The Variation of Indigenous Upland Rice Landraces in Ratchaburi, Thailand Based on Seed Morphology and DNA Sequencing

Prin Phunngam and Nichakorn Pathumrangsan
Department of General Science, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi 70150 Thailand
Email: prin.phunngam@hotmail.com

Natnaree Khambai and Jaruwan Tongjun
Department of Biology, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi 70150 Thailand

Uraiwan Arunyawat
Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900 Thailand
Email: uraiwan.a@ku.ac.th

***Abstract*—In this study, variability of rice landraces in Ratchaburi, Thailand was investigated using seed morphology and DNA sequences. This information is useful for understanding the extent of diverse level of rice landraces in order to provide desirable characters for accomplishing the efficient rice improvement and conservation program. Collected 9 rice landraces were observed using qualitative (awn presence, awn color, lemma and palea color, lemma and palea pubescence, sterile lemma color, sterile lemma length, and seed coat color) and quantitative (1000-grain weight, grain length with and without husk, grain width with and without husk, and ratio length/width with and without husk) characters. Using cluster analysis, all traits divided these rice landraces into two major clusters. Moreover, these rice landraces were also analyzed using single nucleotide polymorphism data based on two putative neutral fragments. This study presented low level of nucleotide diversity for all populations. The statistical tests of neutrality indicated significantly positive departure from neutral equilibrium, which suggested the high intermediate-frequency polymorphisms in these rice landraces.**

***Index Terms*—diversity, DNA sequence, rice landrace, seed morphology**

I. Introduction

Rice landraces (*Oryza sativa* L.) or traditional rice varieties are the considerable staple food crop for indigenous peoples [1] and also the important resources of genetic diversity [2]-[4] such as pests and fungal resistance [4] and potential adaptation to environment [5], [6] which are useful for improvement of cultivated rice varieties [2], [4], [7]. However, presently, these valuable rice landraces have been continuously lost by replacement of modern rice varieties which provide high-yield production and other commercial crops such as sugar cane and rubber tree [3], [7]. To conserve these genetic resources, the appraisal of the genetic variability is considered as the initial step for management [8]

The genetic diversity of rice have been extensively studied in the morphological characters [9]-[13] and molecular techniques such as RAPD (random amplified polymorphic DNA) [14], [15], AFLP (amplified fragment length polymorphism) [16], ISSR (inter simple sequence repeat) [14], [17], SSR (simple sequence repeat) and SNP (single nucleotide polymorphism) [15], [18], [19]. Because of the advance of DNA sequencing technology, especially rapid analysis and economical expense [20]-[24], SNP has been extensively used in many organisms including plants, animals and fungi [22], [25]-[35]. In rice genome, SNPs are abundant and useful for evaluation of genetic diversity and evolutionary process [36]. However, in western Thailand, the assessment of the genetic variability of rice landraces which may provide the basic information for using in breeding program and conservation of genetic resources [2], [37] is still unavailable.

II. Materials and Methods

A. Seed Samples of Rice Landraces

Seed samples of 9 rice landraces were collected from two districts in Ratchaburi province, Thailand. There are four landraces from Suan Phueng (Khaw Nhew Dum, RT01; I-Ngong Yai, RT02; I-Ngong Lek, RT03) and the others from Ban Kha (Nang Pang Near, RT04; Pa Choi, RT05; Bueng Wo Boi, RT06; Khaw Dang, RT07; Chu Purng, RT08).

B. Observations of Seed Morphology

Qualitative (awn presence, awn color, lemma and palea color, lemma and palea pubescence, sterile lemma color,

Manuscript received March 5, 2016; revised November 3, 2016.

doi: 10.18178/joaat.4.1.48-52

sterile lemma length, and seed coat color) and quantitative (1000-grain weight, grain length with and without husk, grain width with and without husk, and ratio length/width with and without husk) characters were observed and scored from all rice landraces based on [38].

C. Analysis of Morphological Data and Clustering

Descriptive statistics based on mean, standard deviation, standard error and coefficient of variation were analyzed from the quantitative characters. Cluster analysis was performed using PAST version 3.10 [39] based on the basic Euclidean distance and unweighted pair-group average (UPGMA) algorithms.

D. DNA Extraction, PCR Amplification and Direct DNA Sequencing

Two seeds of each rice landraces were soaked in distilled water and germinated to 10-day seedling. Unfortunately, one landrace (I-Ngong Lek) was excluded from molecular analysis because of no seed germination. Total young leaves were collected and used for DNA isolation using the modified Genomic DNA Mini Kit (Plant) (Geneaid, Taiwan). The primers were developed from intron flanking-region of gene using EPIC (Exon-Primed Intron-Crossing) method. Gradient PCR was performed for selecting the temperature of amplification (Ta) of each primer. PCR reaction was carried out in a total volume of 25µl containing 1µl genomic DNA, 2.5µl of 10X buffer, 1µl of 50mM $MgCl_2$, 0.25µl of 10mM dNTPs, 0.5µl of each primer (10nmol), 0.3µl of Taq DNA polymerase (Biotechrabbit, Germany) and 18.95µl distilled water. Amplification was conducted in a thermocycler using following program; Initial denaturation at 94 ℃ for 5 min followed by 30 cycles of 94 ℃ for 1 min, Ta for 30s, 72 ℃ for 45s and final extension at 72 ℃ for 10 min. The annealing temperature for *CatA* (Catalase gene) [40] was 59 ℃ and for *Pi54* (Uncharacterized protein; F:GTTGCGTATGAAGGACA TGC, R:AGACCCTCACAGCCTGAAGA) was 59 ℃. Then, the amplified products were separated on 1% agarose gel. PCR products with single band were chosen for purification using Gel/PCR Purification Kit (Favogen, Taiwan). The purified PCR products were directly sequenced in both directions using forward- and reverse-specific primers by Macrogen Inc. (Korea). Forward and reverse sequences of each gene fragment were compared and validated with the retrieved sequence of *Oryza sativa* (*indica*) from the GRAMENE database. The nucleotide sequences were visually determined between base-calling and chromatograms. Sequences were aligned and manually edited using BioEdit version 7.1.9 [41].

E. Analysis of Molecular Data

Data and molecular population genetic analysis were conducted using DnaSP version 5.10.01 [42]. Nucleotide diversity was estimated based on 2 parameters, θ_w [43] and π [44]. Tajima's *D* test [45] was measured skews in the frequency spectrum based on π and θ_w in order to test the neutrality for each locus following the result of cluster analysis. Haplotype diversity was also calculated from each locus.

III. Result

A. Morphological Data and Cluster Analysis

The observation of quantitative traits were performed based on seed morphological characters (Table I), which are 1000-grain weight, grain length with and without husk, grain width with and without husk, and ratio length/width with and without husk. Maximum grain weight (37.00g) was observed in I-Ngong Yai while minimum grain weight (21.76g) was observed in I-Ngong Lek. Maximum grain length (11.13mm) was observed in Khaw Nhew Dum while minimum grain length (9.08 mm) was in I-Ngong Lek. Maximum grain length without husk (8.05mm) was observed in I-Ngong Yai while minimum grain length without husk (6.55mm) was in Bueng Wo Boi. Maximum grain width (3.32mm) was observed in Khaw Nhew Dum while minimum grain width (2.24mm) was observed in Khaw Dang. Maximum grain width without husk (2.94mm) was observed in Nang Pang Near while minimum grain width without husk (2.26mm) was observed in Khaw Dang.

The dendrogram were divided into two major groups by cluster analysis based on quantitative and qualitative traits (Fig. 1). Major cluster (cluster I) consisted of 5 rice landraces while minor cluster (cluster II) consisted of three rice landraces. In major cluster, 4 rice landraces were grouped in two sub-groups and one individual landrace. The first sub-group consisted of I-Ngong Yai and Nang Pang Near based on similarity in color and pubessense of lemma and palea, sterile lemma color, sterile lemma length, seed coat color, 1000-grain weight, grain length and grain width. The second sub-group consisted of Pa Choi and Chu Purng based on similarity in presence of awn, lemma and palea pubessense, sterile lemma color, sterile lemma length, seed coat color, 1000-grain weight, grain length and grain width. Individually, one landrace was separated by the different seed coat color. In minor cluster, 3 rice landraces (I-Ngong Lek, Bueng Wo Boi and Khaw Dang) were grouped based on similarity in lemma and palea pubessense, sterile lemma length, seed coat color and 1000-grain weight. Due to the difference of rice landraces in each population, these divided groups were further used for molecular analysis.

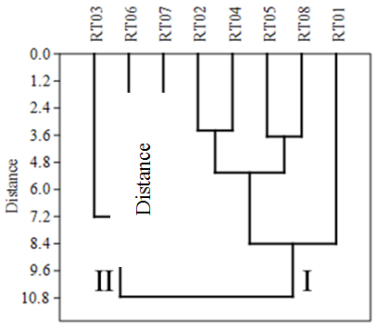

Figure 1. Dendrogram of 8 rice landraces from Ratchaburi province based on the basic Euclidean distance and unweighted pair-group average (UPGMA) algorithms.

B. Molecular Data Analysis

The nucleotide diversity of 8 rice landraces from Ban Kha and Suan Phueng populations was evaluated from a

total of 1,088 nucleotides across 2 non-coding fragments (Table II). The total lengths of the studied loci are 541bp (*CatA*) and 547bp (*Pi54*). The polymorphic sites including 21 single nucleotide substitutions (SNPs) and three large insertion/deletion polymorphisms (indels) were observed. Additionally, low level of nucleotide polymorphism was observed in all studied fragments. For *CatA* gene, nucleotide diversity calculated from populations and clusters was consistent. A significant positive Tajima's *D* value from all populations and clusters indicated an excess of intermediate-frequency polymorphisms. Haplotype was divided into 3 groups. For *Pi54* gene, nucleotide diversity calculated from populations and clusters was also consistent and the excess of intermediate-frequency polymorphisms was also observed. Haplotype was divided into only 2 groups.

TABLE I. DESCRIPTIVE STATISTICS OF 9 RICE LANDRACES FROM RATCHABURI PROVINCE, THAILAND

Trait	Minimum	Maximum	Mean	SD	SE mean	CV
1000-grain weight (g)	21.76	37.00	30.81	5.35	1.89	0.17
Grain length with husk (mm)	9.08	11.13	10.02	0.71	0.25	0.07
Grain length without husk (mm)	6.55	8.05	7.25	0.50	0.17	0.06
Grain width with husk (mm)	2.24	3.32	3.06	0.35	0.12	0.11
Grain width without husk (mm)	2.26	2.94	2.45	0.22	0.07	0.09
Ratio l/w with husk	2.90	4.38	3.31	0.46	0.16	0.14
Ratio l/w without husk	2.66	3.32	2.96	0.22	0.07	0.07

SD = Standard Error; SE mean = Standard Error of Mean; CV = Coefficient of Variation

TABLE II. SUMMARY OF NUCLEOTIDE DIVERSITY AND NEUTRALITY TEST

Locus	Sample category	*n*	*H*	*Hd*	*S*	π	θ_W	*D*
CatA	Total landraces	28	3	0.613	15	0.0131	0.0071	2.75105***
	Ban Kha	20		0.505	12	0.0117	0.0065	2.84498***
	Suan Phueng	8		0.727	15	0.0145	0.0095	2.20252*
	Cluster I	20		0.674	15	0.0139	0.0081	2.63069***
	Cluster II	8		0.571	12	0.0132	0.0089	2.42331***
Pi54	Total landraces	28	2	0.508	6	0.0087	0.0044	2.83562***
	Ban Kha	20		0.505	6	0.0087	0.0048	2.52571***
	Suan Phueng	8		0.571	6	0.0098	0.0066	2.24509*
	Cluster I	20		0.505	6	0.0087	0.0048	2.52571***
	Cluster II	8		0.571	6	0.0098	0.0066	2.24509*

n = Number of sequences used; *H* = Number of haplotype; *Hd* = Haplotype diversity; *S* = Number of segregating sites, π = the average number of difference between all pairs of sequences sampled; θ_W = the number of segregation sites among the investigated sequences; *D* = Tajima's *D* test; *, P < 0.05; **, P < 0.02; ***, P < 0.01

IV. DISCUSSION

The observations of quantitative and qualitative traits observed in 9 rice landraces from Ratchaburi were quite low diversity when compared to rice landraces from West Bengal, India [12]. Seed coat colors of Indian rice landraces (white, light brown, golden yellow and red) were more diverse than rice landraces of Ratchaburi (light brown and purple). Most of rice landraces from India showed the presence of aroma while all rice landraces from Ratchaburi were not observed. However, grain sizes (grain length and grain width) of Indian rice landraces were quite smaller than rice landraces of Ratchaburi. These basic facts of rice diversity could be used as the important resources of desirable characters for conservation and rice improvement program [2].

The relatively low level of nucleotide polymorphism was observed in both studied fragments. Nucleotide variation of *CatA* was higher than nucleotide variation of Pi54. For *CatA* locus, the nucleotide diversity in this study (π = 0.0131) was similar to *Oryza sativa* ssp. *indica* (π = 0.0122), *Oryza rufipogon* (π = 0.0151) and *Oryza barthii* (π = 0.0164) as well as was higher than *Oryza sativa* ssp. *japonica* (π = 0) [40]. Haplotype network of *CatA* showed three groups of sequences; haplotype 1 consisted of 3 rice landraces (Khaw Nhew Dum, Khaw Dang and Chu Purng), haplotype 2 consisted of 1 rice landrace (I-Ngong Yai) and haplotype 3 consisted of 3 rice landraces (Nang Pang Near, Pa Choi and Bueng Wo Boi). For *Pi54* locus, low genetic variation was observed in all groups (Ratchburi, Ban Kha, Suan Phueng, cluster I and cluster II) with small range (π = 0.0087 to 0.0098). Haplotype network of *Pi54* showed two groups of sequences; haplotype 1 consisted of 3 rice landraces (Khaw Nhew Dum, Khaw Dang and Chu Purng), haplotype 2 consisted of 4 rice landrace (I-Ngong Yai, Nang Pang Near, Pa Choi and Bueng Wo Boi). Both haplotype networks showed no relation to the result of cluster analysis. Moreover, the significant positive *D* value in all loci suggested the excess of intermediate-frequency polymorphisms which might indicate bottleneck during domestication process of these rice landraces [40]. Because this study is only the initial information, more samples and gene locus are required for further analysis.

ACKNOWLEDGMENT

This work was financially supported by the Higher Education Research Promotion and National Research University Project of Thailand (HERP-NRU), Office of the Higher Education Commission (under Biodiversity

Research Group, grant number 2558A16162009, year 2015). The author also thanks to EGCB Research Unit, Department of Genetics, Faculty of Science, Kasetsart University.

REFERENCES

[1] S. Chunta, P. Prathepha, T. Jongdee, and B. Jongdee, "Nuances of traditional knowledge in utilization of rice landraces by a farming community in North-Eastern Thailand," *Indian J. Tradit. Know*., vol. 13, no. 3, pp. 473-483, July 2014.

[2] C. Brondani, T. C. O. Borba, P. H. N. Rangel, and R. P. V. Brondani, "Determination of genetic variability of traditional varieties of Brazilian rice using microsatellite markers," *Genet. Mol. Biol*., vol. 29, no. 4, pp. 676-684, 2006.

[3] A. Kobayashi, K. Ebana, S. Fukuoka, and T. Nagamine, "Microsatellite markers revealed the genetic diversity of an Old Japanese Rice Landrace 'Echizen'," *Genet. Resour. Crop Evol.*, vol. 53, no. 3, pp. 499-506, May 2006.

[4] S. G. Ram, V. Thiruvengadam, and K. K. Vinod, "Genetic diversity among cultivars, landraces and wild relatives of rice as revealed by microsatellite markers," *J. Appl. Genet.*, vol. 48, no. 4, pp. 337-345, 2007.

[5] H. K. Parzies, W. Spoor, and R. A. Ennos, "Inferring seed exchange between farmers from population genetic structure of barley landrace Arabi Aswad from Northern Syria," *Genet. Resour. Crop Evol*., vol. 51, pp. 471–478, 2004.

[6] T. Pusadee, P. Oupkaew, B. Rerkasem, S. Jamjod, and B. A. Schaal, "Natural and human-mediated selection in a landrace of Thai rice (*Oryza sativa*)," *Ann. Appl. Biol.*, vol. 165, no. 2, pp. 280–292, Sep. 2014.

[7] T. Pusadee, S. Jamjod, Y. C. Chiang, B. Rerkasem, and B. A. Schaal, "Genetic structure and isolation by distance in a landrace of Thai rice," *Proc. Natl. Acad. Sci*., vol. 106, no. 33, pp. 13880-13885, 2009.

[8] M. A. Toro and A. Caballero, "Characterization and conservation of genetic diversity in subdivided populations," *Philos. Trans. R. Soc. Lond. B. Biol. Sci*., vol. 360, no. 1459, pp. 1367–1378, 2005.

[9] P. Prathepha, "Seed morphological traits and genotypic diversity of weedy rice (*Oryza sativa* f. *spontanea*) populations found in the Thai Hom Mali rice fields of north-eastern Thailand," *Weed Biol. Manag.*, vol. 9, no. 1, pp. 1–9, Mar. 2009.

[10] M. A. Adebisi, F. S. Okelola, M. O. Ajala, T. O. Kehinde, I. O. Daniel, and O. O. Ajani, "Evaluation of variations in seed vigour characters of West African Rice (*Oryza sativa* L.) genotypes using multivariate technique," *Am. J. Plant Sci.*, vol. 4, pp. 356-363, Feb. 2013.

[11] R. C. Rabara, M. C. Ferrer, C. L. Diaz, M. C. V. Newingham, and G. O. Romero, "Phenotypic diversity of farmers' traditional rice varieties in the Philippines," *Agron. J.*, vol. 4, no. 2, pp. 217-241, May 2014.

[12] D. P. Semwal, A. Pandey, D. C. Bhandari, O. P. Dhariwal, and K. Sharma, "Variability study in seed morphology and uses of indigenous rice landraces (*Oryza sativa* L.) collected from West Bengal, India," *Aust. J. Crop Sci*., vol. 8, no. 3, Mar. 2014.

[13] M. Tuhina-Khatun, M. M. Hanafi, M. R. Yusop, M. Y. Wong, F. M. Salleh, and J. Ferdous, "Genetic variation, heritability, and diversity analysis of upland rice (*Oryza sativa* L.) genotypes based on quantitative traits," *Biomed Res. Int.*, vol. 2015, pp. 1-7, Jun. 2015.

[14] W. Qian, S. Ge, and D. Y. Hong, "Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers," *Theor. Appl. Genet.*, vol. 102, no. 2-3, pp. 440-449, 2001.

[15] H. Zhou, Z. Xie, and S. Ge, "Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogon* Griff.) in China," *Theor. Appl. Genet.*, vol. 107, no. 2, pp. 332-339, 2003.

[16] K. Ukoskit, "Genetic diversity of wild rice (*Oryza* spp.) in the northern region of Thailand," *Thammasat Int. J. Sc. Tech.*, vol. 9, no. 4, pp. 11-20, 2004.

[17] S. P. Joshi, V. S. Gupta, R. K. Aggarwal, P. K. Ranjekar, and D. S. Brar, "Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*," *Theor. Appl. Genet.*, vol. 100, no. 8, pp. 1311-1320, 2000.

[18] B. Das, *et al.*, "Genetic diversity and population structure of rice landraces from Eastern and North Eastern States of India," *BMC Genetics*, vol. 14, no. 1, 2013.

[19] N. Singh, *et al.*, "Comparison of SSR and SNP markers in estimation of genetic diversity and population structure of Indian rice varieties," *PloS One*, vol. 8, no. 12, 2013.

[20] R. Halliburton, *Introduction to Population Genetics*, USA: Pearson Education, Inc., 2004.

[21] P. A. Morin, G. Luikart, R. K. Wayne, and the SNP Workshop Group, "SNPs in ecology, evolution and conversation," *Trends Ecol. Evol.*, vol. 19, no. 4, pp. 208-216, 2004.

[22] W. C. Black IV and J. G. Vontas, "Affordable assays for genotyping single nucleotide polymorphisms in insects," *Insect Mol. Biol*., vol. 16, no. 4, pp. 377-387, 2007.

[23] R. Lister, B. D. Gregory, and J. R. Ecker, "Next is now: New technologies for sequencing of genomes, transcriptomes, and beyond," *Curr. Opin. Plant Biol*., vol. 12, no. 2, pp. 107-118, 2009.

[24] A. Vignal, D. Milan, M. S. Christobal, and A. Eggen, "A review on SNP and other types of molecular markers and their use in animal genetics," *Genet. Sel. Evol.*, vol. 34, pp. 275-305, 2002.

[25] M. P. Heaton, *et al.*, "Selection and use of SNP markers for animal identification and paternity analysis in U.S. beef cattle," *Mamm. Genome*, vol. 13, pp. 272-281, 2002.

[26] A. Rafalski, "Application of single nucleotide polymorphisms in crop genetics," *Curr. Opin. Plant Biol.*, vol. 5, pp. 94-100, 2002.

[27] Y. L. Zhu, *et al.*, "Single nucleotide polymorphism in soybean," *Genetics*, vol. 163, pp. 1123-1134, 2003.

[28] Y. Guo and D. C. Jamison, "The distribution of SNPs in human gene regulatory regions," *BMC Genomics*, vol. 6, no. 140, Oct. 2005.

[29] J. M. Seddon, H. G. Parker, E. A. Ostrander, and H. Ellegren, "SNPs in ecological and conservation studies: A test in the Scandinavian wolf population," *Mol. Ecol.*, vol. 14, pp. 503-511, 2005.

[30] D. H. Andersen, *et al.*, "Characterization of 59 canine single nucleotide polymorphisms in the Italian wolf (*Canis lupus*) population," *Mol. Ecol. Notes*, vol. 6, pp. 1184–1187, 2006.

[31] U. Arunyawat, W. Stephan, and T. Staedler, "Using multilocus sequence data to assess population structure, natural selection and linkage disequilibrium in wild tomatoes," *Mol. Biol. Evol.*, vol. 24, no. 10, pp. 2310-2322, 2007.

[32] J. Xu, H. Guo, and Z. L. Yang, "Single nucleotide polymorphisms in the ectomycorrhizal mushroom *Tricholoma matsutake*," *Microbiology*, vol. 153, pp. 2002-2012, 2007.

[33] V. M. Novelli, M. A. Takita, and M. A. Machado, "Identification and analysis of single nucleotide polymorphisms (SNPs) in citrus," *Euphytica*, vol. 138, pp. 227-237, 2004.

[34] A. Cohuet, *et al.*, "SNP discovery and molecular evolution in *Anopheles gambiae*, with special emphasis on innate immune system," *BMC Genomics*, vol. 9, no. 227, 2008.

[35] E. R. A. Cramer, L. Stenzler, A. L. Talaba, C. A. Makarewich, S. L. Vehrencamp, and I. J. Lovette, "Isolation and characterization of SNP variation at 90 anonymous loci in the banded wren (*Thryothorus pleurostictus*)," *Conserv. Genet.*, vol. 9, no. 6, pp. 1657-1660, 2008.

[36] F. A. Feltus, J. Wan, S. R. Schulze, J. C. Estill, N. Jiang, and A. H. Paterson, "An SNP resource for rice genetics and breeding based on subspecies *indica* and *japonica* genome alignments," *Genome Res*., vol. 14, pp. 1812–1819, 2004.

[37] S. Roy, *et al.*, "Genetic diversity and population structure in aromatic and quality rice (*Oryza sativa* L.) landraces from North-Eastern India," *PLoS One*, vol. 10, no. 6, June 2015.

[38] BPGR-IRRI Rice Advisory Committee and International Board for Plant Genetic Resources, *Descriptors for Rice, Oryza Sativa L*, Philippines: International Rice Research Institute, 1980.

[39] Ø. Hammer, D. A. T. Harper, and P. D. Ryan, "PAST: Paleontological statistics software package for education and data analysis," *Palaeontol. Electronica*, vol. 4, pp. 1-31, May 2001.

[40] Q. Zhu, X. Zheng, J. Luo, B. S. Gaut, and S. Ge, "Multilocus analysis of nucleotide variation of *Oryza sativa* and its wild relatives: Severe bottleneck during domestication of rice," *Mol. Biol. Evol.*, vol. 24, no. 3, pp. 875-888, Jan. 2007.

[41] T. A. Hall, "BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nucl. Acids. Symp. Ser.*, vol. 41, pp. 95-98, 1999.
[42] P. Librado and J. Rozas, "DnaSP v5: A software for comprehensive analysis of DNA polymorphism data," *Bioinformatics*, vol. 25, pp. 1451-1452, 2009.
[43] G. A. Watterson, "On the number of segregating sites in genetical models without recombination," *Theor. Popul. Biol.*, vol. 7, pp. 256-276, 1975.
[44] M. Nei, *Molecular Evolutionary Genetics*, New York: Columbia University Press, 1987.
[45] F. Tajima, "Statistical method for testing the neutral mutation hypothesis by DNA polymorphism," *Genetics*, vol. 123, pp. 585-595, 1989.

Prin Phunngam was born in Bangkok, Thailand on 10 November 1983. He obtained Bachelor of Science in Wood Science and Technology and Master of Science in Genetics from Kasetsart University, Bangkok, Thailand. He is now a lecturer at Department of General Science, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi Thailand. His current research interests are population genetics and evolutionary genetics.

Nichakorn Pathumrangsan is a lecturer at Department of General Science, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi Thailand. She is now a PhD student at Sirindhorn International Institute of Technology Thammasat University. She specializes in biodegradable polymer: PLA.

Natnaree Khambai and Jaruwan Tongjun are now undergraduate students at Department of Biology, Faculty of Science and Technology, Muban Chombueng Rajabhat University, Ratchaburi, Thailand.

Uraiwan Arunyawat is an Assistant Professor at Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, Thailand. She obtained her Ph.D. in Population Genetics from University of Munich, Germany and Postdoctoral experience from Institut National de la Recherche Agronomique (INRA), Bordeaux, France. Her current research interests include molecular population genetics and evolutionary genetics of malaria vectors.