Genetic compatibility between *Anopheles lesteri* from Korea and *Anopheles paraliae* from Thailand

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To assess differentiation and relationships between Anopheles lesteri *and* Anopheles paraliae *we established three and five iso-female lines of* An. lesteri *from Korea and* An. paraliae *from Thailand, respectively. These isolines were used to investigate the genetic relationships between the two taxa by crossing experiments and by comparing DNA sequences of ribosomal DNA second internal transcribed spacer (ITS2) and mitochondrial DNA cytochrome* c *oxidase subunit I (COI) and subunit II (COII). Results of reciprocal and F_1-hybrid crosses between* An. lesteri *and* An. paraliae *indicated that they were compatible genetically producing viable progenies and complete synaptic salivary gland polytene chromosomes without inversion loops in all chromosome arms. The pairwise genetic distances of ITS2, COI and COII between these morphological species were 0.040, 0.007-0.017 and 0.008-0.011, respectively. The specific species status of* An. paraliae *in Thailand and/or other parts of the continent are discussed.*

Key words: *Anopheles lesteri* - *Anopheles paraliae* - crossing experiments - second internal transcribed spacer - cytochrome *c* oxidase subunit I - cytochrome *c* oxidase subunit II

The *Anopheles hyrcanus* Group has a wide range of distributions extending from Iberia in Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans. Up until now, at least 27 species have been reported within this group (Harrison & Scanlon 1975, Harbach 2010). It is well known that some species of the Hyrcanus Group are involved in transmission of human diseases, particularly in the Oriental and contiguous parts of the eastern Palaearctic regions. For example, human malaria *Plasmodium vivax* was detected in *Anopheles sinensis*, *Anopheles lesteri*, *Anopheles kleini*, *Anopheles pullus* and *Anopheles belenrae* (Harrison 1973, Ree et al. 2001, Whang et al. 2002, Ma & Xu 2005, Lee et al. 2007, Joshi et al. 2009, 2011, Rueda et al. 2010). Moreover, *Brugia malayi* was found in *An. sinensis* and *An. lesteri* (Sasa 1976) while *Anopheles peditaeniatus* was infected with Japanese encephalitis virus (Zhang 1990, Kanojia et al. 2003). In addition, some members of the Hyrcanus Group have also been considered as economic pests of cattle because of their vicious biting-behaviour and ability to transmit cervid filariae of the genus *Setaria* (Reid et al. 1962, Reid 1968, Harrison & Scanlon 1975).

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An. lesteri has been found in the Philippines (type locality) and the Palaearctic regions (China, Korea and Japan) whereas *Anopheles paraliae* has been detected in the coastal areas of Peninsular Malaysia, Sabah, Sarawak, Brunei, Vietnam and Thailand. However, the taxonomic ambiguity of *An. paraliae* was raised as early as 1959. Morphologically, *An. paraliae* has a narrower apical fringe spot on the wing compared with that of *An. lesteri*, but their immature stages can not be distinguished from each other. Consequently, *An. paraliae* was considered to be a subspecies, *An. lesteri paraliae,* by earlier authors (Sandosham 1959, Reid 1963, 1968, Harrison & Scanlon 1975). Nevertheless, this subspecies was elevated subsequently to species status, i.e., *An. paraliae*, based on distinct characteristics of the adult wings and immature habitats (brackish and/or peaty water) (Harrison et al. 1991). Yet there is no evidence of genetic differences between *An. lesteri* and *An. paraliae*. This article presents the results of crossing experiment and cytogenetic study of these two species and comparative DNA sequence analyses of the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), the cytochrome *c* oxidase subunit I (COI) and subunit II (COII) of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Collection sites - Samples of *An. lesteri* from Korea were caught in a vinyl tent in a rice paddy field of the district of So Rae, Incheon City, northern of the province of Gyeonggi. The *An. paraliae* specimens from Thailand were obtained by using cow-baited traps in three localities, i.e., district of Damnoen Saduak, province of Ratchaburi, district of Pak Panang, province of Nakhon

Si Thammarat and district of Hat Yai, province of Songkhla (Table I). Species identification using F_1-progeny of each iso-female line followed the keys of Rueda et al. (2005) and Rattanarithikul et al. (2006). The distinctive characteristics of wings to separate *An. lesteri* from *An. paraliae* are illustrated in Fig. 1.

Establishment of iso-female lines - Three and five iso-female lines of *An. lesteri* (ilG1, ilG2, ilG3) and *An. paraliae* (ipR1, ipR2, ipN1, ipS1, ipS2), respectively, were established successfully using the methods of Choochote et al. (1983) and Kim et al. (2003). They have been maintained in colonies for more than five consecutive generations in our laboratory and they were used for crossing experiments and comparative DNA sequence analyses.

Crossing experiments - One iso-female line (ilG1) of *An. lesteri* and three iso-female lines (ipR1, ipN1, ipS1) of *An. paraliae* were arbitrarily selected for crossing experiments to determine post-mating reproductive isolation by employing the techniques previously reported by Saeung et al. (2007). Study on salivary gland polytene chromosomes of 4th instar larvae of F_1-hybrids from the crosses followed the techniques of White et al. (1975) and Kanda (1979).

DNA extraction and amplification - Individual F_1-progeny adult females of each iso-female line of *An. lesteri* (ilG1, ilG2, ilG3) and *An. paraliae* (ipR1, ipR2, ipN1, ipS1, ipS2) were used for DNA extraction and amplification. Molecular analysis of ITS2, COI, COII was performed to determine intraspecific sequence variation in *An. lesteri* and *An. paraliae*. Genomic DNA was extracted from adult mosquitoes using the DNeasy® Blood and Tissue Kit (Qiagen). Primers for amplification of ITS2, COI and COII regions followed the methods of Saeung et

TABLE I

Locations, code of iso-female lines of *Anopheles lesteri* and *Anopheles paraliae* and their GenBank accessions

Location	Code of isoline[a]	Length of ITS2 (bp)	DNA region	GenBank accession ITS2	COI	COII	Reference
An. lesteri							
Korea: Gyeonggi	ilG1[a]	448	ITS2, COI, COII	AB733020	AB733028	AB733036	This paper
Gyeonggi	ilG2	448	ITS2, COI, COII	AB733021	AB733029	AB733037	This paper
Gyeonggi	ilG3	448	ITS2, COI, COII	AB733022	AB733030	AB733038	This paper
	-	448	ITS2	EU789791	-	-	Park et al. (2008)
Japan	-	448	ITS2	AB159606	-	-	K Sawabe et al., unpublished observations
Philippines	-	438	ITS2	AY375469	-	-	Wilkerson et al. (2003)
An. lesteri (= *Anopheles anthropophagus*)							
China	-	448	ITS2	AY803792	-	-	Ma and Yang (2005)
	-	438	ITS2	AY375467	-	-	Wilkerson et al. (2003)
An. paraliae							
Thailand: Ratchaburi	ipR1[a]	448	ITS2, COI, COII	AB733023	AB733031	AB733039	This paper
Ratchaburi	ipR2	448	ITS2, COI, COII	AB733024	AB733032	AB733040	This paper
Nakhon Si Thammarat	ipN1[a]	448	ITS2, COI, COII	AB733025	AB733033	AB733041	This paper
Songkhla	ipS1[a]	448	ITS2, COI, COII	AB733026	AB733034	AB733042	This paper
Songkhla	ipS2	448	ITS2, COI, COII	AB733027	AB733035	AB733043	This paper
Anopheles sinensis							
Thailand	-	469	ITS2	AY130473	-	-	Min et al. (2002)
Korea	-	469	ITS2, COI, COII	EU789790	GQ265918	AY130464	Min et al. (2002), Park et al. (2008), Joshi et al. (2009)
Anopheles peditaeniatus							
Thailand	-	463	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote (2011)

a: used in crossing experiments; COI: cytochrome *c* oxidase subunit I; COII: cytochrome *c* oxidase subunit II; ITS2: second internal transcribed spacer.

al. (2007). Polymerase chain reaction (PCR) reaction was performed in total 20 µL volume containing 0.5 U *Ex Taq* (Takara), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer and 1 µL of the extracted DNA. For ITS2, the conditions for amplification consisted of initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1 min and a final extension at 72ºC for 5 min. The amplification profile of COI and COII comprised initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 50ºC for 30 sec and 72ºC for 1 min and a final extension at 72ºC for 5 min. The amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and their sequences directly determined using the BigDye® Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems). The sequence data of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accessions AB733020-AB733043. The ITS2, COI and COII sequences obtained from this study were also compared with deposited sequences available through GenBank (Table I).

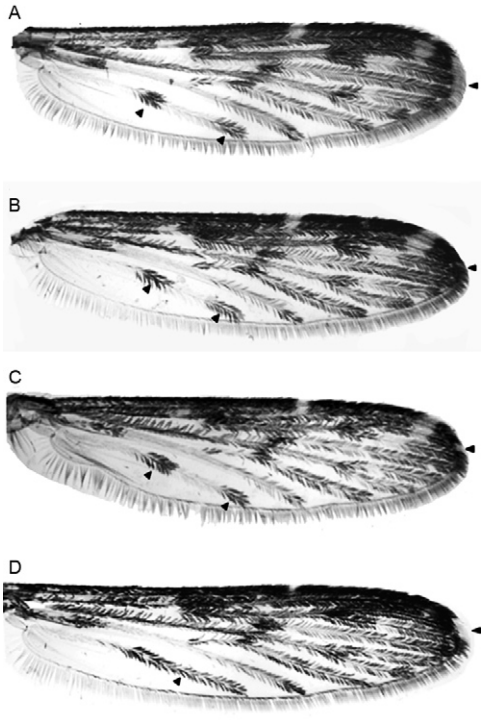

Fig. 1A: wing of *Anopheles lesteri* from Korea showing wide pale fringe spot extending from tip of vein R_1 to R_{4+5} and two dark spots on anal vein (1A); B-D: wings of *Anopheles paraliae* from Thailand showing very narrow pale fringe spot at tip of vein R_2, and two dark spots on 1A similar to that of *An. lesteri* (B), narrow fringe spot at tip of vein R_2 and two dark spots on 1A (C) and moderated fringe spot extending from tip of vein R_{1-3} and one dark spot on 1A (D).

Sequencing alignment and phylogenetic analysis - Sequences of ITS2, COI and COII were aligned using the CLUSTALW multiple alignment program (Thompson et al. 1994). Gap sites were excluded from the following analysis. The Kimura two-parameter method was used to calculate genetic distances (Kimura 1980). Construction of neighbour-joining (NJ) trees (Saitou & Nei 1987) and the bootstrap test with 1,000 replications were performed with the MEGA version 4.0 program (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of one million generations with the nucleotide evolutionary model, GTR+I, which was selected by MrModeltest version 2.3 (Evolutionary Biology Centre, Uppsala University, 2004) as the best-fit model for ITS2, COI and COII. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burnin.

RESULTS

Crossing experiments - Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses between *An. lesteri* from Korea and *An. paraliae* from Thailand are shown in Table II. All crosses yielded viable progenies through the F_2-generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses (repeated twice: experiments 2 and 3, data are not shown). The salivary gland polytene chromosomes of F_1-hybrid larvae from all crosses showed complete synapsis without inversion loops in all chromosome arms (Fig. 2).

Sequence analysis of ITS2, COI and COII regions - The level of genetic distance and number of base substitutions between sequences of the three regions are presented in Tables III-V. Analysis of the ITS2 sequence revealed no intraspecific sequence variation among the three and five iso-female lines of *An. lesteri* and *An. paraliae*, respectively. Comparison of ITS2 sequences indicated that *An. lesteri* differed from *An. paraliae* by 16 base substitutions (pairwise distance = 0.040). In addition, three iso-female lines of *An. lesteri* from Korea were identical with *An. lesteri* from China (= *Anopheles anthropophagus*) (AY803732, AY375467), Japan (AB159606) and Korea (EU789791), but they differed from those of the Philippines (AY375469) by three base substitutions (pairwise distance = 0.007). The average percentages of base composition for the ITS2 sequence of the eight iso-female lines (3 of *An. lesteri* from Korea and 5 of *An. paraliae* from Thailand) were A: 29.9% (29.2-30.5%), T: 24.2% (23.6-24.9%), G: 25.2% (25-25.4%) and C: 20.8% (20.6-20.9%). Percentage of GC content was 46% in *An. lesteri* (448 bp) and 45% in *An. paraliae* (448 bp). All eight sequences differed markedly from *An. sinensis* (pairwise distance = 0.321-0.338) and *An. peditaeniatus* (pairwise distance = 0.550-0.566) (Table III). The analysis of COI (658 bp) among the eight iso-female lines revealed four-nine base substitutions (pairwise distance = 0.007-0.017). On the contrary, *An. lesteri* and *An. paraliae* showed significant differences

TABLE II

Crossing experiments among the four iso-female lines of *Anopheles lesteri* and *Anopheles paraliae*

Crosses (female x male)	Total eggs (n)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
ilG1 x ilG1	345 (155, 190)	65	221 (64.05)	221 (100)	221 (100)	111 (50.23)	110 (49.77)
ipR1 x ipR1	352 (194, 158)	84	296 (84.09)	245 (82.77)	240 (97.96)	98 (40.83)	142 (59.17)
ipN1 x ipN1	380 (190, 190)	86	327 (86.05)	327 (100)	327 (100)	137 (41.90)	190 (58.10)
ipS1 x ipS1	344 (194, 150)	73	186 (54.07)	171 (91.93)	165 (96.49)	61 (36.97)	104 (63.03)
Reciprocal cross							
ilG1 x ipR1	382 (131, 251)	97	332 (86.91)	289 (87.05)	282 (97.57)	169 (59.93)	113 (40.07)
ipR1 x ilG1	393 (187, 206)	98	334 (84.99)	334 (100)	317 (94.91)	190 (59.94)	127 (40.06)
ilG1 x ipN1	402 (182, 220)	60	233 (57.96)	233 (100)	233 (100)	116 (49.79)	117 (50.21)
ipN1 x ilG1	263 (109, 154)	57	147 (55.89)	147 (100)	147 (100)	59 (40.14)	88 (59.86)
ilG1 x ipS1	309 (200, 109)	46	117 (37.86)	107 (91.45)	105 (98.13)	50 (47.62)	55 (52.38)
ipS1 x ilG1	308 (118, 190)	44	114 (37.01)	111 (97.37)	111 (100)	47 (42.34)	64 (57.66)
F_1-hybrid cross							
(ilG1 x ipR1)F_1 x (ilG1 x ipR1)F_1	352 (194, 158)	94	320 (90.91)	320 (100)	314 (98.13)	157 (50)	157 (50)
(ipR1 x ilG1)F_1 x (ipR1x ilG1)F_1	341 (186, 155)	88	290 (85.04)	261 (90)	258 (98.85)	113 (43.80)	145 (56.20)
(ilG1 x ipN1)F_1 x (ilG1 x ipN1)F_1	324 (157, 167)	67	201 (62.04)	201 (100)	201 (100)	101 (50.25)	100 (49.75)
(ipN1 x ilG1)F_1 x (ipN1 x ilG1)F_1	347 (197, 150)	58	180 (51.87)	180 (100)	175 (97.22)	85 (48.57)	90 (51.43)
(ilG1 x ipS1)F_1 x (ilG1 x ipS1)F_1	347 (190, 157)	65	215 (61.96)	213 (99.07)	209 (98.12)	97 (46.41)	112 (53.59)
(ipS1 x ilG1)F_1 x (ipS1 x ilG1)F_1	348 (158, 190)	60	174 (50)	167 (95.98)	164 (98.20)	77 (46.95)	87 (53.05)

a: two selective egg-batches of inseminated females from each cross; *b*: dissection from 100 eggs.

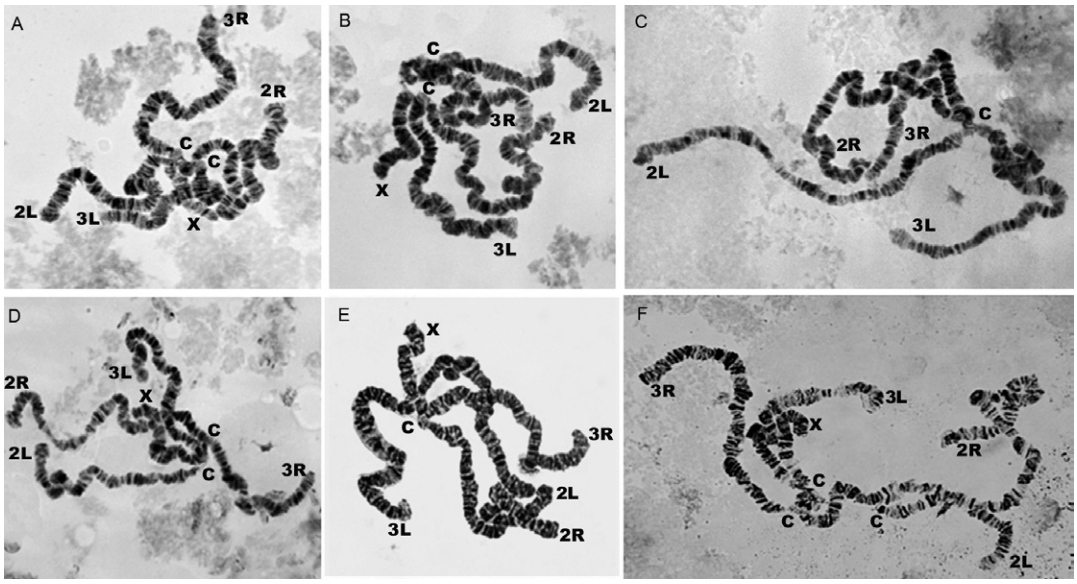

Fig. 2: complete synapsis in all arms of salivary gland polytene chromosome of F_1-hybrid larvae of crosses between *Anopheles lesteri* and *Anopheles paraliae*. A: ilG1 female x ipR1 male; B: ipR1 female x ilG1 male; C: ilG1 female x ipN1 male; D: ipN1 female x ilG1 male; E: ilG1 female x ipS1 male; F: ipS1 female x ilG1 male.

from *An. sinensis* (pairwise distance = 0.034-0.042) and *An. peditaeniatus* (pairwise distance = 0.037-0.041) (Table IV). The analysis of COII (685 bp) among the eight iso-female lines revealed five-seven base substitutions (pairwise distance = 0.008-0.011). These two species also showed significant differences from *An. sinensis* (pairwise distance = 0.039) and *An. peditaeniatus* (pairwise distance = 0.031-0.036) (Table V).

Phylogenetic analysis - The NJ and Bayesian trees of *An. lesteri*, *An. paraliae*, *An. sinensis* and *An. peditaeniatus* were constructed based on the ITS2, COI and COII sequences (Fig. 3). For ITS2, *An. lesteri* (n = 8) and *An. paraliae* (n = 5) were clustered in each monophyletic and well separated from *An. sinensis* and *An. peditaeniatus* with high bootstrap values (93-100%) in both NJ and Bayesian trees. The trees indicated that *An. lesteri* was more closely related to *An. paraliae* (average genetic distances = 0.038) than to the other species. Further, lower sequence divergences (0.000-0.002) were found within the population of each species. For COI and COII, the trees showed that *An. lesteri* was more closely related to *An. paraliae* than to the other species with low level of average genetic distances (0.008-0.011) for both regions, while very low genetic distances (0.003-0.005) were obtained within the population of each species.

DISCUSSION

Crossing experiments using iso-female lines of closely related species of the Oriental *Anopheles* have proven to be a robust systematic procedure for clarifying species status, for example, *Anopheles minimus* and *Anopheles aconitus* (Harrison 1980, Sucharit & Choochote 1982), *Anopheles annularis* and *Anopheles philippinensis* (Choochote et al. 1984), *Anopheles nivipes* and *An. philippinensis* (Klein et al. 1984) and *An. minimus* and *Anopheles flavirostris* (Somboon et al. 2000). These methods are useful for solving taxonomic problems of some sibling species complexes, e.g., *Anopheles dirus* (Baimai et al. 1987), *Anopheles maculatus* (Thongwat et al. 2008), *An. minimus* (Somboon et al. 2001, 2005, Choochote et al. 2002) and *Anopheles barbirostris* (Saeung et al. 2007, 2008, Suwannamit et al. 2009). Likewise, the status of subspecies or cytological races of *Anopheles* can be elucidated by the same approach of cytogenetic study as exemplified in *An. pullus* (= *Anopheles yatsushiroensis*) (Park et al. 2003), *Anopheles vagus* (Choochote et al. 2002), *An. aconitus* (Junkum et al. 2005), *An. sinensis* (Choochote et al. 1998, Min et al. 2002, Park et al. 2008), *An. barbirostris* species A1 (Saeung et al. 2007, Suwannamit et al. 2009), *Anopheles campestris*-like taxon (Thongsahuan et al. 2009) and *An. peditaeniatus* (Choochote 2011). Our findings in this study showed no post-mating reproductive isolation between *An. lesteri* from Korea and *An. paraliae* from Thailand. These results were clearly supported by cytological evidence and DNA analysis. Thus, complete synapsis of salivary gland polytene chromosomes without inversion loops along the entire lengths of all chromosome arms was observed in the F_1-hybrid larvae between *An. lesteri* and *An. paraliae* which strongly indicated genetic compatibility between them.

Analysis of ITS2 sequences of *An. lesteri* from Korea (ilG1, ilG2, ilG3) revealed identical sequences to *An. lesteri* from China (= *An. anthropophagus*), Japan and Korea (genetic distance = 0.000), although they showed little difference from those of the Philippines (genetic distance = 0.007) (Wilkerson et al. 2003, Ma & Yang 2005, Park et al. 2008, K Sawabe et al., unpublished observations). Our results were in agreement with those previously reported by Ma and Xu (2005). Moreover, the low level of pair-

TABLE III

Genetic distance and number of nucleotide substitutions in second internal transcribed spacer sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 ilG1	-	0	0	16	16	16	16	16	0	0	0	0	3	107	108	163
2 ilG2	0.000	-	0	16	16	16	16	16	0	0	0	0	3	107	108	163
3 ilG3	0.000	0.000	-	16	16	16	16	16	0	0	0	0	3	107	108	163
4 ipR1	0.040	0.040	0.040	-	0	0	0	0	16	16	16	16	17	110	111	160
5 ipR2	0.040	0.040	0.040	0.000	-	0	0	0	16	16	16	16	17	110	111	160
6 ipN1	0.040	0.040	0.040	0.000	0.000	-	0	0	16	16	16	16	17	110	111	160
7 ipS1	0.040	0.040	0.040	0.000	0.000	0.000	-	0	16	16	16	16	17	110	111	160
8 ipS2	0.040	0.040	0.040	0.000	0.000	0.000	0.000	-	16	16	16	16	17	110	111	160
9 anthC (AY803792)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	-	0	0	0	3	107	108	163
10 anthC (AY375467)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	-	0	0	3	107	108	163
11 lesJ (AB159606)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	0.000	-	0	3	107	108	163
12 lesK (EU789791)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	0.000	0.000	-	3	107	108	163
13 lesP (AY375469)	0.007	0.007	0.007	0.042	0.042	0.042	0.042	0.042	0.007	0.007	0.007	0.007	-	105	106	163
14 sinK (EU789790)	0.321	0.321	0.321	0.334	0.334	0.334	0.334	0.334	0.321	0.321	0.321	0.321	0.314	-	3	154
15 sinT (AY130473)	0.325	0.325	0.325	0.338	0.338	0.338	0.338	0.338	0.325	0.325	0.325	0.325	0.318	0.007	-	155
16 pedT (AB539061)	0.566	0.566	0.566	0.550	0.550	0.550	0.550	0.550	0.566	0.566	0.566	0.566	0.567	0.520	0.525	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

TABLE IV

Genetic distance and number of nucleotide substitutions in cytochrome *c* oxidase subunit I sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10
1 ilG1	-	1	2	7	6	6	8	7	22	22
2 ilG2	0.002	-	3	8	7	7	9	8	21	21
3 ilG3	0.004	0.006	-	5	4	4	6	5	20	22
4 ipR1	0.013	0.015	0.009	-	1	1	5	0	19	21
5 ipR2	0.011	0.013	0.007	0.002	-	0	4	1	18	20
6 ipN1	0.011	0.013	0.007	0.002	0.000	-	4	1	18	20
7 ipS1	0.015	0.017	0.011	0.009	0.007	0.007	-	5	22	20
8 ipS2	0.013	0.015	0.009	0.000	0.002	0.002	0.009	-	19	21
9 sinK (GQ265918)	0.042	0.040	0.038	0.036	0.034	0.034	0.042	0.036	-	29
10 PedT (AB539069)	0.041	0.039	0.041	0.039	0.037	0.037	0.037	0.039	0.055	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

wise distance (0.040) detected between *An. lesteri* from Korea and *An. paraliae* from Thailand, based on ITS2 sequences, was in accordance with previous reports of different groups of *Anopheles*, e.g., the *Anopheles gambiae* complex (0.4-1.6%) (Paskewitz et al. 1993), *Anopheles dunhami* and *Anopheles nuneztovari* (mean genetic distance = 0.025) (Ruiz et al. 2010), *Anopheles fluviatilis* S and *An. minimus* C (pairwise distance = 0.036) (Singh et al. 2006), *Anopheles kunmingensis* and *Anopheles liangshanensis* (pairwise distance = 0.0381) and *An. pullus* (= *An. yatsushiroensis*) and *Anopheles junlianensis* (pairwise distance = 0.03081) (Hwang 2007). Currently, Calado et al. (2008) showed that *An. nuneztovari* A is not conspecific with *An. nuneztovari* B/C based on COI sequences (genetic distance = 0.00818-0.02071) and *An. dunhami* has been reported as new record in the Brazilian Amazon by comparing sequences with those of *An. nuneztovari* A (genetic distance = 0.01436-0.03343). Similarly, comparative sequences for COI and COII between *An. lesteri* and *An. paraliae* revealed low average genetic distance between them (0.008-0.011). Despite such low genetic distances, phylogenetic trees seem to

TABLE V

Genetic distance and number of nucleotide substitutions in cytochrome *c* oxidase subunit II sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10
1 ilG1	-	1	0	5	5	5	6	5	25	20
2 ilG2	0.002	-	1	6	6	6	7	6	25	20
3 ilG3	0.000	0.002	-	5	5	5	6	5	25	20
4 ipR1	0.008	0.009	0.008	-	0	0	5	0	25	22
5 ipR2	0.008	0.009	0.008	0.000	-	0	5	0	25	22
6 ipN1	0.008	0.009	0.008	0.000	0.000	-	5	0	25	22
7 ipS1	0.009	0.011	0.009	0.008	0.008	0.008	-	5	25	23
8 ipS2	0.008	0.009	0.008	0.000	0.000	0.000	0.008	-	25	22
9 sinK (AY130464)	0.039	0.039	0.039	0.039	0.039	0.039	0.039	0.039	-	29
10 PedT (AB539077)	0.031	0.031	0.031	0.034	0.034	0.034	0.036	0.034	0.045	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

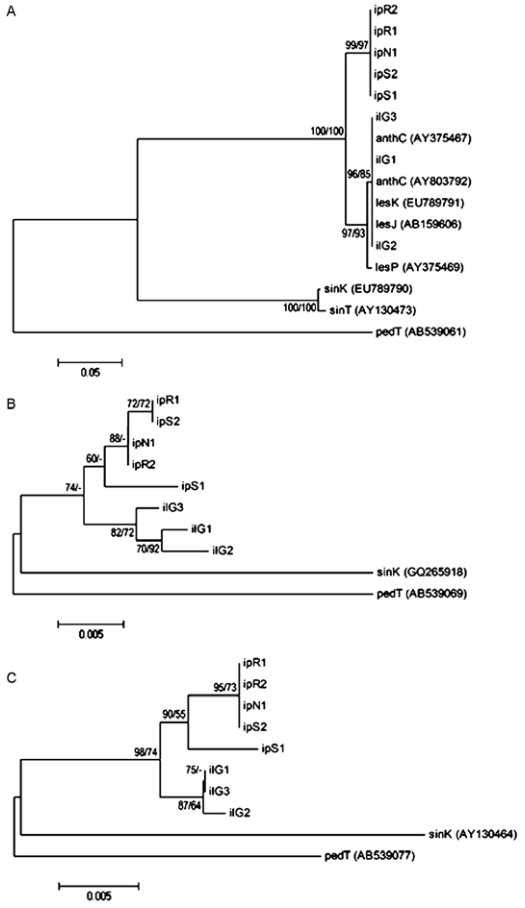

Fig. 3: neighbour-joining (NJ) trees inferred from sequences of three loci. A: second internal transcribed spacer; B: cytochrome *c* oxidase subunit I (COI); C: COII of *Anopheles paraliae*, *Anopheles lesteri*, *Anopheles sinensis* and *Anopheles peditaeniatus*. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). A hyphen (-) shows that the branch did not appear in majority rule (50%) consensus trees of Bayesian analysis. Branch lengths are proportional to genetic distance (scale bar).

indicate that *An. lesteri* and *An. paraliae* were well separated from each other with NJ and Bayesian analyses for three regions, except for the Bayesian tree of COI. Although these two species were distinguished apparently by DNA sequence analysis, they obviously showed genetic compatibility by crossing experiments. Controversy over taxonomic problems with respect to full-fledged species, sibling species and subspecies within a taxon of *Anopheles* has occurred when only data of comparative DNA sequence analyses of certain specific genomic regions were used as first hand criteria for separating them. For example, *An. fluviatilis* S was considered a synonym of *An. minimus* C based on comparison of the D3 domains of 28S (28S-D3) (Harbach 2004, Garros et al. 2005, Chen et al. 2006). However, Singh et al. (2006) carried out molecular analysis on ITS2 and D2-D3 domains of 28S rDNA regions of *An. fluviatilis* S and *An. minimus* C. The authors suggested that these *Anopheles* species did not deserve synonymous status. Hence, crossing experiments between *An. fluviatilis* S and *An. minimus* C using iso-female lines are essential prior to a definite conclusion as to their conspecificity. Our studies using crossing experiments between *An. lesteri* from Korea and *An. paraliae* from Thailand together with data on species distributions, morphological variants, cytology and comparative DNA sequence analyses have clearly indicated that they are conspecific within the taxon *An. lesteri*. Additionally, the population genetic structure will be studied further in order to evaluate the gene flow among *An. lesteri* and *An. paraliae* populations before definitely concluding that *An. lesteri* is a synonym of *An. paraliae*.

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