Mar. Biotechnol. 6, 604–611, 2004
DOI: 10.1007/s10126-004-2300-5

MARINE BIOTECHNOLOGY

Population Structure of Tropical Abalone (*Haliotis asinina*) in Coastal Waters of Thailand Determined Using Microsatellite Markers

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Abstract: Three partial genomic libraries were constructed from genomic DNA of the tropical abalone (*Haliotis asinina*) that was digested with *Alu*I, vortexed/sonicated, and digested with mixed enzyme (*Alu*I, *Hinc*II, and *Rsa*I). The libraries yielded 0.02%, 0.42%, and 1.46% positive microsatellite-containing clones, respectively. Eleven clones each of perfect, imperfect, and compound microsatellites were isolated. Ten primer pairs (*CUHas1–CUHas10*) were analyzed to evaluate their polymorphic level. The numbers of alleles per locus, observed heterozygosity (H_0), and expected heterozygosity (H_e) ranged from 3 to 26 alleles, and varied between 0.27 and 0.85 and between 0.24 and 0.93, respectively. Three microsatellite loci (*CUHas2*, *CUHas3*, and *CUHas8*) were further used for examination of genetic diversity and differentiation of natural *H. asinina* in coastal waters of Thailand. Genetic variabilities in terms of the effective number of alleles (n_e), H_0, and H_e were higher in 2 samples from the Gulf of Thailand (n_e = 9.37, 7.66; H_0 = 0.62, 0.78; and H_e = 0.87, 0.86) than those of one sample (n_e = 6.04; H_0 = 0.58; and H_e = 0.62) derived from the Andaman Sea. Assessment of genetic heterogeneity, including allele frequency comparison and pairwise F_{ST} analysis, indicated interpopulational differentiation, between natural *H. asinina* from the Gulf of Thailand and that from the Andaman Sea ($P < 0.0001$).

Key words: abalone, *Haliotis asinina*, microsatellites, genetic diversity, population differentiation.

INTRODUCTION

Abalones are marine gastropods distributed worldwide along the coastal waters of tropical and temperate areas (Geiger, 2000). Approximately 20 species of abalone are commercially important (Jarayabhand and Paphavasit, 1996). Abalone aquaculture has been established in several countries, but approximately 75% of the world production annually is in China (mainly *Haliotis discus hannai*) and Taiwan (mainly *H. diversicolor supertexta*) (Gordon, 2000).

Three species of tropical abalone, *H. asinina*, *H. ovina*, and *H. varia*, are locally found in Thai waters (Jarayabhand and Paphavasit, 1996). Among these species *H. asinina* is the most promising for aquaculture. Artificially propagated breeding programs and culture techniques for *H. asinina*

Received June 5, 2003; accepted February 18, 2004; online publication March 9, 2005.

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are well developed; however, basic information on genetic population differentiation and levels of genetic diversity of *H. asinina* is necessary to improve the stock selection program and conserve the existing natural gene pool (Klinbunga et al., 2003).

An initial step toward natural stock management of *H. asinina* is to develop molecular genetic markers that can be applied to its genetic management, including determination of stock structure, evaluation of the levels of gene flow, and reconstruction of intraspecific phylogeny. In addition, the ability to determine individuality and parentage of *H. asinina* will provide the means to establish efficient selective breeding programs and to construct genetic linkage maps of *H. asinina* more effectively.

Microsatellites are short, tandemly repeated DNA loci (1–6 nucleotides) arrayed for approximately 10 to 50 copies and abundantly dispersed in eukaryotic genomes. Microsatellite loci exhibiting large numbers of alleles are ideally suited for gene mapping and pedigree analysis (Pepin et al., 1995), whereas loci with lower polymorphic levels can be used for population genetic studies (Wright and Bentzen, 1994).

Microsatellite markers have been successfully developed in several abalone species including *H. rufescens* (Kirby and Powers, 1998), *H. rubra* (Huang and Hana, 1998; Evans et al., 2001), *H. asinina* (Selvamani et al., 2000), *H. discus discus* (Sekino and Hara, 2001), and *H. discus hannai* and *H. kamtschatkara* (Miller et al., 2001).

Genetic diversity and population differentiation of abalone in Thai waters based on microsatellite polymorphism have not been reported. The objective of this study was to develop informative microsatellites in *H. asinina* and to assess the genetic structure of this species in coastal waters of Thailand. We isolated *H. asinina* microsatellites from 3 partial genomic libraries that were constructed using different approaches. Genetic heterogeneity among *H. asinina* populations derived from different areas was investigated using the 3 microsatellite loci.

Materials and Methods

Samples

Specimens of *H. asinina* were collected from 6 samples (Figure 1). Natural abalone include samples from Talibong Island (HATRAW, $N = 28$) located in the west of peninsular Thailand, and Samet Island (HASAME, $N = 12$) and Cambodia (HACAME, $N = 23$) located in the Gulf of Thailand. Samples of hatcheries were offspring of wild broodstock initially established from approximately 100 founders originating in Cambodia (HACAMHE, $N = 15$) and Samet Island (HASAMHE, $N = 10$) and the second generation of *H. asinina* (HAPHIHE, $N = 20$) initially established from approximately 200 founders and maintained at the Aquaculture Department, SEAFDEC, Philippines.

Figure 1. Map of Thailand indicating sampling sites of *H. asinina* used in this study. Dots represent sample locations (except the Philippines sample) from which *H. asinina* was collected. CAM indicates Cambodia; SAM, Samet Island; TRA, Trang.

DNA Extraction

Genomic DNA extracted from a single individual of *H. asinina* (Gulf of Thailand origin) by a proteinase K phenol-chloroform extraction method (Davis et al., 1986) was used

for construction of each partial genomic library. For genotyping of abalone a Chelex-based extraction method (Walsh et al., 1991; Altschmied et al., 1997) was utilized ($N = 108$).

Construction of *H. asinina* Partial Genomic Libraries

Three partial genomic libraries were constructed: an *Alu*I-digested library (5 μg of *H. asinina* genomic DNA digested with 25 U of *Alu*I at 37°C for 2 hours); a vortexed/sonicated genomic library (1 μg of *H. asinina* genomic DNA vortexed for 15 minutes and subsequently sonicated in an Ultrasonic BCGR 5139 bath for 1 hour); and a mixed-enzyme-digested library (6 μg of *H. asinina* genomic DNA digested with 20 U each of *Alu*I, *Hinc*II, and *Rsa*I for 2 hours at 37°C). After agarose gel electrophoresis the resulting 300-bp to 800-bp DNA fragments were excised, eluted out from the gels, and further treated with T4 polynucleotide kinase and Klenow fragment according to conditions recommended by the manufacturer (New England Biolabs). Approximately 150 ng of manipulated genomic DNA of *H. asinina* was ligated to 50 ng of dephosphorylated *Sma*I-digested pUC18 (Amersham Bioscience) overnight at 16°C. The ligation mixture was electrotransformed into *Escherichia coli* XL1-Blue (Dower et al., 1988). Recombinant clones were selected on ampicillin agar plates according to standard protocol (Maniatis et al., 1982).

Screening of Microsatellite-Containing Clones

Transformed clones were transferred onto a piece of Whatman filter paper (#45). The filter paper was hybridized with the γ-^{32}P labeled $(GT)_{15}$ and $(CT)_{15}$ probe and subjected to autoradiography at −80°C for 2 to 4 hours (Grunstein and Hogness, 1975). Positive clones were picked up and cultured individually. Plasmid DNAs were extracted using a boiling method (Holmes and Quigley, 1981).

DNA Sequencing

Plasmid DNAs (500 ng) were sequenced manually using a T7 sequencing kit (Amersham) with universal or reverse primers. The products were analyzed on 8% denaturing polyacrylamide gels at 50 W for 2.5 hours. The sequencing gel was transferred onto a piece of filter paper, dried in vacuo, and subjected to autoradiography overnight at room temperature.

Primer Design and Amplification of Microsatellites

Primer pairs to amplify microsatellite regions were designed using OLIGO 4.0 (National Biosciences; Table 1). Polymerase chain reaction (PCR) was carried out as described by Supungul et al. (2000). PCR products were analyzed on a 6% denaturing polyacrylamide gel at 50 W for 2.5 to 6 hours. After autoradiography allele sizes of each locus were determined by comparison with the M13 sequencing marker (Yanisch-Perron et al., 1985). Cross-species amplification for all loci was tested in *H. ovina* ($N = 5$) and *H. varia* ($N = 5$) under different PCR amplification conditions (annealing temperatures, $MgCl_2$ concentrations, and thermal profiles)

Data Analysis

The number of alleles per locus and observed, and expected heterozygosity were calculated (Nei, 1987). The effective number of alleles (Crow and Kimura, 1965) and allele frequencies at each locus in each sample were calculated. Hardy-Weinberg equilibrium for each locus was examined using the exact test (Rousset and Raymond, 1995). Linkage disequilibrium between loci in each sample and allele frequency distribution between possible sample combinations were compared using the Markov chain approach (Guo and Thompson, 1992). Significance of pairwise F_{ST} values (Weir and Cockerham, 1984) was evaluated. All the calculations described above were conducted using GENEPOP 2.0 (Raymond and Rousset, 1995). The significance levels for multiple tests were adjusted following a sequential Bonferroni approach (Rice, 1989). Cavalli-Sforza and Edwards chord genetic distance (Cavalli-Sforza and Edwards, 1967) was estimated for all possible combinations of samples using the Gendist routine in PHYLIP 3.56c (Felsenstein, 1993).

Results and Discussion

Isolation and Characterization of Microsatellites in *H. asinina*

With the γ-^{32}P-end-labeled GT_{15} probe, the mixed-enzyme-digested library yielded 1.46% positive clones (51 in 3487), While the vortexed/sonicated and *Alu*I-digested libraries gave 0.42% (19 in 4564) and 0.20% (5 in 2510), respectively. Further screening of the vortexed/sonicated library with the $(CT)_{15}$ probe provided another 0.07% of positive microsatellite-containing clones (3 in 4464 clones).

Table 1. Characteristics of Ten Novel Microsatellites in *H. asinina*

Locus[a]	Motif	Primer sequence (5′–3′)	T_a (°C)	Sample size (N)	Size range in (bp)	No. of alleles	H_0	H_e
CUHas1	$(GT)_{17}N_{36}(GT)_{10}$	TCATCTGAGTTAATAAGGGAC TCAGTCATTATCTTAGCGGAG	53	72	258–360	26	0.85	0.93
CUHas2	$(AT)_7(GT)_{37}$	ATGGAAGTCAACAATAGACAGG CCCAGATCAGTTCCACAATAC	57	65	286–340	21	0.68	0.93
CUHas3	$(GT)_{24}(GA)_{18}$	TCCAGACTGCACGTTATTATTCC GCACCCTGTCTCCCTTGAAC	57	71	134–178	13	0.62	0.82
CUHas4	$(GT)_6(TGCA)_4N_{15}(GT)_7$	GTTCCGTTCTACCAATGATCG ACTCGCCGTCGTATACCTAG	57	67	222–250	5	0.40	0.59
CUHas5	$(GT)_{17}$	ATGAACCTCTAATCTAAAGC AGTGCTCTTTACCAATCC	49	72	104–173	19	0.35	0.91
CUHas6	$(GT)_{19}$	CGATGGTGATACGATGATGC ACGGTATGAACATATCGTGAC	57	48	232–240	6	0.75	0.71
CUHas7	$(ACGC)_6$	CTACACCAACATTATCCTG AATCAATAAGTGACTGTCTG	49	48	112–126	3	0.27	0.24
CUHas8	$(AGTG)_{16}$	GTATTACTTGACTTTGAGCC TGTATGTCCTATCACAGCAT	49	72	148–238	19	0.71	0.88
CUHas9	$(GT)_{34}$	TGTCGTAACTCCCATAGCG GGTGTCCATTTATGAATTGAG	53	48	148–240	26	0.81	0.92
CUHas10	$(CA)_{16}CG(CA)_4$	CCACTCACAACAACGCACG AAGGCAGCGAAACCTCACC	53	48	118–160	9	0.42	0.63

[a]GenBank accession numbers are BV096864 to BV096873.

A total of 78 positive clones were sequenced, and 33 microsatellite loci were isolated. The proportions of perfect, imperfect, and compound microsatellites (Weber, 1990) were identical (11 clones accounting for 33.33% of each type of microsatellite).

Fourteen primer pairs were designed, of which 10 (*CUHas1–CUHas10*) worked well in *H. asinina* samples (HACAME, HACAMHE, HASAMHE, and HAPHIHE) with the allelic variations (Table 1, Figure 2). *CUHas1* exhibited the highest variability (26 alleles, $H_0 = 0.85$, $H_e = 0.93$), while the lowest variability was observed at *CUHas7* (3 alleles, $H_0 = 0.27$, $H_e = 0.24$). Linkage disequilibrium between loci was not significant for all combinations of loci after corrections of significance level ($P > 0.0083$). Three microsatellite loci (*CUHas4*, *CuHas6*, and *CuHas7*) were successfully amplified in specimens from the Gulf of Thailand but not in the Andaman Sea sample (HATRAW). Cross-species amplification for *H. ovina* and *H. varia* was not successful at all loci even though a variety of PCR conditions were extensively tested.

The level of genetic variability at each microsatellite locus in this study was as high as that previously reported in *H. rubra* (8–41 alleles, $H_0 = 0.19–0.38$, $H_e = 0.81–0.96$, Huang et al., 2000; 2–16 alleles, $H_0 = 0.14–0.76$, $H_e = 0.40–0.90$, Evans et al., 2001); *H. discus discus* (3–10 alleles, $H_0 = 0.18–0.80$, $H_e = 0.29–0.89$, Sekino and Hara, 2001); and Australian *H. asinina* (2–25 alleles, $H_e = 0.29–0.96$, H_0 was not reported; Selvamani et al., 2000), but lower than microsatellites in *H. kamtschatkana* (20–63 alleles, $H_0 = 0.41–0.89$, $H_e = 0.68–0.96$, Miller et al., 2001).

Nonoverlapping alleles were observed at *CUHas9*; that is, the HATRAW sample possessed smaller alleles (148–162 bp) than those detected in the HACAME sample (184–240 bp) suggesting that this locus has potential to identify the coastal origins of Thai *H. asinina*. However, larger numbers of specimens from other sites of each coastal region should be genetically examined to verify our speculations.

Selvamani et al. (2000) also isolated and characterized 11 microsatellites in *H. asinina*, but the genetic variability of these microsatellites was only examined in the Heron reef population of Australia ($N = 21–41$ per locus). The failure to genotype HATRAW sample originating from the Andaman Sea using *CUHas4*, *CuHas6*, and *CuHas7* primer

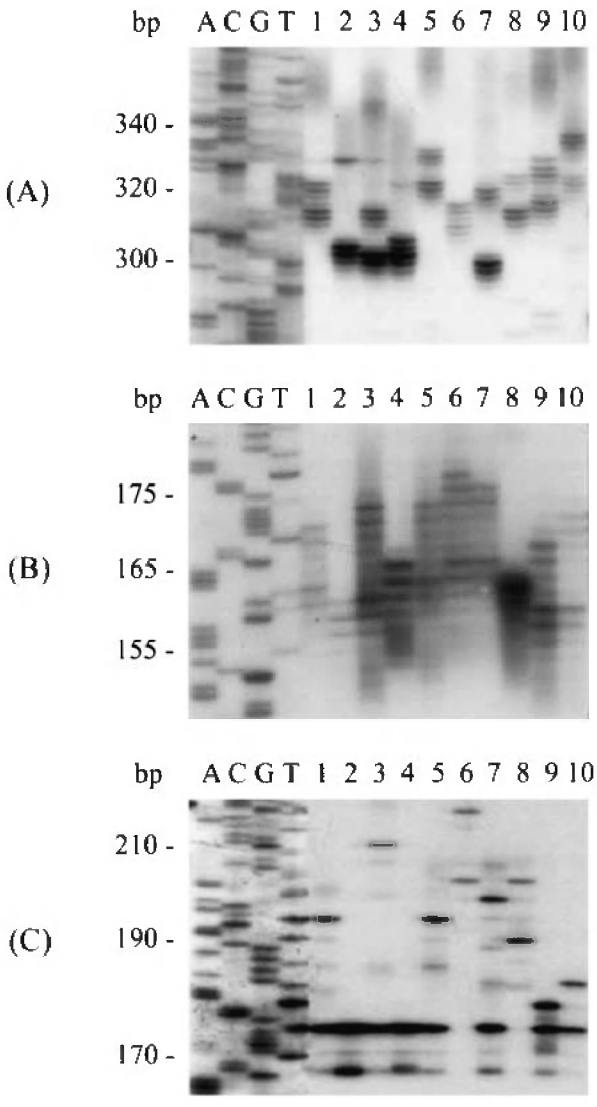

Figure 2. Microsatellite patterns resulting from analysis of *H. asinina* at loci *CUHas2* (**A**), *CUHas3* (**B**), and *CUHas8* (**C**). A sequencing ladder of M13mp18 was used as the size standard (lanes A, C, G, and T).

pairs designed from microsatellites of *H. asinina* from the Gulf of Thailand implied that locus-specific primers of Australian *H. asinina* reported by Selvamani et a1. (2000) should be carefully tested and characterized with Thai *H. asinina* before being used in genetic studies (e.g., the construction of genetic linkage maps and breeding programs) of natural Thai samples and hatchery stocks.

Genetic Diversity of *H. asinina*

Three loci (*CUHas2*, *CUHas3*, and *CUHas8*) were preliminarily tested for population genetic studies of *H. asinina*. The number of alleles, effective alleles, and observed and expected heterozygosity per locus across samples varied from 3.67 to 12.00, 3.58 to 9.37, 0.58 to 0.82, and 0.62 to 0.88, respectively (Table 2).

The HACAME sample exhibited the largest number of alleles and effective alleles per locus (12.00 alleles, $n_e = 9.37$) compared with HASAME (8.33 alleles, $n_e = 7.66$) and HATRAW (8.67 alleles, $n_e = 6.04$). High genetic diversity was also observed in 2 hatchery stocks, HACAMHE (8.67 alleles, $n_e = 8.74$) and HASAMHE (7.67 alleles, $n_e = 7.17$. The HAPHIHE sample exhibited the lowest number of alleles and effective alleles for all loci (3.67 alleles, $n_e = 3.58$). Three instances (HACAME at *CUHas2* and *CUHas3* and HAPHIHE at *CUHas8*) showed significant deviation from Hardy-Weinberg expectations ($P < 0.0001$). Comparable levels of heterozygosity between hatchery stocks (HACAMHE and HSAMHE) and their natural samples (HACAME and HASAME) suggested that founders' contributions in those hatchery stocks were relatively large.

Genetic Heterogeneity of Natural *H. asinina* Samples

On the basis of genetic distance values, the level of genetic differentiation between HASAME and HACAME was low (0.0578), but greater genetic distance was observed between coastal regions (0.1310 and 0.1393, respectively) (Table 3). Genetic heterogeneity analysis and F_{ST} statistics revealed significant genetic population differentiation in natural samples of *H. asinina* ($P < 0.0001$, Table 3) in addition to the results seen in the genetic distance data. A lack of genetic heterogeneity was observed between samples in the Gulf of Thailand (HACAME and HASAME; $P > 0.0083$), but significant population structures were found between those and the Andaman Sea sample (HATRAW) ($P < 0.0001$). As a result, *H. asinina* in coastal waters of Thailand can be differentiated from the Gulf of Thailand and the Andaman Sea stocks.

Klinbunga et al. (2003) determined genetic diversity and population differentiation of the same set of *H. asinina* samples screened in this study by using PCR–restriction fragment length polymorphism of 16S rDNA (mitochondrial DNA). A panmixia was found in natural *H. asinina* populations in Thai waters. In contrast, analysis of genetic differentiation of those samples by randomly amplified polymorphic DNA (RAPD) revealed significant differentiation between the Gulf of Thailand samples HACAME and HASAME and the Andaman HATRAW samples ($P < 0.0001$; Popongviwat, 2001), as well as the results obtained in this study.

Table 2. Mean Number of Alleles, Effective Number of Alleles, and Heterozygosity per Locus of Each Sample of *H. asinina* Across Three Microsatellite Loci (*CUHas2*, *CUHas3*, and *CUHas8*)

Sample	Mean no. of alleles per locus	Effective no. of alleles[a] (n_e)	Mean heterozygosity		Haplotype diversity[b]	
			H_0 ± SD	H_e ± SD	16S rDNA	16S+18S rDNA
Natural						
HACAME	12.00 ± 2.00	9.37	0.62 ± 0.16	0.87 ± 0.05	0.0000	0.3391
HASAME	8.33 ± 0.58	7.66	0.78 ± 0.13	0.86 ± 0.04	0.0000	0.0000
HATRAW	8.67 ± 7.23	6.04	0.58 ± 0.28	0.62 ± 0.27	0.1351	0.7065
Hatchery						
HACAMHE	8.67 ± 1.53	8.74	0.79 ± 0.13	0.88 ± 0.07	0.0000	0.7816
HASAMHE	7.67 ± 0.58	7.17	0.82 ± 0.17	0.86 ± 0.02	0.2731	0.7511
HAPHIHE	3.67 ± 1.53	3.58	0.67 ± 0.58	0.68 ± 0.01	0.0000	0.8359

[a]Mean n_e of 3 loci.
[b]Results from PCR-RFLP analysis assessed for the same sample sets (Klinbunga et al., 2003).

Table 3. Genetic Heterogeneity Analysis, F_{ST} Estimate, and Genetic Distance Between Pairs of *Haliotis asinina* Samples

Pairwise comparison[a]	Probability value[b]			F_{ST}	Genetic distance
	CUHas2	*CUHas3*	*CUHas8*		
HATRAW–HASAME	**0.0009**	**<0.0001**	**<0.0001**	**0.2111***	**0.1393**
HATRAW–HACAME	**<0.0001**	**<0.0001**	**<0.0001**	**0.1977***	**0.1310**
HATRAW–HASAMHE	<0.0001	<0.0001	<0.0001	0.2456*	0.1556
HATRAW–HACAMHE	<0.0001	<0.0001	<0.0001	0.2258*	0.1494
HATRAW–HAPHIHE	<0.0001	<0.0001	<0.0001	0.2870*	0.1486
HAPHIHE–HASAME	<0.0001	<0.0001	<0.0001	0.2004*	0.1649
HAPHIHE–HACAME	<0.0001	<0.0001	<0.0001	0.1785*	0.1564
HAPHIHE–HASAMHE	<0.0001	<0.0001	<0.0001	0.2178*	0.1716
HAPHIHE–HACAMHE	<0.0001	<0.0001	<0.0001	0.1979*	0.1670
HASAME–HACAME	**0.2709^{ns}**	**0.1080^{ns}**	**0.2206^{ns}**	**−0.0052^{ns}**	**0.0578**
HASAME–HASAMHE	0.0100^{ns}	0.0258^{ns}	0.0188^{ns}	0.0455^{ns}	0.0871
HASAME–HACAMHE	0.0310^{ns}	0.0030*	0.0093^{ns}	0.0437^{ns}	0.0767
HACAME–HASAMHE	0.0362^{ns}	0.2380^{ns}	0.5050^{ns}	0.0079^{ns}	0.0521
HACAME–HACAMHE	0.0190^{ns}	0.0216^{ns}	0.1098^{ns}	0.0189^{ns}	0.0531
HACAMHE–HASAMHE	0.9746^{ns}	0.9316^{ns}	0.9273^{ns}	−0.0313^{ns}	0.0113

[a]Comparisons between natural *H. asinina* samples are in bold face.
[b]Probability values of genetic homogeneity between samples. Genetic heterogeneity analysis was conducted based on allele frequency distributions; significance level was further adjusted to $P < 0.0083$ using a sequential Bonferroni method. Superscript ns, indicates not significant; *, $P < 0.0001$.

Nonsignificant genetic heterogeneity between our hatchery stocks (HACAMHE and HASAMHE) and their natural samples (HACAME and HASAME) based on 16S rDNA (Klinbunga et al., 2003), RAPD (Popongwiwat, 2001), and microsatellites (this study) suggests the possibility of using our hatchery stocks to establish stock enhancement programs at the geographic origins of *H. asinina*.

Microsatellite markers developed in this study can be further applied to assist genetic improvement and breeding programs of *H. asinina*, such as determination of correlation between genotypes and survival rates after settlement of larvae, and between growth rate and levels of heterozygosity. In addition, these markers may be useful to identify the stock units of migration ability in natural *H. asinina*. Highly polymorphic levels of microsatellites can also be applied to

parentage analysis, eliminating problems accompanied by traditional selective breeding programs in which offspring of different family lines must be cultured separately.

Acknowledgments

This research was supported by the Thailand Research Funds (TRF) Grant RDG4320015 awarded to S.K. and A.T. The student grant to S.T. was supported by The Thailand Graduate Institute of Science and Technology (TGIST). We also thank 2 anonymous referees for spending their valuable time to review our manuscript.

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