *A. rivularis*, we have used molecular data to ascertain its phylogenetic relationship to known species of *Alocasia*, especially those of the Cuprea Group, and other genera of the tribe Colocasieae, applying the methods employed similarly in previous phylogenetic studies of the Araceae [12-16].

*Corresponding author: Email: hongtruongluu@gmail.com

Table 1. Specimens of sixteen taxa of Araceae species sequenced in this study.

Sequenced voucher number	Taxon	Sequenced voucher number	Taxon
H.T. Van 99	<i>Acorus verus</i>	H.T. Van 54	<i>Homalomena cochinchinensis</i>
H.T. Van 69	<i>Aglaonema simplex</i>	H.T. Van 56	<i>Lasia spinosa</i>
Luu 1110	<i>Alocasia rivularis</i>	H.T. Van 102	<i>Pistia stratiotes</i>
H.T. Van 119	<i>Alocasia odora</i>	H.T. Van 61	<i>Pothos chinensis</i>
H.T. Van 03	<i>Amorphophallus scaber</i>	H.T. Van 70	<i>Rhaphidophora chevalieri</i>
H.T. Van 79	<i>Arisaema roxburghii</i>	H.T. Van 60	<i>Schismatoglottis harmandii</i>
H.T. Van 98	<i>Cryptocoryne ciliata</i>	H.T. Van 104	<i>Scindapsus officinalis</i>
H.T. Van 67	<i>Epipremnum giganteum</i>	H.T. Van 42	<i>Typhonium trilobatum</i>

Table 2. Sequences from the GenBank database used in the study [14].

Taxon	GenBank accession no. (<i>trnL-trnF/matK</i>)
<i>Alocasia beccarii</i>	JQ238731/JQ238817
<i>Alocasia cuprea</i>	JQ238740/JQ238826
<i>Alocasia gageana</i>	JQ238742/EU886580
<i>Alocasia grandis</i>	JQ238743/JQ238828
<i>Alocasia heterophylla</i>	JQ238744/JQ238829
<i>Alocasia hollrungii</i>	JQ238745/JQ238830
<i>Alocasia peltata</i>	JQ238766/JQ238850
<i>Alocasia perakensis</i>	JQ238767/JQ238851
<i>Ariopsis protanthera</i>	AY248947/EU886587
<i>Colocasia esculenta</i>	JQ238804/JQ238890
<i>Englerarum hupnosum</i>	JQ238746/JQ238831
<i>Leucocasia gigantea</i>	JQ238807/JQ238893
<i>Protarum sechellarum</i>	JQ238810/KC466580
<i>Remusatia vivipara</i>	JQ238812/JQ238897
<i>Steudnera assamica</i>	EF517214/JQ238898

Table 3. Primers used in the present study.

Primers (*)	Region	Sequence (5'-3')	References
C (F)	<i>trnL-trnF</i> IGS	GGTCAAGTCCCTCTATCCC	[18]
D (R)	<i>trnL-trnF</i> IGS	ATTTGAACTGGTGACACGAG	[18]
MF (F)	<i>matK</i>	ACCCAGTCCATCTGGAATCTTGGTTC	[19]
MR (R)	<i>matK</i>	CGTACAGTACTTTGTGTTTACGAG	[19]

(*) Direction of primer, F = forward, R = reverse.

Materials and methods

DNA samples taken from fresh leaves of the collected specimen (Luu 1110) and fifteen other taxa of Araceae collected from southern regions of Vietnam were used in this study (Table 1). All respective vouchered specimens were collected following the Kew Botanic Garden protocols [17] and deposited at SGN. Sequences of *trnL-trnF* IGS and *matK* regions of additional species representing typical genera of the tribe Colocasieae from GenBank were also used (Table 2).

The total genomic DNA was extracted from fresh leaf tissues using a Genomic DNA Purification Mini Kit (Thermo, USA). The *trnL-trnF* IGS and *matK* chloroplast DNA regions were amplified using the polymerase chain reaction (PCR). A list of primers is shown in Table 3. The PCR reactions were observed in an Eppendorf Mastercycler Gradient using a volume of 25 µl reaction mixture: 12.5 µl go taq green master mix (Promega, USA), 1.25 µl of each forward and reverse primers (10 µM), 9.5 µl HPLC water, and 0.5 µl DNA template (25 ng). PCR cycles consisted of an initial denaturation for five minutes at 95°C; 35 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 50°C) and extension (1:30 minutes at 72°C); and a final extension at 72°C for 10 minutes. The PCR products were visualised in a 1.5% agarose gel and sent for purification and direct sequencing at Nam Khoa Biotek Company Ltd. (Vietnam) using an ABI 3130 XL Sequencer.

For multiple alignments, the Clustal W [20] was used to recognise the homology between sequences. Phylogenetic analysis was carried out with the software PAUP*4.0a146 [21], using the maximum parsimony and neighbor-joining methods of *Acorus*

verus (Acoraceae) as the outgroup, following Cabrera, et al. [12], Cusimano, et al. [13], and Nauheimer, et al. [15]. The maximum parsimony trees were calculated based on chloroplast sequence data using gaps treated as missing data and heuristic search algorithms [22] with the following parameters: 1,000 random addition sequence replicates, tree bisection and reconnection (TBR) branch swapping, and 10 parsimonious trees held after each replicate [23, 24]; and all characters were equally weighted and treated as unordered [25]. The fit of characters to the trees was also tested by calculating the consistency index (CI), the retention index (RI), and the rescaled consistency index (RC) [26, 27]. The neighbor-joining tree was constructed based on the matrix of pairwise distances between species [28]. The statistical support for phylogenetic trees was carried out using the bootstrap method [29] with 1,000 replicates. The bootstrap values of more than 50% were performed in the discussed trees. The pairwise genetic distances [30] were calculated using the software MEGA6 [31].

Besides that, morphological characteristics were used in the comparison of the new species and its close congeners.

Results

The length of the combined *trnL-trnF* IGS and the *matK* data sets of studied species ranged from 1,160 to 1,179 bp. The entire aligned length of the two regions was 1,459 bp. The phylogenetic analysis of the combined data sets resulted in a parsimonious tree (length: 859 steps, CI: 0.80, RI: 0.67, RC: 0.53) and the neighbor-joining tree (Fig. 1, 2).

In the neighbor-joining tree, *A. rivularis* and other *Alocasia* species were grouped within one clade with a

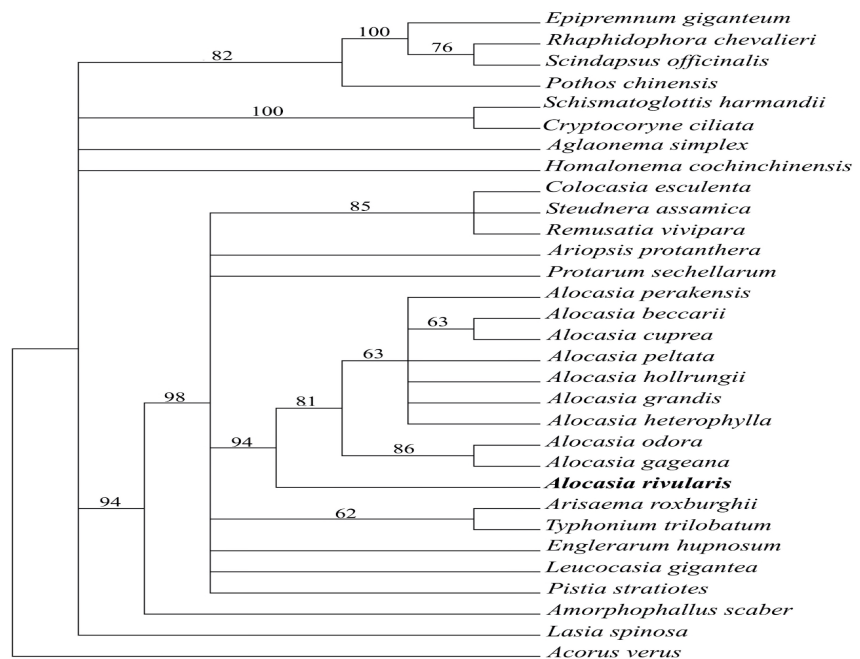


Fig. 1. One of the largest parsimonious trees obtained based on the combined *trnL-trnF* IGS and *matK* data sets. Gaps were treated as missing data. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes. Tree length = 859 steps, CI: 0.80, RI: 0.67, RC: 0.53.

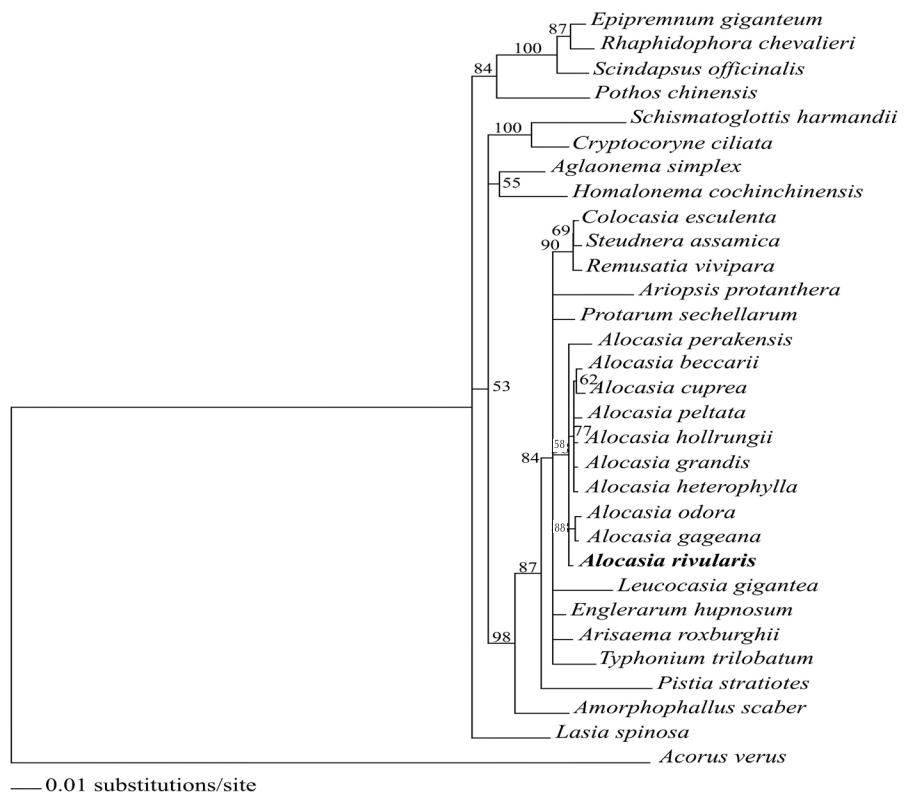


Fig. 2. The neighbor-joining tree obtained from the combined *trnL-trnF* IGS and *matK* data sets. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes.

bootstrap value = 58%. Meanwhile, a very high bootstrap value (94%) was found in the parsimony tree which showed *A. rivularis* as sister to the remaining *Alocasia* species.

Comparing the *trnL-trnF* IGS and *matK* sequences of *A. rivularis* with

those of the Cuprea Group studied (i.e. *A. beccarii*, *A. cuprea*, *A. peltata* and *A. perakensis* which have *trnL-trnF* IGS and *matK* sequences available in the GenBank) after alignment, we found 11 and 8 variable positions among 408 and 730 positions of the *trnL-trnF* IGS

and *matK* sequences, respectively. The number of different substitutions was 5, 6, 7, and 5 in the *trnL-trnF* IGS region and 5, 4, 3, and 6 in the *matK* regions, respectively (Table 4, 5). As results showed, the pairwise genetic distances of *A. rivularis* to any of the *A. beccarii*, *A. cuprea*, *A. peltata*, and *A. perakensis* ranged from 0.008 to 0.009 while those found between each pair of the latter four species range from 0.003 to 0.009 (Table 6).

Table 4. Variable nucleotide positions of the *trnL-trnF* IGS region among *Alocasia* species.

	43	89	184	221	257	258	259	260	305	335	371
<i>A. rivularis</i>	G	C	A	G	-	-	-	-	A	C	G
<i>A. beccarii</i>	A	T	A	G	-	-	T	T	C	C	G
<i>A. cuprea</i>	A	T	C	G	-	-	-	T	C	A	G
<i>A. peltata</i>	A	T	A	C	T	T	T	T	A	C	G
<i>A. perakensis</i>	A	T	A	G	-	-	T	T	A	C	A

Table 5. Variable nucleotide positions of the *matK* region among *Alocasia* species.

	29	97	277	305	373	405	508	711
<i>A. rivularis</i>	C	G	T	C	C	T	A	A
<i>A. beccarii</i>	T	G	T	T	C	C	C	-
<i>A. cuprea</i>	C	G	A	T	C	C	A	-
<i>A. peltata</i>	C	G	T	T	C	C	A	-
<i>A. perakensis</i>	C	T	T	T	T	-	-	-

Discussions

In both analyses, the order of the genera and species are in agreement with those in the previous studies [12-16].

The shown phylogenetical trees obviously indicate that *A. rivularis* is closer to the species of *Alocasia* than to those of the other genera in this study, including *Colocasia esculenta*. As a result, the study species should be placed in *Alocasia* rather than *Colocasia*; which is supported by its morphological

Table 6. Mean pairwise genetic distances among species of the tribe Colocasieae based on the combined *trnL-trnF* IGS and *matK* data sets.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>Alocasia odora</i>																
2. <i>Alocasia gageana</i>	0.003															
3. <i>Colocasia esculenta</i>	0.012	0.012														
4. <i>Staudneria assamica</i>	0.013	0.013	0.001													
5. <i>Remusatia vivipara</i>	0.016	0.016	0.004	0.005												
6. <i>Ariopsis protanthera</i>	0.023	0.023	0.019	0.020	0.023											
7. <i>Protarum sechellarum</i>	0.013	0.013	0.012	0.013	0.011	0.023										
8. <i>Leucocasia gigantea</i>	0.012	0.012	0.011	0.012	0.012	0.022	0.009									
9. <i>Englerarum hypnosum</i>	0.009	0.007	0.008	0.009	0.011	0.019	0.008	0.007								
10. <i>Alocasia rivularis</i>	0.004	0.004	0.011	0.012	0.015	0.022	0.012	0.011	0.008							
11. <i>Alocasia heterophylla</i>	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005						
12. <i>Alocasia grandis</i>	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003					
13. <i>Alocasia beccarii</i>	0.005	0.005	0.015	0.016	0.016	0.026	0.013	0.015	0.012	0.007	0.004	0.004				
14. <i>Alocasia cuprea</i>	0.008	0.008	0.018	0.019	0.019	0.026	0.016	0.018	0.015	0.009	0.007	0.007	0.005			
15. <i>Alocasia holhrungii</i>	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003	0.003	0.004	0.007		
16. <i>Alocasia peltata</i>	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003	0.003	0.004	0.007	0.003	
17. <i>Alocasia perakensis</i>	0.007	0.007	0.016	0.018	0.020	0.027	0.018	0.016	0.013	0.008	0.005	0.005	0.007	0.009	0.005	0.005

characteristics, i.e. 1-loculed and 1- to 2-seeded fruits of 6-8 mm in diameter and ovaries with basal placentation.

The numbers of different substitutions in the *trnL-trnF* IGS and *matK* sequences between *A. rivularis*, and each species of the Cuprea Group (i.e. *A. beccarii*, *A. cuprea*, *A. peltata*, and *A. perakensis*) are equal to or higher than those found between each pair of the latter four species (ranging from 1 to 5 in the *trnL-trnF* IGS and 1 to 3 in the *matK* region). Consequently, the pairwise genetic distance of *A. rivularis* to any of each *A. beccarii*, *A. cuprea*, *A. peltata*, and *A. perakensis*, is equal to or higher than that calculated for each pair of the four latter. Similarly, the genetic distance between *A. rivularis* to any of the other studied *Alocasia* species (i.e. *A. heterophylla* (C.Presl) Merr., *A. grandis* Clemenc. and *A. hollrungii* Engl.) is higher than that found in at least one pair of them (Table 6). Therefore, the newly found species is well phylogenetically separated from all the other mentioned *Alocasia* species.

Morphologically, *A. rivularis* closely resembles *A. peltata*, which is distributed in Borneo, in scattered localities in Sarawak, Brunei, and central Kalimantan. Both species share similar shapes and dimensions of leaves, fruits and seeds as well as the diameter of stem. However, *A. peltata* is readily distinguishable from the Vietnamese congener due to its height of ca. 30 cm, fewer leaves, symmetric leaf blades, an anterior lobe 2-3 times as long as the combined posterior lobes, 5-6 ovuled ovaries, an inflorescence per axil, and obovoid fruiting spathes [4, 11]. Further, *A. peltata* is distributed in the mossy forest floor on ridges at ca. 1,200 m altitude, while our species are found in the dipterocarp-dominated lowland tropical evergreen forest at around 80 m altitude.

The shape of the leaf blades in *A. rivularis* also looks similar to that of the *A. minuscula* A.Hay from Sarawak, but the latter has a smaller size (height of 10-20 cm and stem of ca. 1 cm diameter, very thick and smaller leaf blades (8-13x2-3 cm), more primary lateral veins (8-10 on each side of midrib), striate secondary venation, and shorter petioles (5-10 cm long) with shorter petiole sheaths (ca. 1/7 of petiole length) [4].

Three other *Alocasia* species that have leaf blades similar to those in *A. rivularis* are *A. beccarii* from northwestern Borneo are *A. perakensis* from Peninsula Malaysia and Thailand, and *A. kerinciensis* A.Hay from Sumatra (Indonesia) and but these three latter, like the two compared just above, are readily distinguishable from our new species by their cataphylls interspersed with leaves and symmetric leaf blades. In addition to that, *A. beccarii* and *A. kerinciensis* are different from *A. rivularis* because *A. beccarii* has a small habit (12-28 cm tall), a slender stem that is 5-10 mm in diameter, very long internodes, ca. 2 cm long, and ovoid fruiting spathes, while *A. kerinciensis* has a smaller stem (1 cm diameter, and up to ca. 40 cm long) with internodes that are 5 cm long, an anterior costa with 2-3 primary lateral veins on each side, and smaller ovoid fruiting spathes (ca. 2 cm long) [4, 9]. *A. perakensis* is further distinct from *A. rivularis* in having thick leaf blades, ovoid fruiting spathes, ellipsoid, and red fruits [9].

Taxonomic treatment

Alocasia rivularis Luu, Nguyen-phi & H.T. Van (Fig. 3).

Diagnosis: The new species is morphologically similar to *A. peltata*, *A. minuscula*, *A. beccarii*, *A. kerinciensis*, and *A. perakensis*, and differs in having no cataphylls interspersed with leaves, asymmetric leaf blades with the anterior

lobe 5-6 times as long as combined posterior lobes, two inflorescences per axil, oblong ellipsoid, and longer fruiting spathes.

Type: VIETNAM. Quang Ngai Province, Nghia Hanh District, Hanh Tin Dong Commune, Nui Dau Mountain, 14°52'23.63"N, 108°48'49.48"E, around 80 m in elevation, 14 November 2015, Hong Truong Luu & Hoang Minh Duc Luu 1110 (holotype, SGN; isotypes, SGN & VNMN).

Herbs to 70 cm tall have the following characteristics: *Stem* rhizomatous, elongate, slender, unbranched, and 20-26 mm in diameter, with internodes nearly as wide as long, as well as decumbent and then erect, and often completely exposed. Also having *leaves* ranging from several to 11, clustered at the tips of stems, peltate, and drying brown; *petioles* pale green, glabrous, and 25-35 cm long; petiolar sheath 8-15 cm long, mostly persistent; *blades* narrowly lanceolate to oblong-ovate, asymmetric, sized 16-24x7-8.5 cm, shining, and thinly coriaceous on both surfaces, adaxially dark green, and abaxially pale green; midribs adaxially impressed, and abaxially prominent; *anterior lobes* 12-20 cm long, with 1 cm long acuminate tip, which are 5-6 times as long as the combined posterior lobes; anterior costae with four adaxially impressed, abaxially prominent primary lateral veins on each side (subopposite) diverging at ca. 45-60° which run straight or upcurved into a conspicuous intramarginal vein 1-1.5 mm from the margin; secondary venations inconspicuous to be invisible; *posterior lobes* completely united except for a 1.5-2 mm incision at the extreme base of the leaf; and combined posterior lobes 3-4 cm long, which are widest at the petiole insertion. Also, infructescences two, peduncle 18-24 cm, 6-8 mm in diameter; fruiting spathes which are dark green, oblong ellipsoid,

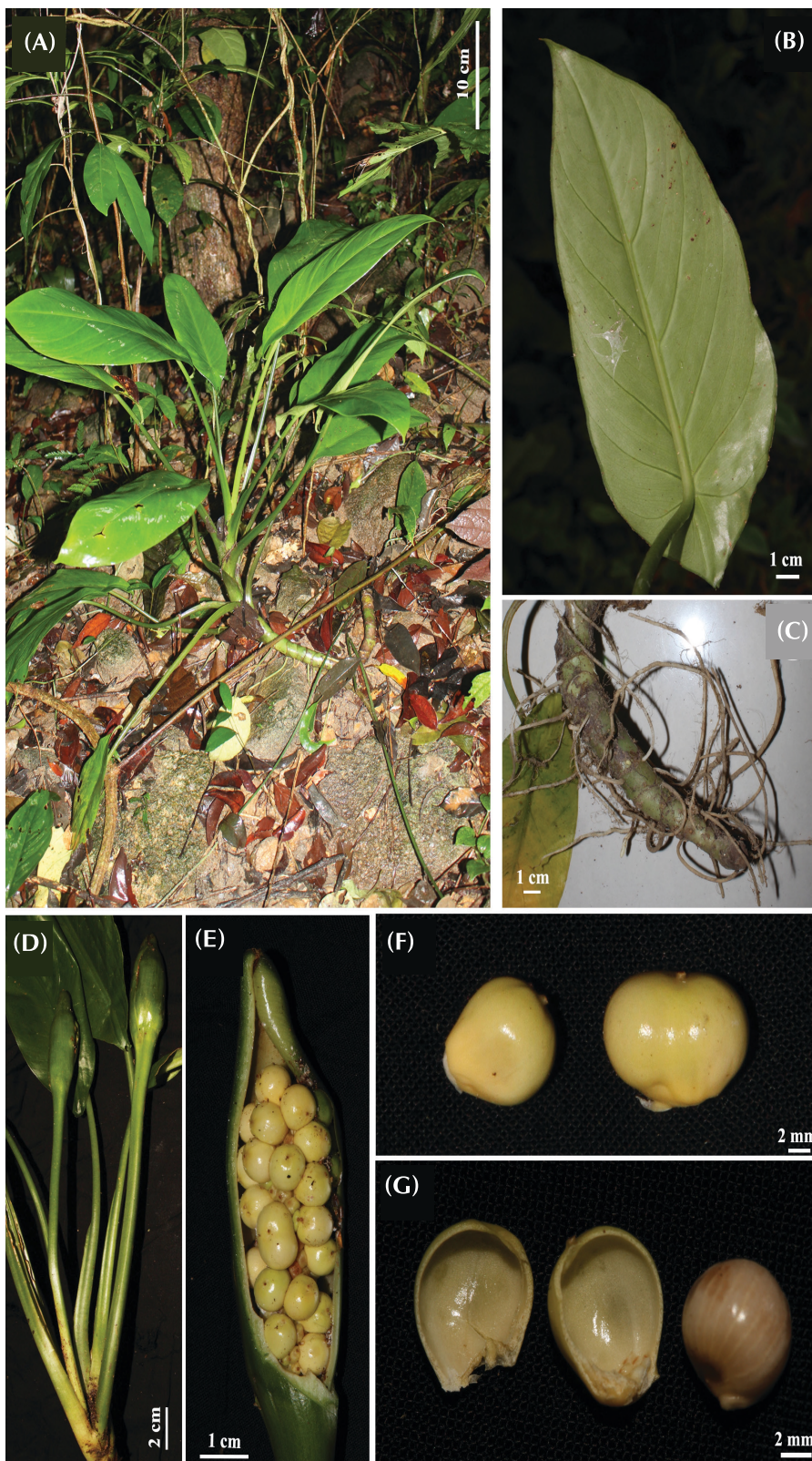


Fig. 3. *Alocasia rivularis* Luu, Nguyen-phi & H.T. Van. (A) The species in the habitat, (B) Leaf blade, abaxial surface, (C) Rhizome, (D) Infructescences, (E) Fruiting spike, (F) Fruits, and (G) Opened berry showing one seed with basal placentation.

6.5-7.5 cm long, ca. 2 cm in diameter, and opened at the middle; fruits obovoid, pale greenish yellow, and usually 7 mm in diameter; and seeds 1 and rarely 2, round, pale pinkish to brownish white, with longitudinal white veins, which are 5-6 mm in diameter.

Habitat: *A. rivularis* is found in the lowland evergreen tropical forests on fertile soils along small streams and ponds; fruits were seen in November.

Distribution: The new species has been found only from the type location.

Etymology: Named to reflect the typical habitat of the species which grows along small streams and ponds.

Based on the available literature [3-10] and the authors' field observations, the known species of *Alocasia* in Vietnam (except those doubtful) can be distinguished by the following key:

1A. Plant epiphytic; spadix with staminodes at the base of the female portion.....2

1B. Plant geophytic; spadix without staminodes at the base of the female portion.....3

2A. Stem tuberous, subglobose
A. evrardii

2B. Stem rhizomatous, elongate
A. vietnamensis

3A. Leaf blade with lateral veins originating almost from base; basal lobes not distinct*A. cucullata*

3B. Leaf blade with lateral veins originating from midrib; basal lobes clearly distinct.....4

4A. Adult leaves asymmetric; posterior lobes almost completely united
A. rivularis

4B. Adult leaves partially peltate or not peltate, symmetric; posterior lobes partially united at base or not.....5

- 5A. Mature plants massive, at least 2 m tall.....6
- 5B. Mature plants not as above.....7
- 6A. Stem erect to decumbent; leaves peltate*A. odora*
- 6B. Stem erect; leaves not peltate
A. macrorrhizos
- 7A. Leaf blade sagittate; posterior lobes triangular.....*A. longiloba*
- 7B. Leaf blade ovate to obovate; posterior lobes rounded.....*A. lecomtei*

Conclusions

The morphological and molecular data as presented above indicate that *A. rivularis* from Quang Ngai Province, Vietnam should be treated as a new good species of *Alocasia*.

ACKNOWLEDGEMENTS

This work was funded partly by the project no. 11/HDTV financed by Quang Ngai Provincial Department of Natural Resource and Environment. The authors are grateful to Mr. Nguyen Dai, Director of Quang Ngai Forest Protection Department and his staff for their kind support for the field trip. The authors thank the anonymous reviewers for their critical comments that helped improve the manuscript.

REFERENCES

[1] H.W. Schott (1832), "Aroidearum synopsis", *Meletemata Botanica*, pp.15-22, Gerold, Vienna.

[2] R. Sweet (1839), *Hortus Brittanicus*, Ed. 3, 3, 631p, G. Don, Ridgway, London.

[3] P.C. Boyce, D. Sookchaloem, W.L.A. Hetterscheid, G. Gusman, N. Jacobsen, T. Idei, V.D. Nguyen (2012), "Araceae", *Flora of Thailand*, **11**, pp.101-321.

[4] A. Hay (1998), "The Genus *Alocasia* (Araceae-Colocasieae) in West Malesia and Sulawesi", *Gardens' Bulletin Singapore*, **50**, pp.221-334.

[5] H. Li, P.C Boyce (2010), "Alocasia", *Flora of China*, **23**, pp.75-79.

[6] V.D. Nguyen (2005), "Araceae", *Checklist of Plant Species of Vietnam*, **3**, pp.871-898, Agriculture Publishing House, Hanoi.

[7] V.D. Nguyen, T.B. Croat, H.T. Luu, C.Y. Lee, J. Lee, R.D. Kok (2013), "Two new species of *Alocasia* (Araceae, Colocasieae) from Vietnam", *Willdenowia*, **43**, pp.293-299.

[8] H. Pham Hoang (2000), "Araceae", *An Illustrated Flora of Vietnam*, **3**, pp.335-367, Youth Publishing House, Ho Chi Minh City [in Vietnamese with English summary].

[9] P.C. Boyce (2008), "A review of *Alocasia* (Araceae: Colocasieae) for Thailand including a novel species and new species records from South-West Thailand", *Thai For. Bull. (Bot.)*, **36**, pp.1-17.

[10] S.J. Mayo, J. Bogner, P.C. Boyce (1997), *The Genera of Araceae*, Royal Botanic Gardens, Kew.

[11] M. Hotta (1967), "Notes on Bornean plants II", *Acta Phytotax Geobot.*, **22**, pp.153-162.

[12] L.I. Cabrera, G.A Salazar, M.W. Chase, S.J. Mayo, J. Bogner, P.D. Vila (2008), "Phylogenetic relationships of aroids and duckweeds (Araceae) inferred from coding and noncoding plastid DNA", *Amer. J. Bot.*, **95**, pp.1153-1165.

[13] N. Cusimano, J. Bogner, S.J. Mayo, P.C. Boyce, S.Y. Wong, M. Hesse, W.L.A. Hetterscheid, R.C. Keating, J.C. French (2011), "Relationships within the Araceae: comparison of morphological patterns with molecular phylogenies" *Amer. J. Bot.*, **98**, pp.1-15.

[14] L. Nauheimer, P.C. Boyce, S.S. Renner (2011), "Giant taro and its relatives: A phylogeny of the large genus *Alocasia* (Araceae)", *Molecular Phylogenetics and Evolution*, **63**, pp.43-51.

[15] L. Nauheimer, M. Dirk, S.R. Susanne (2012) "Global history of the ancient monocot family Araceae inferred with models accounting for past continental positions and previous ranges based on fossils", *New Phytologist*, DOI: 10.1111/j.1469-8137.

[16] L. Nauheimer, P.C. Boyce (2013), "*Englerarum* (Araceae, Aroideae): a new genus supported by plastid and nuclear phylogenies", *Plant. Syst. Evol.*, DOI 10.1007/s00606-013-0914-7.

[17] D. Bridson, L. Forman (1999), *The Herbarium Handbook-Third Edition*, Royal Botanic Gardens, Kew.

[18] P. Taberlet, M.L. Gielly, G. Patou, J. Bouvet (1991), "Universal primers for amplification of three non-coding regions of chloroplast DNA", *Plant molecular Biology*, **17**, pp.1105-1109.

[19] A.J. Fazekas, M.L. Kuzmina, S.G. Newmaster, P.M. Hollingsworth (2012), "DNA barcoding methods for land plants", *Meth. Mol. Biol.*, **858**, pp.223-252.

[20] D.J. Thompson, D.G. Higgins, T.J. Gibson (1994), "Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice", *Nucleic Acids Res.*, **22**, pp.4673-4680.

[21] D.L. Swofford (2002), *PAUP*: Phylogenetic Analysis Using Parsimony (and other methods)*, version 4.0 Beta, Sinauer Associates, Sunderland, Massachusetts.

[22] M. Nei, S. Kumar (2000), *Molecular Evolution and Phylogenetics*, Oxford University Press, New York.

[23] C.J. Harrison, J.A. Langdale (2006), "A step by step guide to phylogeny reconstruction", *Plant J.*, **45**, pp.561-572.

[24] K.S. Yulita, R.J. Bayer, J.G. West (2005), "Molecular study of Dipterocarpaceae: evidence from the *trnL-trnF* and internal transcribed spacer regions", *Plant Species Biol.*, **20**, pp.167-182.

[25] W.M. Fitch (1971), "Towards defining the course of evolution: minimum change for a species tree topology", *Systematic Zoology*, **20**, pp.406-416.

[26] J.S. Farris (1989), "The retention index and the rescaled consistency index", *Cladistics*, **5**, pp.417-419.

[27] A. Kluge, J. Farris (1969), "Quantitative phyletics and the evolution of anurans", *Syst. Zool.*, **18**, pp.1-32.

[28] N. Saitou, M. Nei (1987), "The neighbor-joining method: a new method for reconstructing phylogenetic trees", *Mol. Bio. Evol.*, **4**, pp.406-425.

[29] J. Felsenstein (1985), "Confidence limits on phylogenies: an approach using the bootstrap", *Evolution*, **39**, pp.783-791.

[30] M. Kimura (1980), "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences", *J. Mol. Evol.*, **16(2)**, pp.111-120.

[31] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar (2013), "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0", *Mol. Bio. Evol.*, **30**, pp.2725-2729.

Dendritic cell therapy for cancers and underlying mechanisms involved in cancer development

Thi Xuan Nguyen*, Huy Hoang Nguyen

Institute of Genome Research, Vietnam Academy of Science and Technology

Received 5 January 2017; accepted 14 March 2017

Abstract:

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that affect prime naive T cells and create proper immune responses. The uncontrolled growth of cancer cells often results from the cell's successful inhibition of cytotoxic T lymphocytes. In addition to conventional cancer treatments, including surgery, chemotherapy, and radiation therapy, immunotherapies have been seen to offer promising and innovative cancer treatments. Currently, DC therapy is one of the most popular immunotherapies because it uses tumour antigen-pulsed DC vaccines to fight against cancers. Patients with end-stage cancers treated with DC therapy may extend survival significantly for up to 10 to 15 more years. Researchers worldwide, including in Vietnam, have been focusing on determining the etiology of cancers with an aim to control cancers. One of the major causes of cancer is an increased expressions of proteins, including cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1) which both affect the recruitment of a large number of regulatory T cells and abolishes the presence of cytotoxic T lymphocytes migrating to the tumor sites, and resulting in an immune tolerance. In addition to this, the abnormal activation of the nuclear factor- κ B (NF- κ B) is also an important risk factor for cancer, because its activation leads to transcription of nuclear genes involved in regulating the cell physiological processes, such as maturation, differentiation, proliferation, migration, and invasion. This NF- κ B signal is modulated by bonds among most receptors on immune cell surfaces and their specific ligands. Therefore, investigations of the precise molecular mechanism associated with the regulation of cancer development by DCs and other leukocytes, and the efficiency of cancer therapies have been major challenges for scientists worldwide.

Keywords: *antigen presentation, cancer, cytokine, dendritic cell, dendritic cell therapy, T-cytotoxic.*

Classification number: 3.5

Introduction

In many cancer cases, immune cells, particularly those of antigen-presenting cells, that have not performed their main functions, therefore tumours associated with antigens may not be presented to T and B lymphocytes successfully. Moreover, the expressions of ligand-blocking genes on cancer cell surfaces cause the inhibition of immune

response, leading to an uncontrolled proliferation of cancer cells. Currently, several research studies have focused on immunotherapies using various immune cell types such as natural killer (NK) cells, dendritic cells (DCs), macrophages, and others to suppress the development and metastasis of cancers. However, DC therapy is one of the most common techniques used in cancer therapies

because these cells display more predominant features than other immune cells do. DCs are the most specific tumour antigen-presenting cells to prime naive T lymphocytes initiating needed for immune responses. Mature DCs are characterised by secreting a larger number of inflammatory cytokines and chemokines to promote differentiations of T cells into effector cells. The DC-based cancer immunotherapy aims to induce a recurrence of immune responses in these patients. Accordingly, autologous DCs are pulsed with specific tumour antigens to be mature DCs, which are returned to the donors that show promising preliminary results to cancer treatments. In addition to the roles of DCs in inducing specific T-cytotoxic cells capable of seeking and destroying cancer cells including cancer stem cells, DCs exhibit their ability to activate immune responses as vaccines, resulting in preventing the risk of recurrence and metastasis of cancer cells. Besides this, further investigation of molecular mechanisms involved in regulation of the development of cancers is needed to determine the precise molecular etiologies causing cancers. Currently, issues have been considered extensively by scientists to determine particular answers as soon as possible.

DC biology

DCs are the most effective professional antigen-presenting cells to T lymphocytes to initiate the immune response and remain with immunological memory [1]. DCs are

*Corresponding author: Email: xuannt@igr.ac.vn

present in all lymphoid organs, including spleens, lymph nodes, subcutaneous tissue, intestines, bronchi, and lungs, and therefore these cells show their ability to capture exogenous antigens. In these peripheral tissues, DCs ingest these antigens by endocytosis to become mature DCs, which are characterized by (a) The up-regulation of cell surface molecules including major histocompatibility complex (MHC) and co-stimulatory markers CD80, CD86, CD40 and CD54; (b) The enhanced releases of inflammatory cytokine and chemokine productions of interleukin (IL)-12, IL-6, tumor necrosis factors (TNF)- α , and C-C chemokine receptor type 7 (CCR-7). Mature DCs lose their adhesion and are subsequently recruited to the secondary lymphoid organs to present the antigens to T lymphocytes and differentiate them into effector cells in immune response [1].

Besides this, DCs are also involved in maintaining immune tolerance when they consider antigens as endogenous factors. Similar to the ingestion of exogenous antigens, DCs capture the endogenous factors through their phagocytosis and then lose their adhesion. In contrast to the immunostimulatory effects of DCs, the induction of immune tolerance by DCs (i.e. tolerogenic DCs) is characterized by inhibiting an expression of cell surface molecules and an increase in anti-inflammatory cytokine productions such as IL-10 and transforming growth factor beta (TGF- β) to promote the differentiation of regulatory T cells (T reg) [1, 2]. By this way, the tendency of cancer cells is to invade the discovery of immune cells and subsequently proliferate to increase rapidly in number.

There are many different DC subtypes, including myeloid DCs (mDCs), that are derived from myeloid progenitor cells localised within the bone marrow microenvironment, plasmacytoid DCs (pDCs) are derived from lymphoid progenitor cells in

lymphoid organs, and inflammatory DCs are derived from monocytes. The pDCs are only present in lymphoid organs and rarely expressed in other organs. They are recruited to the lymphoid organs, where they are in an inflammatory state [3]. The functional roles of the pDCs considered as both antigen-presenting cells and stimulators of differentiations of T lymphocytes into effector cells have been in debate and undefined. These DC subtypes share common features including adhesion, antigen-presentation, phagocytosis and migration, however, their immune responses, when exposed to various antigens, are subtype-specific properties [1]. The pDCs treated with viral antigens produce a large amount of interferon (IFN) type 1, such as IFN- α and IFN- β (therefore also known as IFN producing cells), and the mDCs exposed to microbial antigens secrete many inflammatory cytokines such as TNF- α and increase the nitric oxide (NO) synthesis. Various effects of cytokines and chemokines in promoting the differentiation of T lymphocytes into effector cells in the activation of immune response are different from each other [1].

DCs in cancer treatments

Cancer antigens are considered as endogenous factors, therefore, leading to the induction of immune tolerance. Cancer cells are characterized by rapid proliferation and spread throughout the body in the following tendencies: (a) Many T reg cells located at tumor sites contribute to their inhibitory effects on the proliferation of cytotoxic T cells, resulting in an invasion of cancer cells from the monitor of immune system induced by foreign antigens; (b) The up-regulation of inhibitory genes in cancer cells leads to suppressing the activation of signaling receptors on antigen-presenting cell surfaces, thus, these antigens are not presented to T lymphocytes; (c) The induction of cancer cells on the enhanced synthesis of anti-inflammatory cytokines such as IL-10 and TGF- β to suppress the differentiations of T lymphocytes and enhance the release of Fas Ligand (FasL) proteins, which triggers the activation of Fas/FasL signaling pathway in leukocytes causing the apoptotic cell death and inhibiting the immunity [4] (Fig. 1).

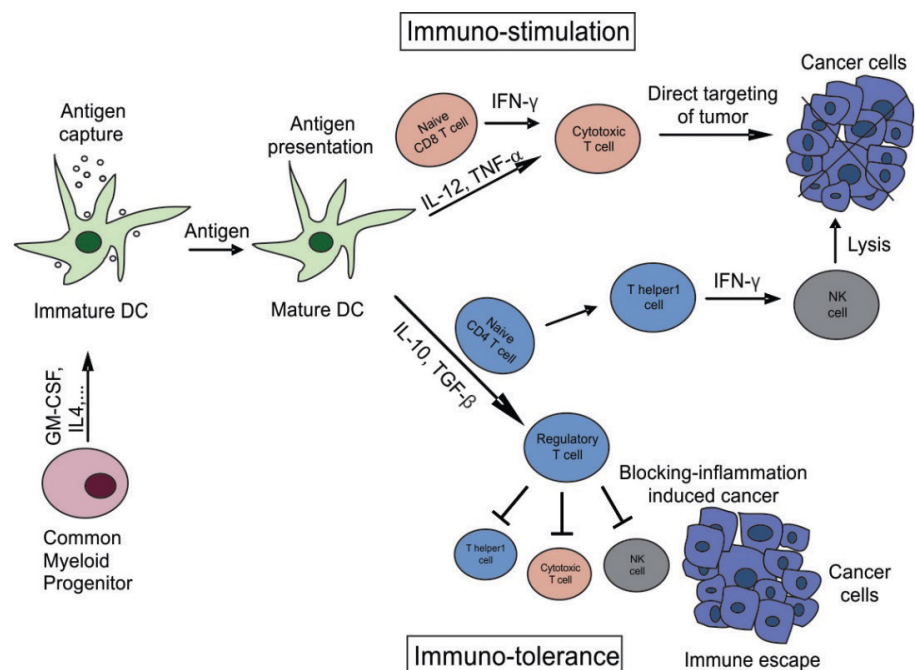


Fig. 1. The regulation of DCs in immune response against cancers.

From investigations using mature DCs as a vaccine, immature DCs should be stimulated with particular antigens derived from cancer cells and subsequently transfused back to their donors with an aim to eliminate cancer cells and prevent the risks of metastasis/recurrence of malignant cells circulated in the body [1]. DC therapies against cancers have been used in clinical laboratory testing in many medical centers. These observations have focused on the identification of the most specific antigens derived from cancer cells to induce the activation of immune response by mature DCs, and followed by the identification of mechanisms underlying the pathogenesis of cancers and determination of targeted cancer therapies.

Technically, DCs are expanded *ex-vivo* for cancer treatments by using peripheral blood mononuclear cells (PBMCs) isolated from blood samples and then PBMCs, which are cultured in the presence of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, TNF- α , and FMS-like tyrosine kinase 3 ligand (Flt3L), to attain DCs. The tumour peptide-induced mature DCs are returned to the donors with the aim to reduce the size of tumours and induce the immunity in patients with cancers. This is currently one of the most common technologies in cancer treatment.

Global researches on cancer

Since 1967, Prof. Okamoto, et al. (Japan), used OK-432 antigen derived from streptococcus to induce activation of DCs in eliminating cancers [5]. In 1995, the DC therapy using melanoma-associated antigen-derived epitopes were treated for patients with melanoma [6]. Currently, clinical applications of DC therapy in treatments of some cancers, such as pancreatic [7], bile duct [8], lung [9], and ovarian [10] cancers, has attained particular success. The results of which indicated that patients with

late-stage cancers or metastatic cancers are treated with either chemotherapy or radiation treatment, and followed by DC therapy, could prolong the longevity as much as 10 to 15 years. In addition to this, other studies also revealed that patients treated with DCs with high MHC class I molecule expression showed a larger number of intraepithelial CD8+ T cells, resulting in improving survival even more [11, 12].

In addition to stimulation of DCs with tumour antigens, necrotic cancer cells have been used to stimulate DCs, which are transfused into tumor-bearing mice, attaining promising results to date [13]. These cells are exposed to ultraviolet light to kill targeted cancer cells and damage their DNA structures, leading to cell necrosis or apoptosis. The flow cytometry method uses annexin V antibody to detect the exposure of phosphatidylserine on the cell surfaces and 7AAD to stain necrotic cell nuclei with the aim to determine whether they are apoptotic or necrotic cells. Several reports indicated that cellular DNA damage of melanoma and B-cell leukemia to induce apoptotic cell death and be followed by exposure to DCs, which are then transfused into cancer patients, leads to activation of cytotoxic T lymphocytes-mediated immune response [14, 15]. In 1998, R.C. Fields, et al., demonstrated that mature DCs triggered by necrotic cancer cells, including breast cancer or sarcoma cells transfused into tumor-bearing mice, resulted in successful immune response to the second exposure of various cancers and inhibition of the metastasis of lung cancer [16]. Currently, the transfusion of mature DCs triggered by nontoxic-targeted cancer cells such as melanoma [17], breast [18], lymph node [19], and several cancers into tumor-bearing mice, has been achieving good consequences. However, no study indicates contributions of these mature DCs to patients with cancers by transfusing them directly to the donors.

Cancer research in Vietnam

In Vietnam, researchers of the Laboratory of Stem Cell Research and Application at the Vietnam National University, Ho Chi Minh City was the first to perform experiments using DCs in mice in 2010. Peripheral blood mononuclear cells (PBMCs) are isolated from blood samples and cultured in a specific pathogen-free condition to obtain therapeutic DCs. The DCs are stimulated with tumour antigens and the activated DCs are then transfused into breast cancer-bearing mice. As a result, the DC therapy, without using chemotherapy or radiation treatment, reduces 80% of the size of breast cancer [13, 20]. However, the application of DC therapy has not clinically been applied yet to treatments of breast cancer patients. In the future, further studies are needed to determine the etiology and molecular mechanisms involved in the regulation of pathogenesis of cancers and would be followed by the discovery of effective cancer drugs.

Molecular mechanisms involving in cancer development

The induction of up-regulated expressions of both cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1) proteins on the leukocyte surfaces by cancer cells is one of the most successful processes to multiply in number and immune escape. Therefore, a large number of T-regs and T helper 17 cells (Th17) are recruited to tumour sites, resulting in the development of immune tolerance and inhibition of the appearance of cytotoxic CD8 T cells and nature killer (NK) cells [21], which aim to suppress the immune response against cancers.

Besides, other studies showed that the expression levels of several receptors, such as toll-like receptors (TLR), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and

TGF- β receptors (TGF- β R), all located at leukocyte surfaces are associated with the development of cancers [22]. Investigations of patients with prostate cancer revealed that the genetic alteration of some genes such as TLR4, TLR1, TLR6, and TLR10, are risk factors for cancers [23, 24]. Similarly, a nucleotide change in the TLR2 gene is also a risk factor for colorectal cancer in mice [25]. Studies on downstream molecules of TLR signalling including myeloid differentiation primary response protein (MyD88) demonstrated that inhibition of the expression of this gene reduces the development of several cancers such as colorectal cancer, melanoma, and liver cancer [26, 27]. The immunotherapy for acute myeloid leukemia using TLR signaling-mediated mature DCs induced by cancer antigens has been achieving high efficiency for the treatment of cancers [28]. Several investigations focused on the role of NLR signaling in the modulation of cancers, as alteration in Nod2 gene is at risk of colorectal cancer [29] and NLR family, pyrin-containing 3 gene (NLRP3)-deficient mice are susceptible to colon and colorectal cancers, mediated through the release of IFN- γ cytokine and activation of signal transducer and activator of transcription (STAT)-1 signaling [30]. In addition to this, a binding between TGF- β , a cytokine produced by leukocytes with TGF- β R, is used to stimulate activation of this signaling leading to blocking the development of early stage cancers; however, facilitating indirectly to the development of late-stage cancers by recruiting a large number of T regs to tumor sites [31]. The induced activation of downstream molecules of TGF- β R including Smad, inhibits the proliferation of cancer cells. Therefore, the abnormal expression of the Smad protein is also a risk factor for several cancers, such as colorectal, prostate, and head and neck cancers [32].

The bindings between receptors located on the leukocyte surfaces and

specific ligands trigger activation of downstream molecules, such as mitogen-activated protein kinases (MAPK) and nuclear factor- κ B (NF- κ B) signalling pathways, is leading to the transcriptions of genes involved in the regulation of cellular physiological processes [33, 34]. Hence, the abnormal activation of NF- κ B signalling causes about 20% of human cancers derived from patients with severe chronic diseases and activation of MAPK signalling in leukocytes, especially DCs, increases the immune response against cancers.

Conclusions

At present, immunotherapies for cancers have attracted special attention by global scientists due to their high safety, efficiency, and not causing suffering from side effects to the patients induced by conventional cancer therapies. The DC therapy technique considered as a vaccine in the treatment of cancers, has been applied extensively since DCs display predominant characteristics than those of other immune cells as follows, the most efficient processing and presenting capacities to T lymphocytes and the release of larger number of inflammatory cytokines compared to other antigen-presenting cells such as macrophages, B cells or NK cells. Therefore, the induction of differentiation of T lymphocytes into effector cells by activated DCs could result in most favourable immune responses. Combined DC therapy in the treatments of cancers after surgery, radiation and chemotherapy has been widely tested with aims for application across broad DC applications in preventing the development of malignant tumours, as well as inducing immunity against cancers. Similarly, the DC-therapy might suppress the threat of cancer recurrences and metastasis of malignant cancer cells to improve longer survivals in patients with end-stage cancers. Further investigations are needed to fine-tune DC protocols

and figure out the most effective ways to abolish cancers and the DC therapy could be among the most promise immunotherapies in future treatments of cancers.

ACKNOWLEDGEMENTS

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-YS.06-2013.21.

REFERENCES

- [1] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, K. Palucka (2000), "Immunobiology of dendritic cells", *Annu. Rev. Immunol.*, **18**, pp.767-811.
- [2] I. Volovitz, S. Melzer, S. Amar, J. Bocsi, M. Bloch, S. Efroni, Z. Ram, A. Tarnok (2016), "Dendritic Cells in the Context of Human Tumors: Biology and Experimental Tools", *Int. Rev. Immunol.*, **35**, pp.116-135.
- [3] H. Yoneyama, K. Matsuno, Y. Zhang, T. Nishiwaki, M. Kitabatake, S. Ueha, S. Narumi, S. Morikawa, T. Ezaki, B. Lu, C. Gerard, S. Ishikawa, K. Matsushima (2004), "Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules", *Int. Immunol.*, **16**, pp.915-928.
- [4] X. Yu, Y. Li, Y. Yu, J. Lei, G. Wan, F. Cao (2016), "Associations between FAS rs2234767 and FASL rs763110 polymorphisms and the risk of lung cancer: a meta-analysis of 39,736 subjects", *Onco Targets Ther.*, **9**, pp.2049-2056.
- [5] H. Okamoto, S. Shoin, S. Koshimura, R. Shimizu (1967), "Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci", *Jpn J. Microbiol.*, **11**, pp.323-326.
- [6] A.B. Bakker, G. Marland, A.J. de Boer, R.J. Huijbens, E.H. Danen, G.J. Adema, C.G. Figdor (1995), "Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro", *Cancer Res.*, **55**, pp.5330-5334.
- [7] M. Okamoto, M. Kobayashi, Y. Yonemitsu, S. Koido, S. Homma (2016), "Dendritic cell-based vaccine for pancreatic cancer in Japan", *World J. Gastrointest. Pharmacol. Ther.*, **7**, pp.133-138.

- [8] M. Kobayashi, T. Sakabe, H. Abe, M. Tani, H. Takahashi, A. Chiba, E. Yanagida, Y. Shibamoto, M. Ogasawara, S. Tsujitani, S. Koido, K. Nagai, S. Shimodaira, M. Okamoto, Y. Yonemitsu, N. Suzuki, M. Nagaya, Therapy DC-vaccine study group at the Japan Society of Innovative Cell (2013), "Dendritic cell-based immunotherapy targeting synthesised peptides for advanced biliary tract cancer", *World J. Gastrointest. Surg.*, **17**, pp.1609-1617.
- [9] H. Takahashi, M. Okamoto, S. Shimodaira, S. Tsujitani, M. Nagaya, T. Ishida, J. Kishimoto, Y. Yonemitsu, Therapy DC-vaccine study group at the Japan Society of Innovative Cell (2013), "Impact of dendritic cell vaccines pulsed with Wilms' tumour-1 peptide antigen on the survival of patients with advanced non-small cell lung cancers", *Eur. J. Cancer*, **49**, pp.852-859.
- [10] M. Kobayashi, A. Chiba, H. Izawa, E. Yanagida, M. Okamoto, S. Shimodaira, Y. Yonemitsu, Y. Shibamoto, N. Suzuki, M. Nagaya, Therapy DC-vaccine study group at the Japan Society of Innovative Cell (2014), "The feasibility and clinical effects of dendritic cell-based immunotherapy targeting synthesised peptides for recurrent ovarian cancer", *J. Ovarian Res.*, **7**, p.48.
- [11] E. Sato, S.H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A.A. Jungbluth, D. Frosina, S. Gnjatic, C. Ambrosone, J. Kepner, T. Odunsi, G. Ritter, S. Lele, Y.T. Chen, H. Ohtani, L.J. Old, K. Odunsi (2005), "Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer", *Proc. Natl. Acad. Sci. USA*, **102**, pp.18538-18543.
- [12] J. Tel, G. Schreibelt, S.P. Sittig, T.S. Mathan, S.I. Buschow, L.J. Cruz, A.J. Lambeck, C.G. Figdor, I.J. de Vries (2013), "Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets", *Blood*, **121**, pp.459-467.
- [13] S.T. Nguyen, H.L. Nguyen, V.Q. Pham, et al. (2015), "Targeting specificity of dendritic cells on breast cancer stem cells: in vitro and in vivo evaluations", *Onco Targets Ther.*, pp.323-334.
- [14] O. Manches, G. Lui, J.P. Molens, J.J. Sotto, L. Chaperot, J. Plumas (2008), "Whole lymphoma B cells allow efficient cross-presentation of antigens by dendritic cells", *Cytotherapy*, **10**, pp.642-649.
- [15] G. Pietra, R. Mortarini, G. Parmiani, A. Anichini (2001), "Phases of apoptosis of melanoma cells, but not of normal melanocytes, differently affect maturation of myeloid dendritic cells", *Cancer Res.*, **61**, pp.8218-8226.
- [16] R.C. Fields, K. Shimizu, J.J. Mule (1998), "Murine dendritic cells pulsed with whole tumour lysates mediate potent antitumor immune responses in vitro and in vivo", *Proc. Natl. Acad. Sci. USA*, **95**, pp.9482-9487.
- [17] N. Werthmoller, B. Frey, R. Wunderlich, R. Fietkau, U.S. Gaipl (2015), "Modulation of radiochemoimmunotherapy-induced B16 melanoma cell death by the pan-caspase inhibitor zVAD-fmk induces anti-tumor immunity in an HMGB1-, nucleotide- and T-cell-dependent manner", *Cell Death Dis.*, **6**, p.e1761.
- [18] I.M. Meraz, D.J. Savage, V. Segura-Ibarra, J. Li, J. Rhudy, J. Gu, R.E. Serda (2014), "Adjuvant cationic liposomes presenting MPL and IL-12 induce cell death, suppress tumor growth, and alter the cellular phenotype of tumors in a murine model of breast cancer", *Mol. Pharm.*, **11**, pp.3484-3491.
- [19] S.K. Hira, I. Mondal, P.P. Manna (2015), "Combined immunotherapy with whole tumour lysate-pulsed interleukin-15-activated dendritic cells and cucurbitacin I promotes strong CD8(+) T-cell responses and cures highly aggressive lymphoma", *Cytotherapy*, **17**, pp.647-664.
- [20] P.V. Pham, N.T. Nguyen, H.M. Nguyen, L.T. Khuat, P.M. Le, V.Q. Pham, S.T. Nguyen, N.K. Phan (2014), "A simple in vitro method for evaluating dendritic cell-based vaccinations", *Onco Targets Ther.*, **7**, pp.1455-1464.
- [21] T. Kitamura, B.Z. Qian, J.W. Pollard (2015), "Immune cell promotion of metastasis", *Nat. Rev. Immunol.*, **15**, pp.73-86.
- [22] H. Clevers (2004), "At the crossroads of inflammation and cancer", *Cell*, **118**, pp.671-674.
- [23] E.M. El-Omar, M.T. Ng, G.L. Hold (2008), "Polymorphisms in Toll-like receptor genes and risk of cancer", *Oncogene*, **27**, pp.244-252.
- [24] J. Sun, F. Wiklund, S.L. Zheng, B. Chang, K. Balter, L. Li, J.E. Johansson, G. Li, H.O. Adami, W. Liu, A. Tolin, A.R. Turner, D.A. Meyers, W.B. Isaacs, J. Xu, H. Gronberg (2005), "Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk", *J. Natl. Cancer Inst.*, **97**, pp.525-532.
- [25] H. Yang, H. Zhou, P. Feng, X. Zhou, H. Wen, X. Xie, H. Shen, X. Zhu (2010), "Reduced expression of Toll-like receptor 4 inhibits human breast cancer cells proliferation and inflammatory cytokines secretion", *J. Exp. Clin. Cancer Res.*, **29**, p.92.
- [26] W.E. Naugler, T. Sakurai, S. Kim, S. Maeda, K. Kim, A.M. Elsharkawy, M. Karin (2007), "Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production", *Science*, **317**, pp.121-124.
- [27] S. Rakoff-Nahoum, R. Medzhitov (2007), "Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88", *Science*, **317**, pp.124-127.
- [28] M. Subklewe, C. Geiger, F.S. Lichtenegger, M. Javorovic, G. Kvalheim, D.J. Schendel, I. Bigalke (2014), "New generation dendritic cell vaccine for immunotherapy of acute myeloid leukemia", *Cancer Immunol. Immunother.*, **63**, pp.1093-1103.
- [29] J.H. Cho (2008), "The genetics and immunopathogenesis of inflammatory bowel disease", *Nat. Rev. Immunol.*, **8**, pp.458-466.
- [30] M.H. Zaki, K.L. Boyd, P. Vogel, M.B. Kastan, M. Lamkanfi, T.D. Kanneganti (2010), "The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis", *Immunity*, **32**, pp.379-391.
- [31] M. Saitoh (2015), "Epithelial-mesenchymal transition is regulated at post-transcriptional levels by transforming growth factor-beta signalling during tumour progression", *Cancer Sci.*, **106**, pp.481-488.
- [32] J. Fang, H. Xu, C. Yang, S. Kayarthodi, R. Matthews, V.N. Rao, E.S. Reddy (2014), "Molecular Mechanism of Activation of Transforming Growth Factor Beta/Smads Signaling Pathway in Ets Related Gene-Positive Prostate Cancers", *J. Pharm. Sci. Pharmacol.*, **1**, pp.82-85.
- [33] L.M. Coussens, Z. Werb (2002), "Inflammation and cancer", *Nature*, **420**, pp.860-867.
- [34] F. Arce, G. Kochan, K. Breckpot, et al. (2012), "Selective activation of intracellular signalling pathways in dendritic cells for cancer immunotherapy", *Anti-cancer Agents Med. Chem.*, **1**, pp.29-39.

Effects of ENSO on cold - air activities and tropical cyclones in Vietnam

Duc Ngu Nguyen*

Centre for Hydro - Meteorological, Environmental
Sciences and Technologies

Received 19 May 2017; accepted 6 June 2017

Abstract:

This paper presents a summary of the results of a study into the effects of ENSO (El Niño and La Niña) on the frequency of cold fronts and tropical cyclones affecting Vietnam. The results show that, during the ENSO periods, cold air activities affecting Vietnam were reduced. The ratio of total positive anomalies to negative anomalies of front frequency was 68% in the case of El Niño, and 60% in the case of La Niña. The frequency of tropical cyclones affecting Vietnam decreased by 28% in the case of El Niño, and increased to 38% in comparison with the multi-year average in the case of La Niña. During the El Niño period, hurricanes affecting Vietnam were often evenly distributed or concentrated in the first half of the typhoon season, but during the La Niña period, hurricanes were concentrated in the second half of the hurricane season.

Keywords: cold air, effects of ENSO, tropical cyclones.

Classification number: 6.2

Introduction

ENSO - El Niño-Southern Oscillation - is an oceanic - atmospheric phenomenon that occurs in the Pacific Ocean, and brings on strong transformations in the weather and climate of many places around the world, and impacts economic and environmental activities [1]. Over the last half of a century (1951-2016), 20 warm ENSO (El Niño) - and 13 La Niña - related occurrences have been identified according to the Oceanic Niño Index - ONI) [2]. Many scientists have been researching this phenomenon, and many programs have been implemented, including surveys gathering empirical research about oceans and the atmosphere, especially the TOGA program (1985-1994) [3-5]. ENSO often causes severe fluctuations of weather in Vietnam, in which the activity of tropical cyclones and cold air is notable.

This paper studies the impact of ENSO on the intrusion of cold air and the effects of typhoons and tropical depressions on Vietnam.

Methodology and Data

Methodology

The main research method used in this study was a standard deviation analysis that contained the following statistical characteristics:

$$1) \text{ Mean: } \bar{X} = \frac{1}{n} \sum_{i=1}^n X_{ti}$$

$$2) \text{ Anomaly: } \Delta X = X_t - \bar{X}$$

Where : $\Delta X_t > 0$, Positive anomaly

$\Delta X_t = 0$, Anomaly 0

$\Delta X_t < 0$, Negative anomaly

3) The method of determining the periods of warm ENSO phase (El Niño) and the periods of cold ENSO phase (La

*Email: nguyenducngu@yahoo.com

Niña): The study compared the correlation coefficient between the average annual sea surface temperature in NIÑO areas with the mean annual air temperature at 14 climate stations representing the climatic zones in Vietnam, in this study, one El Niño episode was defined as a continuous period of no less than six months with a five-month moving average of the standard deviation of monthly sea surface temperature average (SSTA) of NIÑO-3 (150°W-90°W, 5°N-5°S) greater than or equal to 0.5°C. A La Niña episode is a continuous period of no less than six months with a five-month moving average of SSTA in the NIÑO-3 area less than or equal to -0.5°C.

Data

- Monthly average sea surface temperature data and anomaly in the NIÑO period of 1950-2000.
- Southern Oscillation Index (SOI) in the period of 1876-2001 [6].
- Data on climatic elements including sea level pressure at four stations in Hanoi, Da Nang, Vung Tau, and Con Dao; cold fronts and typhoons affected Vietnam during the periods of 1950-2000 [7-9].

Results and discussions

The El Niño and La Niña episodes during the period of 1956-2000

As was stated above, during 1956-2000, there was an occurrence of 12 El Niño episodes: 1957/1958, 63/64, 65/66, 68/70, 72/73, 76/78, 79, 82/83, 86/88, 91/92, 93, and 97/98, and eight La Niña episodes: 64/65, 67/68, 70/71, 73/74, 75/76, 84/85, 88/89, and 98/00. The 1997/1998 El Niño season had the highest SSTA value of 3.9°C, which occurred in December 1997 and was considered the strongest El Niño season out of the above mentioned El Niño episodes. The 1998/2000 La Niña had the largest negative SSTA value of -1.6°C, recorded in January 2000, which was considered the strongest La Niña occurrence in the above mentioned La Niña season [10]. These two ENSO batches are used in the case study.

The impact of ENSO on the operation of cold air in Vietnam

Fluctuations of atmospheric pressure:

Checking fluctuations of atmospheric pressure in Hanoi, Da Nang, Vung Tau, and Con Dao for the 1997/1998 El Niño and 1998/2000 La Niña showed that suitable for SOI, the strongest fluctuations, decrease in the north (Fig. 1 and 2). In the El. period of 97/98, the pressure anomaly of the stations was positive (as opposed to the SOI index) and increased according to El's development cycle. Until the peak stage and then gradually decrease in the period of recession El. During

the 98/2000 La Niña, the pressure anomaly of the stations is positive and fluctuate in the La. Cycle, corresponding to the volatility of the SOI.

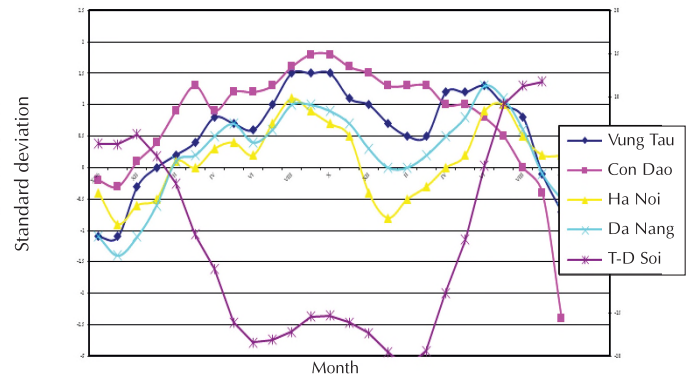


Fig. 1. Variation of the standard deviation of sea level pressure of moving average of five months in El Niño.

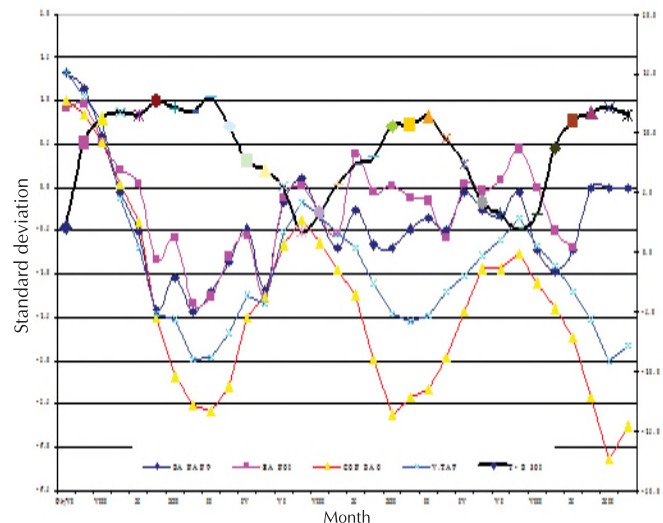


Fig. 2. Variation of the standard deviation of sea level pressure of moving average of five months in La Niña 1998/2000.

Fluctuations of cold fronts affect Vietnam:

Cold front activity affecting Vietnam is reflected through the frequency of cold fronts and the cold front seasons in Hanoi. The impact of ENSO including the El Niño and La Niña's influences were analyzed on a basis of the data on the anomaly of cold front frequency and unusual behavior of cold seasons.

1) Standard of frequency and standard of cold fronts season

The period of 1956-2000 had 1.303 cold fronts, which averages at 29.1 phase for a year, and 2.42 cold fronts per month (Table 1). That is a typical value for the frequency of cold fronts in a month, assuming the number of fronts distributed throughout the year for 12 months. So the cold season in

Vietnam can be considered to be from October to May the next year. June and September are considered as transition months, and July and August are the interruptions of cold fronts.

Table 1. Frequency of cold front over Hanoi from 1956 to 2000.

Months	1	2	3	4	5	6	7	8	9	10	11	12	Year
Total	181	144	148	120	121	68	6	7	61	131	160	156	1303
Average	4.0	3.2	3.3	2.7	2.7	1.5	0.13	0.16	1.4	2.9	3.6	3.5	29.1

2) The effect of El Niño on the frequency of cold fronts

In the 150 months of El Niño, during the period of 1956-2000, there were 356 cold fronts throughout Hanoi. On average, each month there had 2.37 cold fronts, less than the average monthly amount. Although the number of El Niño occurrences had fewer cold fronts, at approximately equal to the number of El Niño cold fronts, the number of El Niño months was negative anomalies than that number of El Niño months that had positive anomalies. The negative anomaly occurs much in the last months of the cold season (II, III, IV, V) and two months of cold interruption (VII, VIII). On the contrary, the positive anomaly is greater than the negative anomaly in the first few months of winter and winter. This leads to the conclusion that, under El Niño conditions, the cold front season has a higher frequency in the first half of winter and is more likely to end sooner than normal (Table 2).

Table 2. Anomaly of cold front frequency in El Niño months.

Sign of anomaly	1	2	3	4	5	6	7	8	9	10	11	12	Total
Positive	7	4	1	2	5	7	2	1	7	9	9	6	60
Negative	4	7	7	10	8	6	11	12	8	4	4	7	88
No Anomaly	2	0	0	0	0	0	0	0	0	0	0	0	2
Total	13	11	8	12	13	13	13	13	14	13	13	13	150

3) La Niña's influence on cold fronts

In the 107 months of La Niña, during the period of 1956 to 2000, there were 243 cold fronts in Hanoi. On average, La Niña had 2.27 cold front phases, less than the normal average during those months (Table 3). As in the El Niño months, the number of La Niña months had a negative effect on cold fronts, more than the positive anomaly. Almost all months that had negative anomalies are superior, especially during the half months after winter. Only in May, October, and December, opposite situation was observed, in which the positive anomalies were greater than the negative anomalies. In addition, the La Niña season of 1998-2000, which lasted for only four months (in 18 months of existence), was the period of interruption and transition of the cold front season but still very little cold front.

Table 3. The anomaly of cold front frequency in La Niña months.

Sign of anomaly	1	2	3	4	5	6	7	8	9	10	11	12	Total
Positive	0	2	2	3	5	3	0	4	4	7	4	8	42
Negative	4	6	6	5	2	6	8	4	5	4	7	3	60
None	5	0	0	0	0	0	0	0	0	0	0	0	5
Total	9	8	8	8	7	9	8	8	9	11	11	11	107

An overview of the ENSO cold-front relationship:

- In the El Niño and La Niña periods, the frequency of cold fronts usually decreased, especially in the last months (for El Niño) and the second half of the seasons (for La Niña).

- During the El Niño and La Niña periods, the cold front seasons usually began no sooner or later than usual, but sometimes ended sooner than usual.

- In the El Niño and La Niña periods, cold front interruptions were usually longer than in normal years. During the El Niño years, the number of colder waves deepened further south during the La Niña years. In the years of El Niño and La Niña, the number of cold fronts affecting our country is less than normal. The ratio of total the positive and negative anomaly of the front frequency in Hanoi for the months of the year was only 68% in the case of El Niño and 60% in the case of La Niña. The time interruption of a front is longer, especially in the last months of the cold front season and therefore, the cold front season ended earlier than normal.

The impact of ENSO on the occurrences of tropical cyclones in Vietnam

The standard number of storms and storm seasons in Vietnam:

During the period of 1956-2000, there were 311 storms and tropical depressions (here-in-after referred to as "storms"), and their impact on Vietnam averaged 6.9 attacks per year and 0.58 each month (Table 4). In this study, the mean values of monthly and yearly storms during the 1956-2000 monitoring period were considered to be monthly and yearly storms. If the hurricane season is a period of months with an average of 0.33 times/month or more (at least an average of 3 years with 1 storm), the storm season in Vietnam is from June to December.

Table 4. The average of number storm for per month and year.

Months	1	2	3	4	5	6	7	8	9	10	11	12	Year
Number of storm	0.0	0.02	0.07	0.07	0.11	0.67	0.73	1.16	1.42	1.33	1.00	0.33	6.91

*Impact of ENSO on the occurrences of storms:***- Hurricanes affect Vietnam under El Niño conditions**

During the period of 1956-2000, there were 12 El Niño periods with a total of 150 El Niño months and 63 storms. On average, there were 0.42 typhoons in Vietnam for each El Niño month, less than the average number of 0.16 attacks (about 28%). Most of the storms affecting Vietnam during the El Niño months of the hurricane season are less than the monthly standard (negative anomaly). Hence, the negative anomaly of the year is also significantly greater than the positive anomaly (Table 5). Thus, the number of hurricanes affecting Vietnam under El Niño conditions is less than normal.

Table 5. Sign of anomaly storm in El Niño months of the hurricane season.

Month	6	7	8	9	10	11	12	Year
Positive anomaly	3	7	4	5	1	2	3	25
Negative anomaly	9	5	8	9	12	11	10	64
Total	12	12	12	14	13	13	13	89

- Hurricanes affect Vietnam under La Niña conditions

During the period of 1956-2000, there were nine occurrences during the La Niña phase with a total of 107 La Niña occurrences and 86 storms. On average, each La Niña month during this period had 0.8 storms affecting Vietnam, more than the standard average of 38% and twice the number of storms affecting Vietnam under El Niño conditions. The difference between the positive anomaly and the negative anomaly is not much but the absolute value of the positive anomaly is significantly greater than the absolute value of the negative anomaly, so the number of storms in the months and in both The hurricane season under La Niña condition is still higher than the average (Table 6).

Table 6. Sign of storm anomaly in La Niña month of the hurricane season.

Month	6	7	8	9	10	11	12	Year
Positive anomaly	4	3	3	4	8	6	3	31
Negative anomaly	3	5	5	5	3	5	8	34
Total	7	8	8	9	11	11	11	65

Conclusions

1. The phenomenon of ENSO clearly affects the distribution of pressure in Vietnam. During the El Niño cycles, the sea surface pressure at Vietnam stations had increased. The sea level pressure anomaly is positive, increasing according to the El Niño development cycles, as opposed to negative values and

the process of the SOI. In the La Niña cycles, the situation is reversed; the monthly pressure anomalies are negative, as opposed to the positive and the SOI. As far south, these effects are clearer.

2. The frequency of cold fronts that affects Vietnam in periods of warm ENSO and cold ENSO are less trivial. The frequency of cold fronts decreased much in the last months of the season in the case of El Niño and the second half of the cold season in the case of La Niña. The cold front season ends earlier than usual.

3. The frequency of storms affecting Vietnam in El Niño was 28% less than usual, while in La Niña, the frequency of storms affected Vietnam more than usual by about 38% and nearly twice the frequency of storms affected during the El Niño waves. During the El Niño periods, typhoons affecting Vietnam are often evenly distributed or concentrated in the first half of the typhoon season, while during the La Niña periods, typhoons are concentrated in the second half of the hurricane season.

REFERENCES

- [1] Duc Ngu Nguyen (2002), *Impact of ENSO on weather, climate, environment and socio-economic in Vietnam*, Synthesis report of the independent scientific research national project, **Vol.1**, Hanoi.
- [2] Climate Prediction Center (1998), "Quantifying, detecting and monitoring ENSO", *World Climate News*, **No.13**, p.5.
- [3] J.L. Evans (1990), "Envisaged impacts of enhanced greenhouse warming on tropical cyclones in the Australian region", *CSIRO Division of Atmospheric Research Technical Paper*, **No.20**, pp.1-31.
- [4] Kevin E. Trenberth (1996), "El Niño definition", *Exchanges*, **1(3)**, pp.6-8.
- [5] World Climate Research Programme (1995), *CLIVAR: A study of climate variability and predictability: Science Plan*, WCRP 89, WMO/TD No.690.
- [6] Bureau of Meteorology, Australia (1999), *Southern Oscillation Index (SOI) Archives*, Copyright Commonwealth of Australia.
- [7] Department of Hydro-meteorological Forecasting, and National Center for Hydro-meteorological Forecasting, *The frequency of typhoon and tropical depression landing data and direct impacts on Viet Nam, Characteristics activity of tropical cyclones and tropical depression annual in the period of 1956-2000*.
- [8] Department of Hydro-meteorological Forecasting, and National Center for Hydro-meteorological Forecasting, *The cold air frequency data (cold fronts) affecting Vietnam, Characteristics of annual cold air in the period of 1956-2000*.
- [9] Viet Nam Institute of Meteorology, Hydrology and Climate Change, *Standard climate data for the period of 1961-2010*.
- [10] Japan Meteorological Agency (1996, 1997), *Climate change monitoring report*.

Current and projected climate change in the Mekong Delta

Van Thang Nguyen*, Van Khiem Mai, Van Thang Vu, Dang Mau Nguyen

Vietnam Institute of Meteorology, Hydrology and Climate change

Received 10 May 2017; accepted 5 June 2017

Abstract:

This article evaluates trends in past and projected future climate change at the Mekong Delta. The study is based on updated observation data gathered up to 2014, and the latest climate change scenarios published by the Ministry of Natural Resources and Environment (MONRE). The results show that the mean annual temperature increased by 0.3 to 0.9°C, and annual rainfall increased by 5 basis points to 20% at most observation stations during the years 1958-2014. In comparison to the baseline (1986-2005), temperatures were projected to increase by between 1.3 to 1.4°C in the middle of the century and by between 1.7 to 1.9°C at the end of the century, under the medium scenario (RCP4.5). According to the high scenario of RCP8.5, temperatures likely increased by 1.8 to 2°C in the middle of the century and 3.4 to 3.6°C at the end of the century. Annual rainfall is expected to increase from 10 to 20% in the mid-21st century under both RCP4.5 and RCP8.5 scenarios; increase of 30% in a part of the northern Mekong Delta under the RCP8.5 scenario.

Keywords: climate change, Mekong Delta, projected climate, rainfall, temperature.

Classification number: 6.2

Introduction

The Mekong Delta is the largest river delta in Vietnam, located in the Mekong River Basin with a total natural area of about 3.96 million ha. This delta has a coastline ranging over 700 km, contiguous to Cambodia in the northwest, to South Vietnam in the east, to the East Sea in the northeast, to the Pacific Ocean in the south, and to the Gulf of Thailand in the west [1]. This region is favourable to marine economic development, exploitation, aquaculture, and consumption and export. The Mekong Delta is the largest agricultural development area in the country, contributing considerably to the region's total food production. However, due to its low elevation and flat terrain, as well as its location within tropical monsoon

climates, this area is vulnerable to climate change [1].

In addition to this, freshwater resources in the Mekong Delta are strongly influenced by hydropower dams in the Mekong River System, especially during the dry season. Typically, severe droughts and saltwater intrusion from late 2015 to early 2016 in the Mekong Delta was caused by a prolonged El Nino phenomenon. According to the Intergovernmental Panel on Climate Change (IPCC), the Mekong Delta is one of three deltas classified to have extreme vulnerability of impact from sea level rise caused by climate change; this list also includes the Ganges River Delta of the Brahmaputra River (Bangladesh) and Nile River (Egypt) [2].

There have been a number of studies

into the effects of climate change in the Mekong Delta since the 1990s [3, 4]. In recent years, the assessment of the impact of climate change, as well as its consequences, has been investigated by many authors [2, 5-15]. However, most of these studies assess the impact of climate change according to national scales, and the Mekong Delta is the only part of the assessments. In general, most research is primarily based on the IPCC's Greenhouse Gas Emission Scenario, published in 2007 [5, 6, 11, 12, 14]. In 2016, MONRE has updated the impact assessment of climate change and climate change projections at the national scale based on updated data (till 2014) and a new approach from Representative Concentration Pathways - RCPs announced by IPCC in 2013 [7].

The purpose of this study is to calculate, analyse, and evaluate trends in climate change ranging from the past to projected impacts at the Mekong Delta, based on the latest climate change studies from MONRE and IPCC. This study will update important information about climate change for use to assess the impact and vulnerability of the Mekong Delta to climate change.

Data and methodology

Data

Observation data:

Observation data includes datasets of climatic variables gathered from 1958-2014 at 11 stations in the Mekong Delta: Moc Hoa, My Tho, Cao Lanh, Ba Tri, Cang Long, Chau Doc, Can Tho, Soc Trang, Rach Gia, Bac Lieu, and Ca Mau.

Model data:

The models used for constructing climate change scenarios include: (i) the AGCM/MRI model of Meteorological Research Institute in Japan, (ii) the PRECIS model of Met Office Hadley Centre in UK, (iii) the CCAM model of the Commonwealth Scientific and

*Corresponding author: Email: nvthang.62@gmail.com

Industrial Research Organization (CSIRO) in Australia, (iv) the RegCM model of the Abdus Salam International Centre for Theoretical Physics (ICTP) in Italy, (v) and the cLWRF model of National Centers for Environmental Prediction (NCEP) in USA. These models were used to simulate the regional climate in the baseline period (1986-2005) and projected regional climate at the middle (2046-2065), and at the end (2080-2099) of the 21st century (Table 1).

In this study, linear regression was used to identify the trends in climate variables during the period of 1961-2014. Linear regression of a predicted (y) at time (t) can be described as follows:

$$y = a_0 + a_1 t$$

$$a_0 = \bar{y} - a_1 \bar{t}, \quad a_1 = r_{yt} \frac{S_y}{S_t}$$

$$S_y = \sqrt{\sum_{i=1}^n (y_i - \bar{y})^2}, \quad S_t = \sqrt{\sum_{i=1}^n (t_i - \bar{t})^2}$$

Table 1. Models used for constructing climate change scenarios.

No.	Models	GCMs	Resolution	Available Data Period			
				Baseline	RCP4.5	RCP6.0	RCP8.5
1	CCAM	ACCESS1-0	10 km	1970-2005	2006-2099	x	2006-2099
2		CCSM4					
3		CNRM-CM5					
4		GFDL-CM3					
5		MPI-ESM-LR					
6		NorESM1-M					
7	RegCM	ACCESS1-0	20 km	1980-2000	2046-2065 2080-2099	x	2046-2065 2080-2099
8		NorESM1-M					
9	Precis	HadGEM2-ES	25 km	1960-2005	2006-2099	x	2006-2099
10		GFDL-CM3					
11		CNRM-CM5					
12	CLWRF	NorESM1-M	30 km	1980-2005	2006-2099	x	2006-2099
13	MRI-20km_A	NCAR-SST	20 km	1984-2003	x	x	2080-2099
14	MRI-20km_B	HadGEM2- SST					
15	MRI-20km_C	GFDL - SST					
16	MRI-20km_D	SST					

x: No data.

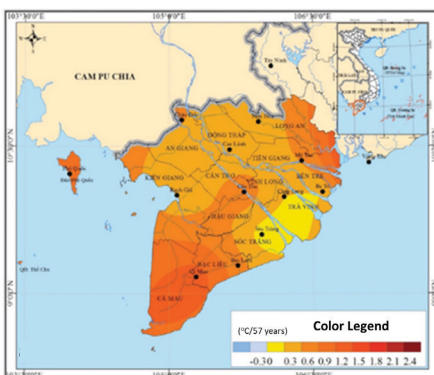


Fig. 1. Observed trend of annual temperature (°C) during the period of 1961-2014.

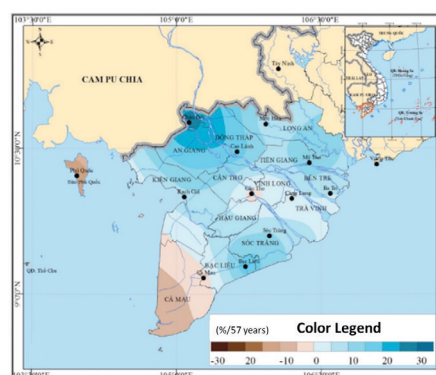


Fig. 2. Observed trend of annual rainfall (%) during the period of 1961-2014.

Methodology

Identify the trends of climate change in the past:

In which $\bar{y}$, $\bar{t}$ are means, and S_y , S_t are standard deviations of y and t , respectively; r is linear correlation

coefficient between y and t . The increased and decreased trends of $y(t)$ are identified by the slope a_1 .

Climate change projections:

Projected climate change was calculated based on the ensemble method of 16 members derived from 16 models in Table 1. The variability of variables was defined as follows:

- Determine the magnitude of changes in temperature (°C):

$$\Delta T_{\text{future}} = T_{\text{future}}^* - \overline{T_{1986-2005}^*}$$

- Determine the magnitude of changes in rainfall (mm):

$$\Delta R_{\text{future}} = \frac{(R_{\text{future}}^* - \overline{R_{1986-2005}^*})}{\overline{R_{1986-2005}^*}} * 100$$

In which: ΔT_{future} is the difference between future temperature and the temperature taken at the baseline period (°C), T_{future}^* is the future temperature (°C), $\overline{T_{1986-2005}^*}$ is the average temperature of the baseline period (°C); ΔR_{future} is the difference between the rainfall in the future and the rainfall at the baseline period (%), R_{future}^* is the future rainfall (mm), $\overline{R_{1986-2005}^*}$ is the rainfall in the baseline period (mm).

Results and discussions

Climate change in the past

Temperature:

The annual average temperature had an increasing trend across the Mekong Delta, with increases of 0.3 to 0.9°C during the period of 1958-2014. The increasing trend in temperature at the southern areas increased faster than that of the areas in the north, except for a portion in the northeast region of the Mekong Delta (Fig. 1).

Rainfall:

Annual rainfall increased by 5 to 20% in the period from 1958 to 2014 in the majority area of the Mekong Delta. As well, there was a decrease of less than 10% in rainfall over the southern

region (Can Tho and Ca Mau, Fig. 2).

Climate extremes:

Daily maximum temperature (TXx) has been seen to vary heterogeneously, and some stations had a slightly increasing trend, while others had a decreasing trend (Soc Trang, Can Tho, Cao Lanh, Rach Gia, and Ca Mau). In contrast, daily minimum temperature (TNn) increased at most of the stations, with increases at a range from of 0.01°C/10 years (at My Tho station) to 1.36°C/10 years (at Chau Doc station). Along with a decreasing trend at TXx, the number of days with the high temperatures of over 35°C (SU35) decreased at most stations in the Mekong Delta. Heavy rainfall increased more than average rainfall. The maximum 1-day rainfall (Rx1day), the maximum 5-day rainfall (Rx5day), and the number of days with precipitation exceeding 95% (R95P) had increases in most localities. Dry conditions appeared more and more often, especially during the dry season.

Climate change projections

Changes in the mean values of climate variables:

Changes in average temperature at the middle and the end of the century, in comparison to those of the baseline period, are presented in Fig. 3 and 4. The figures exhibit the most likely increases in temperature across the whole Mekong Delta. The increasing temperatures seen in the provinces of the Mekong Delta are quite similar to each other; however, the increasing rate of temperatures is slightly higher in the parts of the northeastern and southern Mekong Delta.

- Temperature:

According to the RCP4.5 scenario, the annual average temperature likely increases by 1.3 to 1.4°C in the middle of the century (Fig. 3A) and 1.7 to 1.8°C at the end of the century (Fig. 3B) in comparison to the baseline period.

Under the RCP8.5 scenario, compared with the baseline period, the annual average temperature likely

increases by 1.8 to 2.0°C in the middle of the century (Fig. 4A) and 3.4 to 3.6°C at the end of the century (Fig. 4B).

- Rainfall:

According to the RCP4.5 scenario: When compared to the baseline period,

annual rainfall at the mid-21st century will likely increase by 0 to 20% with increases in rainfall over the Northern Delta (e.g. Long An, Dong Thap, Tien Giang, and Ben Tre, seen in Fig. 5A). At the end of the 21st century, an increase in rainfall is typically 10 to 20%; and the

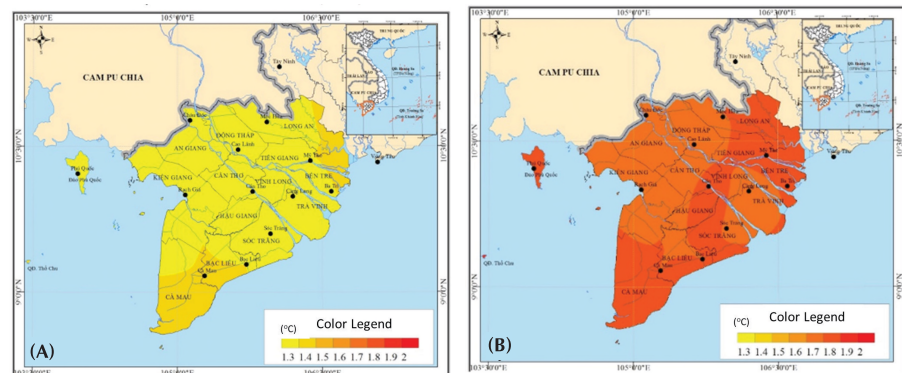


Fig. 3. Projected changes in annual temperature (°C) for the middle (A) and the end (B) of 21st century compared to the baseline period under RCP4.5 scenario from an ensemble of models.

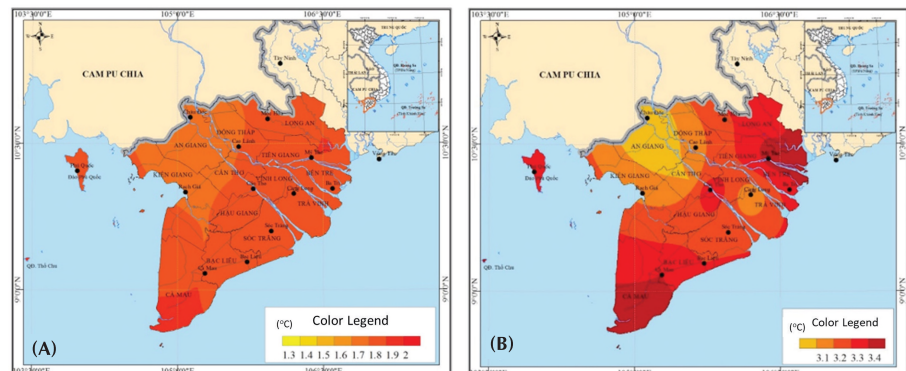


Fig. 4. Projected changes in annual temperature (°C) for the middle (A) and the end (B) of 21st century compared to the baseline period under RCP8.5 scenario from an ensemble of models.

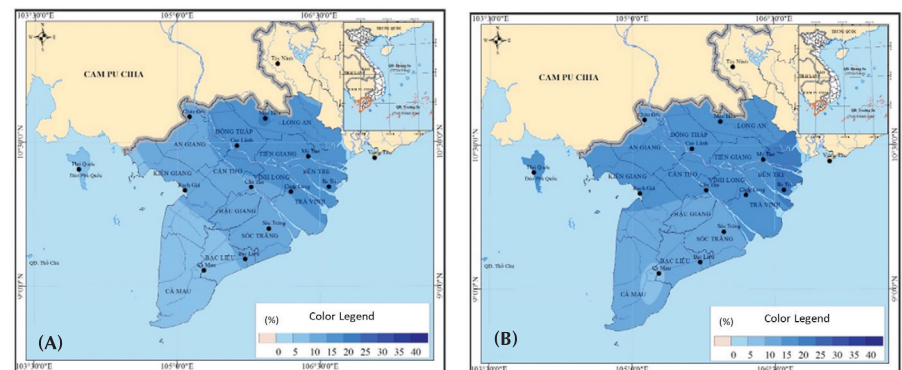


Fig. 5. Projected changes in annual rainfall (%) for the middle (A) and the end (B) of 21st century compared to the baseline period under RCP4.5 scenario from an ensemble of models.

Northern provinces will have a higher increase in rainfall (Fig. 5B).

According to the RCP8.5 scenario: Compared to the baseline, rainfall in the mid-21st century will likely increase by 10-20% (Fig. 6A). By the end of the 21st century, annual rainfall will likely

increase higher than that of the mid-century, with a typical increase of 10 to 30% (Fig. 6B).

Changes in climate extremes:

- Extreme temperature:

Changes in average maximum temperature at the end of the 21st century:

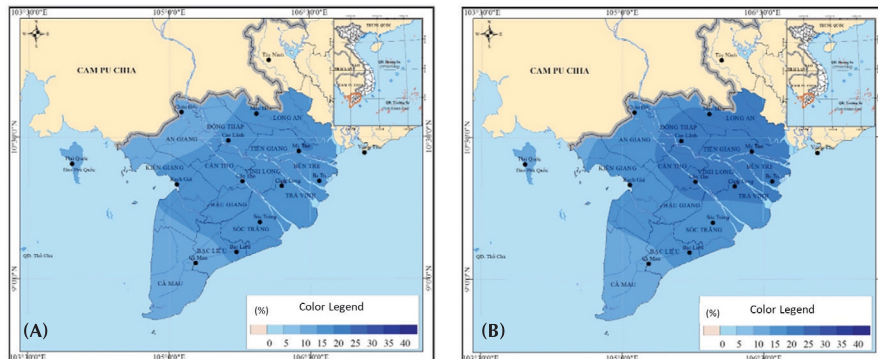


Fig. 6. Projected changes in annual rainfall (%) for the middle (A) and the end (B) of 21st century compared to the baseline period under RCP8.5 scenario from an ensemble of models.

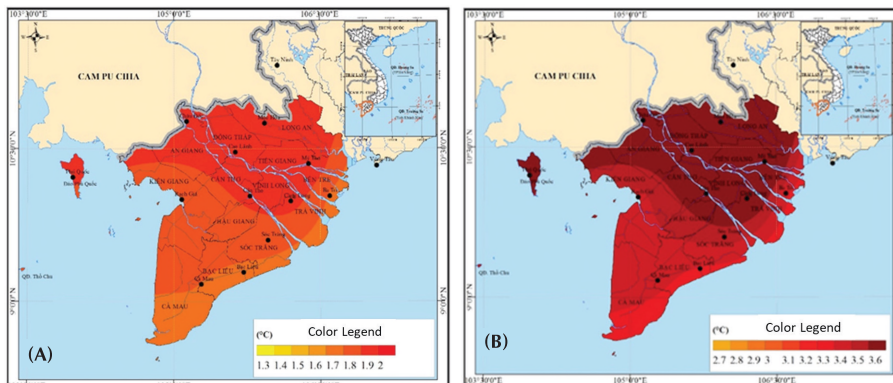


Fig. 7. Projected changes in annual mean maximum temperature (°C) for the end of 21st century compared to the baseline period according to RCP4.5 (A) and RCP8.5 (B) scenarios from an ensemble of models.

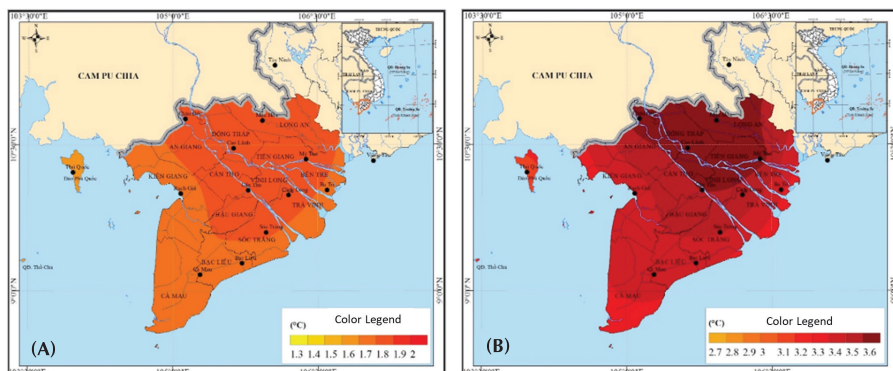


Fig. 8. Projected changes in annual mean minimum temperature (°C) for the end of 21st century compared to the baseline period according to RCP4.5 (A) and RCP8.5 (B) scenarios from an ensemble of models.

Compared to the baseline period, the average maximum temperature will likely increase by 1.8 to 2°C as seen with the RCP4.5 scenario (Fig. 7A), and by 3.5 to 3.8°C as seen with the RCP8.5 scenario (Fig. 7B). Accompanied with an increase of maximum temperature, the number of hot days is likely to increase by 10 to 20 days as predicted by the RCP4.5 scenario or by 20 to 40 days as based on the RCP8.5 scenario by the end of the 21st century.

Change in average minimum temperature at the end of the 21st century: Compared to the baseline period, the average minimum temperature will likely increase by 1.8 to 1.9°C as seen with the RCP4.5 scenario (Fig. 8A) and by 3.4 to 3.6°C as seen with the RCP8.5 scenario (Fig. 8B).

- Extreme rainfall:

Compared to the baseline, the maximum 1-day rainfall (Rx1day) and the maximum 5-day rainfall (Rx5day) are expected to increase in the future. According to the RCP4.5 and RCP8.5 scenarios, Rx1day increased by 10 to

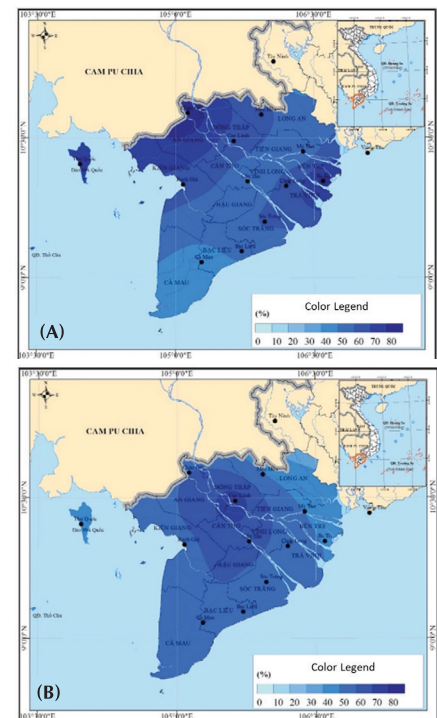


Fig. 9. Projected changes in Rx1day (mm) for the end of 21st century compared to the baseline period according to RCP4.5 (A) and RCP8.5 (B) scenarios from an ensemble of models.

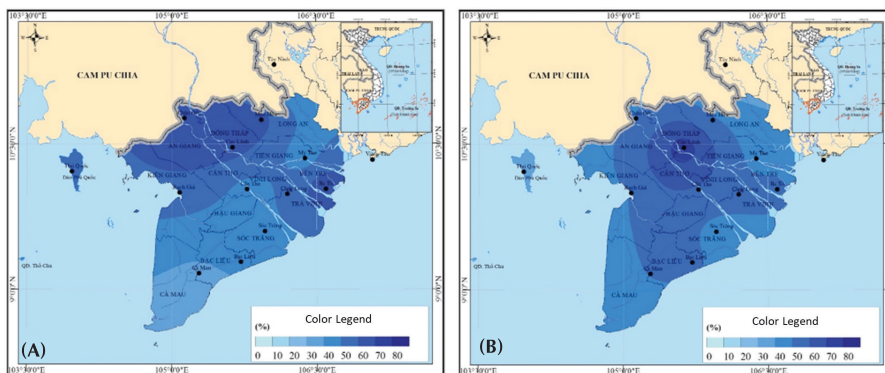


Fig. 10. Projected changes in Rx5day(mm) for the end of 21st century compared to the baseline period according to RCP4.5 (A) and RCP8.5 (B) scenarios from an ensemble of models.

40% and Rx5day increased by 20 to over 40% (Fig. 9 and 10) by the end of the 21st century.

Conclusions

This study presents the results of an assessment of past and projected impacts of climate on the Mekong Delta, based on updated data (up to 2014) and the latest climate change scenarios from the MONRE; the results show that:

1) *The indicators of climate change in the Mekong Delta:*

The annual average temperature in the Mekong Delta Region increased during the period of 1958-2014, with a typical increase within a range of 0.3 to 0.9°C. In which, the temperature increased higher in localities such as Long An, Hau Giang, Bac Lieu, Ca Mau, and Phu Quoc. Minimum temperature has obviously increasing trends at most stations.

Annual rainfall increased by 5-20% in most provinces of the Mekong Delta during the period of 1958-2014.

2) *Climate change projections:*

- **Temperature:** According to the RCP4.5 scenario, the average annual temperature will likely increase by 1.3 to 1.4°C in the mid-21st century and by 1.7 to 1.9°C at the end of the 21st century. According to the RCP8.5 scenario, the average annual temperature will likely

increase by 1.8 to 2°C in the mid-21st century and 3.4 to 3.6 at the end of the 21st century. The average maximum temperature increases higher than the average minimum temperature and the increasing trend gradually reduces from northern to southern regions of the Mekong Delta.

- **Rainfall:** When compared to the baseline period, rainfall based on RCPs (RCP4.5 and RCP8.5) likely increases by 5 to 15% at the middle and at the end of the 21st century, in which the increase in the North is higher than in the Southern Delta. At the end of the century, Rx1day increased by 10 to 40%, while Rx5day increased by 20 to over 40%.

REFERENCES

[1] Vietnam Institute of Meteorology, Hydrology and Environment (2010), *Impacts of climate change on water resources and adaptation measures in Mekong Delta*, Project final report.

[2] IPCC (2007), *Climate change 2007: The Scientific basis, contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change*, Cambridge University Press, United Kingdom and New York, NY, USA.

[3] Duc Ngu Nguyen, Trong Hieu Nguyen (1991), *Climate change and their impacts in Vietnam for past 100 years - Natural and human*, Truth Publishing House, Hanoi.

[4] Duc Ngu Nguyen, Trong Hieu Nguyen (1999), *The status of climate change in Vietnam for next decades*, Vietnam Institute of Meteorology and Hydrology.

[5] MONRE (2009), *Scenario of climate change and sea level rise for Vietnam*, Vietnam Publishing House of Natural Resources, Environment and Cartography (NARENCA).

[6] MONRE (2012), *Scenario of climate change and sea level rise for Vietnam*, NARENCA.

[7] MONRE (2016), *Scenario of climate change and sea level rise for Vietnam*, NARENCA.

[8] Duc Thanh Ngo, Van Tan Phan (2012), "Non-parametric test for trend detection of some meteorological elements for the period 1961-2007", *Journal of Science*, Vietnam National University, Hanoi, **28(3S)**, pp.129-135.

[9] Duc Ngu Nguyen - Chief author (2008), *Climate change*, Science and Technics Publishing House, Hanoi.

[10] Nguyen Van Thang, et al. (2015), *Changes in climate extremes and impacts on the natural physical environment*, NARENCA, pp.84-139.

[11] Van Thang Nguyen, et al. (2010), *Study the effects of climate change on natural conditions, natural resources and propose strategic solutions for prevention, mitigation and adaptation for sustainable socio-economic development in Vietnam*, Final report KC08.13/06-10, Vietnam Institute of Meteorology, Hydrology and Environment, Hanoi, 330 pages.

[12] Van Thang Nguyen, et al. (2013), "Climate change in Mekong Delta and adaptations", *Journal of Science*, Vietnam National University, Hanoi, **25**, pp.187-194.

[13] Van Thang Nguyen, et al. (2017), "Changes in climate extreme in Vietnam", *Vietnam Science and Technology*, **1(1)**, pp.79-87.

[14] Van Tan Phan, et al. (2010), *Study the impacts of global climate change on climate extreme factors and events in Vietnam, predictability and strategic response*, Final report KC08.29/06-10.

[15] Thanh Hang Vu, Thi Thu Huong Chu, Van Tan Phan (2009), "Trend of maximum daily rainfall in Vietnam during 1961-2007", *Journal of Science*, Vietnam National University, Hanoi, **25(3S)**, pp.423-430.

Vietnam Journal of Science, Technology and Engineering (VJSTE): Manuscript Template

General Instructions on using this template and submitting a manuscript to VJSTE: Thank you for preparing a manuscript for submission to VJSTE. Using this template, or following the guidelines below, will help us in processing your paper. Our goal is to be able to identify each section of your manuscript so that we can accurately record the title, authors, abstract, etc. and also be able to enrich it by including reference links and an accurate layout.

You can submit your paper at <http://vietnamscience.vn>

So that we can identify the parts of your paper, and even if you do not use our template, please begin each section with the specific key words listed below. Please do not use paragraph breaks in the title, author list, or abstract.

Title

Authors

Affiliations

Abstract

One Sentence Summary

Main Text

Acknowledgements

References

Several of these headings are optional, for example, not all papers will include a one-sentence summary, figures or tables.

Please use the .docx format (all version after Word 2007).

More specific formatting instructions are provided in the actual template, which follows.

Title: How to Format a Science Paper (replace with your real title)

Authors: The author list should be one single paragraph (no breaks). Authors should be listed by first name or initial followed by last name and separated by commas. Use superscript numbers to link affiliations, and symbols *†‡ for author notes. For example, X. Jones¹*, P. Smith^{1,2}

Affiliations:

¹Affiliations should be preceded by superscript numbers corresponding to the author list, and each affiliation should end with a period.

²Each affiliation should be a separate paragraph.

Correspondence to: include the email addresses or telephone number of the corresponding author(s). Please use the asterisk () symbol for the corresponding author information.

Abstract: The abstract should be about 100-150 words, and organized in this structure: An opening sentence that sets the question that you address and is comprehensible to the general reader, background content specific to this study, results, and a concluding sentence. It should be one paragraph only.

One Sentence Summary: A brief summary of the main result of your paper, without excessive jargon and less than 150 characters.

Main Text: In general, this should include a brief (1-2 paragraph) introduction, followed by a statement of the specific scope of the study, followed by results and then interpretations. Please avoid statements of future work or claims of priority, and avoid repeating the conclusions at the end.

All of the Figures and Tables should be cited in order (which should be cited as, for example, figure S1, and table S1). You can include page breaks if you would like to place the figures within the text.

Subheads: These can be included in Research Articles or Reviews and should be brief.

Equations can be included. We do not recommend using the native Word 2007, 2008, 2010 or 2011 equation editor. This can in some cases produce less reliable MathML, the online markup language we use, which may result in display errors. Instead, use the legacy equation editor in word (Insert menu; select insert object; select word equation) or use Mathtype (recommended). If you enter equations in simple LaTeX, check that they will convert accurately (Word 2007 and higher can convert simple LaTeX equations).

Fig. #: (Begin each figure caption with a label, “**Fig. 1.**” for example, as a new paragraph.).

Fig. 1. The figure caption should begin with an overall descriptive statement of the figure followed by additional text. They should be immediately after each figure. Figure parts are indicated with capital letters (**A**). If you prefer, you can place both the actual figures and captions logically through the text near where they are cited rather than at the end of the file (but not both). If a paragraph in the main text begins with the name of a figure, write out “Figure” in full (e.g., <para>“Figure 1 shows....”).

Fig. 2. You can place graphics above each equation as part of this file.

Table #: (Begin each table caption with a label “**Table 1.**”, etc.) as a new paragraph.

Table 1. Start this caption with a short description of your table. Format tables using the Word Table commands and structures. Do not create tables using spaces or tabs characters.

Acknowledgments: The acknowledgments should include a statement about where the data reported in the paper are presented, archived, or available. If in an archive, include the accession number or a placeholder for it. Please also include relevant funding information such as grant numbers and funding agencies. You can also include a statement of author contributions here. The acknowledgements can be multiple paragraphs.

References:

References should be cited in bracket with a number [1]. Multiple reference citations are separated by commas [2, 3] or if a series, dashes [4-6].

References are listed in numerical order, and in the same order in which they are cited in text. The reference list should include all and only those references you have cited in the text. The reference list appears at the end of the paper.

Please include the above heading “References”.

Please do not mix in references with explanatory notes.

The reference consist of: Family name(s) and initials of all authors (year of publication), “Titles of journal articles” (optional), *Title of journal, book or other publication*, **volume(number)**, page numbers.

Example of a reference list:

[1] T. Binzoni, T.S. Leung, D. Boggett, D.T. Delpy (2002), “A new near infrared laser-Doppler flowmeter for deep tissue perfusion monitoring”, *MAGMA*, **14**, pp.74-75.

[2] T. Binzoni, T.S. Leung, D. Boggett, D.T. Delpy (2003), “Non-invasive laser Doppler perfusion measurements of large tissue volumes and human skeletal muscle blood RMS velocity”, *Phys. Med. Biol.*, **48(1)**, pp.2527-2549.

ISSN 2525-2461

Vietnam Journal of Science, Technology and Engineering

JUNE 2017 • VOL.59 NUMBER 2

EDITORIAL COUNCIL

STANDING MEMBERS:

Bao Chau Ngo (*University Avenue Chicago, USA*)
Gia Khanh Pham (*Chairman of Vietnam Organ Transplant, Vietnam*)
Thuc Tran (*Vietnam Panel on Climate Change, Vietnam*)
Van Hieu Nguyen (*Vietnam Academy of Science and Technology, Vietnam*)

MEMBERS:

Akos Redey (*University of Pannonia, Hungary*)
Chi Buu Bui (*Vietnam Academy of Agricultural Sciences, Vietnam*)
Clark Gedney (*Bio Media Center for Instructional Design, Biological Sciences, Purdue University, USA*)
Dang Xuan Tran (*Hiroshima University, Japan*)
Dindo Campilan (*International Center for Tropical Agriculture (CIAT) - Asia Regional Office, Philippines*)
Dinh Duc Nguyen (*University of Engineering and Technology (UET) - Vietnam National University, Hanoi, Vietnam*)
Edgar Andreas Bohner (*VTT Technical Research Centre of Finland, Finland*)
Franz Nestmann (*Institute for Water and River Basin Management, Karlsruhe Institute of Technology, Germany*)
Gopalakrishnakone P (*National University Health System, Singapore*)
Henry Nguyen (*University of Missouri, Columbia, USA*)
Hung Viet Pham (*University of Science - Vietnam National University, Hanoi, Vietnam*)
John Nieber (*University of Minnesota, USA*)
Pierre Darriulat (*European Organization for Nuclear Research; Vietnam Academy of Science and Technology, Vietnam*)
Stefan Norra (*Institute of Applied Geosciences, Karlsruhe Institute of Technology, Germany*)
Tran Binh Le (*University of Science and Technology of Hanoi, Vietnam*)
Tri Nang Tran (*University of Minnesota, USA*)
Truong Duc Pham (*University of Birmingham, UK*)
Tu Bao Ho (*Japan Advanced Institute of Science and Technology, Japan*)
Van Suong Hoa (*Concordia University, Canada*)
Van Thang Nguyen (*Vietnam Institute of Meteorology, Hydrology and Climate change, Ministry of Natural Resources and Environment, Vietnam*)
Van Toi Vo (*Tufts University, Massachusetts, USA; Vietnam National University in Ho Chi Minh City, Vietnam*)

EDITORIAL OFFICE

113 Tran Duy Hung Str., Cau Giay Dist., Hanoi
Tel: (84.4) 39436793; Fax: (84.4) 39436794
Email: khcnvn@most.gov.vn
Website: vietnamscience.vn

EDITOR-IN-CHIEF

Ngoc Bao Dang

DEPUTY EDITOR

Thi Hai Hang Nguyen
Thi Huong Giang Nguyen

HEAD OF EDITORIAL BOARD

Thi Minh Nguyet Pham

HEAD OF ADMINISTRATION

Ngoc Quang Hung Luong

ART DIRECTOR

Thi Luan Dinh

PUBLICATION LICENCE

No.1153/GP-BTTTT 26th July 2011

No.2528/GP-BTTTT 26th December 2012

No.592/GP-BTTTT 28th December 2016