Molecular identification of two morphologically similar *Eulecanium* species: *E. giganteum* and *E. kuwanai* (Hemiptera: Coccidae)

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Abstract—Most species of the genus *Eulecanium* Cockerell (Hemiptera: Coccidae) are important economic pests for ornamental plants and fruit trees. Two morphologically similar species, *Eulecanium giganteum* Shinji and *E. kuwanai* Kanda, are distributed mainly in China and are quite difficult to identify because of the paucity of distinguishing characteristics, which can only be observed in slide-mounted young, adult females. Furthermore, we demonstrate here that the species occur in sympatry and on many of the same host plants. Mitochondrial cytochrome c oxidase I (COI) and the D2–D3 expansion segments of 28S rDNA were used for accurate identification of these two *Eulecanium* species from 19 different locations in China. The average K2P distances of COI sequences were 0.47% in *E. kuwanai* and 0.32% in *E. giganteum,* and the interspecific divergences varied from 7.23% to 8.34%. Neighbour-joining (NJ) trees of COI and 28S rDNA revealed two distinct non-overlapping clusters, respectively. Meanwhile, "best close match" analysis also showed that 100% of individuals were classified successfully using COI and 28 S sequences. Differentiating between *E. giganteum* and *E. kuwanai* is challenging when using ecological and morphological traits. In contrast, identification using DNA diagnostics appears to be very effective, especially when slide-mounted specimens are difficult to obtain.

Eulecanium Cockerell (Hemiptera: Coccidae) comprises ~50 species and subspecies globally; most species in this genus are important economic pests that are distributed worldwide (Hamon and Williams 1984; Ben-Dov 1993; Hodgson 1994; Ben-Dov *et al.* 2010). They feed on the sap of plants and excrete a sticky substance (honeydew) that induces sooty mould growth (Tang 1977; Xie *et al.* 2006). Due to great economic losses in fruit trees and woody ornamentals, *Eulecanium* species have been extensively studied for decades (Hamon and Williams 1984; Tang 1991; Hodgson 1994; Wang 2000, 2001; Li *et al.* 2002; Zhang 2004; Xie *et al.* 2006). A high degree of morphological similarity is found among the different taxa of *Eulecanium* species (Hodgson 1994; Ben-Dov and Hodgson 1997), and preparation of slides is time-consuming, both of which make *Eulecanium* species difficult to identify. The taxonomic keys are based on the adult female stage; thus, the identification of nymphs is usually impossible, because of a general absence of taxonomic keys.

In China, *E. giganteum* Shinji and *E. kuwanai* Kanda, two morphologically similar species, are sympatrically distributed in the northern regions, and each is polyphagous on many of the same hosts (Fig. 1). Although *E. giganteum* was

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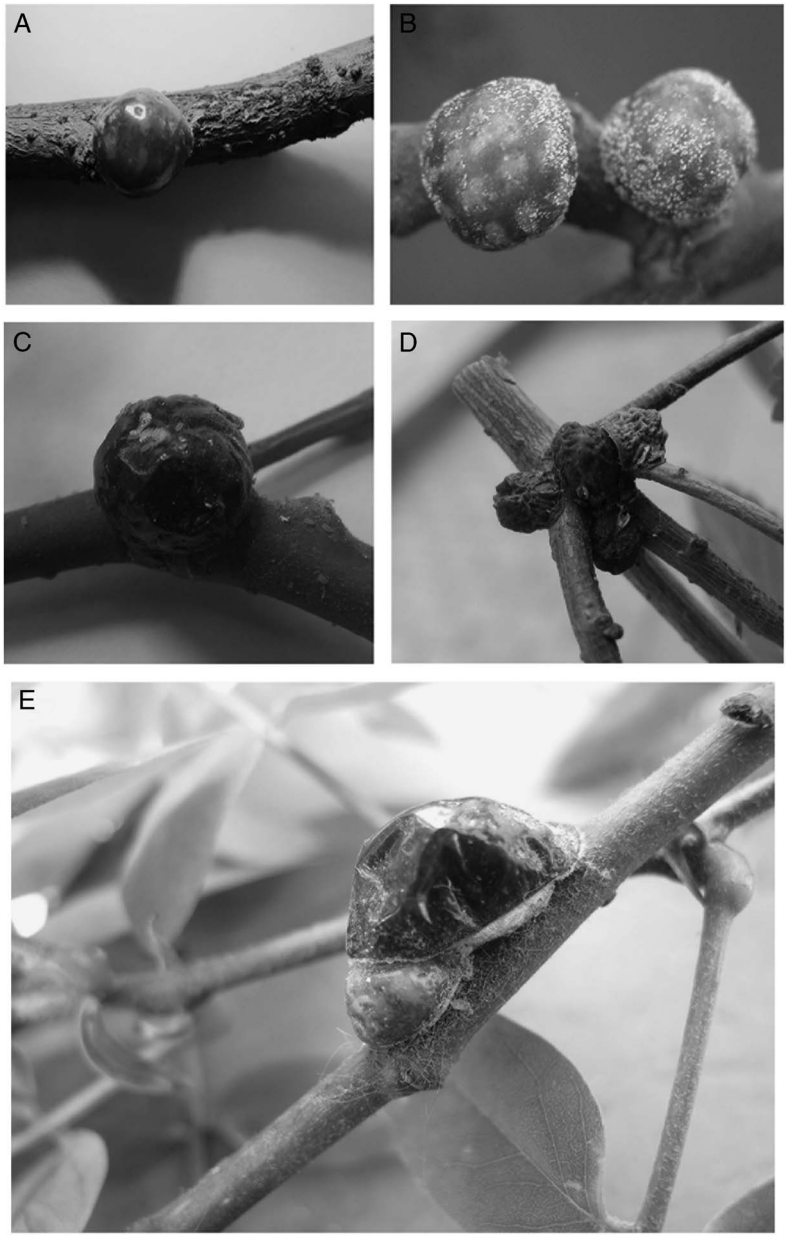
Fig. 1. Different appearance of adult females of *Eulecanium giganteum* and *Eulecanium kuwanai*. (A) *E. giganteum*; (B) *E. kuwanai*; (C) dead individual of *E. giganteum*; (D) dead individual of *E. kuwanai*; (E) larger individual is *E. giganteum* smaller individual is *E. kuwanai*.



recorded in Morioka of Japan (Shinji 1935) and the Primorsky Krai of Russia (Borchsenius 1955; Danzig 1980), there have been no documented sightings in these regions in recent decades. In contrast, they have been found in the northern area of China (Tang 1991; Wang 2001; Xie *et al.* 2006). All have the same life history type of one generation per year. They overwinter as second instar nymphs on twigs. The crawlers emerge in early April and grow quickly. The second instar nymphs mature to mate in May, and the population size of newly hatched crawlers rapidly increases in June. From June to September, large numbers of crawlers feed on leaves and damage

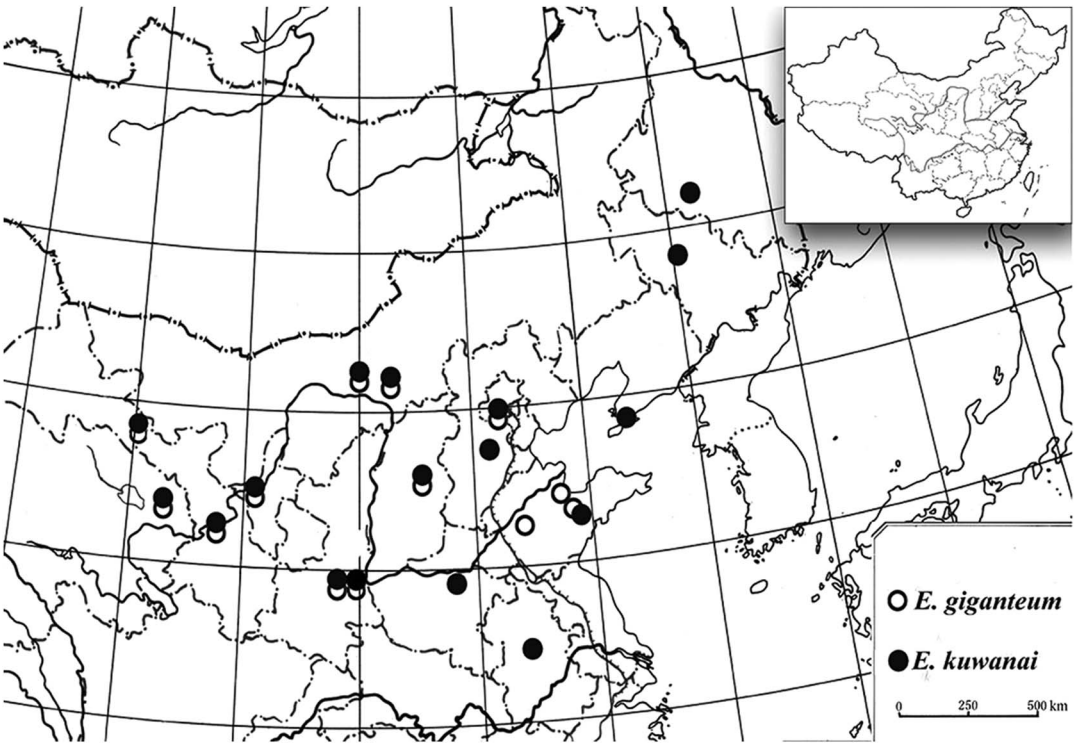
host plants; the second instar nymphs then return to the twigs for winter dormancy (Xie 1998; Wang 2000). For the reasons mentioned above, it is difficult to distinguish these two species, because they often appear together on the same host plants and even on the same twigs.

Owing to the serious damage they cause to garden trees, *E. giganteum* was listed in the catalogue of quarantine pests for plants imported to the People's Republic of China in 2007. Although the size and colouration of the adult female body were important traits for delimiting these two morphologically similar species (Xie 1998), these traits are not always stable. Variations in the shape, size, and colouration of the adult female body on different host plants have been observed in *Eulecanium* species (Ben-Dov and Hodgson 1997). Tang (1991) recognised two distinct species, since dead individuals of *E. kuwanai* versus *E. giganteum* are wrinkled versus smooth. Moreover, sympatric occurrence and overlap of the host range render classification of the two taxa more difficult. Shi and Lü (1989) determined that *E. giganteum* and *E. kuwanai* are two different ecological types (wrinkled and smooth individuals) of the same species, with the different phenotypes resulting from varying population densities. The accurate identification of these two morphologically similar species, especially at immature stages, will require a new tool.

The barcode region of the cytochrome oxidase I (COI) gene is a proven, useful marker for effectively discriminating between closely related insects (Hebert *et al.* 2004a, 2004b; Hajibabaei *et al.* 2006; Mikkelsen *et al.* 2007; Lee *et al.* 2011). There are also some studies that have identified scale insects using the COI gene (Ball and Armstrong 2007; Pieterse *et al.* 2010; Park *et al.* 2011; Abd-Rabou *et al.* 2012; Beltra *et al.* 2012; Deng *et al.* 2012; Sethusa *et al.* 2014). Thus, the barcode region of the COI gene was selected as a potential marker for delimiting these two species. The 28S gene was analysed to expand the amount of information available for species identification (Deng *et al.* 2012; Sethusa *et al.* 2014).

In this study, we collected *E. giganteum* and *E. kuwanai* individuals from 19 different locations in China (Fig. 2). COI and 28S rDNA sequences

Fig. 2. Sampling sites in China. Detail sampling information is shown in Supplementary Appendix B.



were obtained to classify these two morphologically similar species. One aim of this work was to determine whether morphological identification of *E. giganteum* and *E. kuwanai* based on dorsum morphology (wrinkled or smooth) during the post-reproductive stage is consistent with the results of molecular identification. The other goal was to confirm that molecular identification is effective for classifying crawlers and eggs. For describing in detail materials and methods, see Supplementary Appendix A.

A total of 192 COI sequences were obtained. The final length of the constructed COI sequence was 718 base pairs. The frequencies of adenine (A) and thymine (T) were high (A = 41.8%, C = 12.6%, G = 6.8%, T = 38.8%), and no insertions or deletions were observed. The intraspecific divergences of *E. giganteum* sequences were 0–0.98%, with a mean divergence of 0.32%. For *E. kuwanai*, the intraspecific divergence range was identical to that of *E. giganteum*, with 0.47% mean divergence. The interspecific divergence of COI sequences varied from 7.23% to 8.34%, with a mean value of 7.60%. A 28S amplicon was recovered from 192 individuals. The final alignment of the 28S sequence was 897 base pairs in length. The mean intraspecific divergence of *E. kuwanai* (0.038%) was greater than that of *E. giganteum* (0.00%), with an average interspecific variation of 1.30%.

In the COI neighbour-joining tree (Fig. 3A), all individuals were split into two clusters with high bootstrap values (> 90). One cluster was comprised of four haplotypes from 96 *E. giganteum* COI sequences, while the other included six haplotypes from 96 COI *E. kuwanai* sequences. The 28S neighbour-joining tree (Fig. 3B) was almost identical to that of the COI sequence, indicating that both *E. kuwanai* and *E. giganteum* were identified unambiguously. Based on all of the intraspecific pairwise distances, the cutoff values of COI (0.84%) and 28S (0.11%) were obtained using the TaxonDNA programme. The proportions of successful identifications using "best match" and "best close match" all reached 100% of the samples for COI and 28S genes. As predicted, all individuals identified by the habitus of post-reproductive females (wrinkled or smooth) were consistent with the molecular identification results (Supplementary Appendix B).

According to the information in Table 1, the distribution of these two species overlapped almost everywhere except for in three provinces (Heilongjiang, Jilin, and Xinjiang). The distribution limit of *E. kuwanai* was further north than that of *E. giganteum*, and *E. giganteum* individuals were observed in Heilongjiang and Jilin. In contrast, Xinjiang was not included within the western distribution range of *E. kuwanai*, even though there were many potential host plants, such as *Sophora japonica* Linnaeus (Fabaceae), *Ulmus pumila* Linnaeus (Ulmaceae), and *Ziziphus jujube* Miller (Rhamnaceae), in that area. Comparing host ranges, *E. giganteum* had a greater host range (15 species) than that of *E. kuwanai* (eight species). However, *S. japonica*, *U. pumila*, and *Z. jujuba* were the main host plants for both of these two species.

In this study, two morphologically similar species of *Eulecanium* were identified based on the mitochondrial COI gene and ribosomal 28S DNA sequences. In the COI sequence analysis, the interspecific divergence between these two closely related species was 7.60%, whereas intraspecific variation was very low (mean 0.39%). High interspecific variation and low intraspecific variation were also observed in other scale insects such as *Ceroplastes* Gray (Hemiptera: Coccidae) species (12.19% and 0.42%, respectively; Deng *et al.* 2012) and armored scales and mealybugs (10.09% and 0.97%, respectively; Park *et al.* 2011). A significant decrease in genetic variation from congeneric individuals to conspecific individuals indicated that a clear barcoding gap existed and could be used to differentiate these two species. In other genera, two closely related species were reported to differ by 2% in *Planococcus* Ferris (Hemiptera: Pseudococcidae) (Rung *et al.* 2008) and 5% in *Ceroplastes* (Deng *et al.* 2012), suggesting that the 7.60% interspecific divergence in COI between *E. kuwanai* and *E. giganteum* could be used to correctly classify them. According to the TaxonDNA results of the COI and 28S sequences, all individuals were identified correctly. Our results suggest that *E. kuwanai* and *E. giganteum* were identified successfully using molecular identification methods, and that they are not two different ecological types (wrinkled versus smooth individuals) of the same species. It is impractical to distinguish these two species based on the habitus of post-reproductive females, since species identification requires accurate and efficient methods for identifying organisms in various developmental stages. Molecular identification

Fig. 3. (A) COI and (B) 28S neighbour-joining trees of these two *Eulecanium* species, using Kimura-2-parameter distance. Bootstrap values for each haplogroup are calculated in MEGA4.0 with 1000 replicates and species are shown in the right column. Some identical sequences from same locations are removed in order to construct simplified trees.

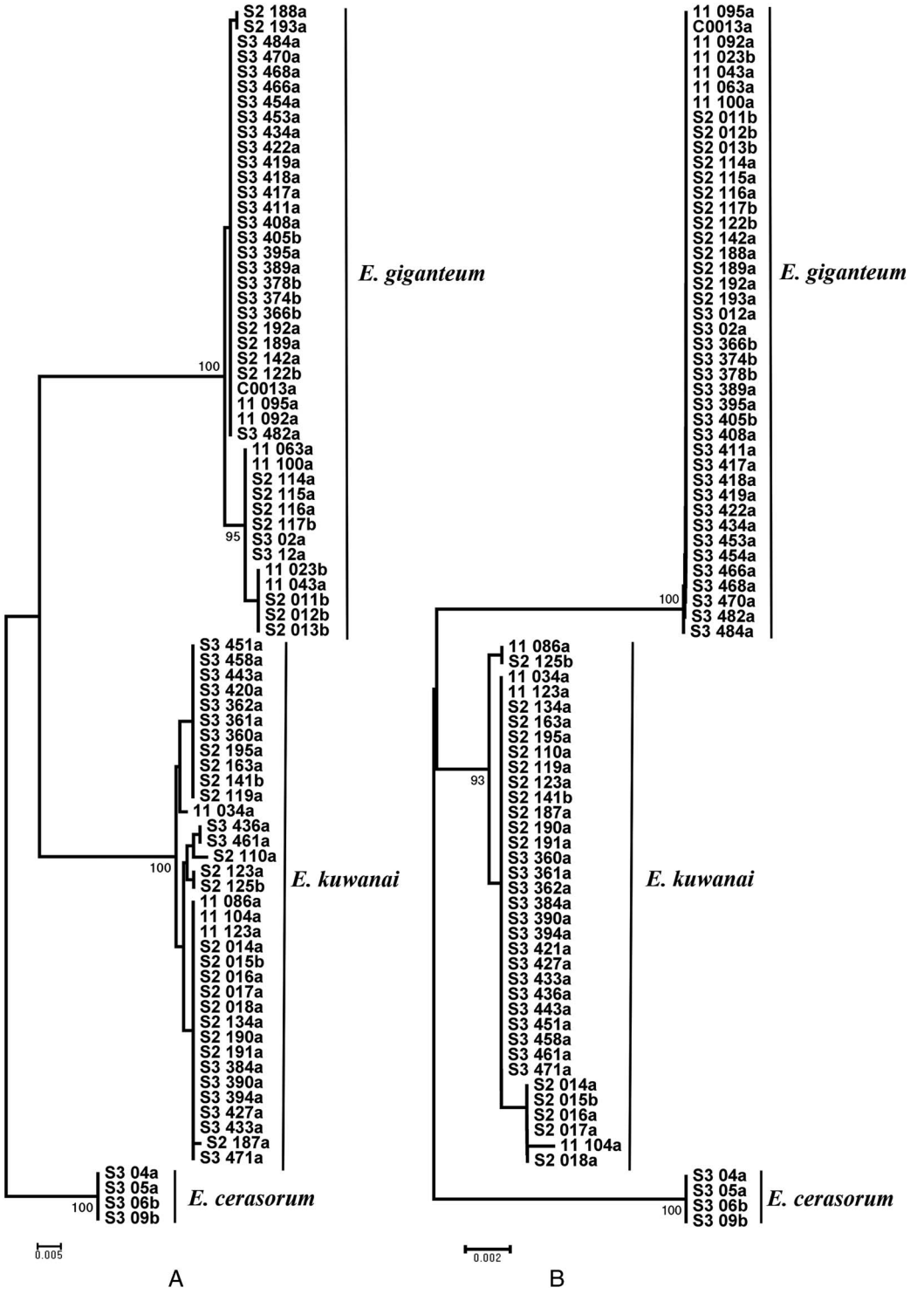


Table 1. A comparison of locations in China and host plants of *Eulecanium* species.

Host plants		Locations (province)	
Eulecanium giganteum	*Eulecanium kuwanai*	*Eulecanium kuwanai*	*Eulecanium giganteum*
Acer elegantulum	/	Anhui	Anhui
Albizia julibrissin	/	Beijing	Beijing
Albizia julibrissin	/	Gansu	Gansu
Armeniaca vulgaris	/	Hebei	Hebei
Broussonetia papyrifera	/	Heilongjiang	/
/	*Carya cathayensis*	Henan	Henan
Juglans regia	*Juglans regia*	Inner Mongolia	Inner Mongolia
Koelreuteria paniculata	/	Jilin	/
Populus species	*Populus* species	Liaoning	Liaoning
/	*Prunus cerasifera*	Ningxia	Ningxia
Populus tomentosa	/	Qinghai	Qinghai
Rosa species	/	Shaanxi	Shaanxi
Salix babylonica	*Salix babylonica*	Shandong	Shandong
*Sophora japonica**	*Sophora japonica**	Shanxi	Shanxi
*Ulmus pumila**	*Ulmus pumila**	/	Xinjiang
Wisteria sinensis	/		
*Ziziphus jujuba**	*Ziziphus jujuba**		

/ The locations where *E. giganteum* or *E. kuwanai* are not recorded.
* Main host plants for these two species.

using the COI and 28S genes can be used to identify *Eulecanium* species correctly.

Eulecanium giganteum seriously threatens jujube production in Xinjiang, which is the main jujube production area of China and contained more than 300 000 ha of production area in 2012 (Li and Xu 2013). Although *E. kuwanai* has not been reported in Xinjiang so far, the possibility of *E. kuwanai* being introduced into Xinjiang still exists. Jujube is an important host plant for *E. kuwanai*. Sympatric occurrence of these two species were observed in other regions, excluding Heilongjiang, Jilin, and Xinjiang, indicating the ecological niche of *E. kuwanai* is similar to that of *E. giganteum*. It is necessary to provide a rapid and accurate diagnostic method to identify these two species for quarantine, to prevent further invasions into non-infested areas of China. Most specimens collected in the field are not suitable for slide mounting, because the typical teneral stage is rather short and followed by a general swelling of the body and a hardening of the dorsum (Hodgson 1994). Molecular techniques provide an effective and rapid method to distinguish the immature individuals. Further studies that include specimens of *E. giganteum* and *E. kuwanai* from their type localities are needed to verify true conspecificity of the individuals used in this study.

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Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.4039/tce.2015.42

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