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Phylogenetic Position of the Endemic Large Carpenter Bee of the Ogasawara Islands, *Xylocopa ogasawarensis* (Matsumura, 1912) (Hymenoptera: Apidae), Inferred from Four Genes

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The Ogasawara (Bonin) Islands are oceanic islands of volcanic origin located in the northwestern Pacific Ocean about 1,000 km south of the Japanese mainland. A large carpenter bee, *Xylocopa* (*Koptortosoma*) *ogasawarensis*, is endemic to the islands but its closest relative is unknown. The Ogasawara Islands are geographically closest to the Japanese Archipelago, but this area is inhabited only by species of a different subgenus, *Alloxylocopa*. Thus, *X. ogasawarensis* is commonly thought to have originated from other members of *Koptortosoma*, which is widely distributed in the Oriental tropical region. In this study, we investigated the origin of *X. ogasawarensis* using a phylogenetic analysis of *Xylocopa* based on four genes: mitochondrial cytochrome oxidase subunit I (COI) and cytochrome b (Cyt b), and nuclear elongation factor-1α (EF-1α) and phosphoenolpyruvate carboxykinase (PEPCK). A combined analysis of the four genes strongly suggests that *Koptortosoma* is a large, polyphyletic group, within which *Alloxylocopa* is embedded. *Xylocopa ogasawarensis* emerged as the species most closely related to *Alloxylocopa* and not to Oriental species of *Koptortosoma*. Contrary to previous views of the origin of *X. ogasawarensis*, our results suggest that *X. ogasawarensis* and *Alloxylocopa* share a common origin and diverged after they colonized the island regions of East Asia.

Key words: *Koptortosoma*, large carpenter bee, molecular phylogeny, oceanic island, Ogasawara (Bonin) Islands, *Xylocopa*

INTRODUCTION

Isolated oceanic islands that have never been connected to a continental landmass harbor a remarkable diversity of endemic organisms as a result of local evolution and speciation. The Ogasawara (Bonin) Islands are typical oceanic islands of volcanic origin located in the northwestern Pacific Ocean about 1,000 km south of the Japanese mainland and comprise four island chains from north to south: the Muko-jima Islands, the Chichi-jima Islands, the Haha-jima Islands, and the Kazan Islands. The insect fauna of the Ogasawara Islands consists of 1,281 species, of which 26.4% are endemic to this area (Ohbayashi et al., 2003). Nine native bee species occur in the Ogasawara Islands, all endemic (Ohbayashi et al., 2003).

A large carpenter bee, *Xylocopa* (*Koptortosoma*) *ogasawarensis* (Matsumura, 1912), is one of the endemic bee species in the Ogasawara Islands and occurs on Chichi-jima and Haha-jima. *Xylocopa ogasawarensis* exhibits conspicuous sexual dimorphism in pubescence color, as is typical of other members of the subgenus (Sugiura, 2008). *Koptortosoma* occurs from South Africa through the Middle East and India and into Australia and includes 196 species comprising >40% of the global diversity of *Xylocopa* (469 species; Michener, 2007). Leys et al. (2002) performed a phylogenetic analysis based on nuclear and mitochondrial DNA sequences that suggested that *Koptortosoma* is not a monophyletic group, and is roughly divided into Oriental-Australian and Ethiopian groups.

More than 50 species of *Koptortosoma* occur throughout the Oriental region (Hurd and Moure, 1963). However, the subgenus has not been recorded in the Japanese Archipelago, which is the closest landmass to the Ogasawara Islands and is inhabited instead by four species of the subgenus *Alloxylocopa*. Thus, until now, it has been proposed that the ancestor of *X. ogasawarensis* occurs in the Oriental tropical region where *Koptortosoma* species are abundant (Yasumatsu, 1955; Hirashima, 1981). However, this hypothesis has not been verified by using a phylogenetic approach. In this study, we investigated the phylogenetic relationships of *X. ogasawarensis* to infer its origin

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based on sequences of four genes: mitochondrial cytochrome oxidase subunit I (COI) and cytochrome b (Cyt b), and nuclear elongation factor-1α (EF-1α) and phosphoenolpyruvate carboxykinase (PEPCK).

MATERIALS AND METHODS

Sampling

We used sequence data available from previous studies (Leys et al., 2000, 2002; Leijs and Hogendoorn, 2008) and added original sequences for *X. ogasawarensis* and six species from Japan and Laos (Table 1). Because higher subgeneric relationships within *Xylocopa* have been well established previously (Leys et al., 2002; Leijs and Hogendoorn, 2008), we focused our analysis on the well-defined monophyletic clade of ~10 Old World subgenera, including *Koptortosoma* and *Alloxylocopa* (Oriental+African clades in Leys et al., 2002; Table 1). *Xylocopa* (*Xylocopoides*) *virginica* (Linnaeus, 1771) and *X.* (*Proxylocopa*) *olivieri* Lepeletier, 1841, were used as outgroups to this clade. The subgeneric classification follows Michener (2007).

Molecular protocols

We preserved a foreleg of each bee in 99% ethanol at –20°C prior to DNA extraction. Legs were ground with pestles in reaction tubes and incubated in extraction buffer containing 470 μl of CTAB, 30 μl of 10% SDS, and 15 μl of proteinase K (20 mg/ml) at 55°C for 20 h. After incubation, the DNA was extracted with a phenol-chlorophorm solution, precipitated with ethanol, and stored in 20 μl of distilled water. The COI and Cyt b regions were amplified by using the UEA7/UEA10 and cb1/cb2 primer pairs of Leys et al. (2000), respectively, and the PEPCK region was amplified by using the primers G146F and G147R of Leys et al. (2002). The F1 copy of the EF-1α region was amplified using the primers, 5'-AA[A/G] TA[C/T] GCC TGG GT[A/G] [C/T]T[G/T] GA[C/T] AAG CT-3' (forward) and 5'-T [C/T]TT [A/C/G/T]GG CGA [C/G/T]GG [C/T]TC [C/G/T]A[A/G] CAT GT-3' (reverse), which were newly designed for this study based on previously available *Xylocopa* EF-1α sequences.

PCR amplifications were performed in 20-μl reaction mixtures containing 0.4 pmol each primer, 0.2 mM each dNTP, 1X Ampdirect buffer (Shimadzu, Kyoto, Japan), 0.4 U ExTaq polymerase (TaKaRa,

Table 1. Sampling details of the specimens used in the present study. Newly sampled species are indicated in bold. Locality is unavailable for species treated in Leijs and Hogendoorn (2008). Sequence data are missing where GenBank accession numbers are not given.

Subgenus		GenBank accession numbers			
Species	Locality	COI	Cyt b	EF-1α	PEPCK
Outgroup					
Proxylocopa (*P.*)					
olivieri Lepeletier, 1841	Israel	AY005240	AY005267	AY005294	AY005321
Xylocopoides (*Xc.*)					
virginica virginica (Linnaeus, 1771)	USA, Alabama	AY005231	AY005258	AY005285	AY005312
Ingroup					
Alloxylocopa (*A.*)					
***albinotum* Matsumura, 1926**	Japan, Yonaguni-jima Is.	EU722505	EU722498	EU445979	EU445993
***amamensis* Sonan, 1934**	Japan, Akuseki-jima Is.	EU722503	EU722496	EU445977	EU445991
***appendiculata circumvolans* Smith, 1873**	Japan, Yaku-shima Is.	EU722502	EU722495	EU445976	EU445990
***flavifrons* Matsumura, 1918**	Japan, Okinoerabu-jima Is.	EU722504	EU722497	EU445978	EU445992
Koptortosoma (*K.*)					
***ogasawarensis* Matsumura, 1912**	Japan, Haha-jima Is.	EU722506	EU722499	EU445984	EU445998
nigrita (Fabricius, 1775)	South Africa	AY005238	AY005265	AY005292	AY005319
caffra (Linnaeus, 1767)		EU180083	EU180102	EU180112	
pubescens Sponola, 1838	Israel	AY005236	AY005263	AY005290	AY005317
aruana Ritsema, 1876	Australia, Queensland	AY005234	AY005261	AY005288	AY005315
scioensis Gribodo, 1884	Zimbabwe	AY005237	AY005264	AY005291	AY005318
lieftincki Leys, 2000	Australia, Queensland	AY005235	AY005262	AY005289	AY005316
confusa Perez, 1901	Indonesia, Bali Is.	EU180077	EU180096		EU180124
sp. 1	Laos	EU722507	EU722500	EU445985	EU445999
sp. 2	India	EU180081	EU180100	EU180110	EU180126
***caerulea* (Fabricius, 1804)**	Laos	EU722508	EU722501		EU446002
provida Smith, 1863		EU180088	EU180105	EU180116	EU180129
nigroclypeata Rayment, 1935		EU180074	EU180093		EU180121
nobilis tricolor Smith, 1858		EU180078	EU180097	EU180108	EU180125
parvula Rayment, 1935		EU180073	EU180092		EU180120
disconata Friese, 1914		EU180075	EU180094		EU180122
smithii Ritsema, 1876		EU180085	EU180104	EU180114	EU180128
dimidiata Lepeletier, 1841		EU180079	EU180098	EU180109	
flavicollis (DeGreer, 1778)		EU180082	EU180101	EU180111	
watmoughi Eardley, 1983		EU180087	EU180115		
waterhousei Leys, 2000		EU180076	EU180095		EU180123
Mesotrichia (*M.*)					
latipes (Drury, 1773)	Indonesia, Bali Is.	AY005241	AY005268	AY005295	AY005322
acutipennis Smith, 1854	Thailand	AY005242	AY005269	AY005296	AY005323

Otsu, Japan), and 0.5 μl unquantified DNA template solution. Reaction conditions were: 5 min at 94°C; 30 cycles of 94°C for 30 s, 45°C (COI, Cyt b, and EF-1α) or 50°C (PEPCK) for 30 s, and 72°C for 1 min; and a final extension step for 7 min at 72°C. PCR products were excised from agarose gels and purified by using a NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany). Sequencing was performed by using the PCR primers and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA) and electrophoresed on an ABI 3100 sequencer (Perkin-Elmer).

Phylogenetic analysis

Because PEPCK sequences contained introns, they were aligned by using Clustal X (Thompson et al., 1997) and subsequently checked by eye for obvious misalignments. However, approximately 60% of the aligned PEPCK intron positions could not be aligned unambiguously and thus were excluded from the analysis. Gaps within PEPCK sequences were treated as missing data. The COI, Cyt b, and EF-1α sequences contained no introns, and thus the alignment was straightforward. Phylogenetic trees were obtained both together and separately for each gene by using the maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods. MP analysis was performed by using PAUP* 4.0b10 (Swofford, 2002) and by conducting heuristic searches with 100 random addition analyses with equal character weights and tree-bisection-reconnection (TBR) branch swapping. Clade support for the MP analyses was assessed by bootstrap analysis of 1000 pseudoreplicates. Prior to the ML analysis, an appropriate model of base substitution and model parameters were obtained by using Modeltest 3.0 (Posada and Crandall, 1998). ML heuristic searches were conducted by using PAUP* with 10 random addition analyses and TBR branch swapping, and nodal support was assessed by bootstrap analysis with 1,000 replications in PhyML (Guindon and Gascuel, 2003). For the Bayesian analysis, we used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), with best-fit models inferred by Modeltest. In the combined analysis of the four genes, substitution parameters were estimated separately for each gene by using the 'unlink' command. Markov chain Monte Carlo simulations were run for two million generations, with trees sampled every 1000 generations for a total of 2001 trees, and posterior probabilities of topology, branch lengths, and parameter estimates were obtained after omitting the first 1001 trees as burn-in.

RESULTS

Analyses of individual partitions and the combined data

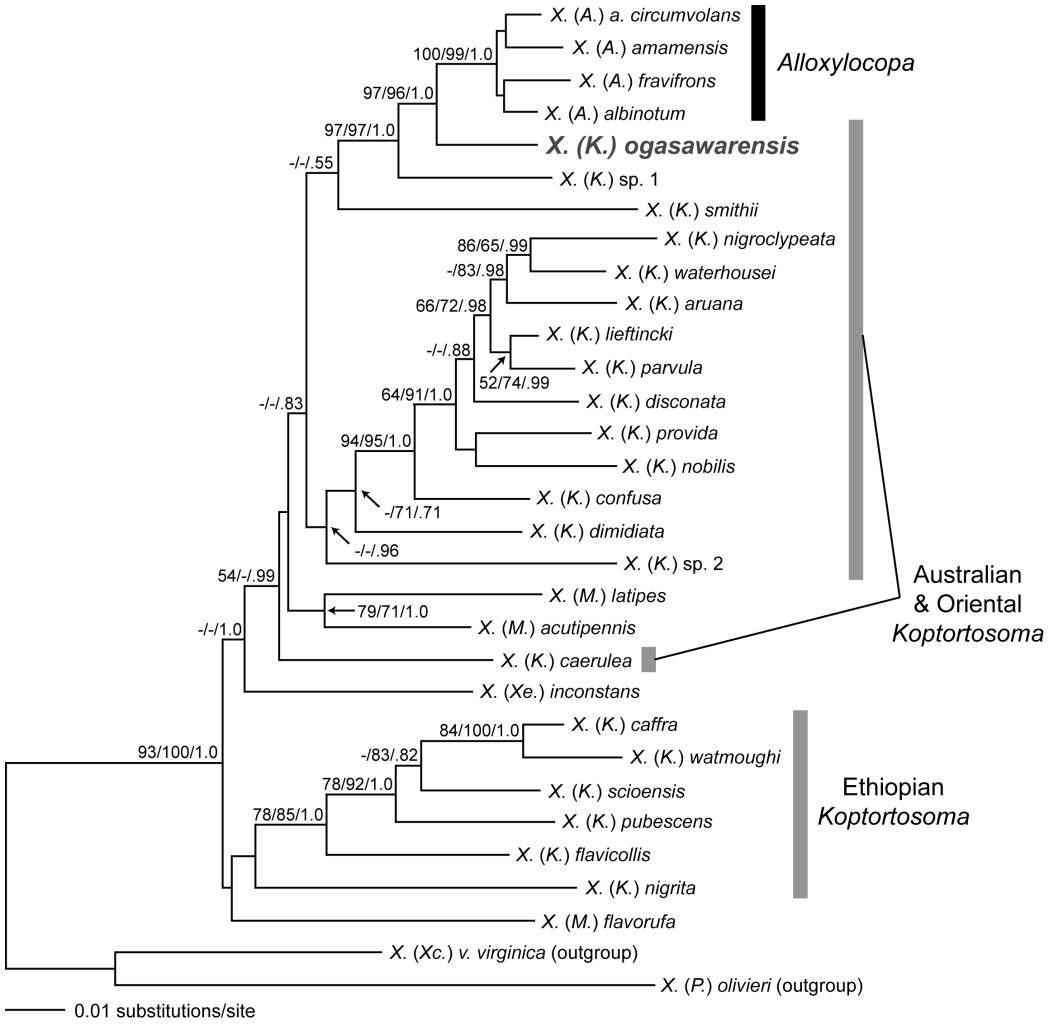

Fig. 1. Maximum likelihood phylogeny based on the combined COI, Cyt b, EF-1α and PEPCK data set. Numbers above branches represent maximum parsimony and maximum likelihood bootstrap values (in percent; shown only when >50%) followed by Bayesian posterior probabilities (shown only when >0.5). Abbreviations of subgenera follow those in Table 1.

set produced similar topologies; here we focus on the results of the combined analysis of the four genes. The combined four-gene data matrix was 1970 bp long, with 601 variable and 351 parsimony-informative positions. The MP analysis resulted in two equally parsimonious trees (tree length=1,485, consistency index [CI]=0.517, retention index [RI]=0.513), which are essentially similar to the ML phylogeny (-ln likelihood=10267.185; Fig. 1) and differed only regarding the relationships of closely related species. The Bayesian analyses also produced an overall similar topology. Consistent with previous studies (Leys et al., 2002; Leijs and Hogendoorn, 2008), the subgenus *Koptortosoma* was recovered as a polyphyletic group, within which *Alloxylocopa* and the remaining six subgenera are embedded. The *Koptortosoma* species were roughly divided into Afrotropical and Oriental-Australian species, as was previously suggested (Leys et al., 2002). The results strongly suggest that *Xylocopa ogasawarensis* is the sister group to the four Japanese *Alloxylocopa* species (Fig. 1).

DISCUSSION

The results of our phylogenetic analysis strongly suggest that *Alloxylocopa* is embedded within polyphyletic *Koptortosoma*, and that *X. ogasawarensis* is most closely related to *Alloxylocopa*, rather than to the Oriental *Koptortosoma*. *Alloxylocopa* includes the four sampled Japanese species together with the two (sub)species not represented in our data (*X. a. appendiculata* Smith, 1852, which is a subspecies of the Japanese *X. a. circumvolans*, and *X. phalothorax* Lepeletier, 1841). The two taxa not sampled both occur in continental China and Korea; thus, all *Alloxylocopa* species are known from the East Asian region that is geographically closest to the Ogasawara Islands. These data suggest that the common ancestor of *X. ogasawarensis* and *Alloxylocopa* most likely diverged after it had colonized the islands of East Asia (Fig. 2). It is possible that our sampling of *Koptortosoma* was not sufficient to identify the true sister species of *X. ogasawarensis*. Nevertheless, our results do not provide evidence for an independent colonization of the Japanese island region by phylogenetically distant *Xylocopa* lineages, as was previously suggested (Yasumatsu, 1955; Hirashima, 1981), but instead lend strong support for the close affinity of *X. ogasawarensis* to *Alloxylocopa* species distributed in the Japanese mainland and Ryukyu Archipelago.

A previous morphological examination of *Xylocopa* subgenera (Minckley, 1998) suggested that species of *Alloxylocopa* share overall similar morphologies with species of *Koptortosoma*, and this is reflected in the results of our phylogenetic analysis. The most significant morphological differences between the two groups are the sexual color dimorphism in *Koptortosoma* and enlarged eyes in

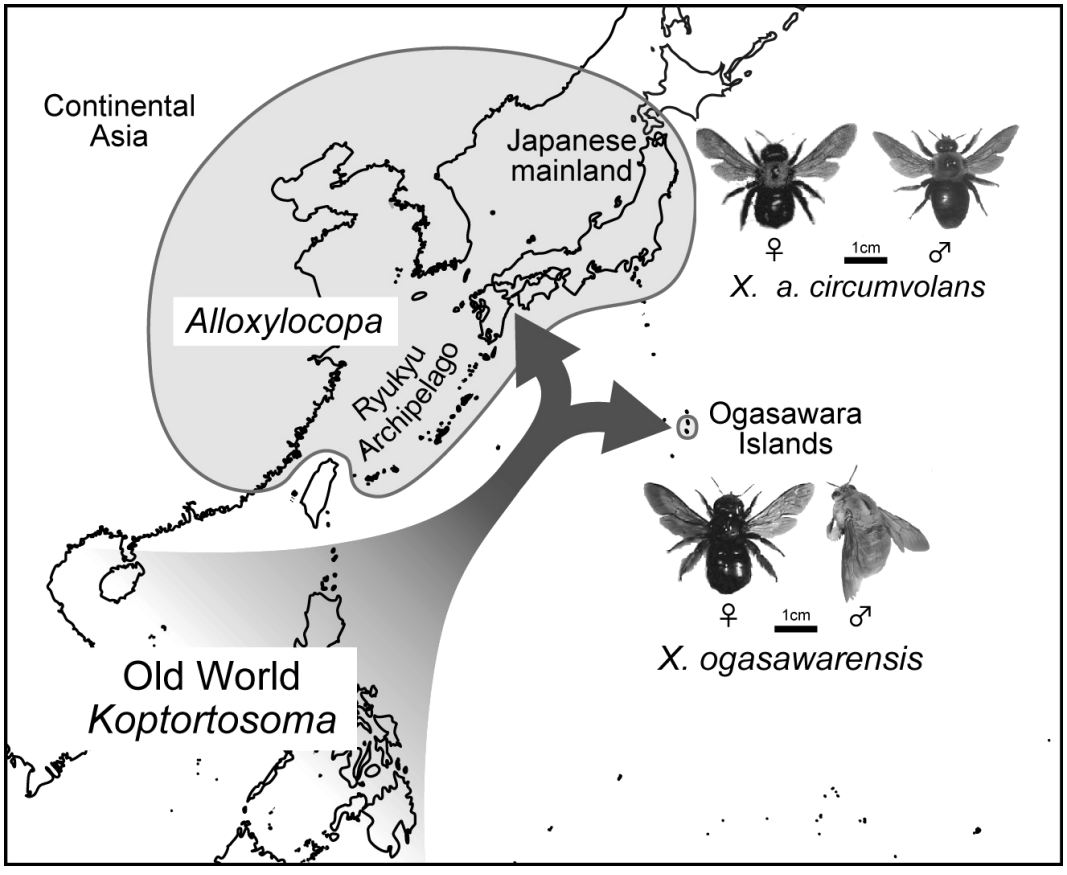

Fig. 2. Geographic distributions of the four Japanese *Alloxylocopa* species and *X. ogasawarensis*, and the hypothetical process of colonization of the Japanese islands by these bees. Species of the Old World *Koptortosoma* and *X. ogasawarensis* have conspicuous sexual dimorphism in pubescence color that is typical of the subgenus.

Alloxylocopa males. However, these characters are tightly associated with male mating strategies and do not necessarily reflect phylogenetic relationships (Leijs and Hogendoorn, 2008). Other morphological characters, including the male genitalia, are otherwise not strikingly different among these two groups and related subgenera (Hurd and Moure, 1963; Minckley, 1998); thus, the non-monophyly of *Koptortosoma* and the sister-group relationship of *Alloxylocopa* and *X. ogasawarensis* do not necessarily require a complex explanation of morphological evolution.

Bees are known as poor crossers of oceanic barriers (Michener, 1979), but a few *Xylocopa* species have succeeded in colonizing oceanic islands (Hurd and Moure, 1963). The most likely mechanism by which *Xylocopa* bees colonize oceanic islands is by floating in drift logs of the trees in which these bees had constructed their nests. This is evidenced by overrepresentation of wood- or stem-nesting bees in the oceanic island bee fauna worldwide (Michener, 1979). The distance between the Ogasawara Islands and the closest continental landmass is about 1,000 km, which is comparable to cases of other oceanic islands inhabited by endemic *Xylocopa* species: the Galápagos Islands, situated 1,000 km west of Ecuador, and Revillagigedo Islands, located 650 km off the western Mexican coast (Hurd and Moure, 1963). Thus, although anthropogenic dispersal cannot be ruled out entirely, *X. ogasawarensis* likely colonized the islands by itself after it had split from the shared ancestor of *Alloxylocopa* and *X. ogasawarensis* (Fig. 2).

Regarding other native bees of the Ogasawara Islands, Cronin (2004) studied the phylogenetic relationships of East Asian *Ceratina* species and found that the endemic *Ceratina* (*Neoceratina*) *boninensis* Yasumatsu, 1955 is most closely related to *C.* (*Neoceratina*) *dentipes* Friese, 1914, which occurs in the Ryukyu Archipelago. A morphological investigation of species of Megachilinae (Ikudome and Yamane, 1990) also suggested that they share similarities with species of the East Asian region. Taken together, our results provide additional evidence suggesting that the endemic bee fauna of the Ogasawara Islands has a close affinity to that of East Asia, especially the island regions of the Japanese mainland and the Ryukyu Archipelago.

The terrestrial fauna of the Ogasawara Islands is characterized by high endemism and morphological distinctiveness as compared to their continental relatives. As a result, some species have been classified in separate genera that are endemic to the Ogasawara Islands. However, recent molecular phylogenetic studies suggest that endemic animals of the Ogasawara Islands are not as genetically distinct as suspected from their morphological distinctiveness, and those that have been assigned generic rank probably do not merit such status. For example, the endemic dragonfly genus *Boninagrion* is nested within *Ischnura*, which is widely distributed in the East Asian region (Karube et al., 2004). Also, land snails of the endemic genus *Mandarina* are very closely related to *Euhadra* of the Japanese mainland (Chiba, 1999). Thus, future molecular phylogenetic studies may identify previously unsuspected origins for the endemic fauna of the Ogasawara Islands.

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