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On the evolution of the species complex *Pachycondyla chinensis* (Hymenoptera: Formicidae: Ponerinae), including the origin of its invasive form and description of a new species

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Abstract

Ants are one of the most successful and widespread organisms in the world. Although ants of the genus *Pachycondyla* (Hymenoptera: Formicidae: Ponerinae) are predominantly tropical in distribution, *Pachycondyla chinensis* (Emery) is especially abundant in temperate zones in Asia. Recently, *P. chinensis* has also become an abundant invasive ant species in the United States. However, it was well-known that the *Pachycondyla chinensis* species complex remained unresolved. Our molecular and morphological results allow us to distinguish two species in the species complex: the species *P. chinensis* and the new cryptic species *P. nakasujii* **sp. nov.**, and these two species are widely and sympatrically distributed and abundant in temperate forests in Japan. Moreover, phylogenetic analysis showed that *P. chinensis* has been introduced into the United States from Japan. In conclusion, our finding of the new species *P. nakasujii* suggests that much remains undiscovered even in biologically fascinating and well-studied organisms.

Key words: *Pachycondyla nakasujii*, cryptic species, invasive ant, phylogeography

Introduction

The ants arose more than a hundred million years ago and eventually spread around the world (Brady *et al.* 2006; Moreau *et al.* 2006; Moreau 2009), and subsequently diversified, with differences among biogeographic regions in their species, genera and even subfamilies (Hölldobler & Wilson 1990; Bolton *et al.* 2006). Recently, humans have begun to, however unintentionally, transport ants outside of their native range, with more than a hundred and perhaps hundreds of such introduced species now established and a smaller subset invasive (McGlynn 1999). Such invasive ants often pose problems for the well-being of natural communities and ecosystems, agricultural production, and public health (Kemp *et al.* 2000; Holway *et al.* 2002; Klotz *et al.* 2005; Strayer *et al.* 2006).

Mirroring the pattern for ants more generally, the genus *Pachycondyla* (Hymenoptera: Formicidae: Ponerinae) evolved no later than 75 million years ago, when the continents had separated (Bolton *et al.* 2006; Brady *et al.* 2006; Moreau *et al.* 2006). Species of the genus then apparently dispersed from one continent to another before further diverging into the 209 extant species now recognized (Bolton *et al.* 2006; Mackay & Mackay 2006; Yamane 2007). Ants of the genus *Pachycondyla*, like many ponerine ants, are now predominantly tropical in distribution, with few exceptions (Bolton *et al.* 2006). As a consequence, the origin of *Pachycondyla* species with temperate distributions is particularly interesting. *Pachycondyla chinensis* (Emery) (formerly *Brachyponera chinensis*), widely distributed from Far Eastern Asia to Southeast Asia, is

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one such species (Chou & Terayama 1991; Zhenghui 1994; Japanese Ant Database Group 2003; Eguchi *et al*. 2005; Bolton *et al*. 2006). Also interesting in this context is the recent discovery that *P. chinensis* has become an abundant invasive ant species in the United States (Smith 1934; Bolton *et al*. 2006; Leath *et al*. 2006; Nelder *et al*. 2006; Guénard & Dunn 2010).

The genus *Pachycondyla* is extremely diverse with respect to morphology and is likely to be polyphyletic (Ward 2007). However, recent molecular analyses include only a limited number of taxa of the genus *Pachycondyla* (e.g. Ohnishi *et al*. 2003; Astruc *et al*. 2004; Brady *et al*. 2006; Moreau *et al*. 2006; Ouellette *et al*. 2006). Thus, many species complexes in *Pachycondyla* remain unresolved (e.g. Lucas *et al*. 2002; Wild 2005). *Brachyponera*, which is currently synonymized with the genus *Pachycondyla* (Bolton *et al*. 2006), seems a well-defined and morphologically consistent group (Yamane 2007). Unfortunately, no phylogenetic studies based on DNA sequence data have been conducted on *Brachyponera* and, therefore it remains tangled in a suite of species complexes (Yamane 2007).

The Japanese and Ryukyu archipelagoes, in which the *Pachycondyla chinensis* species complex is found, as well as Taiwan, form an island-arc in Far Eastern Asia. The Japanese archipelago consists mainly of Hokkaido, Honshu, Shikoku, and Kyushu, and the Ryukyu archipelago consists mainly of Amami-Oshima Island, Okinawa Island, and Iriomote Island from north to south. *Pachycondyla chinensis* is widely distributed in the temperate (Honshu, Shikoku, and Kyushu) and subtropical (the Ryukyu archipelago) zones except for the subarctic zone (Hokkaido) in Japan. It is common in southern Honshu and the more southern Japanese islands (Japanese Ant Database Group 2003). *Pachycondyla chinensis* and the closely related species *P. luteipes* (Mayr) (formerly *B. luteipes*) are sympatrically distributed in Iriomote Island and nearby islands including Taiwan (Chou & Terayama 1991; Japanese Ant Database Group 2003).

A complicating feature of *P. chinensis* is that it displays great morphological variation, such that morphological studies have proven difficult. Brown (1958) famously suggested this group might not ever be understood. And so, where did this species evolve and what is its current phylogenetic structure? Here, we use data on the mitochondrial cytochrome *c* oxidase subunit I gene (COI) to understand the evolution of the only temperate *Pachycondyla* species and the genetic structure underlying its great and complicated morphological variation.

Material and methods

Collection and preservation of specimens. We collected a total of 47 mature ant colonies of the *Pachycondyla chinensis* species complex: 38 in the native range (Honshu, Shikoku, Kyushu, Amami-Oshima, Okinawa, and Iriomote Island, Japan, and Taiwan) and 9 in the invaded range (North Carolina, South Carolina, and Georgia, USA). We also collected a total of 9 mature ant colonies of the closely related species *P. luteipes* on Amami-Oshima Island and Iriomote Island, Japan, and Taiwan. Nests of all of these ants were most commonly found in rotten wood or leaf litter. Workers, queens, and males were extracted with an aspirator from the nest and preserved in 99.5% ethanol for DNA extraction or in 70% ethanol for morphological study. We haphazardly selected two workers from each colony, one for DNA extraction and one for morphological study.

Phylogenetic analysis. A 565-bp fragment of COI corresponding to positions 1683 to 2247 in the *Drosophila yakuba* Burla mitochondrial genome (Clary & Wolstenholme 1985) was used for phylogenetic analysis. DNA was extracted from individual worker heads using a Chelex protocol modified from Walsh *et al*. (1991). The primers CI-13 (5'-ATAATTTTTTTTATAGTTATACC-3') and CI-14 (5'-GTTTCTTTTTTTCCTCTTTC-3'), designed by Hasegawa (unpubl.), were used for polymerase chain reaction (PCR). The primers '3' ends correspond to positions 2002+ (CI-13) and 2568- (CI-14) in the mitochondrial DNA sequence of *Apis mellifera* Linnaeus (Crozier & Crozier 1993). PCR consisted of 40 cycles of denaturing at 94°C for 30 s, annealing at 48.5°C for 30 s, and extension at 72°C for 40 s and resulted in an amplification product of 608-bp. PCR products were sequenced in both directions (Big Dye Terminator cycle sequencing, electrophoresis on an ABI 3100; Applied Biosystems, USA). The primer sequences were removed and the remaining 565-bp COI portion was used. The COI region of the species complex

Pachycondyla chinensis and four other ant species were used for phylogenetic analysis. Consensus sequences were aligned using the ClustalX algorithm (Thompson *et al*. 1997) from the BioEdit 7.0.4.1 sequence editor (Hall 1999) and corrected manually. Bayesian inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with the GTR + G model selected by the hierarchical Likelihood Ratio Test (hLRT) in MrModeltest 2.3 (Nylander, 2004). *Cryptopone sauteri* (Wheeler) (GQ264538) was used as an outgroup. Two runs with four chains of Markov chain Monte Carlo iterations were performed for 2,000,000 generations, when the average standard deviations of split frequencies were below 0.01 (the first 25% of the generations as burn-in). Trees were kept for every 100 generations, and the latest 75% of the trees were used to calculate 50% majority-rule trees and to determine the posterior probabilities. All mitochondrial sequence data of ants were deposited in the DDBJ/EMBL/GenBank mitochondrial sequence database. Accession numbers are provided in Fig. 1.

Morphological study. A total of 48 workers (including the holotype specimen of *P. chinensis* borrowed from the Museo Civico di Storia Naturale "Giacomo Doria", Genova, Italy) were measured using an Olympus stereo microscope with a digital imaging system (FLVFS-LS, Flovel, JAPAN). Seven measurements were taken: Head width (HW), the maximum width of the head in frontal view, posterior to and not including the eyes; Head length (HL), in frontal view, from the maximum posterior extention of the head to the anterior clypeal margin; Scape length (SL), the length of the antennal scape, not including the basal condyle; Eye length (EL), the maximum diameter of the eye in lateral view; Weber length (WL), the distance from the anterior face of the pronotum to the maximum posterior extension of the metapleuron, in lateral view; Mesosoma width (MW), the maximum width of the mesosoma, in dorsal view; Dorsal petiolar width (PW), the maximum width of the petiole in dorsal view. To compare biometric data, we used a non-parametric test (Mann-Whitney *U*-test; Statistica 03J, StatSoft, USA).

Results

Phylogenetic analysis

The phylogenetic tree based on mitochondrial sequences of the COI region illustrated that the *Pachycondyla chinensis* species complex contains at least two species: the species *P. chinensis* and the new cryptic species *P. nakasujii*. *Pachycondyla chinensis*, *P. nakasujii*, and *P. luteipes* each have posterior probability support =1.00. The *P. chinensis* clade is composed of three subclades. Two of the subclades constitute a temperate lineage and the third subclade is composed of the subtropical lineage (Fig. 1).

All nine sequences of *P. chinensis* in the United States were identical to each other (GenBank accession numbers GQ264547–GQ264549, GQ2645463–GQ264565, and GQ264570–GQ264572 for Georgia, South Carolina, and North Carolina, respectively) and were identical with eight sequences of *P. chinensis* in the temperate zones in Japan (GenBank accession numbers GQ264541, GQ264544, GQ264546, GQ264552, GQ264557, GQ264559, GQ264560, and GQ264568). The phylogenetic analysis showed that *P. chinensis* in the United States was contained in a well-supported cluster together with *P. chinensis* in the temperate zones in Japan (0.98 posterior probability support) (Fig. 1).

Morphological studies

All morphological traits measured were statistically significantly different ($P < 0.05$) between *P. nakasujii* and *P. chinensis*. All traits measured were significantly smaller in *P. nakasujii* than in *P. chinensis* with one exception: PW is larger in *P. nakasujii* ($P < 0.00001$, $P < 0.00001$, $P < 0.00001$, $P < 0.00001$, $P < 0.00001$, $P < 0.00001$, and $P < 0.05$ for HW, HL, SL, EL, WL, MW, and PW, respectively) (Fig. 2).

Bivariate plots of morphometric measurements of PW vs. HW clearly differentiate between *P. nakasujii* and *P. chinensis* (including the holotype) (Fig. 3).

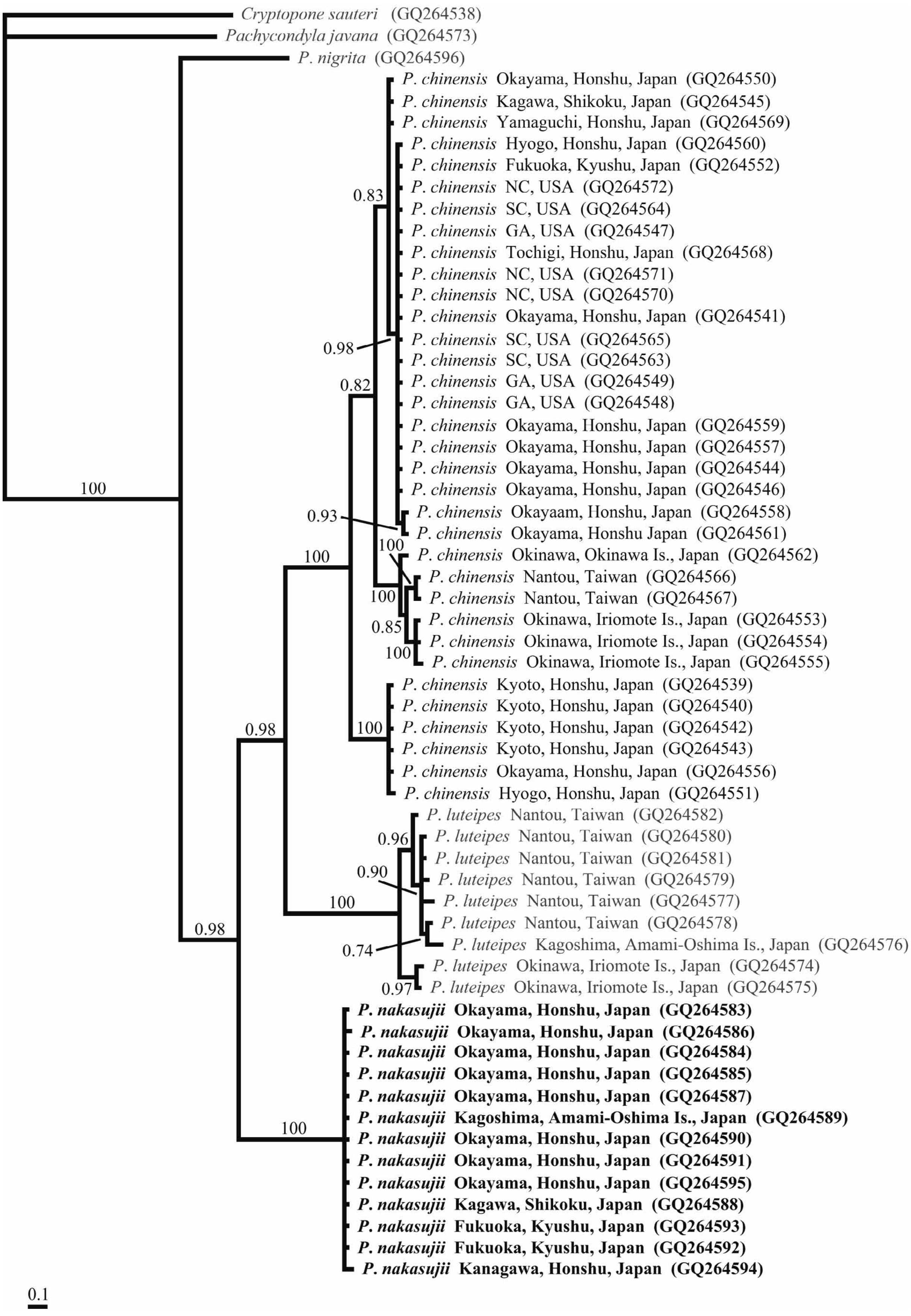

FIGURE 1. Bayesian phylogenetic tree of the species complex *Pachycondyla chinensis* based on mitochondrial sequences of the COI region. The corresponding posterior probabilities (≥0.70) are shown by the branch. The horizontal bar represents a distance of 0.1 substitutions per site. GenBank accession numbers are shown in parentheses.

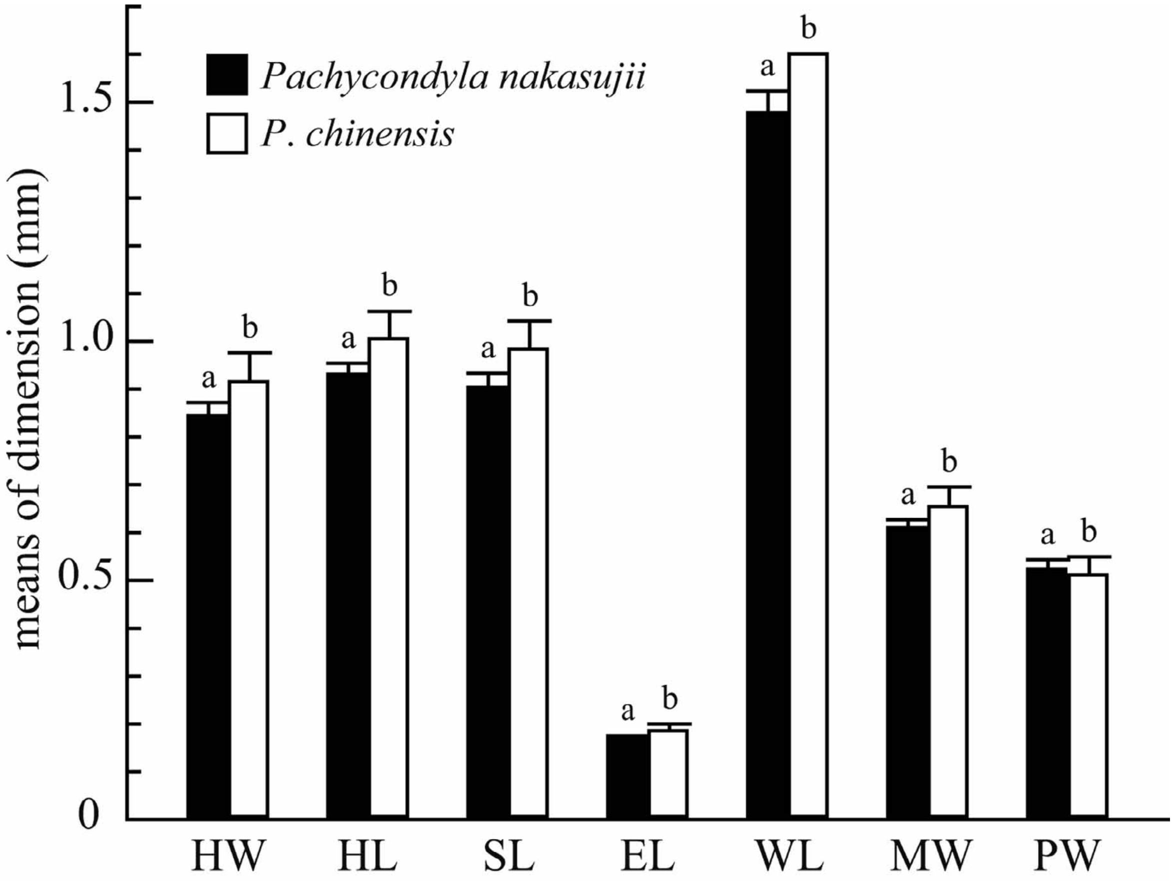

FIGURE 2. Mean (± SD) of the biometric measurements of *P. nakasujii* and *P. chinensis* (n = 13 and 35, respectively).

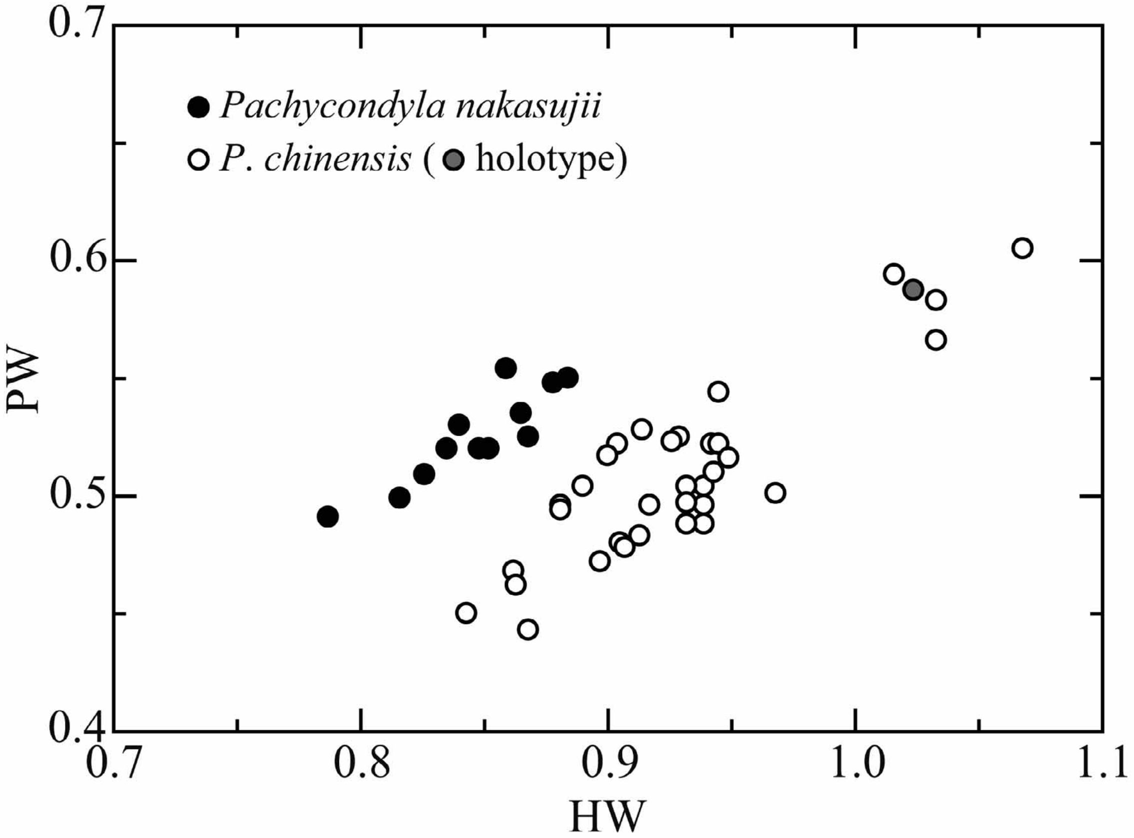

FIGURE 3. Bivariate plots of PW by HW measurements (mm) of *P. nakasujii* and *P. chinensis*.

Species description

Pachycondyla nakasujii sp. nov.

(Figs 4, 5)

Worker: *Measurements* (mm) holotype (paratypes, n = 15): HW 0.83 (0.79–0.85); HL 0.91 (0.88–0.94); SL 0.87 (0.83–0.92); EL 0.15 (0.14–0.18); WL 1.42 (1.36–1.47); MW 0.60 (0.59–0.64); PW 0.52 (0.49–0.55). *Structure*. Head slightly longer than wide, weakly round-sided behind eyes, with weakly emarginate posterior margin in full-face view. Mandible with 6 larger and 4 smaller teeth on the masticatory margin, and with distinct basal fovea. Antenna long, scape surpassing the posterior margin of head by less than 1/4 of its total length in full-face view; all funicular segments longer than wide. Pronotal dorsum bluntly demarcated from lateral face; seen from above anterolateral corner round. Mesonotum distinctly demarcated from pronotum and propodeum by deep furrows, posteriorly weakly emarginate. Mesopleuron with a somewhat vestigially transverse groove. In profile propodeum demarcated by a distinct obliquely running suture from metapleuron; posterior face well demarcated from lateral face by a rather sharp ridge, but separation from dorsal face indistinct. Petiole thin and high, with weakly convex dorsal margin in profile; in posterior view petiolar scale round and slightly higher than wide, with convex dorsal margin. *Sculpture*. Dorsum of head minutely and densely punctate. Clypeus with much more superficial sculpture and shiny except for sculptured median portion. Mandible finely striatopunctate except for the area around apex and along mesal margin smooth with large sparse punctures. Dorsum of pronotum finely punctate; its lateral face and mesonotum with still finer punctation; mesopleuron almost smooth and shiny, with rugulae in lower area; upper portion of metapleuron punctate, with smooth interspaces, lower portion rugose, with smooth interspaces. Dorsolateral face of propodeum irregularly sculptured and matte, posterior face smooth except for margins and upper portion weakly sculpututred. Petiole anteriorly micropunctate, posteriorly smooth. Gastral segments densely and weakly punctate evenly over the surface. *Pilosity*. Body covered with decumbent pubescence except for mesopleuron, metapleuron, and posterior faces of propodeum and petiole that are almost bare. Standing hairs sparse; gastral tergites 1 and 2 each with more than ten standing hairs. *Coloration*. Body dark brown to blackish; gaster slightly lighter than head and mesosoma. Antennal funiculus, mandible, subpetiolar process and legs yellowish brown to orangish.

Queen: *Structure*. Body distinctly larger than the worker, with the head width ranging from 0.86 to 0.87 mm. Eye much larger than the worker; EL = 0.22–0.25 mm. Ocelli small, ca. 0.07 mm in maximum diameter; distance between posterior ocelli greater than that between anterior and posterior ocelli. Mesopleuron above always with a distinct transverse groove. Compared with the worker, petiole relatively thin, seen from above almost lacking dorsal flat portion. *Sculpture*. Body sculpture much as in the worker except for distinctly punctate metapleuron. *Pilosity*. Body covered with decumbent pubescence except for mesopleuron, and posterior faces of propodeum and petiole that are almost bare. Eye with short fine standing hairs. Mesosoma dorsally and gastral tergites with numerous long standing hairs. *Coloration*. Much as in the worker.

Male: *Structure*. Head including eyes much wider than long. Eye large; EL = 0.26–0.27 mm, strongly convex, mesally weakly emarginate. Clypeus transverse with anterior and posterior margins truncate; labrum anteriorly produced as a narrow lobe. Mandible long and fragile, not opposable, basally wide and suddenly narrowed toward bluntly pointed apex. Ocelli small, ca. 0.08 mm in maximum diameter; distance between posterior ocelli much greater than that between anterior and posterior ocelli. Antenna long; scape twice as long as pedicel and shorter than segment 3. Pronotum short; mesoscutum large, with weak notauli and sharp parapsidal lines; mesopleuron large, with a wide transverse furrow which is striate on the bottom; scutellum dorsally convex with an anterior transverse zone which is much lower than the main disc; axilla extensively concave; metanotum short. Propodeum rounded; dorsal, lateral and posterior faces not clearly differentiated. Petiole relatively low, in profile rather strongly narrowed above, seen from back almost as wide as high, with dorsal margin almost straight; subpetiolar process flat, with obtuse posterior tooth in profile. *Sculpture*. Head superficially micropunctate. Pronotum, mesoscutum, metanotum and metapleuron weakly sculptured; mesopleuron extensively smooth and strongly shiny; axilla coarsely rugose. Propodeum more strongly and densely sculptured, but on its posterior face sculpture somewhat weaker. Anterior and posterior faces of

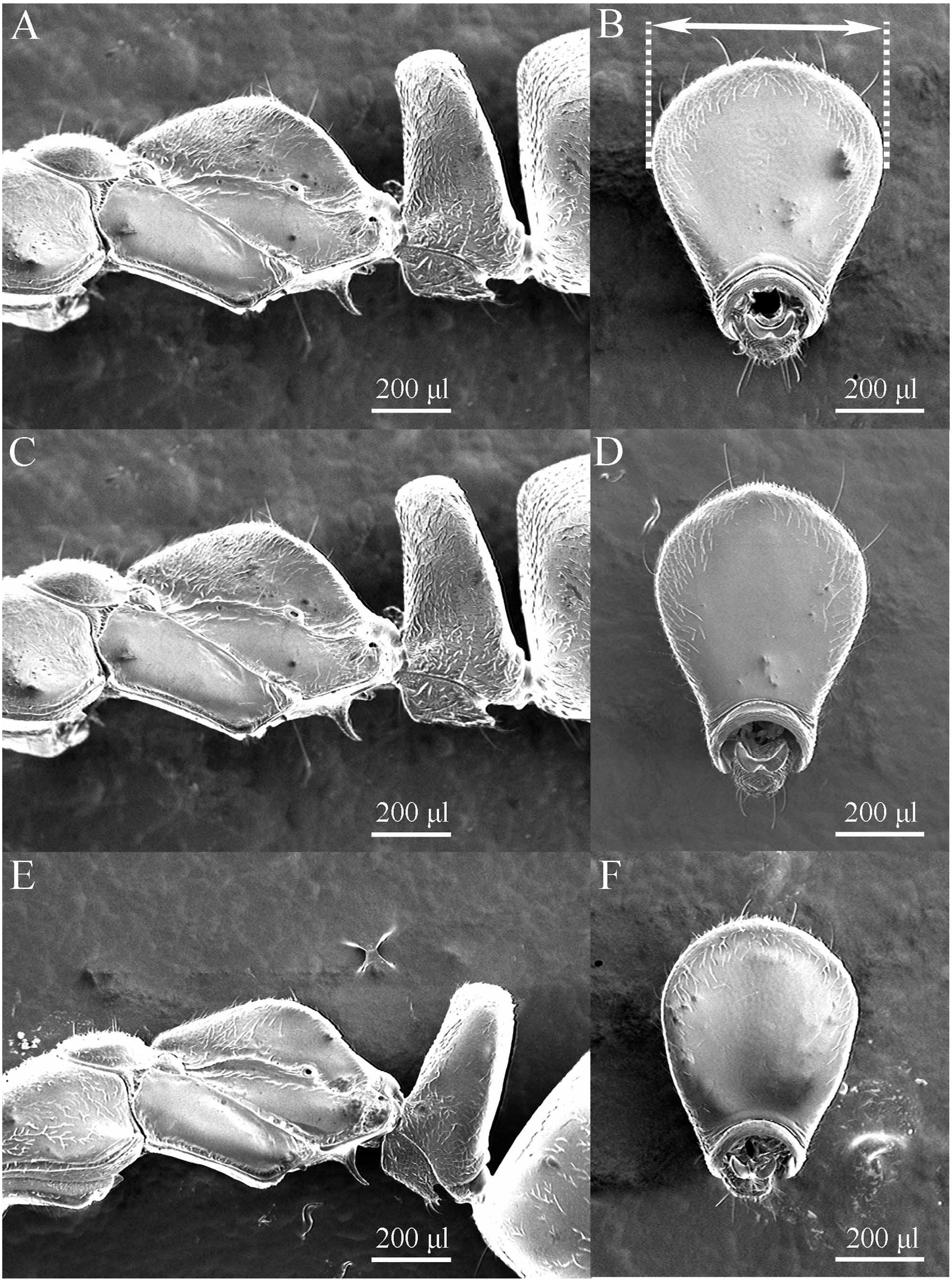

FIGURE 4. Characters of worker propodeum and petiole. The left side of worker propodeum and petiole of *P. nakasujii* (A), *P. chinensis* (C) and *P. luteipes* (E), and the posterior face of worker petiole of *P. nakasujii* (B), *P. chinensis* (D) and *P. luteipes* (F) (Scanning electron microscopy photographs). Horizontal white arrow: PW. Also see Japanese Ant Database Group (2003).

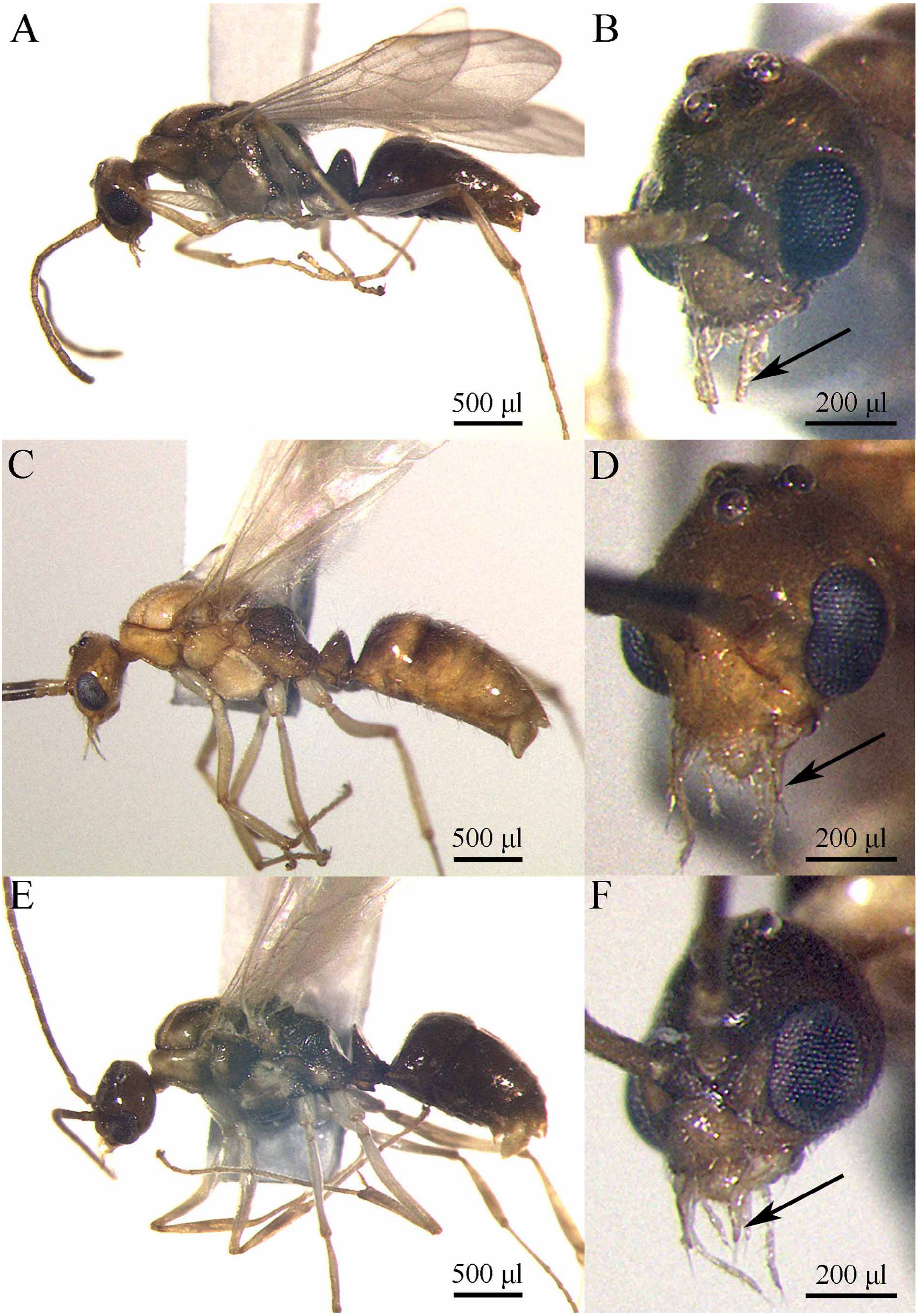

FIGURE 5. Characters of male coloration and mandible of *P. nakasujii* (A, B), *P. chinensis* (C, D) and *P. luteipes* (E, F). Black arrows indicate the mandible.

petiole densely and irregularly sculptured; posterior face very weakly sculptured and somewhat shiny. Gaster only superficially sculptured. *Pilosity.* Body covered with decumbent pubescence except for mesopleuron, and posterior faces of propodeum and petiole that are almost bare. Petiole extensively with dense obliquely standing short hairs on its anterior and lateral faces; subpetiolar process below with standing hairs. Outer margin of forewing and hindwing with dense short fringe. *Coloration*. Body brown to pale brown, with ivory white legs.

Type material examined. Holotype: worker, JAPAN, Okayama Prefecture, Okayama, Mt. Handayama, 34°41’47”N, 133°55’32”E, 22 m, 5 vi 2007, (coll. T. Yashiro). Paratypes: 3 queens and 8 workers from the same locality, 5 vi 2007, (coll. T. Yashiro); 3 males and 7 workers, JAPAN, Okayama Prefecture, Wake, Mt. Tenjinyama, 34°50’56”N, 134°07’47”E, 206 m, 4 vii 2008, (coll. T. Yashiro).

Type depository. The holotype (NSMT-I-Hym 51990) is deposited in the National Museum of Nature and Science, Tokyo. Paratypes are deposited in the National Museum of Nature and Science, Tokyo, Japan; the Osaka Museum of Natural History, Osaka, Japan; the Museo Civico di Storia Naturale “Giacomo Doria”, Genova, Italy; the Natural History Museum, London, England; and the Museum of Comparative Zoology, Harvard University, Cambridge, USA.

Additional material examined. 5 workers, JAPAN, Kanagawa Prefecture, Yokosuka, Hayama, 35°15’37”N, 139°36’23”E, 150 m, 7 v 2008, (coll. M. Yashiro); 1 queens and 5 workers, JAPAN, Kagawa Prefecture, Takamatsu, Nishiueta, 34°13’53”N, 134°4’38”E, 86 m, 14 x 2007, (coll. T. Yashiro); 1 queens and 5 workers, JAPAN, Fukuoka Prefecture, Tagawa, Mt. Hikosan, 33°28’52”N, 130°55’10”E, 994 m, 11 x 2007, (coll. T. Yashiro).

Etymology. This species is named in honor of Professor Emeritus Fusao Nakasuji, a notable Japanese entomologist and an authority on integrated pest management, who supervised the undergraduate and master's theses of one of the authors (Toshihisa Yashiro) at Okayama University.

Remarks. This species belongs to the *Pachycondyla chinensis* species complex. It is easily distinguished from the sympatric species *P. chinensis* in the worker by having a proportionally wider petiole (PI [Pronotal index: PW/HW x 100] = 60–64 in *P. nakasujii*, PI = 48–58 in *P. chinensis*) (Fig. 4B,D) and in the male by having much darker coloration and the well developed mandibles (Fig. 5A–D). Moreover, the worker of this species is easily distinguished from another closely related species, *P. luteipes*, by having a proportionally wider petiole (PI = 60–64 in *P. nakasujii*, PI = 52–58 in *P. luteipes*) (Fig. 4B,F) and a sculptured propodeum (largely smooth in *P. luteipes*) (Fig. 4A,E).

Pachycondyla nakasujii nests in rotten wood or leaf litter on the ground. It is common in Honshu, Shikoku, and Kyushu, Japan, where it is sympatric with *P. chinensis* (Fig. 6). Although *P. nakasujii* and *P. chinensis* are sympatrically distributed in temperate zones, *P. nakasujii* is rare in dry and disturbed area, where *P. chinensis* remains common. Both species have been observed to nest near termite nests frequently and to carry termites back to their nests as food.

Discussion

Our results allow us to distinguish two species in the *Pachycondyla chinensis* species complex: *P. chinensis* and the new cryptic species *P. nakasujii* (Figs 1, 2, 3). These species are widely distributed and abundant in Japan (Figs 1, 6). It is perhaps surprising that one can find a new and abundant species even in a highly developed region of Japan where there are many myrmecologists and broad interest among citizens in ants and ant biology, an indication that much remains undiscovered, nearly everywhere, even for well-studied taxa.

Pachycondyla nakasujii and *P. chinensis* are sympatrically distributed in temperate forests. The range of *P. nakasujii* and the more tropical and subtropical *P. luteipes* (Chou & Terayama 1991; Japanese Ant Database Group 2003; Bolton *et al*. 2006; Eguchi *et al*. 2005) are largely disjunct, overlapping only on Amami-Oshima Island (Fig. 1). Our record of *P. luteipes* from Amami-Oshima Island is new, despite the large number of entomologists who have collected on the island (Fig. 1), a discovery perhaps best explained by a recent invasion of *P. luteipes*. The complicated phylogenetic relationships within *P. chinensis* are possibly due in part to the apparent ease with which this clade is introduced to new habitats (Fig. 1), like other invasive ant species

(Suarez *et al*. 2001). Additional samples and analyses of *P. chinensis* and closely related species from Korea, mainland China, and Southeast Asia would be useful to better understand the origin and evolution of these few *Pachycondyla* species with temperate distributions.

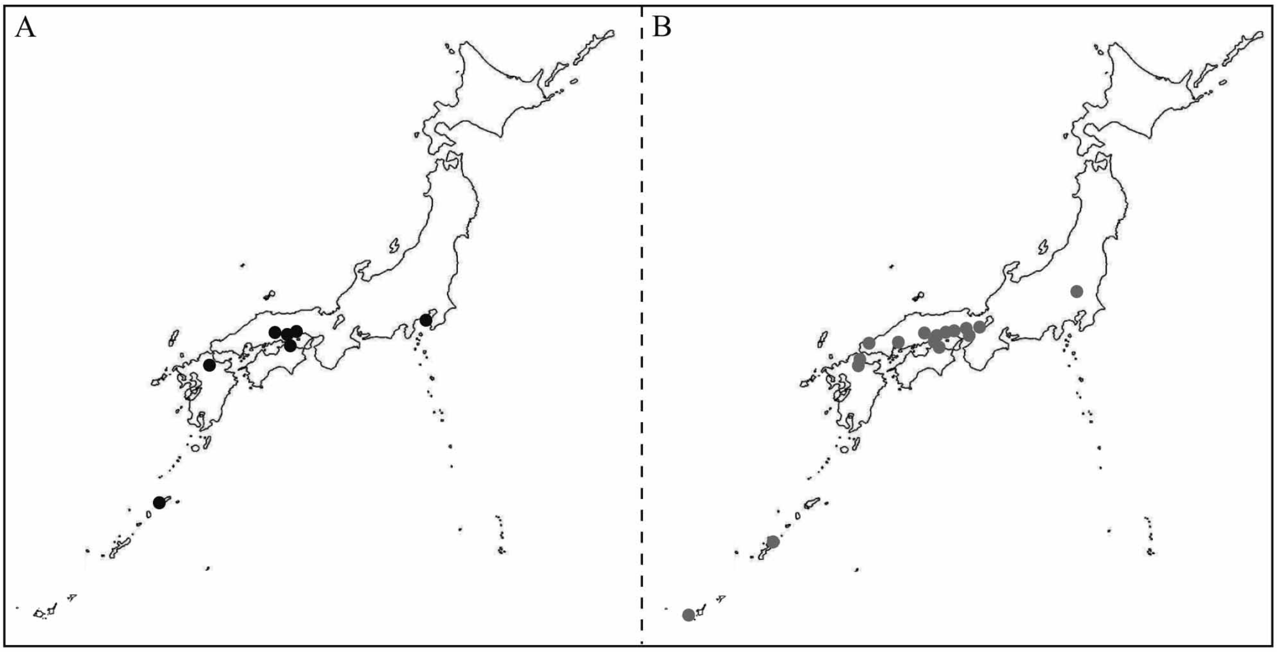

FIGURE 6. Currently known distribution of *P. nakasujii* (A) and *P. chinensis* (B) in Japan. Each dot may indicate more than one site if they are located less than 20 km apart.

Our phylogenetic tree showed no significant molecular difference between *P. chinensis* from the United States and those from the temperate zones in Japan, suggesting *P. chinensis* has been introduced into the United States from Japan (Fig. 1). It is noteworthy that *P. chinensis* is probably now the sixth introduced ant species in the United States mainland to come from Japan, which has a small geographic area relative to that of most regions from which species might originate (Smith 1979; MacGown *et al*. 2005; Steiner *et al*. 2006; Fisher & Cover 2007; Kjar & Sunian 2007; Ivanov & Milligan 2008; Fig. 1). It is perhaps not surprising that many species might be introduced from Japan to the eastern United States, given the relatively similar climates of the two regions (see Koppen Climate Map, Köppen 1923) and the long history of shipping routes connecting the regions. What is surprising is that the pattern is not symmetrical. No ant species are known to have been introduced from the United States to Japan (Japanese Ant Database Group 2003). A similar pattern has been observed for introduced plant species (Guo *et al*. 2006). Cremer *et al*. (2008) recently demonstrated that key invasive traits (e.g. mating system, body-size, queen number, and recognition efficiency in ants) are sometimes already present in the native range of what are to become invasive species. It is interesting to wonder whether there are attributes of the Japanese fauna that predispose it to being invasive or conversely traits of the North American fauna that predispose it to being invaded.

Several aspects of the *Pachycondyla chinensis* species complex have been studied in detail, including its specialized predation on termites (Teranishi 1929), consequences for public health (Bircher 2005; Klotz *et al*. 2005; Leath *et al*. 2006; Nelder *et al*. 2006; Lee *et al*. 2009), and the introduction into new habitats (Smith 1934; Brown 1958). However, this species complex contains two species that differ in body size and apparently life history. Identifying which species were the subjects in previous studies will necessary to understand how our existing knowledge of the *Pachycondyla chinensis* species complex maps onto our new evolutionary understanding of the group. Gotoh and Ito (2008) reported the seasonal cycle of changes in the colony structure of what we regard here as *P. nakasujii* (based on the morphological data in their paper) and thus provided useful information about the biology of *P. nakasujii*. Whether *P. chinensis* shows similar seasonal changes in colony structure remains unknown.

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