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Confirmation of *Aedes koreicus* (Diptera: Culicidae) in Belgium and description of morphological differences between Korean and Belgian specimens validated by molecular identification

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Abstract

In 2008, specimens resembling *Aedes* (*Finlaya*) *koreicus* (Edwards) (also *Ochlerotatus koreicus* or *Hulecoeteomyia koreica*) were found in Belgium during a national mosquito survey (MODIRISK). Small but consistent differences were, however, observed between the specimens described from Peninsula Korea and those found in Belgian. To achieve the correct identification a detailed morphological comparison was made between the Belgian specimens and reference material from Korean mainland and island populations housed at the Smithsonian Institution (Walter Reed Biosystematics Unit (WRBU), Washington, USA). The identification was furthermore supported by molecular evidence based on the ND4 region (mtDNA) of available Korean and Belgian mosquito specimens. Morphological and molecular comparison confirmed the initial identification of *Aedes koreicus*. Based on morphological characteristics, the species collected in Belgium most likely originated from Jeju-do, an island south of the Korean Peninsula. The observed dissimilarities between Korean and Belgian specimens resembled a number of morphological differences mentioned previously between female adults collected on the Korean Peninsula and Jeju-do. This is the first report of *Aedes koreicus* outside its natural distribution range. A correct and rapid identification of new invading and spreading vector species is crucial for the implementation of effective control measurements. Hence a correct and easy accessible description of all possible variations of species arriving in new areas is highly recommended. Therefore, a comparative morphological study on the Smithsonian material of the species from Korean mainland, island population and from Belgium is given, pictures of the main aberrant characteristics and scanning electron microscope images of all stages of the species are included and molecular confirmation of the identification based on the mtDNA ND4 region is provided.

Key words: *Aedes koreicus*, morphological comparison, egg structure, molecular identification, ND4, Republic of Korea, Belgium

Introduction

Species of the genus *Aedes* Meigen in general are known for their invasive potential since the eggs of this genus can tolerate long desiccation periods therefore surviving transport across international borders. This trait has not only an impact on native biodiversity but also on human health as numerous members of this genus are potent vectors for different mosquito-borne diseases (Cook *et al.* 2005; Kearney *et al.* 2009). In the last decade, several exotic *Aedes* species were reported from central and northern Europe; including *Aedes* (*Stegomyia*) *albopictus* (Skuse) (or *Stegomyia albopicta*), *Aedes* (*Ochlerotatus*) *atropalpus* (Coquillett) (or *Ochlerotatus atropalpus*) (Scholte *et al.* 2009, 2010), *Aedes* (*Finlaya*) *j. japonicus* (Theobald) (also *Ochlerotatus j. japonicus* or *Hulecoeteomyia j. japoni-*

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cus) (Schaffner *et al*. 2004; Schaffner *et al*. 2009; Versteirt *et al*. 2009) and *Aedes (Stegomyia*) *aegypti* (also *Stegomyia aegypti*) (Scholte *et al.* 2010). Considering the increased risk on introduction of exotic mosquito species due to the intensification of global traffic, a rapid and correct identification is critical. Hence, good descriptions of possible invading species are extremely significant, even more so when taking into account that diagnostic characteristics of species can differ between populations. Although recent publications (Reinert 2000; Reinert *et al.* 2004, 2008, 2009; Shepard *et al*. 2006) indicate justified reasons for the reclassification of the genus *Aedes* (especially elevating *Ochlerotatus* to generic rank), the traditional classification has been used in present paper with the newer alternatives in parentheses. In 2008, an exotic species resembling *Aedes* (*Finlaya*) *koreicus* (Edwards) (also *Ochlerotatus koreicus* or *Hulecoeteomyia koreica*) was found in Belgium during a national survey. Specimens of this species found in Belgium displayed small but consistent differences between *Ae. koreicus* specimens from Korean Peninsula described by Tanaka *et al*. (1979). Complicating morphological identification even more is the possible presence of sibling species and species groups as observed frequently in *Anopheles* species (Rueda *et al*. 2009). Previous morphological and molecular studies indicate the close relationship between *Ae. koreicus* and members of the *Ae. japonicus* complex (Tanaka *et al.* 1979; Widdel *et al.* 2005; Cameron *et al.* 2010), even contesting the current commonly accepted construct for this complex. Tanaka *et al.* (1979) described overlapping ranges of all differentiating morphological characteristics found in adults of the two species. Recent molecular work with microsatellites as indicators of evolutionary distance between species, confirmed this strong relationship between the species as already seen using only sequence data (Widdel *et al*. 2005; Cameron *et al.* 2010).

In this contribution, a morphological and molecular comparative study is made to obtain a correct identification of the species and to provide a detailed morphological description of it by comparing it with reference material from the Korean Peninsula, Korean islands (Republic of Korea, ROK) and Belgium.

Material and methods

Specimen collection and morphological identification. Adults and immature stages were collected in two locations in Maasmechelen (Belgium, 50.9941°N, 5.6182°E) near the National Park "Hoge Kempen" and the industrial area of Maasmechelen (50.9959°N, 5.6208°E) during 2008 and 2009. Adults were trapped at both sites using two Mosquito Magnet Liberty Plus traps (MMLP, Woodstream Corporation, Lititz, PA, USA) and two CDC Gravid traps (Frommer Updraft, J.W. Hocke company, Gainesville, FL, USA). All traps were placed in such a way that they had minimal influence on each other (Latin square principle) and operated for 48 h at two-week intervals from 20 April to 19 October 2009. Larvae were collected on different occasions between June and November 2008 and 2009 using 500 ml dippers, small sieves and a pipette. They were transported alive to the laboratory in vials labeled with site-specific identification details. Larvae were killed by a thermal shock with hot water (60ºC) and stored in 80% ethanol. Different artificial habitats (tyres, plastic cups, rusted pots, etc.) as well as natural sites (puddles, road tracks, etc.) were searched in an area of ca. 1.5 km² around the adult collection sites. Eggs were sampled by use of artificial oviposition sites (black 1 liter flower pots) filled with an infusion-baited mixture (described by Scott *et al.* 2001), and with a polystyrene float (5x5 cm) as oviposition support (Scott & Crans 2003).

Morphological identification of larvae and adults was done using a stereoscopic microscope and the identification keys of Tanaka *et al*. (1979), Schaffner *et al*. (2001) and Becker *et al*. (2003). A comparison with international reference material housed at the Smithsonian Institution (WRBU), Washington, DC, USA, was made.

Molecular identification. Eggs, larvae and both reared- and wild-collected adult specimens were used for the molecular identification to verify and confirm the morphological identification. DNA extraction of individual mosquitoes was performed on one to four legs whereas whole immature stages were used, utilizing the protocol described by Collins *et al*. (1987). A fragment of the mitochondrial COI (Cytochrome Oxidase I) gene, the mitochondrial ND4 (Nicotinamide adenine dinucleotide dehydrogenase subunit 4) gene, and the entire ribosomal ITS2 (Internal transcribed spacer 2) region was amplified for 8 individuals and positive amplifications were subsequently sequenced. The COI region was amplified using the barcode primers of Folmer *et al*. (1994), the ND4 region by primers designed by Fonseca *et al*. (2001) and the ITS2 region was amplified using primers described by Proft *et al*. (1999). Amplification of the COI region was performed in a 20-µl reaction mixture containing 10 µl 2x Phire® Animal Tissue PCR Buffer, 0.5 µM of each primer, 0.4 µl of Phire® Hot Start II DNA Polymerase and 1 µl of template. Cycling conditions were as follows: initial denaturation at 98°C for 5 min, 40 cycles of denaturation at

98°C for 5 sec, primer annealing at 50°C for 5 sec, and primer extension at 72°C for 20 sec. Final extension was performed at 72°C for 1 min and cooling down at 4°C. The protocol of Fonseca *et al*. (2001) and Proft *et al*. (1999) was followed for the amplification of, respectively, the ND4 and ITS2 region. All amplification products were checked on a 2% agarose gel and visualized after ethidium bromide staining on an Image master VDS (Amersham Pharmacia, Uppsala, Sweden). Positive PCR products were sequenced (Genoscreen, Lille, France) and sequence data were edited and aligned with BioEdit and were compared with data available in CBOL (www.barcodinglife.org) and GenBank. Pairwise and overall distance between the obtained sequences and similar ones in CBOL or/and GenBank was calculated using Mega 5.05 (Tamura *et al*. 2011). The sequences were deposited in GenBank with accession numbers: JF430391 for the ITS2, JF430392 for the ND4 and JF430393 for the COI region of *Ae. koreicus*.

In addition, a recently developed rapid assay based on the ND4 region to discriminate *Ae. koreicus* from other known invasive *Aedes* species (Cameron *et al*. 2010) was applied using the same specimens. The assay combines universal primers for the group of known invasive *Aedes* species, as well as one unique primer for *Ae. koreicus* and generates a single DNA band of 465 bp in *Ae. aegypti* L., *Ae. albopictus* and *Ae. j. japonicus* whereas individuals of *Ae. koreicus* display a band at 283 bp and a band at 465 bp. In this assay, we included *Ae. aegypti* and *Ae. albopictus* from Vietnam and Cambodia next to *Ae. j. japonicus* and *Ae. koreicus* from Belgium.

Scanning electron microscopy. Adults, larvae and eggs were examined and photographed with an Environmental Scanning Electron Microscope (ESEM) FEI Quanta-200. Eggs collected from the polystyrene oviposition supports in the field, and adults were kept dry (silica gel) in the lab until critical point drying. Larval material was kept in 80% ethanol (EtOH) and was dehydrated in different concentrations of EtOH and eventually fixated in formaldehyde before critical point drying. Afterwards, all specimens were mounted on stubs with sticky tape and coated with gold under high vacuum evaporation.

Results

Morphological comparison

A detailed comparative morphological study of *Aedes* specimens from Maasmechelen (Belgium) and reference material from the Smithsonian Institute (available at the Walter Reed Biosystematics Unit—WRBU, Washington, USA) aided to correctly identify the species as *Ae. koreicus*. All Belgian individuals showed however consistent morphological differences compared with specimens from the Korean Peninsula but resembled to specimens from Jeju-do, which is an island situated south of the Peninsula (Tanaka *et al*. 1979). The main aberrant characteristic for adults was the pattern on hindtarsomere 5 (Table 1). This feature was also present in several individuals from Jeju-do at the Smithsonian collection. Because differences between Korean and Belgian specimens occurred especially in the coloured scales on the pedicel, the overall colour of the scales on the thorax, the scale pattern on the abdominal terga and in the presence of a pale band on hindtarsomeres 4-5, these characteristics are described more in detail below. An overview of these morphological differences is given in Table 1. The description of the Belgian species is based on adult females as males are similar. Specimens were deposited at the Royal Belgian Institute of Natural Sciences (Brussels, Belgium).

Examined species: Adults, Belgium: 5 females and 10 males; adults Jeju-do (WRBU field material): KS78-104, KS78-107, KS87-3, KS87-5, KS87-100, KS87-101, KS87-102, KS87-103, KS87-105 and KS87-117.

Adult (Figs 1–4). Pedicel: According to Edwards (1917), the first antennal joint has “small flat pale scales” but Tanaka *et al*. (1979) described the pedicel as “brown; mesal side dark, covered with small broad scales, most often (14/23) with more pale scales than dark ones, sometimes (8/23) all scales pale, rarely (1/23) dark scales more abundant than pale ones”. In all Belgian specimens, the pedicel was dark, with dorsal and lateral spots of pale scales. The pedicel of specimens examined from Jeju-do show the same pattern as the Belgian individuals.

TABLE 1. Main characters that distinguish adult females of Belgian and Korean *Aedes koreicus* and *Aedes j.japonicus* (from Tanaka *et al*. 1979) (indicated with *) and/or field specimens studied by the first author (▪).

Characteristics	*Aedes koreicus*			*Aedes j. japonicus* * ▪
	Belgium ▪	Korean Peninsula *	Jeju-do Island* ▪	
Head/vertex	With pale erected forked scales	Erect forked scales frequently entirely dark, if pale scales than between 1-4	Erect forked scales almost always pale: 1-10	Numerous erect forked scales, often entirely dark, otherwise with variable numbers of pale scales
Thorax/postpronotum	Numerous pale falcate scales	Covered with broad pale scales, occasionally falcate scales present	Numerous pale falcate scales present, scales narrower	Covered with broad pale scales, almost no dark scales present
Abdomen	Basomedian and basolateral pale areas; variation: only basomedian or only basolateral pale areas present	Very thin basomedian pale band and always basolateral pale spots	Very thin basomedian band and white basolateral spots on anterior terga	Always basomedian and basolateral pale areas
Hindtarsomere 4	Basal pale band	Basal pale band	Basal pale band	Dark, sometimes with some pale scales
Hindtarsomere 5	Basal pale band	Entirely dark; sometimes with a few pale scales	Basal pale band	Entirely dark
Postbassal pale band of hindfemur	Missing	Missing	Missing	Present and mostly complete
Subspiracular area	20-30 broad pale scales	20-30 broad pale scales	20-30 broad pale scales	No pale scales

Thorax (Fig. 1): Edwards (1917) described the mesonotum as “dark covered with blackish brown and golden-yellow scales”, the latter arranged in “three not very sharply defined lines” forming: “1) a continuous margin to the mesonotum, 2) a median longitudinal line forking just in front of the scutellum, and 3) a pair of short lines on the anterior half and a long pair on the posterior half bent outwards at the suture”. According to Tanaka *et al*. (1979), the mesonotum is covered with “narrow curved dark and yellowish brown scales”, the latter forming “a rather broad median stripe, a narrow anterior dorsocentral stripe, a posterior dorsocentral stripe and a supraalar patch”. Tanaka *et al*. (1979) describes the median stripe on the mesonotum “bifurcating along the prescutellar margin where the scales become paler”. The Belgian specimens showed a dark scutum covered with blackish brown and distinct silver-white scales. Otherwise, the scales are arranged in the same pattern as described by Tanaka *et al*. (1979). Several Jeju-do specimens that were examined from the reference collection of the Smithsonian Institution showed yellowish-brown scales arranged as described by Tanaka *et al*. (1979).

FIGURE 1. Scutum and scutellum of Belgian *Aedes koreicus.*

Abdomen (Fig. 2): After Edwards (1917), the dorsal side of the abdomen is covered with “dark scales”, the terga having ”brilliant pale basolateral spots and isolated small, dull pale median basal bands, which are joined with the basolateral spots on tergum VIII”. According to Tanaka *et al*. (1979), tergum I bears dorsally a “median patch of dark scales, occasionally pale scales intermixed”. Tanaka *et al*. (1979) describes terga II–IV as having ”dark scales with basolateral patches of pale scales, occasionally these spots develop into short basal bands but were not fused with basolateral patches”, tergum VIII as having usually “a basal pale bands but not fused with basolateral patches”, and tergum VIII as having usually “a basal pale band”.

FIGURE 2. Abdominal patterns in Belgian *Aedes koreicus.*

In the Belgian species the dorsal side of the abdomen is covered with dark scales whereas a high degree of variation was observed in the pattern of pale scales. Most frequently, two patterns were seen: (1) tergum I bears a very thin basomedian pale band, terga II–VII also have basolateral pale patches; (2) terga I–IV have only basomedian pale bands, terga V–VII have a small basomedian pale band and basolateral pale spots. On tergum VIII, the basomedian pale band and basolateral pale spots are fused. However, in some specimens only a basomedian pale band or only basolateral pale spots are present on all or a few terga.

Specimens from Jeju-do examined at the WRBU have a very thin basomedian pale band on most terga; sometimes pale basolateral spots can be seen especially on terga IV–VII; in some species the basomedian pale band and basolateral pale spots were fused on tergum VIII.

Legs (Figs 3–4): According to Edwards (1917), the forelegs are ”dark” except for “1) the femora which has a pale patch on the proximal half of the anterior surface, and a pale apex and 2) tarsomeres 1–3 which have a narrow basal pale ring”. The midlegs and hindlegs are characterized as “overall similar”. However the midfemora are described as “ventrally pale to the apex”. The pale bands on tarsomere 1–3 are described as “slightly broader”. Moreover there is a “very narrow pale ring” on tarsomere 4. The hindfemora are determined as “pale on their proximal half except for a narrow ventral dark band”; the apex is characterized as “more broadly pale than on other legs” and the tarsal rings are “broader with a few pale scales at the base of the fifth hindtarsomere”. Tanaka *et al*. (1979) gave a similar description. The authors characterized the hindtarsomeres 1–4 as having “distinct basal pale bands” and mentioned that hindtarsomere 5 occasionally displays an “incomplete basal pale band or dorsal pale scales at base”.

FIGURE 3. Hindleg of Belgian *Aedes koreicus.*

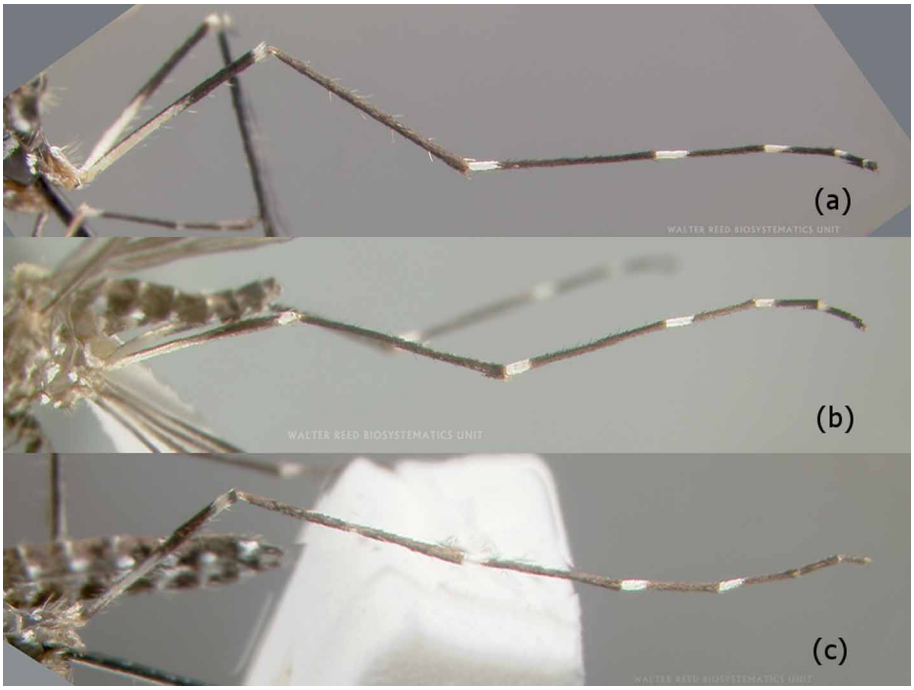

FIGURE 4. Differences in hindleg ornamentation of (a) *Aedes koreicus* from Belgium, (b) *Aedes koreicus* from peninsular Korea and (c) *Aedes j. japonicus* from Belgium.

In Belgian specimens, all legs show the same patterns; femora with a large pale patch on the proximal half of the fore- and midlegs and on 0.67 of the hindleg, apex and other joints are also pale. Remarkably, hindtarsomeres 4 and 5 have a clear basal pale band. Jeju-do specimens show also these characteristics although this pale basal band on hindtarsomere 5 is small and interrupted.

Larva, fourth-instar (Fig. 5). The larva was not described by Edwards (1917) but was described by Tanaka *et al*. (1979) from typical specimens collected on the Korean Peninsula. Larvae from the Belgian population show the same discriminating characteristics described by Tanaka *et al*. (1979), which distinguish them from *Ae. j. japonicus*. The absence of detached simple pecten spines and the complex form of the apical spines on the saddle in *Ae. koreicus* are especially different in the two species. The combination of all characteristics that separate typical *Ae. koreicus* larvae from typical *Ae. j. japonicus* larvae are given by Tanaka *et al*. (1979).

FIGURE 5. SEM photo of the saddle and siphon of *Aedes koreicus.*

Egg (Fig. 6). The egg is dark, cigar-shaped and tapers posteriorly; chorionic cells appear primarily pentagonal and variable in size. It is not clear if the depression on lateral side in all egg specimens studied is an artefact or a natural characteristic.

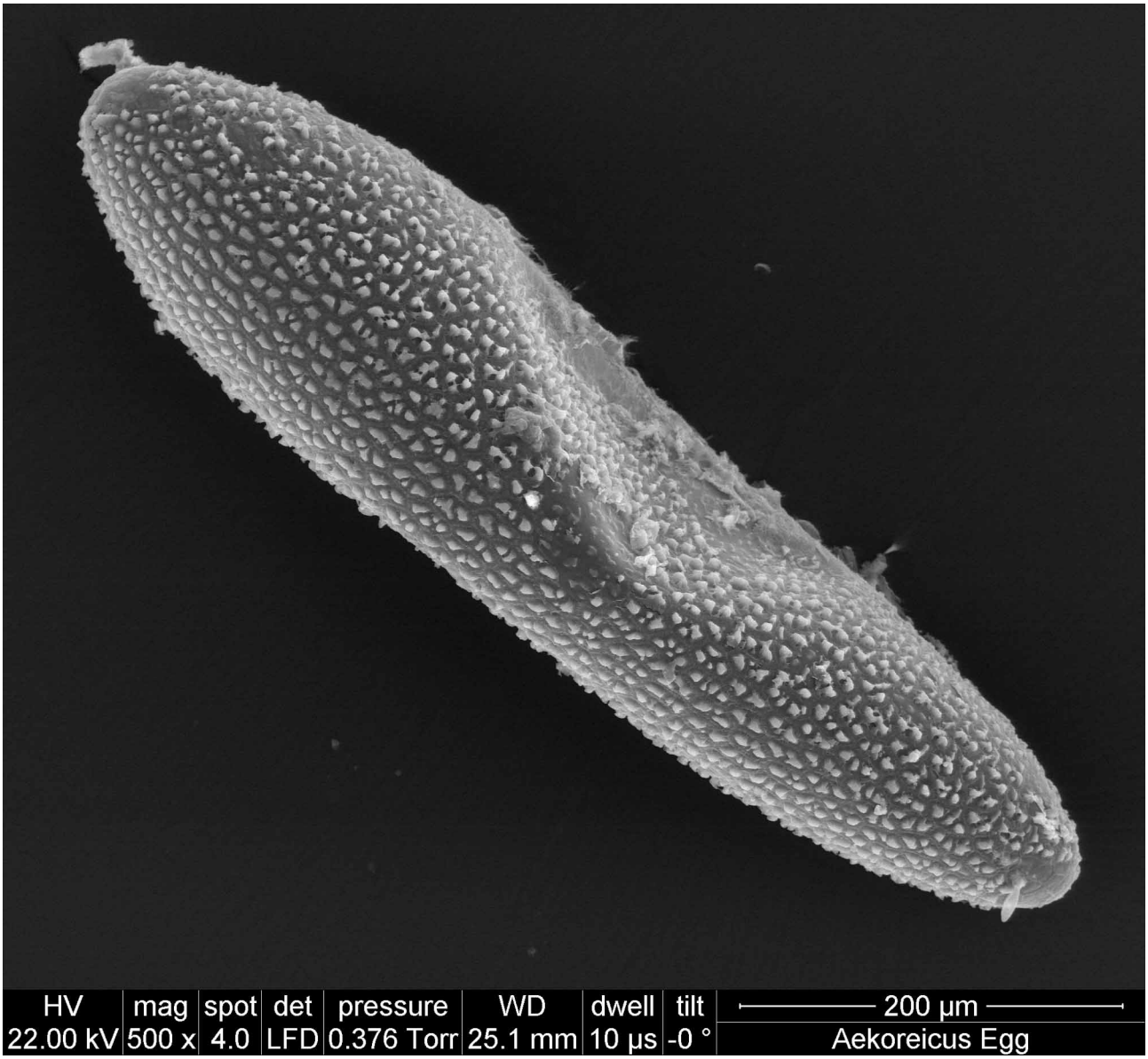

FIGURE 6. SEM photo of an egg of *Aedes koreicus.*

Molecular identification

The total size of the amplified fragments of the COI, ITS 2 and ND4 regions in all Belgian adult specimens was 709, 412 and 465 bp respectively. None of the sequenced positive PCR amplifications showed similarities with sequences available in GenBank. The morphological identification was confirmed by comparing ND4 sequence data from Belgium specimens to the ND4 sequence from material in the Smithsonian collection. The similarity for the ND4 sequence of the Belgian and Korean specimens of the Smithsonian collection was 99%, pairwise distance between sequences varied between 0.003 and 0.011 and overall distance was 0.007.

The rapid assay developed by Cameron *et al.* (2010) proved to be a useful tool to separate *Ae. koreicus* from other invasive *Aedes* species, due to the double band displayed at 283 bp and 465 bp for all *Ae. koreicus* individuals tested. All other *Aedes* species included in the analysis displayed a single band.

Discussion

Small but consistent morphological differences between populations of a species can cause severe identification problems. This is certainly the case for the Belgian founder population of *Ae. koreicus*. Despite distinct differences found between characteristics described in the literature from the type specimen and other specimens, a correct identification could be made based on an elaborate morphological and molecular study on reference collections from different institutions.

Tanaka *et al*. (1979) mentioned a number of morphological differences between adult females collected on the Korean Peninsula and Jeju-do that correspond to the observed differences between Korean type specimens and Belgian specimens. The erect forked scales on the vertex are usually entirely dark in the mainland population whereas most Jeju-do specimens showed a mix of dark and pale scales. The latter population had also more crescent-shaped scales on the anteprontotum compared to the Korean Peninsula population. Morphological study of the Smithsonian Institute material revealed a remarkably high number of specimens from Jeju-do that have hindtarsomere 5 with an incomplete basal pale band, which is not the case for the mainland specimens. This characteristic was present in all individuals of the Belgian population. Moreover, this population not only showed the characteristics of the Jeju-do specimens, it also exhibited a large variation in abdominal patterns, showing a combination of basomedian and basolateral pale bands. Based on the remarkable resemblance with this population, it could be assumed that the species was imported from Jeju-do. Tanaka *et al*. (1979) stated that observed differences between specimens from the Korean Peninsula and Jeju-do could be due to clinal variation. Although most aberrant characteristics between Belgian and Korean specimens could be caused by isolation or founder effects, others, such as differences in the color of scales on the scutum, could be due to aging or preservation, although even dry Belgian specimens retained the silver-white scales, or due to environmental factors. Also genotype- environment interactions during the invasion process cannot be ruled out (Erfmeier & Bruelheide 2010).

The initial identification of *Ae. koreicus* was also hampered by the fact that this species resembles *Ae. j. japonicus*. According to Tanaka *et al*. (1979), the ranges of morphological variation of all the differentiating characteristics in the adults and larvae of the two species overlap. However, these characters show distinctly different tendencies of variation. *Aedes koreicus* differs from *Ae. j. japonicus* in the following characteristics: the pedicel usually has more pale than dark scales, often all scales are pale; the postpronotum usually has a few broad dark scales; the subspiracular area usually has a scale-patch; and hindtarsomere 4 always has a complete basal pale band.

In addition to morphological similarity, sequence data (ND4, COII, and D2) (Widdel *et al*. 2005; Cameron *et al*. 2010) also indicates that *Ae. j. japonicus* is closely related to *Ae. koreicus*. The COI region is widely used as an identification tool for many organisms, striving for the rapid and inexpensive generation of molecular species "tags". However, misleading results can be generated if the species concerned contain nuclear copies of mtDNA (Numts) as these may amplify in addition to, or even instead of, the authentic target mtDNA (Hlaing *et al*. 2009). This phenomenon was recently observed in *Aedes aegypti* where the COI gene sometimes reveals this ambiguity and the ND4 gene is amplified instead. Based on this ND4 region, a rapid assay was developed to separate *Ae. koreicus* from *Ae. j. japonicus* (Cameron *et al*. 2010). The results obtained from Belgian specimens of both species confirm this latter method. Such molecular diagnostics may in the future be crucial to distinguish known invasive *Aedes* species.

Distinguishing closely related species is not easy, even for trained taxonomists, but is of critical importance when dealing with invasive mosquito species that are potential disease vectors. Considering the important need for rapid and correct identifications and the difficulties this sometimes poses, good descriptions of possible invasive species are extremely important. Furthermore, it has to be taken into account that local adaptations, as isolation by distance, can have an effect on the genotype as well as the phenotype of the new founder population, leading to possible variations in morphology. Both the morphological and molecular data indicate the close relationship between the two Oriental invasive aedines and show the importance of a rapid and accurate identification. This morphological description of differing characters between originally described *Ae. koreicus* specimens and Belgian ones, combined with molecular identification based on the mtDNA ND4 region, will be useful in invasive mosquito monitoring programs worldwide.

Based on this study, the invasive species in Belgium was correctly identified as *Ae. koreicus* and a hypothesis on its origin was conceived, which has implications for import surveillance.

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