Cryptic Species of Parasitoids Attacking the Soybean Aphid (Hemiptera: Aphididae) in Asia: *Binodoxys communis* and *Binodoxys koreanus* (Hymenoptera: Braconidae: Aphidiinae)

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ABSTRACT Collections of parasitoids attacking the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), in South Korea yielded specimens that were originally identified as *Binodoxys communis* (Gahan) (Hymenoptera: Braconidae). We report here on laboratory studies indicating that this population is actually a previously unknown species of *Binodoxys*. Four classes of comparisons were made between the Korean population and a Chinese population that also had been identified as *B. communis*. The comparisons included 1) mating trials coupled with behavioral observations and spermathecal examinations, 2) assessment of nucleotide divergence at two mitochondrial and two nuclear gene loci, 3) patterns of host use, and 4) reassessment of morphological characters. These studies revealed premating reproductive isolation of the two populations and minor nucleotide differences in mitochondrial cytochrome oxidase I sequences and nuclear internal transcriber spacer 1 sequences, providing strong indications that they are different species. Subtle morphological differences also were discovered that confirmed that the Chinese population corresponds to *B. communis*, whereas the Korean population does not. We propose the name *Binodoxys koreanus* Starý, sp. n. for the Korean population. The two species exhibited similar host ranges in the laboratory, the most notable exception being that *B. koreanus*, sp. n. is better able to develop in a population of *Aphis craccivora* Koch that harbors the bacterial endosymbiont *Hamiltonella defensa* Moran, which seems to strongly interfere with the development of *B. communis*. We discuss the implications of our results for biological control introductions against the soybean aphid in North America.

KEY WORDS cryptic species, *Binodoxys communis*, *Binodoxys koreanus*, soybean aphid, parasitoids

When two populations are classified as belonging to the same species based upon morphological characters, but are subsequently recognized as separate species, they are often designated as "cryptic species" (Bickford et al. 2007). Despite being morphologically very similar or indistinguishable, cryptic species are genetically divergent and may differ in several behavioral or physiological traits. An understanding of cryptic species relationships is critical for characterization of speciation, biodiversity, and community structure and also has important implications for ecological interactions such as host specialization and interspecific competition (Zhang et al. 2004, Kankare et al. 2005a, Smith et al. 2008, Forbes et al. 2009). Conservation efforts, in particular, depend upon an understanding of cryptic species relationships (Bickford et al. 2007) as does the success and safety of biological control of pest and weed species (De Bach 1969, Rosen 1986, Clarke and Walter 1995, Beard 1999, Stouthamer et al. 2000a, Madeira et al. 2001, Pinto et al. 2003, Meyling and Eilenberg 2007).

Recognition has been growing over the past decade that numerous lineages of insect parasitoids are composed of complexes of cryptic species and also that cryptic parasitoid species often attack different host species (Heimpel et al. 1997; Fernando and Walter 1997; Kankare et al. 2005a,b; Smith et al. 2006, 2007, 2008; Heraty et al. 2007; Abrahamson and Blair 2008; Bernardo et al. 2008; Kathirithamby 2009). Cryptic speciation also has been found to be associated with host specialization in herbivorous insects (Hebert et al. 2004, Scheffer et al. 2004, Blair et al. 2005, Stireman et al. 2005, Abrahamson and Blair 2008, Thompson 2008). The scale of discovery of new cryptic species in

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these and other studies has been so great that estimates of global species diversity are having to be increased. A particularly important breakthrough has come from studies in which parasitoids were reared from caterpillars collected in Costa Rican rain forests over the past 30 yr. Adult parasitoids were identified morphologically and then genotyped using cytochrome oxidase I (COI) barcode sequences, revealing 15 new cryptic species of the tachinid genus *Belvosia* beyond 17 original morphological species, and a staggering 142 new cryptic species of microgastrine braconids beyond 171 morphological species (Smith et al. 2006, 2008). Similar upward adjustments in diversity are being made for *Cotesia* parasitoids of checkerspot and related butterflies (Kankare et al. 2005a,b).

Nucleotide divergences between cryptic species of parasitoids in genes that tend to vary among species range from <1% among some species of *Aphelinus*, *Trichogramma*, and *Cotesia* (Stouthamer et al. 2000b, Kankare et al. 2005b, Heraty et al. 2007) to 10% or more in some *Encarsia* and *Ageniaspis* species as well as the strepsipteran genus *Caenocholax* (Alvarez and Hoy 2002, Monti et al. 2005, Kathirithamby et al. 2007). Reproductive isolation in laboratory mating trials is obviously consistent with separate species, but it is not a requirement for cryptic (or other) species status (Bickford et al. 2007). Allopatric populations may diverge genetically and behaviorally before evolving reproductive isolation, as barriers to gene flow are not under selection in allopatry. For example, some allopatric populations of *Aphelinus* spp. remain reproductively compatible while differing in host-use patterns, subtle morphological traits and genetic sequences, whereas pairs of sympatric populations prove reproductively incompatible in laboratory mating trials (Wu et al. 2004, Heraty et al. 2007).

Here, we report on a new cryptic species within the aphid parasitoid genus *Binodoxys* (Hymenoptera: Braconidae: Aphidiinae). Two purported geographic strains of *Binodoxys communis* (Gahan) (Hymenoptera: Aphidiinae) were collected in China and South Korea as potential biological control agents of the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), which invaded North America from Asia in 2000 (Heimpel et al. 2004, Wyckhuys et al. 2007a). Routine laboratory studies of these two populations suggested that they were reproductively incompatible, and we report here on formal studies to determine whether they are, in fact, cryptic species. These studies include mating trials, sequencing of mitochondrial and nuclear DNA of the two populations, a comparison of host-use patterns, and a reexamination of morphological features. Our studies suggest strongly that the Chinese population is *B. communis* but that the Korean population is a cryptic species closely related to *B. communis* and we propose the name *Binodoxys koreanus* Starý, sp. n.

Materials and Methods

Origin of Parasitoids. Specimens of *Binodoxys* were reared from parasitized *A. glycines* collected by K.A.H. in commercial fields and in research plots of soybean, *Glycine max* (L.) Merr., in Heilongjiang Province, northeastern China, and from several provinces in South Korea. Commercial fields in Heilongjiang were often relatively large, sometimes many hectares, whereas in Korea, the fields were nearly always small plots, often no more than border rows planted alongside other crops. Populations of *A. glycines* were very low and highly dispersed at all sites at the time of collections in late August 2002 (China) and August 2003 (Korea). The mummies obtained from collections were shipped by express mail to the USDA–ARS Beneficial Insect Introduction Research laboratory in Newark, DE, where laboratory cultures were established from emergent adults. Individuals of *Binodoxys* composed 74% of the aphidiine braconids from Heilongjiang, China, that were alive upon arrival, or subsequently emerged in Newark, and 30% of those from Korean material. Because of the relatively small number of live *Binodoxys* adults obtained from individual sites, adults from multiple sites were pooled to establish one culture from China in 2002 (from seven males and 33 females) and one culture from Korea in 2003 (from 10 males and 17 females). Both parasitoid cultures were transferred to a quarantine laboratory at the University of Minnesota (Minnesota Department of Agriculture/Minnesota Agricultural Experiment Station Quarantine Facility) in 2003, where they were maintained in continuous culture in separate rooms on *A. glycines* on soybean (25°C, 75% RH, and a photoperiod of 16:8 [L:D] h) (see Wyckhuys et al. 2008 for general rearing protocol). Individuals from both populations were identified as *B. communis* based upon the keys found in Starý and Schlinger (1967) and Chen and Shi (2001). We will argue below that the Chinese population is indeed *B. communis* but that the Korean population is a new species. Until then, we will refer to the entities as the Chinese and Korean *Binodoxys* populations, respectively.

Reproductive Compatibility. We set up reciprocal crosses of the Chinese and Korean populations of *Binodoxys* by establishing between- and within-population crosses ($n = 10$ in both cases). We ensured that both males and females were unmated before the experiments by isolating mummies in gelatin capsules (size 00) until emergence of adults. Individuals used in all crosses were between 24 and 48 h old. Males and females were placed into gel capsules as single pairs for 1 h to allow mating, and then introduced into *A. glycines*–soybean microcosms identical to those used for parasitism assays described below (the cages were kept at 25°C, 75% RH, and a photoperiod of 16:8 [L:D] h). Both females and males were placed into the microcosms to allow mating in the event it did not occur in the gel caps. Female parasitoids were allowed to oviposit for 24 h before being removed from the microcosms. As mummies formed, they were isolated individually in clear gelatin capsules (size 00). We counted the mummies produced per microcosm, and the number of male and female adults emerging. Due to haplodiploid sex determination, production of female offspring is evidence of mating, and the produc-

tion of only male offspring is consistent with virgin oviposition (Heimpel and de Boer 2008). Data were square-root transformed and analysis of variance (ANOVA) was used to assess significance of sex ratio differences among mating treatments. In addition, the numbers of total offspring produced (males + females) per microcosm were square root-transformed and analyzed using a factorial ANOVA with "*Binodoxys* population" and "cross type" as factors This analysis was done to evaluate the possibility that male origin would affect female oviposition behavior.

Although the results of this experiment suggested that *B. communis* and the Korea population were reproductively isolated (see Results), it was unclear whether isolation occurred before or after mating. To address this, we observed individual mating behavior in the context of the reciprocal and within-population crosses. Matings were established in clear gelatin capsules (size 00) and observed under a dissecting microscope at 10–50×. Based on preliminary observations, four male behaviors were noted and timed: 1) male wing fanning, 2) chase (when a male followed a female), 3) mounting attempt, and 4) copulation (the male bends his abdomen toward the female genitalia and genital contact occurs). In addition, three other male behaviors were timed: walking, grooming, and resting. Depending upon the particular mating combination, 19–26 replicates were used. Observations began when a male and a female were introduced into the gelatin capsule and lasted for 5 min or until copulation occurred. Each female that had close contact with a male during either a mounting attempt or copulation event was dissected and the spermatheca examined under a compound microscope at 1,000× magnification to detect the presence of sperm. Dissections were done 4 h after the presumed sexual contact, a time determined on the basis of preliminary observations to be sufficient for spermatozoa to reach the spermatheca. In a separate experiment, single unmated male and female parasitoids corresponding to the two between-population reciprocal crosses and the two within-population crosses were placed together in a gel capsule for 1 h. After this time, the females were dissected and their spermathecae checked for the presence of sperm as described above (10 replicates per cross).

Comparison of DNA Sequences. *16S rDNA and ITS1.* DNA was isolated from parasitoids that were killed by freezing and stored in microcentrifuge tubes at −80°C. DNA was extracted from individual females of each *Binodoxys* population using the Puregene DNA isolation tissue kit (Gentra Systems, Minneapolis, MN). Polymerase chain reaction (PCR) amplifications were carried out in 50-μl volumes using Gotaq Green mix (Promega, Madison, WI) with 25 μl of Gotaq, 0.5 μl of each primer (20 μM) (forward and reverse), 22 μl of water, and 2 μl of DNA sample. All PCR products were run on 2% agarose gels, stained in ethidium bromide, and visualized with UV light. Bands of ≈1000-bp (internal transcriber spacer [ITS]1), 400-bp (16S) were cut from the gel, purified using Ultrafree-DA columns (Micron Bioseparations, Billerica, MA), and sequenced directly. Sequencing of these fragments was performed at the University of Minnesota (BioMedical Genomics Center-DNA Sequencing and Analysis Facility).

COI and D2 Expansion Area. DNA was extracted from parasitoids using a Chelex/Proteinase K DNA extraction method. Individual parasitoids preserved in 95% ethanol were allowed to dry briefly in a 1.5-ml microcentrifuge tube and were then ground with a sterile pestle in 100 μl of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) and 2 μl of proteinase K (20 mg/ml; Amresco, Solon, OH). Samples were incubated at 55°C for 2–4 h followed by 99°C for 10 min. Tubes were spun at 14,000 rpm for 4 min, and the supernatant was transferred to a new tube and stored at −20°C. PCR amplifications (25 μl) were performed in a MJ Mini Thermocycler (Bio-Rad Laboratories) with 2.5 μl of 10× PCR buffer (Invitrogen, Carlsbad, CA), 2.5 μl of 50 mM $MgCl_2$, 0.25 ml of 25 mM dNTPs (Invitrogen), 1.0 μl of each forward and reverse primer (10 μM), 17.9 μl of double-distilled H_2O, and 0.1 μl of *Taq* DNA polymerase (Invitrogen). PCR products were visualized by gel electrophoresis on 2% agarose gels stained with GelRed (Biotium Inc., Hayward, CA) and purified with ExoSAP-IT (exonuclease i/shrimp alkaline phosphatase method; USB Corp., Cleveland, OH). Purified PCR products were sequenced at the University of Hawaii at Manoa (Advanced Studies of Genomics, Proteomics, and Biotechnology Sequencing Service). Information on primers and PCR conditions are provided in Table 1. Ten individuals per *Binodoxys* population were sequenced for each region amplified. All PCR reactions included a negative control (mix without DNA).

Parasitoid Host Range. Twenty aphid species were tested as potential hosts for the Korean population in identical manner to tests performed on the Chinese population that were reported previously (Desneux et al. 2009b). The parasitism assays for the Chinese and Korean populations were done over the same period (April 2004–January 2006) and under identical conditions within the quarantine laboratory. The aphids were reared in the quarantine laboratory on their respective host plants at 25°C, 75% RH, and a photoperiod of 16:8 [L:D] h (see Desneux et al. 2009b for details). Table 2 provides the aphid species, host plants, and phylogenetic relationships among the aphid species. These aphid species were chosen in part because they cover a broad phylogenetic range. Four species native to North America were included to estimate the potential risk to native species posed by *Binodoxys* if introduced to control soybean aphid (Wyckhuys and Heimpel 2007; Wyckhuys et al. 2007a,b, 2009; Desneux et al. 2009b).

In preparation for parasitism assays, mummified *A. glycines* were removed from soybean leaves and kept individually in plastic 6-cm-diameter petri dishes until emergence of adults. Females were mated within 24 h of emergence and supplied with a droplet of diluted honey (80%) before use in experiments. Parasitoids used for all experiments were 24–48 h old, naïve with respect to host encounters, and used only once.

Table 1. Molecular markers, primers (forward [F], reverse [R]), and PCR conditions used for amplifying the mitochondrial (mt) and nuclear (nuc) sequences of individuals from the Chinese and Korean populations

Gene	Primer	PCR condition
16S (mt)	F: 5′-TTA CGC TGT TAT CCC TAAA-3′[a] R: 5′-CGC CGT TTT ATC AAA AAC ATG T-3′[b]	Initial denaturation: 94°C for 4 min 35 cycles: 94°C for 30 s, 50°C for 1 min, 72°C for 1 min Final step: 72°C for 7 min
COI (mt)	F: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′[3] R: 5′-GGTCAACAAATCATAAAGATATTGG-3′[c]	Initial denaturation: 94°C for 2 min 35 cycles: 94°C for 15 s, 50°C for 30 s, 72°C for 45 s Final step: 72°C for 2 min
D2 (nuc)	F: 5′-AGT CGT GTT GCT TGA TAG TGC AG-3′[d] R: 5′-TTG GTC CGT GTT TCA AGA CGG G-3′[d]	Initial denaturation: 94°C for 3 min 35 cycles: 94°C for 45 s, 55°C for 30 s, 72°C for 1.5 min Final step: 72°C for 30 min
ITS 1 (nuc)	F: 5′-TAC ACA CCG CCC GTC GCT ACT A-3′[e] R: 5′-ATG TGC GTT CRA AAT GTC GAT GTT CA-3′[e]	Initial denaturation: 94°C for 4 min 35 cycles: 94°C for 30 s, 60°C for 50 s, 72°C for 1.5 min Final step: 72°C for 2 min

[a] Kambhampati and Smith (1995).
[b] Simon et al. (1994).
[c] Folmer et al. (1994).
[d] Chen et al. (2004).
[e] Ji et al. (2003).

Individual female parasitoids were each exposed to aphids for 24 h on their respective host plants, which were potted and covered by plastic cylindrical cages (diameter, 11 cm; height, 21 cm) (see Table 2 for sample sizes). Fifty aphids of mixed instars were placed per plant, and cages were kept in a growth chamber at 25°C, 75% RH, and a photoperiod of 16:8 [L:D] h. Newly developed mummies were isolated individually in clear gelatin capsules (size 00). We counted the mummies produced per plant, and the number of male and female adults emerging. The numbers of parasitoid mummies produced per plant, and the numbers of emergent adults, were square-root transformed to homogenize variances, and then compared among aphid species using ANOVA followed by Tukey's test to separate means. The proportions of adults emerging from mummies were arcsine-square-root transformed and compared among aphid species using ANOVA (Tukey's test as post hoc analysis). We used log-likelihood goodness-of-fit tests to evaluate the hypothesis that observed sex ratios for the Korea population on each aphid species differed from 0.5 as in Heimpel and Lundgren (2000).

We compared the numbers of mummies (*t*-tests), proportional emergence (chi-square tests) and sex ratio (chi-square tests) on each aphid host species in the presence of the Korean population (this study) with those of the same aphids in the presence of the Chinese population (data presented in Desneux et al. 2009b).

Morphological Characterization of the Korean Population. The original description of *B. communis* done by Gahan (1926) in Taiwan using material obtained

Table 2. Aphid species, phylogenetic relationships, host plants, and replication in the host use experiment.

Phylogeny	Species[a]	Host plant species	Replications	Tribe
	Rhopalosiphum padi	*Hordeum vulgare*	12	
	Rhopalosiphum maidis	*Hordeum vulgare*	10	
	Schizaphis graminum	*Hordeum vulgare*	10	
	Aphis rumicis	*Rumex altissimus*	9	
	Aphis craccivora	*Vicia fabae*	13	Aphidini
	*Aphis asclepiadis**	*Asclepias syriaca*	10	
	Aphis glycines	*Glycine max*	15	
	Aphis gossypii	*Gossypium hirsutum*	14	
	*Aphis monardae**	*Monarda fistulosa*	11	
	*Aphis oestlundi**	*Oenothera biennis*	11	
	Aphis nasturtii	*Solanum tuberosum*	20	
	Aphis nerii	*Asclepias incarnata*	11	
	Myzus persicae	*Brassica oleracea*	11	
	Diuraphis noxia	*Hordeum vulgare*	10	
	*Uroleucon leonardi**	*Echinacea purpurea*	10	
	Macrosiphum euphorbiae	*Solanum tuberosum*	10	Macrosiphini
	Sitobion avenae	*Hordeum vulgare*	9	
	Acyrthosiphon pisum	*Vicia fabae*	11	
	Lipaphis erysimi	*Brassica oleracea*	10	
	Aulacorthum solani	*Solanum tuberosum*	10	

[a] All aphid species are in the family Aphididae and subfamily Aphidinae Phylogeny is from information in von Dohlen et al. (2006), Coeur d'Acier et al. (2007), and unpublished data.

Asterisks indicate aphid species native to North America. All aphids were field-collected in the vicinity of St. Paul, MN, with the exception of *D. noxia*, and *A. gossypii*, which were shipped from the USDA–ARS quarantine laboratory at the Beneficial Insect Introductions Research Unit in Newark, DE.

Table 3. Proportions of individuals of the Chinese and Korean *Binodoxys* populations exhibiting different mating behaviors during the between- and within-population crosses

Cross	Male wing fanning	Chase	Mounting attempt	Copulation	Mating success
♂ Chinese × ♀ Korean	0.69ns	0.46*	0.27**	0**	0**
♂ Chinese × ♀ Chinese	0.82	0.82	0.77	0.36	0.36
♂ Korean × ♀ Chinese	0.79ns	0.63 ns	0.37ns	0**	0**
♂ Korean × ♀ Korean	0.84	0.79	0.63	0.26	0.26

The comparisons between within-strain and between-strain crosses are based on Fisher exact tests (ns, not significant; $*P < 0.05$, $**P < 0.01$).

from *A. gossypii* Glover (the host of the holotype specimen) enabled a redescription by Starý and Schlinger (1967), which has been followed by subsequent authors (Paik 1976, Chen and Shi 2001). A preliminary identification of *Binodoxys* species sampled in Korea referred this material to *B. communis* but experiments showing reproductive incompatibility (see Results) provoked a more detailed morphological examination that resulted in determination of a new species.

Specimens preserved in 95% ethanol were separated into two groups. The first group was dry-mounted for collections, and for determination of body size and color. The second group was slide mounted to examine morphological characters. Specimens were washed in distilled water, then transferred to 10% KOH for 2 min and then rewashed and mounted in Fauré-Berlese medium.

Results

Reproductive Compatibility. Reciprocal crosses between the Chinese and Korean populations yielded only male offspring (20.0 ± 4.4 males for Chinese females held with Korean males and 16.4 ± 4.0 males for Korean females held with Chinese males; $F_{1,18} = 0.24$; $P = 0.648$). In contrast, the within-population crosses produced both male and female offspring (Chinese population: males, 13.9 ± 3.8; females, 16.7 ± 4.6 and Korean population: males, 13.3 ± 3.5; females, 17.5 ± 4.3). Within-population crosses produced significantly more total offspring than between-population crosses ($F_{1,39} = 5.34$; $P = 0.027$), but there was no significant effect of *Binodoxys* population or the interaction between population and cross type (*Binodoxys* population: $F_{1,39} = 0.13$, $P = 0.718$; and interaction: $F_{1,39} = 0.10$, $P = 0.750$).

No copulation was observed during any of the behavioral observations of Chinese/Korean population pairs. The males exhibited courtship behavior (wing fanning and chase) and to a lesser extent mounting attempts, but copulation did not occur (Table 3). When encountering Korean females, similar proportions of Chinese and Korean males exhibited wing fanning, chasing, and mounting attempts. In contrast, significantly higher proportions of Chinese than Korean males exhibited chasing and mounting attempts when encountering Chinese females (Table 3). Courtship behaviors and mating attempts were observed at high rates during the within-population crosses but successful copulation occurred only in 36 and 26% of cases for Chinese and Korean populations, respectively. When copulation was observed (in within-population crosses), the mean time that elapsed before a mating attempt was 64.3 ± 22.6 s for the Chinese population and 61.3 ± 42.5 s for the Korean population ($t = 0.95$, $P > 0.05$). The spermathecae of all females of the between-population crosses were empty 4 h after the crosses were initiated, whereas spermatozoa were visible in spermathecae of females from the within-population crosses (Fig. 1) for which copulation had been observed.

When males and females from the four possible crosses were placed into gel caps for 1 h, spermatozoans were found in the spermathecae of females from both of the within-population crosses, but neither of the between-population crosses.

Comparison of Mitochondrial DNA (mtDNA) and rDNA Sequences. A 417-bp fragment of the mitochondrial 16S rRNA gene was amplified from all individuals from which DNA was isolated. The sequences of these fragments were identical among the individuals within both populations (GenBank accession FJ024082). A 646-bp fragment was obtained for the D2 expansion area of 28S rRNA (GenBank accessions FJ798199 and FJ798200). DNA sequences were similarly identical among all individuals of both populations. The ITS1 region was sequenced in its entirety (765 bp) and differed at position 519 with an insertion of a triplet of nucleotides (GAT) in Korean population. The rest of the ITS1 region was identical between the two populations (Chinese population: GenBank accession FJ811824). For the COI gene, a 709-bp PCR fragment was obtained for all individuals examined and sequences were consistent among individuals of a given population. A single nucleotide substitution was observed between the Chinese (GenBank accession FJ798201) and Korean populations (GenBank accession FJ798202); the Chinese population displayed the nucleotide C, whereas the Korea population displays the nucleotide T at position 489.

Parasitoid Host Range. Mummy and adult production differed significantly among aphid species offered to females of the Korean population (Fig. 2A; mummy: $F_{19,208} = 15.20$, $P < 0.001$ and adult: $F_{19,208} = 14.73$, $P < 0.001$). The Korean population produced the most mummies and adults on *A. glycines*, *Aphis monardae* Oestlund, *Aphis gossypii* Glover, *Aphis oestlundi* Gillette, and *Aphis nasturtii* Kaltenbach. In contrast, relatively few mummies and adults were produced on *Aphis rumicis* L., *Aphis craccivora* Koch, *Rhopalosiphum maidis* (Fitch), *Sitobion avenae* (F.), and *Aphis*

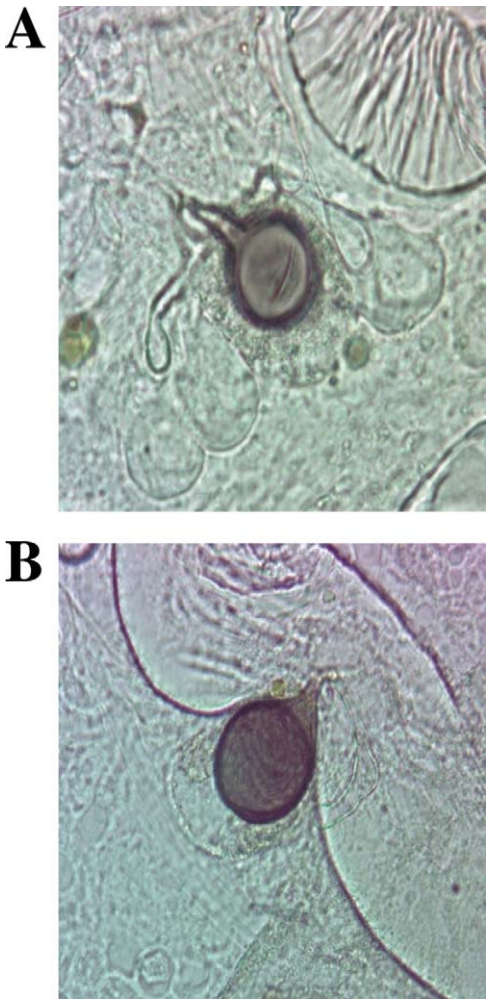


Fig. 1. Photographs of a spermatozoan-free spermatheca (A) and a spermatheca containing spermatozoans 4 h after mating (B). Magnification, 1,000×. (Online figure in color.)

nerii (Boyer de Fonscolombe) and no mummies were observed on *Macrosiphum euphorbiae* (Thomas), *Rhopalosiphum padi* (L.), *Acyrthosiphon pisum* (Harris), *Lipaphis erysimi* (=*L. pseudobrassicae*) (Kaltenbach), *Myzus persicae* (Sulzer), and *Uroleucon leonardi* (Olive).

Emergence rates of Korean population adults differed significantly among aphid species as well ($F_{14,100}$ = 1.84; P = 0.045) with *A. glycines* and *Schizaphis graminum* (Rondani) exhibiting the highest adult emergence rates (0.87). The parasitoid sex ratio was significantly female-biased only in *Aulacorthum solani* but significantly male-biased in *Aphis asclepiadis* Fitch, *A. monardae*, *A. gossypii*, *A. oestlundi*, *A. nasturtii*, *A. craccivora*, and *S. graminum* (Fig. 2A).

The Korean population yielded significantly fewer mummies than the Chinese population did on *A. monardae* and *A. asclepiadis*, but significantly more on *A. oestlundi* and *A. craccivora* (Fig. 2). The mummy emergence rates were significantly higher for the Korean population than the Chinese population for *A. glycines*, *A. monardae*, and *A. gossypii*, which were the three species that produced the most mummies for the Korean population. Parasitoid sex ratios differed significantly between the two populations on *A. monardae* and *S. graminum* (less male-biased in the Korean population) as well as on *A. gossypii* (more male-biased in the Korean population).

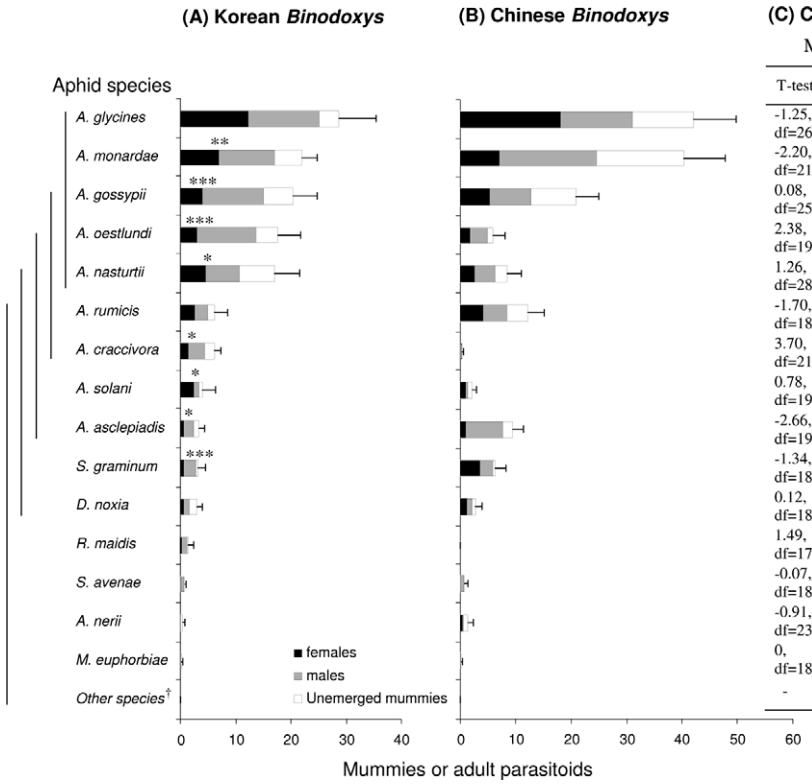
Description of *B. koreanus* Starý, n. sp. Information on reproductive isolation and genetic differentiation led us to conclude that the Chinese and Korean populations should be classified as separate species. Further morphological analysis suggested that the Chinese population indeed belonged to *B. communis*, but that the Korean population represented a new species. The species is named according to its area of distribution, Korea; *Binodoxys koreanus* Starý, n. sp. Deposition: Holotype-female—U.S. National Museum (USNM), Washington, D.C. Paratypes : USNM, University of Minnesota and the National Institute of Agricultural Science and Technology, Suwon, S. Korea, and coll. P. Starý (České Budějovice—17 females: 9-dry mounted, 8 as slides; 28 males).

The new species resembles *B. communis* but differs in characters of the petiole. The spiracular tubercles are distinct and acutely pointed and the petiole is distinctly constricted between the spiracular and secondary tubercles (Fig. 3A). In contrast, the petiole in *B. communis* bears poorly distinguishable spiracular tubercles, and dilates slightly and gradually to the secondary tubercles (Fig. 3B).

Female. Eyes large; width of malar space equal to one sixths of eye length. Tentorial index (=tentorio-ocular line/inter-tentorial line) equal to one fourth. Maxillary palpi four-segmented, labial palpi two-segmented. Antennae 11-segmented, very slightly thickened toward the apex. F1 is 3 times longer than wide, with no placodes. F2 2.5 times longer than wide with one placode.

Mesonotum smooth, notauli distinct in the fore ascendent part, with sparse setae before the prescutellar furrow. Propodeum (Fig. 3C) with complete central pentagonal areola, carinae somewhat irregular with some short adventive carinae. Forewing (Fig. 3D) with triangular pterostigma 3 times longer than wide, metacarpus half as long as pterostigma, radial vein as long as pterostigma. Petiole (Fig. 3A) twice as long as its width at the spiracles, spiracular tubercles relatively prominent and distinct, secondary tubercles obtusely prominent. Petiole distinctly constricted between spiracular and secondary tubercles, slightly wider at the secondary tubercles than at the spiracular tubercles. Genitalia: Ovipositor sheaths (Fig. 3E) narrow, anal prongs straight, slightly arcuate apically, with four to five upper marginal setae and five to eight lower marginal setae, upper setae about twice as long as lower, and two apical setae of equal length. Body length between 1.4 and 1.6 mm.

Coloration. Head brown, lower border of gena and clypeus yellow. Mouthparts yellow, apices of mandibles brown. Antennae brown, scape, pedicel, and flagellar segments one and two distinctly yellow to



(C) Comparison of the two populations

	Mummies		Emergence		Sex ratio
T-test	*P*	X^2	*P*	X^2	*P*
-1.25, df=26	0.222	5.07	**0.024**	1.83	0.177
-2.20, df=21	**0.039**	20.13	**<0.001**	5.52	**0.019**
0.08, df=25	0.939	1057	**0.001**	9.85	**0.002**
2.38, df=19	**0.028**	0.79	0.375	2.62	0.105
1.26, df=28	0.217	3.10	0.079	0.13	0.719
-1.70, df=18	0.107	2.74	0.098	0.13	0.718
3.70, df=21	**0.001**	0.20	0.655	1.40	0.236
0.78, df=19	0.444	1.29	0.257	0.06	0.805
-2.66, df=19	**0.015**	0.55	0.459	0.24	0.626
-1.34, df=18	0.198	0.99	0.319	8.35	**0.004**
0.12, df=18	0.902	2.26	0.133	0.32	0.571
1.49, df=17	0.153	-	-	-	-
-0.07, df=18	0.945	0.02	0.896	0.02	0.897
-0.91, df=23	0.370	0.32	0.572	0.97	0.324
0, df=18	1	0	1	-	-
-	-	-	-	-	-

Fig. 2. (A) Mean numbers adult female and male offspring as well as unemerged mummies produced per aphid species for the Korean population, in descending order of total mummies. Means for total mummies produced by aphid species subtended by lines do not differ ($P > 0.05$; ANOVA with Tukey's post hoc test). Asterisks indicate deviation from a 0.5 sex ratio: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). †Other species included *A. pisum*, *L. erysimi*, *M. persicae*, *R. padi*, and *U. leonardi*. (B) The same data for the Chinese population, from Desneux et al. (2009b). (C) Statistical analyses for comparison of the two populations. *P* values below 0.05 are in bold.

yellow brown. Mesosoma brown, propleura and prosternum yellow, propodeum with yellow-brown patterns. Wings hyaline, venation brownish, tegulae brown. Forelegs yellow, middle legs with femora and parts of tibiae brownish, hind femora and tibiae brown except basal margin of hind tibiae. Coxae and trochanters yellow, hind coxae partially brown, apices of all legs brown. Petiole and the basal spot on tergite 2 yellow, the rest of metasoma brown. Ovipositor sheaths and prongs dark brown.

Male. Similar to females, except antennae 13-segmented, coloration somewhat darker than in the female (base of antennae brown).

Discussion

A Korean population of *Binodoxys* that was originally identified as *B. communis* based upon morphological keys is reproductively isolated from a population of *B. communis* collected in Northeastern China. We discovered minor differences in nucleotide sequences and host use patterns between the populations and subtle morphological differences as well. In previous studies, we documented substantial differences in handling and stinging times of *A. glycines* by these two species (Desneux et al. 2009a) as well as slight differences in development times (S. Acheampong, K. Wyckhuys, and G.E.H., unpublished.). Based upon these lines of evidence, we argue that the Korean population is actually a separate species and propose the name *B. koreanus* Starý, sp. n.

As noted in the introduction, there has been an explosion in the discovery of cryptic species in parasitoid lineages (recent examples include Kankare et al. 2005a,b; Smith et al. 2006, 2007, 2008; Heraty et al. 2007; Kathirithamby 2009). Our study represents the strongest data to date in support of cryptic species within the aphidiine braconids although allozyme evidence consistent with cryptic species has been reported for *Aphidius ervi* Haliday and related species (Unruh et al. 1989, Atanassova et al. 1998). Earlier work on *Trioxys*, a genus closely related to *Binodoxys*, also was suggestive of cryptic species. In this case, two populations were originally classified as *Trioxys utilis* Muesebeck until it was discovered that they had quite different host and habitat ranges, and partial reproductive compatibility, which led to further systematic work and the separation of one population as *Trioxys pallidus* (Haliday) (Hall et al. 1962). Thus, our study adds to the

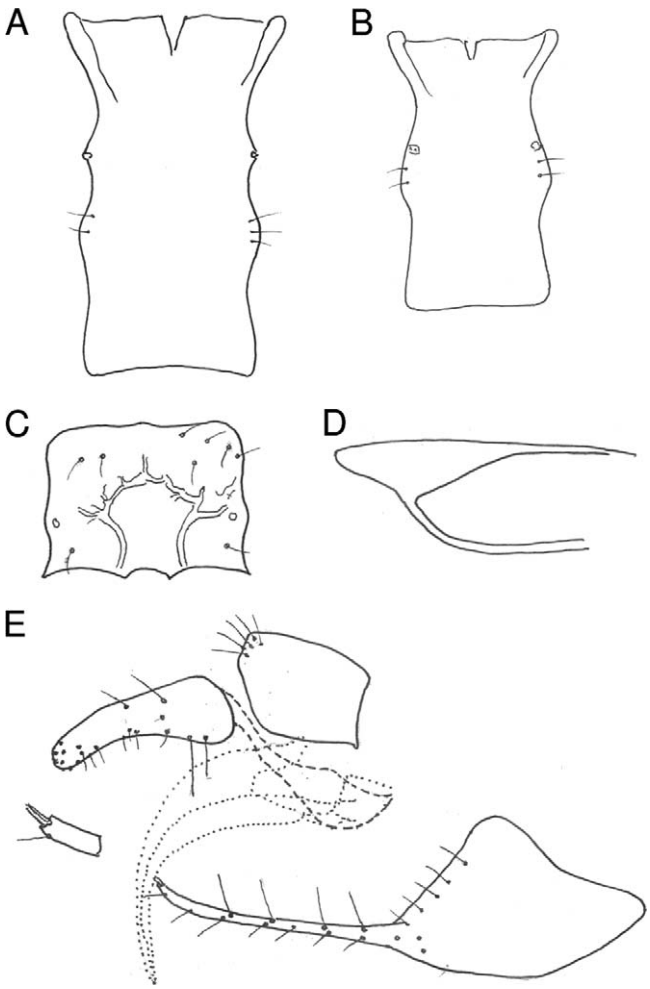


Fig. 3. (A) Petiole of female *B. koreanus* n. sp. female paratype. (B) Petiole of *B. communis.* (C) Propodeum of *B. koreanus* n. sp., female paratype. (D) Forewing (detail) of *B. koreanus* n. sp. female paratype. (E) Genitalia of *B. koreanus*, n. sp. female paratype.

burgeoning literature on cryptic species in parasitoids in general, and aphidiine braconids in particular.

Our finding that *B. communis* and *B. koreanus*, sp. n. are cryptic species comes despite nearly identical sequences at the mitochondrial COI gene. The portion of the COI sequence that we used is the popular DNA barcoding region that has been used to identify cryptic species in other species, including parasitoids (Smith et al. 2006, 2007, 2008). Our finding of cryptic species despite nearly identical barcode sequences may indicate recent divergence of the two species and also demonstrates that widely used barcoding sequences are not always sensitive enough to separate cryptic species. Two cryptic species of leafminer parasitoids in the encyrtid genus *Ageniaspis* similarly showed virtually no nucleotide divergence in COI sequences, although three other molecular markers showed substantial levels of genetic divergence between the two species (Hoy et al. 2000, Alvarez and Hoy 2002).

Although cryptic species are by definition morphologically indistinguishable based upon information available at the time of the initial identification, further study often reveals previously-unrecognized morphological differences (Schlick-Steiner et al. 2007). This has been the case for a number of other cryptic parasitoid species (Clarke 1993, Woolley et al. 1994, Manzari et al. 2002, Giorgini and Baldanza 2004). In our case, the new species *B. koreanus*, sp. n. keyed out to *B. communis* using the taxonomic works of *Binodoxys* spp. in the Far East (Starý and Schlinger 1967, Chen and Shi 2001), but upon further inspection the subtle morphological differences that are reported on here were identified.

We found little evidence that *B. communis* and *B. koreanus*, sp. n. specialize on different host species, as has been found in other complexes of cryptic parasitoid species (Heimpel et al. 1997; Fernando and Walter 1997; Kankare et al. 2005a,b; Smith et al. 2006, 2007, 2008; Heraty et al. 2007; Abrahamson and

Blair 2008; Bernardo et al. 2008; Kathirithamby 2009). Both parasitoid species performed best on soybean aphid, which is the host that both were collected and reared on. However, some differences in host-use patterns were found. In particular, *B. koreanus*, sp. n. produced more mummies on *A. craccivora* than did *B. communis*. The colony of *A. craccivora* used for these assays harbored the bacterial endosymbiont *Hamiltonella defensa* Moran, and previous studies suggested that these endosymbionts were probably involved in resistance of *A. craccivora* to *B. communis* (Desneux et al. 2009b) as is the case for *H. defensa* in the pea aphid, *Acyrthosiphon pisum* (Oliver et al. 2003, 2005). That *B. koreanus*, sp. n. was able to parasitize this host with some success suggests that it is at least partially able to overcome the resistance conferred by this endosymbiont. As is the case for *B. communis* (Desneux et al. 2009b), *B. koreanus*, sp. n. offspring production seems to match the phylogenetic proximity of aphid species to *A. glycines*, the most suitable host in the host range experiment. All of the most suitable hosts are members of the genus *Aphis*, including two species that are new associations for *B. koreanus*—both *A. monardae* and *A. oestlundi* are native to North America. Exceptions to the phylogenetic trend involved aphids (*A. asclepiadis* and *A. nerii*) on plants with toxic plant compounds implicated in reducing parasitoid fitness (Desneux et al. 2009b).

Bickford et al. (2007) reasoned that cryptic species would be prevalent in taxa with little selection for morphological differentiation, because initial species designations are based upon morphological characters. They discussed two conditions that might lead to a paucity of morphological differentiation: nonvisual mate recognition and morphological stasis. Along these lines, Henry (1994) made a convincing argument that cryptic speciation is particularly likely in insects that use acoustic signaling as mating signals because the subtle differences in sound needed to differentiate one species from another do not necessitate morphological differentiation. We have no evidence that *Binodoxys* species use acoustic signaling in courtship, but it seems likely that chemical communication is used, which can similarly produce differences among species without visual differentiation. Cryptic species of aphid parasitoids in the genus *Aphelinus* use female-produced trail pheromones as mating cues (Fauvergue et al. 1995, Kazmer et al. 1996), which could in principle allow the evolution of reproductive isolation without noticeable morphological change. Turning to the second condition of Bickford et al. (2007)—morphological stasis—it has been argued that selection for morphological stasis is expected in parasites that have a particularly intimate relationship with their hosts. Schonrogge et al. (2002) singled out koinobiont parasitoids, along with true parasites and social parasites, as being particularly susceptible to cryptic speciation. *Binodoxys* species are koinobiont parasitoids, as are many parasitoid groups that have been found to contain cryptic species complexes (Hoy et al. 2000; Kankare et al. 2005a,b; Quicke et al. 2006; Smith et al. 2006, 2007, 2008; Heraty et al. 2007; Kathirithamby 2009). However, cryptic species have been found in idiobiont parasitoid lineages as well (Clarke 1993, Fernando and Walter 1997, Woolley et al. 1994, Heimpel et al. 1997, Pinto et al. 2003, Bernardo et al. 2008), and in members of the genus *Eretmocerus* (Hymenoptera: Aphelindiae), which are facultative koino/idiobionts (Hunter et al. 1996, Jones and Greenberg 1998).

In conclusion, we have identified a new *Binodoxys* species attacking soybean aphid in Asia. Releases of *B. communis* have been ongoing in the North Central United States since 2007 (Wyckhuys et al. 2007a; G.E.H., unpublished). *B. koreanus*, sp. n. is one of several species currently being held in quarantine that could potentially also be released against soybean aphid (Wyckhuys et al. 2007a; P.S. et al., unpublished). Should *B. koreanus*, sp. n. be released alongside the closely related *B. communis* in North America? Because *B. koreanus*, sp. n. and *B. communis* exhibit premating reproductive isolation, the production of low-fitness hybrid offspring is very unlikely to occur. It is conceivable, however, that interspecific courtship could occur, and this could entail some time costs and increase the fraction of females that remain unmated. We also found that females exposed to heterospecific males produced fewer total offspring than females exposed to conspecifics males, a result consistent with previous findings that virgin females of other aphidiine braconids produced fewer offspring than mated females (Michaud 1994). The broader questions of whether a release of this second species would result in more effective biological control overall, or whether safety to nontarget organisms would be affected, is beyond the scope of this study.

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