Molecular Phylogenetics of *Aedes japonicus*, a Disease Vector That Recently Invaded Western Europe, North America, and the Hawaiian Islands

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ABSTRACT We used two mitochondrial loci (nicotinamide adenine dinucleotide dehydrogenase subunit 4 and cytochrome oxidase II) and a nuclear locus (28S-D2 spacer) for a total of 1337 bp to evaluate the relationships among the four subspecies of *Aedes* (*Finlaya*) *japonicus* Theobald. *Ae. j. japonicus* was recently introduced into the United States and has been expanding rapidly. We also included in our analysis a morphologically very closely related species, *Aedes* (*Finlaya*) *koreicus* Edwards, as well as three more distantly related species: *Aedes* (*Finlaya*) *togoi* Theobald, *Aedes* (*Finlaya*) *hatorii* Yamada, and *Aedes* (*Aedimorphus*) *vexans* Meigen. We found that the four subspecies in the *Ae. japonicus* complex are genetically quite distinct but seem to form a monophyletic group that surprisingly also includes *Ae. koreicus*, suggesting the need for a taxonomic reconsideration of the group. We also found that the two southern subspecies are more closely related to each other than to any of the remaining subspecies or to *Ae. koreicus* and may indicate an ancient north–south split of the lineage. Considering the overlap between *Ae. j. japonicus* and *Ae. koreicus*, but the stronger association between *Ae. koreicus* and humans, we are surprised it also has not expanded from its original range. As a proactive reaction to this possibility, we designed and tested a DNA-based rapid assay to differentiate *Ae. koreicus* from some of the species with which it may be confused in the United States. These *Aedes* are putative vectors of several important viral encephalitides.

KEY WORDS cytochrome oxidase II, nicotinamide adenine dinucleotide dehydrogenase subunit 4, ITS-D2 spacer, *Aedes koreicus*, *Ochlerotatus japonicus*

The genus *Aedes* (Diptera: Culicidae) includes the principal vectors of yellow fever, dengue, and aperiodic lymphatic filariasis (Foster and Walker 2002). This is a diverse taxon currently containing 921 species (www.mosquitocatalog.org; but also see http://mosquito-taxonomic-inventory.info/valid-species-list and Reinert 2000, Reinert et al. 2004, 2006, 2008), which is in need of a detailed examination (Black 2004, Savage and Strickman 2004, Tabachnick 2005). A few species in this group of mosquitoes have historically expanded their global distribution in association with humans (Tabachnick and Powell 1979). Many *Aedes* are container breeders that take advantage of water accumulated in human refuse and/or have an egg stage that can withstand dehydration. In particular, members of this genus seem to be especially able to exploit the current extensive worldwide trade in used tires and similar containers (Lounibos 2002). The introduction of the African *Aedes (Stegomyia) aegypti* to the New World heralded epidemics of yellow fever as well as dengue fever (Tabachnick and Powell 1979), and the introduction of *Ae.* (*Stg.*) *albopictus* exacerbated the scourge of dengue fever in Central and South America, if not in North America (Gratz 2004, Paupy et al. 2009), and has become the primary vector of chikungunya fever virus, leading to recent epidemics in Asia and Europe (de Lamballerie et al. 2008).

Aedes (*Finlaya*) *japonicus japonicus* Theobald (Diptera: Culicidae) was first collected outside its native range of northeast Asia (Tanaka et al. 1979) in 1998 when it was collected in the United States (Peyton et al. 1999, Andreadis et al. 2001). Although it is unclear when it was first introduced to the United States, the fact that extensive collections aimed at

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detecting the presence of *Ae. albopictus* (Moore et al. 1990) in 1992 failed to uncover *Ae. j. japonicus* argues that it must have been introduced since 1992 (Andreadis et al. 2001). *Ae. japonicus* has expanded in North America from three states in 1998 (Connecticut, New York, and New Jersey) to a current total of 31 (Alabama, Connecticut, Delaware, Georgia, Hawaii, Iowa, Illinois, Indiana, Kentucky, Massachusetts, Maryland, Maine, Michigan, Minnesota, Missouri, North Carolina, New Hampshire, New Jersey, New York, Ohio, Oregon, Pennsylvania, Rhode Island, South Carolina, South Dakota, Tennessee, Virginia, Vermont, Washington, Wisconsin, and West Virginia) as well Quebec, Canada (Larish and Savage 2005, Widdel et al. 2005, Saenz et al. 2006, Bevins 2007, Morris et al. 2007, Hughes et al. 2008,Neitzel et al. 2009). The species is very common in northeastern states, e.g., Pennsylvania, Connecticut, and New York (Andreadis et al. 2001, Falco et al. 2002); and although it is inexorably expanding south and into the Midwest, its presence there is still very localized (Joy 2004, Roppo et al. 2004, Qualls and Mullen 2006, Bevins 2007). Breeding populations also were found in France in 2000 (Schaffner et al. 2003) and in Belgium in 2002 (Versteirt et al. 2009).

Ae. (*Fin.*) *japonicus* is currently composed of four geographically distinct subspecies: *Ae. j. japonicus*, *Ae. j. shintienensis* Tsai & Lien, *Ae. j. yaeyamensis* Tanaka, Mizusawa & Saugstad, and *Ae. j. amamiensis* Tanaka, Mizusawa & Saugstad. The subspecies *Ae. j. japonicus* is very common in Palearctic Japan and shows little morphological variation there. *Ae. j. japonicus* also is found in Korea and throughout China (Lu et al. 1997), although it is not clear whether this is the only subspecies found there. Common on Yaeyama Gunto, the southern most islands of the Ryukyu Archipelago, *Ae. j. yaeyamensis* is quite distinct from the other subspecies, although the diagnostic hind femur scale pattern clearly overlaps *Ae. (Fin.) koreicus*, a closely related species found in Korea and China, making misidentification likely (Fig. 1). Tanaka et al. (1976) attributes the morphological differences between *Ae. j. yaeyamensis* and the other subspecies to it having become isolated earlier in the evolution of the complex. *Ae. j. shintienensis*, which occurs in Taiwan, closely resembles its northern neighbor *Ae. j. yaeyamensis* in the aedeagus, implying a possible common ancestor. *Aedes j. amamiensis* is found in Amami Ôshima, the northernmost Islands of the Ryukyu Archipelago, and although not as clearly defined morphologically as *Ae. j. yaeyamensis* it is still considered a distinct subspecies. Interestingly, only two specimens collected in Okinawa, in central Ryuku Archipelago, have been identified as *Ae. japonicus.* These specimens were morphologically similar to *Ae. j. amamiensis* (Toma and Miyagi 1981, 1986).

Close examination of the morphological evidence indicates there are insufficient diagnostic traits to reliably separate the subspecies in the *Ae. japonicus* complex from each other and from *Ae. koreicus.* As adults, the primary character separating them is the degree of development of the sub-basal dark band

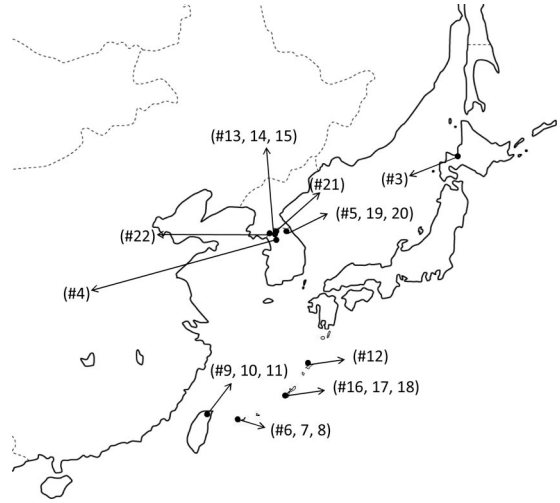

Fig. 1. Sampling locations in East Asia. Numbers refer to those in Table 1.

(Fig. 1). Additional morphological characters identifying specimens as *Ae. koreicus* have a generous amount of overlap with *Ae. japonicus* and the overlap in characters could lead to possible misidentification of adult *Ae. japonicus* (especially *yaeyamensis*) as *Ae. koreicus* (Tanaka et al. 1979).

Substantial similarity of morphological characters, in particular the male genitalia, suggests that *Ae. koreicus* may be more closely related to *Ae. japonicus* than suggested previously. The phylogenetic structure of the genus *Aedes*, including subgenus *Finlaya*, has been thus far largely without molecular data. We therefore sought to test for concurrence of molecular and morphological characters.

Our objectives were to examine the relationships between members of the *Ae. japonicus* complex both to understand patterns of evolution within the genus *Aedes* as well as to develop better diagnostic markers. *Ae. j. japonicus* and *Ae. koreicus* have been shown to be efficient laboratory and field vectors of several encephalitides, including West Nile virus and Japanese encephalitis (Tanaka et al. 1979; Takashima and Rosen 1989; Turell et al. 2001; Sardelis et al. 2002a,b; Kutz et al. 2003, Sardelis et al. 2003), which makes understanding and attempting to curb their expansion across the World critical.

Materials and Methods

For phylogenetic analysis, we sequenced three gene regions in 20 specimens collected from East Asia and two from the United States. We included specimens from the four subspecies of *Ae. japonicus* as well as specimens of *Ae. koreicus*, *Aedes* (*Finlaya*) *togoi* Theobald, *Aedes* (*Finlaya*) *hatorii* Yamada, and *Aedes* (*Aedimorphus*) *vexans* Meigen, with the latter three species as outgroups (Fig. 2; Table 1). Specimens were collected, identified based on morphology, and stored either dry or in ethanol, before DNA extraction. Total

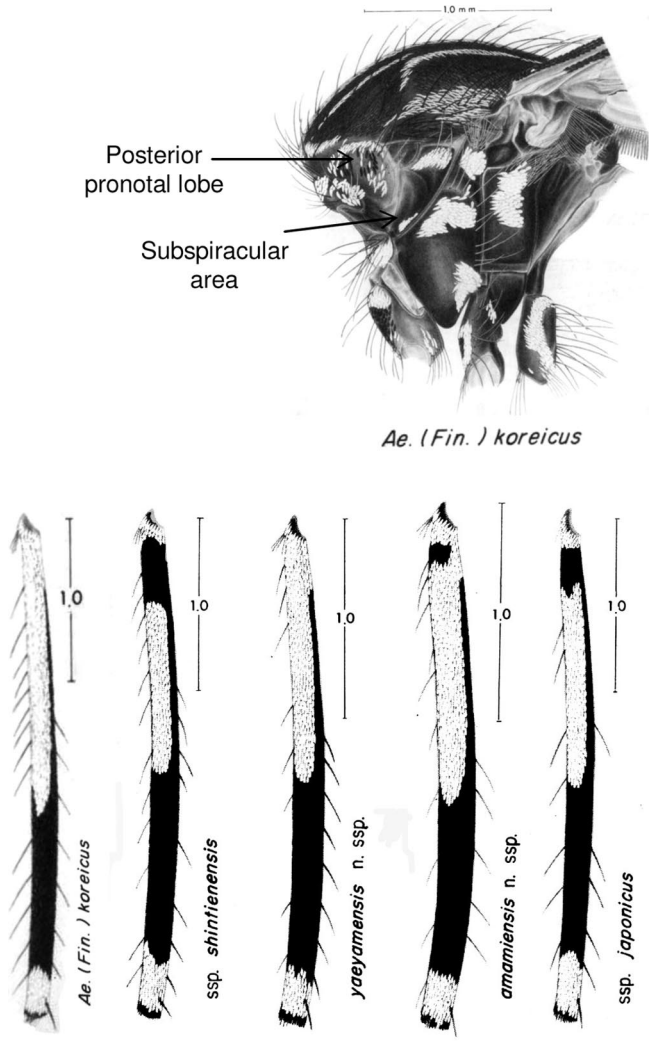

Fig. 2. Comparison of the thorax and hind femora of *Ae. koreicus* and *Ae. japonicus* subspecies. In *Ae. koreicus*, the posterior pronotal lobe has dark scales in 89.2% of specimens, whereas in *Ae. j japonicus* 7.3% have them. The subspiracular area has a distinct patch of pale scales in 87.8% of *Ae. koreicus*, whereas 93% of *Ae. j japonicus* lack any scales in this area (Tanaka et al. 1979). In addition, larvae of *Ae. koreicus* always lack detached simple pectin teeth, a character that is very rare in *Ae. japonicus* s.l. Pictures modified from Tanaka et al. (1979) (drawings by S. Shibata).

genomic DNA was extracted from individual whole mosquitoes by using a phenol chloroform extraction method as described in Fonseca et al. (2000).

DNA was amplified from each specimen at two mitochondrial loci, nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) and cytochrome oxidase II (COII), and one nuclear locus, 28S ribosomal subunit spacer 2 (D2). For ND4, we used the primers N4J-8502D (5′-CGTAGGAGGAGCAGCTATATT-3′) and N4N-8944D (5′-AAGGCTCATGTTGAAGCTCC-3′) as described in Fonseca et al. (2001). Partial COII sequences were obtained using the primers Pierre (5′-AGTTCATCTCCTTTAATAGAACA-3′) and Barbara (5′-TGGTCAATGTTCAGAAATTTGTGG-3′) modified from Simon et al. (1994). The D2 variable expansion region of 28S rRNA was amplified using primers D2 F (5′-AGTCGTGTTGCTTGATAGTG-3′) and D2R (5′-CTTGGTCCGTGTTTCAAGAG-3′) from Sallum et al. (2002). Each reaction was carried out in a 50-μl volume, with final concentrations of 1× polymerase chain reaction (PCR) buffer, 300 nM of each primer, 250 mM of each dNTP, 2 mM $MgCl_2$ (2.5 mM for COII), 1 mg/ml BSA (0.4 mg/ml for D2), 0.05 U of *Taq* Gold polymerase (Applied Biosystems, Foster City, CA) and 5 ng of DNA. The PCR amplification consisted of a 10-min denaturation at 96°C, 40 cycles of 40 s at 94°C, 40 s at 55°C (50°C for COII and 48°C for D2), and 1 min at 72°C ending with a final extension step of 10 min at 72°C.

Cycle sequencing was performed using Big Dye 3.1 chemistry (Applied Biosystems) after cleaning the

Table 1. Sample ID, collection location and source of specimens used in the phylogenetic analyses (all specimens were collected between 1997 and 2000)

	ID	Species	Country	Region	Received from
1[a]	Aej 3	*Ae. j. japonicus*	USA	Long Island, NY	S. Campbell (Suffolk Vector Control, Yaphank, NY)
2	Aej 67	*Ae. j. japonicus*	USA	Franklin Co, PA	B. Pagac (Army Center for Health Promotion and Prevention Medicine-North, Fort Meade, MD)
3	Aej 33	*Ae. j. japonicus*	Japan	Sapporo	I. Takashima (Hokkaido University, Sapporo, Japan)
4	Aej 154	*Ae. j. japonicus*	Korea	Gyeonggi-Forest Station	M. Mogi (Saga Medical School, Saga, Japan)
5	Aej 193	*Ae. j. japonicus*	Korea	Gangwon Do	H.-C. Kim (U.S. Army, Republic of Korea)
6	Aejya 79	*Ae. j. yayaemensis*	Japan	Iriomotejima, Yaeyama Islands	I. Miyagi (University of the Ryukyus, Okinawa, Japan)
7	Aejya 80	*Ae. j. yayaemensis*	Japan	Iriomotejima, Yaeyama Islands	I. Miyagi
8	Aejya 81	*Ae. j. yayaemensis*	Japan	Iriomotejima, Yaeyama Islands	I. Miyagi
9	Aejsh 138	*Ae. j. shintienensis*	Taiwan	Tsouko, Sanhain twp, Taipe County	J. C. Lien (National Taiwan University, Taipei, Taiwan)
10	Aejsh 139	*Ae. j. shintienensis*	Taiwan	Tsouko, Sanhain twp, Taipe County	J. C. Lien
11	Aejsh 140	*Ae. j. shintienensis*	Taiwan	Tsouko, Sanhain twp, Taipe County	J. C. Lien
12	Aejam 386	*Ae. j. amamiensis*	Japan	Amami & Omacr;shima, Ryukyu Islands	M. Mogi
13	Aek 3	*Ae. koreicus*	Korea	CampCasey, Gyeonggi Do	D. Claborn (Uniformed Services University of Health Sciences, Bethesda, MD)
14	Aek 4	*Ae. koreicus*	Korea	CampCasey, Gyeonggi Do	D. Claborn
15	Aek 5	*Ae. koreicus*	Korea	CampCasey, Gyeonggi Do	D. Claborn
16	Aet 1	*Ae. togoi*	Japan	Okinawa	I. Miyagi
17	Aet 2	*Ae. togoi*	Japan	Okinawa	I. Miyagi
18	Aet 5	*Ae. togoi*	Japan	Okinawa	I. Miyagi
19	Aeho 1	*Ae. hatorii*	Korea	Gangwon Do	H.-C. Kim
20	Aeho 2	*Ae. hatorii*	Korea	Gangwon Do	H.-C. Kim
21	Aev 1	*Ae. vexans*	Korea	Pochon, Gyeonggi Do	H.-C. Kim
22	Aev 2	*Ae. vexans*	Korea	Daeseong-dong, Gyeonggi Do	H.-C. Kim

[a] Numbers correspond to sample numbers indicated on the map in Fig. 2.

PCR products using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Cycle sequencing products were cleaned with Sephadex columns (Princeton Separations, Adelphia, NJ) before being run on an ABI3700.

Sequences were edited using Sequencher 4.2.2 (GeneCodes, Ann Arbor, MI) and aligned using CLUSTALW (Thompson et al. 1994) with manual adjustments made in BioEdit 7.0.9 (Hall 1999). For each locus, the model of nucleotide evolution that best fit the data were determined in MrModeltest as implemented in MrMTgui 1.0 (Posada and Crandall 1998, Nylander 2004, Nuin 2006), using the Akaike Information Criterion (AIC) and supported by the hierarchical likelihood ratio test. The model selected for the ND4 data set was a HKY+G model, for COII it was a GTR+I+G model, and for D2 a GTR+I model. Bayesian phylogenetic analysis was conducted using these models in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) for each locus separately and for a combined partitioned data set. One cold and three heated chains (temperature = 0.075) were run for 5 million generations in two independent MCMC searches. Trees were sampled every 1,000 generations with the first 500 sampled trees discarded as burn-in. Posterior probabilities for each node were obtained with 50% majority consensus. For comparison, maximum parsimony (MP) trees were also constructed for each locus and the combined data set. MP analysis was performed in PAUP* 4.0b10 (Swofford 2000) by using a heuristic search with tree bisection reconnection (TBR) branch swapping. Branch support was estimated using 10,000 replicate bootstrap resamplings. For D2 there were very small differences between some taxa, thus an alternative method to search the treespace was used as described by Edgecombe et al. (2000). This method performed 1,000 random addition sequence replicates sampling three trees per iteration. Bootstrapping was performed in a similar manner. Shortest trees and 50% majority consensus trees were observed in FigTree 1.2.2 (Rambaut 2009). To examine the robustness of the nodes of the combined parsimony tree, we calculated the Bremer support in TREEROT version 3 (Sorenson and Franzosa 2007). The percentage difference within and between species was calculated by counting the number of nucleotide differences, in BioEdit 7.0.9.0 (Hall 1999), over the total number of bases sequenced.

Results

Sequences were obtained for all 22 specimens at three loci: ND4 (348 bp; GenBank accessions GU229919–GU229934), COII (509 bp; GU229893–GU229908), and D2 (480 bp; GU229909–GU229918). Under the MP analysis, the combined tree had 243 parsimony-informative characters (ND4, 73; COII, 118; and D2, 50), resulting in two trees (14,4,NA due to method used) of length 444 (146, 226, and 68). Following Bayesian analysis the standard deviation of

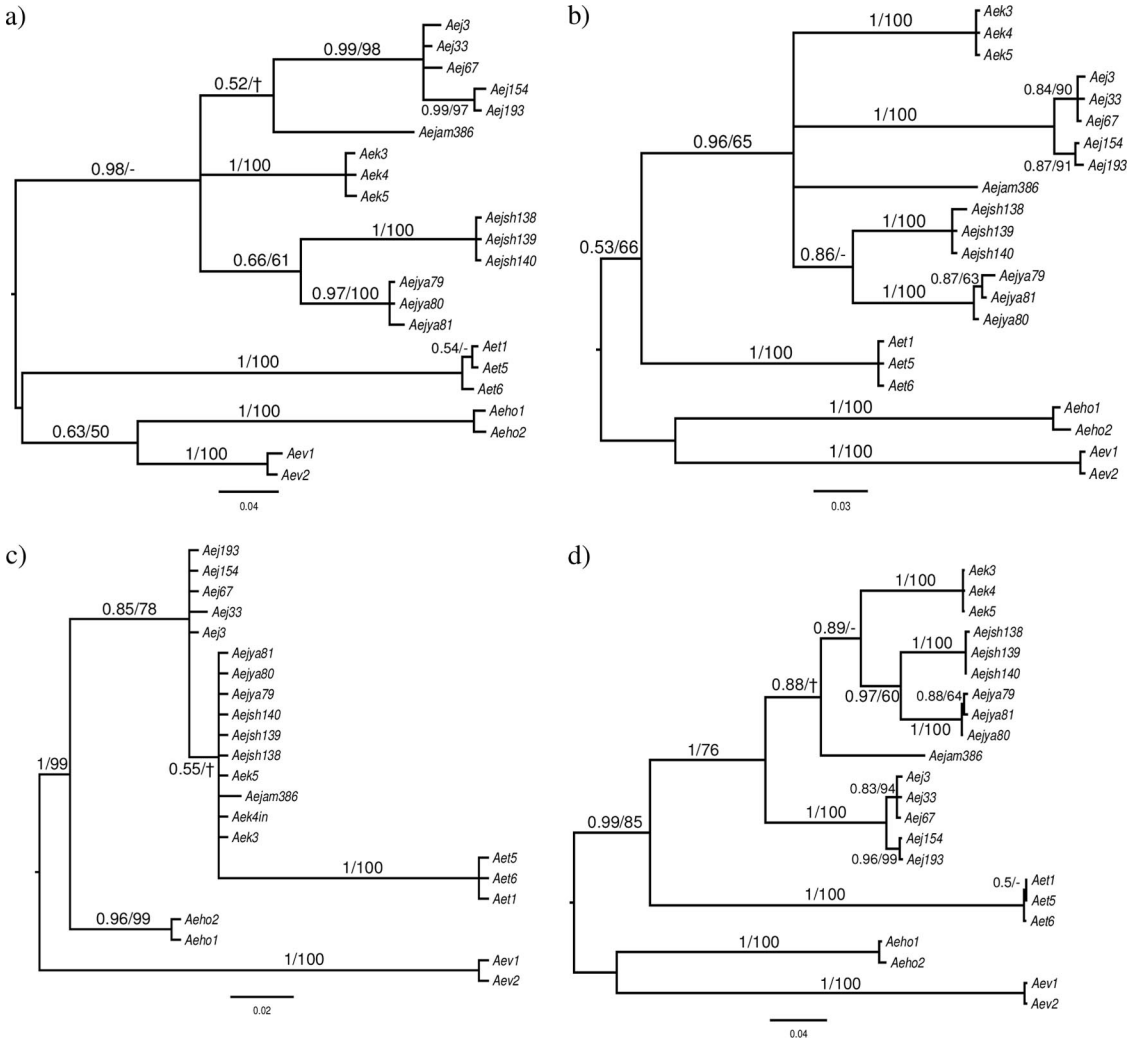

Fig. 3. Bayesian 50% majority rule consensus trees for ND4 (a), COII (b), D2 sequence data (c), and the combined data set (d). For each node, the Bayesian posterior probability and parsimony bootstrap support are shown (- indicates <50% support; † indicates the node was not recovered by that method).

the split frequencies was <0.005 and the average value for the assessed potential scale reduction factors was 1.00 for all loci, indicating that convergence was met in all cases.

In the combined tree *Ae. japonicus* s.l. and *Ae. koreicus* form a monophyletic group with strong posterior probability and moderate bootstrap support (Fig. 3d). The Bayesian analysis fully resolved the relationships between the taxa, grouping the two southern subspecies, *Ae. j. shintienensis* and *Ae. j. yaeyamensis*, and joining them with A*e. koreicus* and *Ae. j. amamiensis* as a sister taxa to *Ae. j. japonicus.* The MP analysis, however, did not resolve the relationships between the subspecies. This was further highlighted by low and negative Bremer support values within this clade indicating conflicting signals from the three genes. Overall the Bremer support values were higher for the two mitochondrial genes (ND4, 69.6; COII, 91.5; and D2, 44.9).

Individually the gene trees do not show such a clear pattern. For the mitochondrial markers, ND4 and COII, Bayesian and MP analyses gave very similar results (Fig. 3a and b), with a few nodes not recovered using MP. A monophyletic grouping of *Ae. japonicus* s.l. and *Ae. koreicus* was recovered, but within this grouping the relationships between the subspecies are unresolved with the exception of *Ae. j. shintienensis* and *Ae. j. yaeyamensis*, which cluster together. For ND4, the MP tree has low bootstrap support for placing *Ae. togoi* outside the *japonicus-koreicus* clade, thus leaving the relationships between all the taxa unclear. In all cases, multiple individuals from within a taxa grouped together. In contrast, the D2 phylogeny failed to recover the subspecies (Fig. 3c) due to very small differences between these taxa (Table 2). The Bayesian and MP trees disagreed in the placement of *Ae. togoi*, which under a Bayesian framework, was placed in the *japonicus-koreicus* clade, although at a large

Table 2. Percentage nucleotide difference between the taxa based on the number of nucleotide differences over the total length of the sequences averaged over specimens[a]

	Aev	Aeho	Aet	Aek	Aej	Aejam	Aejsh	Aejya
Aev	**0.1**	7.9	10.0	7.9	7.9	8.1	8.1	8.1
Aeho	9.7	**0.4**	8.8	4.4	4.4	4.6	4.6	4.6
Aet	9.7	10.3	**0.0**	6.5	6.7	6.9	5.3	5.3
Aek	10.8	11.2	10.3	**0.2**	1.2	0.8	0.5	0.5
Aej	10.7	11.4	9.5	9.2	**0.9**	1.1	0.7	0.7
Aejam	9.6	10.6	9.0	8.3	8.6	—	0.8	0.8
Aejsh	9.8	10.7	10.3	8.0	8.9	7.9	**0.1**	0.0
Aejya	9.6	11.4	10.5	8.7	8.5	7.7	6.3	**0.3**

[a] The mitochondrial genes are combined in the bottom triangle, and the nuclear D2 marker is in the top triangle. Within-species variation is in bold on the diagonal calculated over all three markers. The species abbreviations are the same as in Table 1.

distance from these species. The MP analysis placed *Ae. togoi* outside the *japonicus-koreicus* clade with bootstrap support for the clade of 68%. In addition, MP recovered an *Ae. j. japonicus* clade with bootstrap support of 73%, leaving the remaining subspecies and *Ae. korecicus* unresolved.

The minimum percent difference among subspecies in the *Ae. japonicus* complex at the mitochondrial loci was 6.3%, comparable to that between any of the subspecies and *Ae. koreicus* (Table 2). Indeed, even comparisons with the three outgroup species did not yield considerably higher percent differences. For the D2 locus, comparisons between members of the *Ae. japonicus* complex and the outgroup species, *Ae. togoi*, *Ae. hatorii*, and *Ae. vexans*, produced high percent differences; but again, *Ae. koreicus* yielded similar values to those obtained in comparisons among subspecies.

***Ae. koreicus* Assay.** To aid in the identification of *Ae. koreicus*, we designed a molecular assay based on the ND4 sequences obtained in this study. At site 151 of our ND4 sequence alignment (corresponding to site 181 in GenBank sequences DQ470164–470154), the three *Ae. koreicus* have a T, whereas the five *Ae. j. japonicus* have a G and the other species have an A; thus, this site was chosen to design a primer unique to *Ae. koreicus* (ND4korF 5′-CCCCATTTAACCC-CCAATAT-3′). The identification assay can be performed as a multiplex PCR with the primers N4J-8502D(F) and N4N-8944D(R), ND4korF, with the conditions used to amplify ND4 (described above) adjusting the final concentration of the two forward primers to 0.2 μM. The assay gives a single band of 465 bp in *Ae. j. japonicus* (13 samples tested), *Ae. j. shintienensis* (3), *Ae. j. yaeyamensis* (3), *Ae. j. amamiensis* (1), *Ae. togoi* (2), *Ae. hatorii* (2), and *Ae. vexans* (1) as well as *Ae. atropalpus* (1) and *Ae. triseriatus* (3). We tested 26 specimens of *Ae. koreicus*, both field collected and museum specimens (Smithsonian Institution, Washington, DC) from the southern Korea Peninsula. All displayed a band of 283 bp as well as a the expected 465-bp band. We also compared the ND4 haplotypes found in *Ae. koreicus* with all the haplotypes recovered from *Ae. j. japonicus* across the World (26 haplotypes in >300 specimens, Fonseca et al. 2001, Fonseca et al. 2010) and found that this SNP was consistent. Thus, this assay should provide a useful tool for the early identification of *Ae. koreicus*.

Discussion

The primary conclusion of this study is that the four subspecies in the *Ae. japonicus* complex are genetically quite distinct, averaging ≈8% nucleotide differences at the two mitochondrial loci. Furthermore, they seem to form a monophyletic group that surprisingly also includes *Ae. koreicus*.

We chose the genetic regions for this analysis based on previous knowledge of their polymorphism (Simon et al. 1994, Fonseca et al. 2001). ND4 was used successfully in population level analysis of *Ae. japonicus* both from their natural range in Japan and introduced populations in the United States (Fonseca et al. 2001, 2010). Among the 29 Japanese specimens of *Ae. j. japonicus* collected from Nagasaki and Saga (Kyushu), Hiroshima and Tokyo (Honshu), and Sapporo and Chitose (Hokkaido). Fonseca et al. (2001) identified only 11 polymorphisms at the ND4 locus. An analysis that includes all these specimens results in an ND4-tree with the same topology as the tree presented here (data not shown). Preliminary analysis of the mitochondrial locus COII showed a similarly high reported interspecific variation and low intraspecific variation as expected (Cook et al. 2005). Finally, the 28S ribosomal D2 spacer is a known slow mutating nuclear locus, suited for deeper phylogenetic information (Sallum et al. 2002). We choose three species to use as outgroups: *Ae. togoi* and *Ae. hatorii*, which belong to the same subgenus as *Ae. japonicus*, and *Ae. vexans*, which is more distantly related. All three species are found in the Palearctic and Oriental regions.

Although the combined Bayesian tree is completely resolved into dichotomies, the analysis of parsimony and all individual gene trees return polytomies involving the members of the *Ae. japonicus* complex and *Ae. koreicus*. This result likely stems from differences in the way Bayesian and MP approaches manage polytomies (Lewis et al. 2005). Although it is possible that further loci might reduce the level of polytomy, it is equally plausible that the trees reflect a quasi-simultaneous split of the original population into isolated units possibly related to the complex geology and tectonics of Japan and surrounding regions (Taira 2001). A split into northern and southern lines, that independently colonized the Ryukyu archipelago, would explain why the two southern subspecies are more closely related to each other than to any of the other species as well as the seemingly disjunct distribution across the archipelago.

The consistent relationship observed between *Ae. koreicus* and the subspecies of *Ae. japonicus* in all molecular markers used, suggests that a new taxonomic construct for the *Ae. japonicus* complex should be considered, in which the subspecies are raised to species or *Ae. koreicus* is reclassified as a subspecies. Although all the morphologically differentiating characteristics found in adults and larvae of *Ae. koreicus*

and *Ae. japonicus* s.l. overlap (Tanaka et al. 1979), mating incompatibilities have been demonstrated between *Ae. koreicus* and *Ae. j. japonicus* (Miyagi and Lee 1975). *Ae. j. japonicus* and *Ae. koreicus* also display important behavioral differences where they are sympatric: in southern Korea Peninsula, *Ae. j. japonicus* is primarily a forest and rural dwelling mosquito, whereas *Ae. koreicus* is better adapted to urban environments (Tanaka et al. 1979). Our sampling did not include specimens from mainland China, where other sibling species, subspecies, or even intermediate forms may exist. However, the current evidence of strong genetic isolation among the subspecies of *Ae. japonicus* and *Ae. koreicus,* as well as evidence of ecological isolation in sympatry, does fulfill many of the required criteria for species level recognition (i.e., biological, ecological, and evolutionary). Indeed, as summarized in DeQueiroz (2007), "... any evidence of lineage separation is sufficient to infer the existence of separate species."

It is perhaps surprising, or just a matter of chance, that the most recent introduction to the United States was *Ae. j. japonicus* and not *Ae. koreicus.* There is clear evidence that *Ae. japonicus* has been introduced multiple times both into the United States (Fonseca et al. 2010) and across the world (Laird et al. 1994, Schaffner et al. 2009); therefore, there is the distinct possibility that *Ae. koreicus* will also begin spreading across the world. In anticipation of such an event, we developed a DNA-based rapid assay to differentiate *Ae. koreicus* from some of the species with which it may be confused. Besides *Ae. japonicus,* in the United States these include *Ae. atropalpus* and *Ae. triseriatus* two endemic mosquito species that colonize the same habitats. The assay requires minimal laboratory supplies because the unique extra band in *Ae. koreicus* specimens can be easily visualized on an agarose gel. The phylogenetic analysis of the *Ae. japonicus* complex led us to the reexamination of their taxonomic status, as well as the recognition of another potentially invasive species, *Ae. koreicus.*

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