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. 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. 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 2004, 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* 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, Andreasis et al. 2001). Although it is unclear when it was first introduced to the United States, the fact that extensive collections aimed at...
detecting the presence of *Ae. albopictus* (Moore et al. 1990) in 1992 failed to uncover *Ae. 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, 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).

*Aedes (Fin.) japonicus* is currently composed of four geographically distinct subspecies: *Ae. japonicus*, *Ae. japonicus*, *Ae. japonicus*, *Ae. japonicus*. These specimens were morphologically distinct 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. 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.

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

**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 *Aedes 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
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’-CGTAGGAGGAGCAGTTATATT-3’) and N4N-8944D (5’-AAGGCTCATGTGTAAGCTCC-3’) as described in Fonseca et al. (2001). Partial COII sequences were obtained using the primers Pierre (5’-AGTTCATCTCCTTTAATAAGACA-3’) and Barbara (5’-TGGTCAATGTTCAAAATTITGGG-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’-CTTGTCCTGTTTCAAGAG-3’) from Sallum et al. (2002). Each reaction was carried out in a 50-μl volume, with final concentrations of 1X polymerase chain reaction (PCR) buffer, 300 nM of each primer, 250 mM of each dNTP, 2 mM MgCl₂ (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

![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).](https://academic.oup.com/jme/article-abstract/47/4/527/992149)
Following Bayesian analysis the standard deviation of
the standard deviation of posterior probabilities
was 0.075)

Under the MP analysis, the combined tree had 243
shortest trees and 50% majority consensus
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 Bre-
mer 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 se-
quenced.

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*   | Aej 3            | Ae. j. japonicus | USA                     | S. Campbell (Suffolk Vector Control,YPAPH, NY) |
| 2    | Aej 67           | Ae. j. japonicus | USA                     | B. Pagac (Army Center for HealthPromotion and Prevention Medicine-North, Fort Meade, MD) |
| 3    | Aej 33           | Ae. j. japonicus | Japan                   | I. Takashima (Hokkaido University,Sapporo, Japan) |
| 4    | Ae 154           | Ae. j. japonicus | Korea                   | M. Mogi (Saga Medical School, Saga, Japan) |
| 5    | Ae 193           | Ae. j. japonicus | Korea                   | H.-C. Kim (U.S. Army, Republic of Korea) |
| 6    | Aejya 79         | Ae. j. yapaemensis| Japan                   | I. Miyagi (University of the Ryukyus, Okinawa, Japan) |
| 7    | Ae 50            | Ae. j. yapaemensis| Japan                   | I. Miyagi |
| 8    | Aejya 51         | Ae. j. yapaemensis| Japan                   | I. Miyagi |
| 9    | Aejsh 138        | Ae. j. shintienensis| Taiwan                   | J. C. Lien (National Taiwan University, Taipei, Taiwan) |
| 10   | Aejsh 139        | Ae. j. shintienensis| Taiwan                   | J. C. Lien |
| 11   | Aejsh 140        | Ae. j. shintienensis| Taiwan                   | J. C. Lien |
| 12   | Aejam 386        | Ae. j. amamiensis| Japan                   | M. Mogi |
| 13   | Aek 3            | Ae. koreicus     | Korea                   | D. Claborn (Uniformed Services University of Health Sciences, Bethesda, MD) |
| 14   | Aek 4            | Ae. koreicus     | Korea                   | D. Claborn |
| 15   | Aek 5            | Ae. koreicus     | Korea                   | D. Claborn |
| 16   | Aet 1            | Ae. togoi        | Japan                   | I. Miyagi |
| 17   | Aet 2            | Ae. togoi        | Japan                   | I. Miyagi |
| 18   | Aet 5            | Ae. togoi        | Japan                   | I. Miyagi |
| 19   | Aeho 1           | Ae. hatorii      | Korea                   | H.-C. Kim |
| 20   | Aeho 2           | Ae. hatorii      | Korea                   | H.-C. Kim |
| 21   | Ae 1             | Ae. vexans       | Korea                   | H.-C. Kim |
| 22   | Ae 2             | Ae. vexans       | Korea                   | H.-C. Kim |

* Numbers correspond to sample numbers indicated on the map in Fig. 2.

Results

Sequences were obtained for all 22 specimens
at three loci: ND4 (348 bp; GenBank accessions
GU229919–GU229934), COII (509 bp; GU229993–
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

PCR products using the QIAquick PCR purification kit
(QIAGEN, Valencia, CA). Cycle sequencing products
were cleaned with Sephadex columns (Princeton Separ-
arations, 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 7.0.9.0 (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
Bayesian phylogenetic analysis was conducted using these models in MrBayes 3.1.2 (Ron-
quist and Huelsenbeck 2003) for each locus sepa-
rately and for a combined partitioned data set. One
cold and three heated chains (temperature
0.075) were run for 5 million generations in two inde-
pendent 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 con-
sensus. 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 tree-
space was used as described by Edgecombe et al.
(2000). This method performed 1,000 random addi-
tion 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 Bre-
mer 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
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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
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 *Ae. 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
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. koreicus 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 C and the other species have an A;
thus, this site was chosen to design a primer unique
to Ae. koreicus (ND4korF 5'-CCCATTTAACC
C-3'). The identification assay can be performed as
a multiplex PCR with the primers N4-5502D(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.
shihueiensis (3), Ae. j. yaejamaensis (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 Insti-
tution, 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 haplo-
types recovered from Ae. j. japonicus across the World
(26 haplotypes in >300 specimens, Fonseca et al. 2001,
and all individual gene trees return polymorphisms 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 poly-
tomies (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-simul-
taneous 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 distri-
bution 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 taxo-
nomic 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 char-
acteristics found in adults and larvae of Ae. koreicus

### 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

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

* 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.

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 suc-
cessfully 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 mito-
chondrial locus COII showed a similarly high reported
interspecific variation and low intraspecific variation
as expected (Cook et al. 2005). Finally, the 28S ribo-
somal 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 involv-
ing the members of the Ae. japonicus complex and Ae.
koreicus. This result likely stems from differences in
the way Bayesian and MP approaches manage poly-
tomies (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-simul-
taneous 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 distri-
bution across the archipelago.
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 sympathy, 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, 2001; 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 sympathy, 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.”

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