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Morphological and genetic variation of the pine shoot tunnel beetle *Placusa pinearum* (Staphylinidae) in China

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ABSTRACT. *Placusa pinearum*, a newly described beetle species found living in pine shoot tunnels bored by the pine tip moth, *Dioryctria rubella* (Pyralidae), has potential as a vector to transport biological agents for controlling this moth pest of pine trees because of the high degree of niche overlap (co-occurrence) between them. In order to help determine the suitability of potential biological control vectors, it is useful to obtain knowledge concerning the intraspecific variation of the vector. We examined *P. pinearum* collected from 14 different geographical populations in China using morphological and molecular markers. An UPGMA dendrogram based on morphological characters showed divergence of populations of *P. pinearum* in a comparison of beetles from southwestern and 3 other geographic regions (central, northwestern, and eastern regions). We also compared 965-nucleotide sequences of the mitochondrial cytochrome oxidase subunit I gene from 56 individuals; 19 haplotypes were identified based on 28 polymorphic sites in this region. A Bayesian phylogenetic tree showed significant genetic divergence among the different populations in eastern China. In addition, absence of shared haplotypes, coupled with high pairwise F_{ST} values, demonstrated

significant genetic divergence between the populations from the southwest and the other 4 main geographical regions (eastern, southern, central, and northwestern regions). Generally, we found the morphological divergence to be congruent with genetic divergence in these *P. pinearum* populations. This information should be useful for selection of suitable source materials in the species gene pool for future biological control programs.

Key words: *Placusa pinearum*; Morphological variation; COI; Genetic structure; Intraspecific divergence; Biological control

INTRODUCTION

One hundred and forty-three species of the genus *Placusa*, belonging to the subfamily Aleocharinae (Coleoptera: Staphylinidae), have been described globally, 9 of which can be found in China (Newton et al., 2005; Gao et al., 2011). The genus is characterized by the 4-4-5 tarsal formula and occupation of the subcortical habitat of trees. We recently reported that a new species, *Placusa pinearum*, which also belongs to the subfamily Aleocharinae, lives in the pine shoot tunnels bored by the larva of the pine shoot moth *Dioryctria rubella* Hampson (Gao et al., 2011).

D. rubella, a well-known major borer pest on the shoots of young pine trees, causes great economic damage in pine tree nurseries in China. The host pine trees of *D. rubella* include Masson's pine (*Pinus massoniana* Lambert), loblolly pine (*P. taeda* Linnaeus), Chinese white pine (*P. armandii* Franchet), Chinese red pine (*P. tabulaeformis* Carrière), Korean pine (*P. koraiensis* Siebold and Zuccarini), Yunnan pine (*P. yunnanensis* Franchet), Scots pine (*P. sylvestiris* Linnaeus var. *mongolica* Litvinov), black pine (*P. thunbergii* Parlatore), slash pine (*P. elliottii* Engelmannii), and dragon spruce (*Picea asperata* Masters) (Ji et al., 2008).

Controlling the pine shoot moth *D. rubella* is difficult due to its concealed habitat. Studies on the fauna of arthropods inside pine shoot tunnels have revealed that many arthropod species live in the tunnels; among them, the beetle *P. pinearum* has been found most frequently. In addition, a high degree of niche overlap (co-occurrence) between the pine shoot moth *D. rubella* and the beetle *P. pinearum* has been previously reported (Gao et al., 2010), which triggered our interest as to whether this beetle species can be used as an effective carrier to transport biological control agents for controlling pine shoot moths. Previously, Gross et al. (1994) used honeybees to deliver nuclear polyhedrosis virus and successfully controlled the corn earworm, and Peng et al. (1998) reported that *Trichogramma dendrolimi* Matsumura used as a vector carrying cytoplasmic polyhedrosis virus controlled the forest pest insect *Dendrolimus punctatus* Walker.

To develop biological control programs, laboratory artificial mass production of biological control agents needs to consider first the selection of a suitable source colony from field-collected materials, abundant genetic variation, which has been considered an important factor for successful colonization, and establishment of exotic biological control agents (Krafsur et al., 2005). To avoid genetic decay in laboratory stocks, Mackauer (1980) mentioned that a sufficient number of founding individuals (less than 50) was needed in mass production procedures to avoid bottlenecks. In other words, those well-adapted biotypes can expand their geographic ranges or be matched to local climate conditions for successful colonization in a new habitat. Therefore, more attention shoud be focused on the genetic diversity, genetic structure of a candidate wild population of biological control agents. Especially if genetic divergence of biological control agents from different geographical regions is very weak, we

can select any source colony in the species gene pool. Conversely, if the genetic divergence is distinct, the selection of a local source colony may then be appropriate.

P. pinearum is widely distributed in an area of 1900 km ranging from the east to the west of China and from 23° to 33°N latitude. Although the *P. pinearum* populations in different geographical regions live in the same microhabitat, they prefer habitation in different pine tree species. For example, in eastern China, *P. pinearum* prefers living inside the shoot tunnels of Masson's and loblolly pine, whereas in southwestern China, it prefers those of Yunnan pine. Moreover, while the beetle specimens collected from different geographical regions share morphological similarities in genitalia, tergite 8, tarsal formula, and mouthpart structures, those collected from the Provinces of Jiangsu, Anhui, Hunan, Guangdong, and Shanxi have yellow elytra and those from the Provinces of Sichuan and Yunnan have black elytra.

Previous reports have shown that morphological polymorphism is a response to the host nutritional, chemical, and physical structures (Heard, 2012). Host preference may allow insects to feed more effectively on the host plants, which in turn results in restriction of gene flow between populations and promotes host race formation of the feeding insects (Magalhães et al., 2007). Studies have also suggested that morphological divergence between populations is a result of widespread reproductive isolation and local adaptation (Sota et al., 2007) and may be a starting point of a speciation process (Rudh et al., 2007).

Considering the potential use of *P. pinearum* as a carrier of microbial control agents against pine shoot moth, it is essential to understand the intraspecific differentiation between populations of such a biocontrol vector insect. The objective of our study was to assess the morphological divergence as well as genetic diversity and genetic divergence between different *P. pinearum* populations in China and analyze the relationship between their morphological and genetic divergence.

MATERIAL AND METHODS

Specimen collection and identification

P. pinearum beetles were collected over a 4-year period (2008-2011) from 14 geographical locations across a latitudinal range of 1900 km in China (Figure 1). Information regarding sampling sites, collection dates, and host pine trees is provided in Table 1.

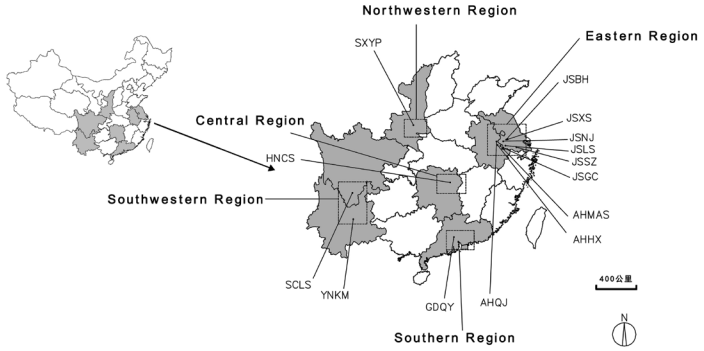

Figure 1. Geographical map showing locations and abbreviated names of *Placusa pinearum* samples from China. For abbreviations, see Table 1.

Table 1. List of sampling sites, dates, and host plants of *Placusa pinearum* investigated in this study as well as GenBank accession number for each haplotype defined.

Geographical region (No. of samples)	GPS coordinates	Sampling site (code)	Host species	Collected by	Collection date	Haplotype code (No. of individuals)
Eastern region (N = 41)	N = 31°39'01.79" E = 118°59'29.26"	Jiangsu Lishui (JSLS)	*P. taeda* Linnaeus	Ji Baozhong; Gao Jiangyong	June 2008	HAP-6 (2) HAP-9 (4) HAP-10 (2)
	N = 31°22'17.12" E = 119°03'39.52"	Jiangsu Gaochun (JSGC)	*P. massoniana* Lambert	Ji Baozhong; Gao Jiangyong	August 2008	HAP-2 (5) HAP-9 (1) HAP-11 (1) HAP-12 (1) HAP-13 (1)
	N = 32°07'19.79" E = 119°11'51.49"	Jiangsu Xiashu (JSXS)	*P. taeda* Linnaeus	Gao Jiangyong	August 2008	HAP-2 (2) HAP-6 (1) HAP-9 (3) HAP-10 (1)
	N = 32°03'36.34" E = 118°49'40.48"	Jiangsu Nanjing (JSNJ)	*P. thunbergii* Parlatore	Ji Baozhong; Gao Jiangyong	April 2009	HAP-6 (1) HAP-14 (1)
	N = 32°08'26.89" E = 118°58'25.76"	Jiangsu Baohua (JSBH)	*P. massoniana* Lambert	Gao Jiangyong	October 2011	HAP-2 (1) HAP-15 (1) HAP-16 (1)
	N = 31°16'32.89" E = 120°25'14.31"	Jiangsu Suzhou (JSSZ)	*P. massoniana* Lambert	Gao Jiangyong; Sun Zhenjun	September 2010	HAP-3 (1) HAP-9 (1)
	N = 31°39'00.54" E = 118°33'33.07"	Anhui Maanzhan (AHMAS)	*P. thunbergii* Parlatore	Gao Jiangyong	June 2010	HAP-9 (2)
	N = 32°03'46.40" E = 118°00'54.68"	Anhui Quanjiao (AHQJ)	*P. massoniana* Lambert	Wang Guoxing	August 2010	HAP-2 (5) HAP-9 (1)
	N = 31°46'55.85" E = 118°07'23.04"	Anhui Hexian (AHHX)	*P. massoniana* Lambert	Zhang Kai; Gao Jiangyong	May 2011	HAP-17 (1) HAP-18 (1)
Central region (N = 4)	N = 28°24'24.96" E = 113°01'48.01"	Hunan Changsha (HNCS)	*P. massoniana* Lambert	Wang Guoxing; Gao Jiangyong	August 2009	HAP-1 (1) HAP-2 (1) HAP-3 (2)
Southern region (N = 4)	N = 23°49'10.29" E = 113°11'10.88"	Guangdong Qingyuan (GDQY)	*P. massoniana* Lambert	Liu Jianfeng	September 2009	HAP-3 (2) HAP-5 (1) HAP-6 (1)
Southwestern region (N = 5)	N = 25°07'04.50" E = 102°41'09.29"	Yunnan Kunming (YNKM)	*P. armandii* Franchet	Zhao Jiejun	September 2009	HAP-8 (2)
	N = 27°49'17.03" E = 102°23'15.49"	Sichuan Liangshan (SCLS)	*P. armandii* Franchet	Sun Chongde	May 2009	HAP-4 (3)
Northwestern region (N = 2)	N = 33°47'26.87" E = 109°01'50.58"	Shanxi Yingpan (SXYP)	*P. tabuliformis* Carrière	Gao Jiangyong	October 2010	HAP-7 (1) HAP-19 (1)

All adult specimens were carefully examined for external and internal characteristics (Gao et al., 2011) under a stereoscopic microscope (MZ 16; Leica Microsystems, Switzerland). Identification of specimens was based on the shape of tergite 8, the aedeagus, and the median portion of the spermathecal stem.

Morphometric variables and analysis

Seven measurements were obtained from 87 individuals of *P. pinearum* with an ocular micrometer under a stereomicroscope at a magnification of 40 or 100X. These measurements included head width (HW), head length (HL), pronotum width (PW), pronotum length (PL), elytral width (EW), elytra length (EL), and body length (BL). To eliminate size variation among individuals, data (Table 2) were log-normalized (Audisio et al., 2001). The measurements were then used to calculate 6 log-transformed ratios, that is, head length by head width (HL/HW), pronotum length by pronotum width (PL/PW), elytra length by elytra width (EL/

EW), head length by body length (HL/BL), pronotum length by body length (PL/BL), and elytra length by body length (EL/BL) (Klimaszewski et al., 2008). Elytral color (EC) was also used as a categorical variable, with the value "1" indicating black and "0" indicating yellow (Stapel et al., 2008). Cluster analysis of the ratios and categorical variables was subsequently performed by the SPSS12.0 software, as described by Ribera and Nilsson (1995).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from the forebody (head and thorax) of specimens using the DNeasy Tissue kit (Qiagen, Bio Basic Inc., Markham City, ON, Canada) following the manufacturer protocol. A fragment of the mitochondrial cytochrome oxidase subunit I (mtDNA COI) gene was amplified using the primer pair: TY-J-1460 (5'-TACAATTTATCGCC TAAACTTCAGCC-3') and C1-2416ra (5'-GCTAATCATCTAAAAATTTTAATTCC-3') (Elven et al., 2010). All PCR amplifications were set up in 50-µL reaction mixtures containing 1.7 µL DNA extract as the template, 0.3 µL 25 µM dNTP mixture, 5 µL 10X PCR buffer, 1 µL 10 µM of each primer, 1 µL 5 U/µL DNA Taq polymerase, and 40 µL ddH_2O. PCR was performed in a thermal cycler (Eppendorf AG 22331, Hamburg, Germany), with an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, annealing temperature at 56°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR products subjected to electrophoresis on a 1.5% agarose gel were stained with ethidium bromide and visualized by UV transillumination. PCR products purified using the EZ-10 Spin Column DNA Gel Extraction kit (Bio Basic Inc.) were sequenced on an ABI 3730 automated sequencer with the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), following manufacturer instructions.

Sequence alignment and analysis

Sequences were aligned using ClustalW (Thompson et al., 1994), as implemented by the BioEdit (2.0) software. The sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis for sequence similarity searches. The DnaSp (5.1) software was used to calculate the number of both mitochondrial haplotypes and polymorphic sites and to perform the Fu and Li D neutrality test (Rozas et al., 2003). A median-joining network analysis was also performed using NETWORK 4.5 (Bandelt et al., 1999). Edited sequences of each haplotype were deposited in GenBank (JQ837822, JQ765747, JQ805146-JQ805147, JQ815556-JQ815564, and JQ82229-JQ822234) (Table 3). Haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987) were determined using ARLEQUIN (3.1) (Schneider et al., 1997), and the significance of the fixation index (F_{ST}) was also assessed by a nonparametric analysis with 1000 permutations using ARLEQUIN (3.1) (Excoffier et al., 1992).

The genetic structure of population subdivision was examined by analysis of molecular variance (AMOVA) using ARLEQUIN (3.1) (Excoffier et al., 1992). The populations were grouped either by geographic locations or by host species. For the geography-based analysis, populations were categorized into eastern, southern, central, northwestern, and southwestern groups. Genetic variation was also partitioned into 4 groups on the basis of the host plant: loblolly pine, Masson's pine, black pine, and Chinese white pine. Samples from Shanxi province (SXYP) were excluded from both the geographical and host species analyses due to the small sample size.

Table 2. Measurements of morphological characteristics of *Placusa pinearum* specimens.

Sampling site code	JSGC	JSNJ	HNCS	YNKM	JSSZ	SCLS	JSLS	GDQY	AHMAS	JSBH	AHHX	JSXS	SXYP	AHQJ
No. of individuals	9	4	10	3	3	4	7	4	5	6	9	12	3	8
Head width (HW)	0.29 ± 0.024	0.31 ± 0.033	0.30 ± 0.028	0.34 ± 0.003	0.27 ± 0.002	0.29 ± 0.072	0.32 ± 0.008	0.28 ± 0.009	0.31 ± 0.012	0.31 ± 0.014	0.30 ± 0.020	0.31 ± 0.029	0.33 ± 0.013	0.31 ± 0.010
Head length (HL)	0.24 ± 0.017	0.23 ± 0.038	0.24 ± 0.021	0.31 ± 0.003	0.22 ± 0.023	0.20 ± 0.033	0.23 ± 0.015	0.19 ± 0.015	0.24 ± 0.011	0.23 ± 0.017	0.22 ± 0.025	0.23 ± 0.026	0.26 ± 0.011	0.23 ± 0.015
Pronotum width (PW)	0.35 ± 0.047	0.38 ± 0.056	0.34 ± 0.026	0.41 ± 0.001	0.32 ± 0.037	0.35 ± 0.087	0.39 ± 0.014	0.34 ± 0.019	0.38 ± 0.014	0.37 ± 0.018	0.32 ± 0.022	0.39 ± 0.044	0.39 ± 0.004	0.39 ± 0.017
Pronotum length (PL)	0.23 ± 0.040	0.26 ± 0.050	0.25 ± 0.015	0.29 ± 0.005	0.22 ± 0.064	0.23 ± 0.059	0.27 ± 0.015	0.22 ± 0.008	0.26 ± 0.012	0.26 ± 0.014	0.21 ± 0.016	0.26 ± 0.025	0.29 ± 0.002	0.26 ± 0.017
Elytra width (EW)	0.45 ± 0.054	0.48 ± 0.069	0.47 ± 0.038	0.56 ± 0.003	0.39 ± 0.020	0.43 ± 0.094	0.49 ± 0.015	0.43 ± 0.021	0.48 ± 0.018	0.47 ± 0.031	0.45 ± 0.042	0.48 ± 0.058	0.49 ± 0.007	0.48 ± 0.018
Elytra length (EL)	0.34 ± 0.033	0.34 ± 0.067	0.36 ± 0.054	0.42 ± 0.007	0.29 ± 0.039	0.32 ± 0.044	0.35 ± 0.013	0.33 ± 0.006	0.35 ± 0.019	0.35 ± 0.013	0.30 ± 0.038	0.35 ± 0.046	0.37 ± 0.001	0.35 ± 0.019
Body length (BL)	2.11 ± 0.324	2.27 ± 0.394	2.20 ± 0.176	1.71 ± 0.03	1.89 ± 0.273	1.79 ± 0.214	2.39 ± 0.083	1.87 ± 0.141	2.45 ± 0.043	2.28 ± 0.222	2.36 ± 0.071	2.31 ± 0.281	2.40 ± 0.025	2.31 ± 0.197
Elytra color (EC)	Yellow	Yellow	Yellow	Black	Yellow	Black	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow

All measurements (shown as mean ± standard deviation) are given in millimeters.

Table 3. Haplotype frequency (N) and distribution among *Placusa pinearum* populations in China.

Haplotypes	N	Regions	Variable sites	GenBank accession No.
HAP-1	1	Central	GGGGGTGTTCGTGCGCCTTTAATACAAT	JQ765747
HAP-2	14	Central, Eastern	A.	JQ815564
HAP-3	5	Central, Eastern, Southern	A. . . AC. . . . . C. T. . . . . G. . . . TA	JQ822229
HAP-4	3	Southwestern	A. . . A. . . . . C. T. . . . . T . . .	JQ815557
HAP-5	1	Southern	A. . . A. . C. . . C. T. . . . . G. . GT. . .	JQ815558
HAP-6	5	Eastern, Southern	A. . . A. . . . . T.	JQ815560
HAP-7	1	Northwestern	A. . . A. . . . . T. . T. . C.	JQ805147
HAP-8	2	Southwestern	A. . . A. . . . . C. T.	JQ815556
HAP-9	12	Eastern	AA . .A. . . . TA. AT. . . . . C.	JQ815559
HAP-10	3	Eastern	A. . . A. . . . . T.	JQ822230
HAP-11	1	Eastern	A. . . A. . . . . A.	JQ822231
HAP-12	1	Eastern	A. A. A. . . . . T.	JQ822232
HAP-13	1	Eastern	A. . AA. . . . TA. AT. . . . . C. . G. .	JQ822233
HAP-14	1	Eastern	A. . AAC. . . . . C. T. . . . . G. . . . TA	JQ822234
HAP-15	1	Eastern	A. . . . . A. . . A.	JQ805146
HAP-16	1	Eastern	A. . . . . C. . . . . C.	JQ815563
HAP-17	1	Eastern	A. . . A. A.	JQ815561
HAP-18	1	Eastern	A. . . A. . . . . G.	JQ815562
HAP-19	1	Northwestern	A. . . . . T.	JQ837822

Furthermore, the correlation between genetic and geographical distances was determined using a Mantel test (1000 permutations) with ARLEQUIN (3.1) (Schneider et al., 2000). The demographic history of *P. pinearum* populations was also inferred from the mtDNA COI data using the coalescent Bayesian skyline plot model (Drummond et al., 2005), as implemented by the BEAST (1.5.3) program and visualized using the Tracer (1.5) program. The Bayesian tree and divergence time among *P. pinearum* populations were generated using the Bayesian relaxed clock method in BEAST (1.5.3) (Drummond and Rambaut, 2007).

RESULTS

Morphological variation

BL ranged from 1.71 in YNKM to 2.45 mm in AHMAS (Table 2), averaging 2.22 mm. The head, pronotum, and elytra were all transverse and usually less than twice as wide as long. The ranges of the measurements were as follows: HL, 0.19-0.31 mm; HW, 0.27-0.34 mm; PL, 0.21-0.29 mm; PW, 0.32-0.41 mm; EL, 0.29-0.42 mm; and EW, 0.39-0.56 mm. These observations indicated that the specimens from different geographical regions had similar size variations. In contrast, their EC was polymorphic. The EC of the specimens collected from YNKM (N = 3) and SCLS (N = 4) was black, whereas that of specimens collected from other sampling sites (N = 80) was unanimously bronze yellow (Table 2).

Morphology-based tree

The dendrogram (Figure 2) constructed with unweighted pair group method with arithmetic averages using Euclidean distances indicated that 14 populations of *P. pinearum* could be divided into 3 geographically distinct groups: Group 1, populations from the JSXS, JSLS, AHMAS, SXYP, AHQJ, JSBH, JSNJ, HNCS, AHHX, and JSGC regions; Group 2, populations from the GDQY, JSSZ and SCLS regions; and Group 3, populations from YNKM.

The populations in Groups 1 and 3 had yellow and black elytra, respectively, whereas Group 2 included populations with both yellow and black elytra (Table 2). While Group 1 corresponded geographically to the eastern, central, and northwestern regions in China, Group 3 was from the southwestern regions and Group 2 contained a population from each of the southern, eastern, and southwestern regions.

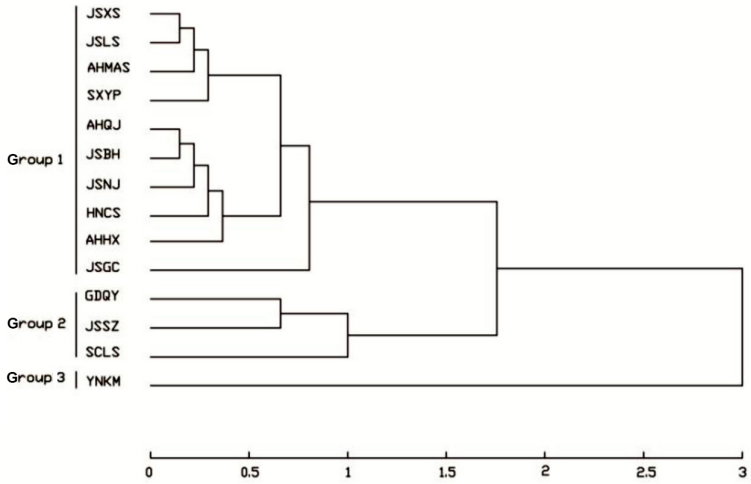

Figure 2. UPGMA dendrogram of Euclidean distances calculated from 6 log-transformed ratio variables and 1 categorical variable among *Placusa pinearum* populations.

Genetic diversity

A total of 56 mtDNA COI sequences (965 nucleotides) of *P. pinearum* were analyzed in this study. The data sets comprised 28 variable sites (14 parsimony informative sites and 14 singleton variable sites), which accounted for 2.9% of all nucleotides. The average base composition of T, C, A, and G in the mtDNA COI sequence was 37, 16, 31, and 16%, respectively, indicating that the sequence was predominantly AT-rich (A + T, 68%; G + C, 32%). In addition, no deletion or insertion of bases was observed.

These 56 mtDNA COI sequences yielded 19 haplotypes (Table 3), including 12 unique ones (HAP-1, HAP-5, HAP-7, HAP-11, HAP-12, HAP-13, HAP-14, HAP-15, HAP-16, HAP-17, HAP-18, and HAP-19) and 7 shared ones (HAP-2, HAP-3, HAP-4, HAP-6, HAP-8, HAP-9, and HAP-10), among which HAP-4 and HAP-8 were notably exclusively shared by individuals from the southwestern region. In contrast, 3 haplotypes (HAP-2, HAP-3, and HAP-6) were shared by the individuals from the eastern and south+central regions. HAP-2 and HAP-9, the most common and the second most common haplotypes, were shared by 14 and 12 individuals, respectively.

Among these 12 unique haplotypes, 8 were found in the populations from the eastern region (JSGC, JSNJ, JSBH, and AHHX), 2 were from the northwestern region (SXYP), 1 was from the southern region (GDQY), and 1 was from the central region (HNCS). In contrast, the populations from the southwestern region (SCLS and YNKM) had no unique haplotype.

Moreover, the populations from the southwestern region showed the lowest π

(0.000622) and *h* (0.600), whereas those from the southern region had the highest π (0.006390) and those from the northwestern region the highest *h* (1.000) (Table 4).

Table 4. Statistics of population of *Placusa pinearum* variation in the COI gene sequences.

Regional grouping of populations	Sampling site	No. of individuals	No. of haplotypes	Haplotype diversity (*h*)	Nucleotide diversity (π; mean ± SD)
Eastern	JSXS, JSNJ, JSBH, JSLS, JSGC, JSSZ, AHMAS, AHQJ, AHHX	41	13	0.8134 ± 0.0413	0.004508 ± 0.002527
Southern	GDQY	4	3	0.8333 ± 0.2224	0.006390 ± 0.004590
Southwestern	SCLS, YNKM	5	2	0.6000 ± 0.1753	0.000622 ± 0.000681
Northwestern	SXYP	2	2	1.0000 ± 0.5000	0.005181 ± 0.005676
Central	HNCS	4	3	0.8333 ± 0.2224	0.005354 ± 0.003910

Genetic structure

When populations were grouped by geographical location, AMOVA result showed that the majority of molecular variation (70.30%, F_{ST} = 0.29696, P < 0.0001) was within populations, whereas only a small part (10.83%, F_{CT} = 0.10835, P > 0.05) was between groups (Table 5). When populations were grouped by host species, similar AMOVA results were obtained (within populations, 71.94%, F_{ST} = 0.28064, P < 0.0001; between groups, 11.40%, F_{CT} = 0.11397, P < 0.05). These results indicated that there was no distinct geographical structure of genetic variation between different groups of *P. pinearum* when 13 populations (not including SXYP) were grouped by either host species or geographical location. Moreover, the Mantel test showed that there was no significant correlation between the F_{ST} values and geographical distances (km) (r = 0.372, P = 0.229 > 0.05; data not shown).

Table 5. Analysis of molecular variance (AMOVA) of *Placusa pinearum* populations in the context of different regions and host species.

Source of variation	d.f.	SSD	Percentage of total variance[1]	Variance component[2]	P value[3]
Geographical regions					
Among groups	3	3.124	10.83	F_{CT} = 0.10835	0.07331
Among population within groups	9	6.317	18.86	F_{SC} = 0.21153	0.00000
Within population	41	13.651	70.30	F_{ST} = 0.29696	0.00000
Host species					
Among groups	3	3.643	11.40	F_{CT} = 0.11397	0.01271
Among population within groups	9	5.799	16.67	F_{SC} = 0.18810	0.00293
Within population	41	13.651	71.94	F_{ST} = 0.28064	0.00000

d.f. = degrees of freedom; SSD = sum of squared differences. [1]Percentage of total variance contributed by each component; [2]variance component estimates; [3]probability of obtaining a more extreme component estimate by chance alone.

However, significant population differences were found between the southwestern and the other regions. The pairwise F_{ST} values were 0.31136 (P < 0.05) between the populations from the southwestern and eastern regions, 0.38446 (P < 0.05) between the southwest and central regions, 0.21664 (P < 0.05) between the southwest and southern regions, and 0.58567 (P < 0.05) between the southwest and northwest regions (Table 6). Thus, these findings indicated that there was significant genetic divergence between the southwest and the other 4 main geographical regions.

Table 6. Pairwise F_{ST} values between 5 populations of *Placusa pinearum*.

Geographical regions	Central region	Southwestern region	Southern region	Northwestern region	Eastern region
Central	-	-	-	-	-
Southwestern	0.38446*	-	-	-	-
Southern	-0.13333	0.21664*	-	-	-
Northwestern	0.02671	0.58567*	0.07617	-	-
Eastern	0.19051	0.31136*	0.27681*	0.01278	-

*Significant F_{ST} value ($P < 0.05$).

Genetic divergence and population demography

For the phylogenetic analyses, *Placusa* sp (from GenBank accession No. GQ980883) was selected as the outgroup. The Bayesian phylogenetic tree (Figure 3) shows that 19 haplotypes of *P. pinearum* among 56 individuals from 14 populations were clearly separated into 4 clades. Clade 1 comprised 2 haplotypes found among 13 individuals from 6 sampling sites (JSXS, JSLS, JSGC, JSSZ, AHMAS, and AHQJ) in the eastern region. The phylogenetic tree indicated that clade 1 was the most divergent group (100% node-supported), which was also supported by the haplotype network (Figure 4), in which the haplotypes HAP-9 and HAP-13 (in clade 1) with 5 and 6 mutation steps, respectively, were significantly different from HAP-10 (in clade 3).

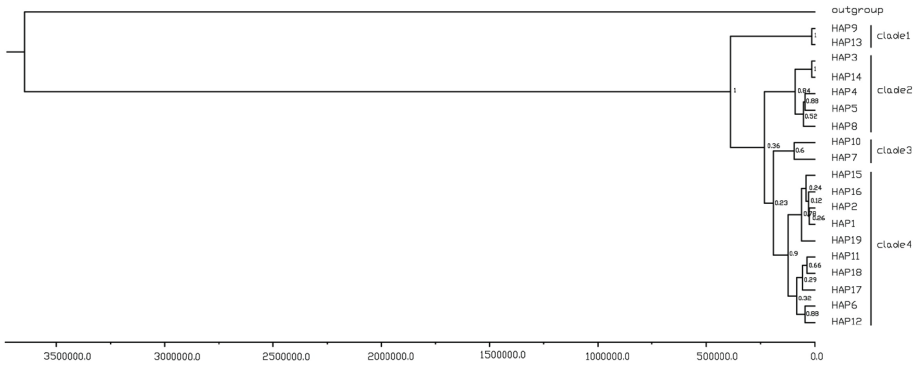

Figure 3. Bayesian tree and the divergence time (years before the present time) of *Placusa pinearum* populations performed using BEAST (1.5.3). Branch values represent posterior probability support.

Clade 2 comprised 5 haplotypes found among 12 individuals from 6 sampling sites (GDQY, HNCS, JSNJ, JSSZ, SCLS, and YNKM), which were widely spread in the southern, central, eastern, and southwestern regions. Clade 3 consisted of 2 haplotypes, which were found among only 4 individuals from 3 sampling sites in the eastern (JSXS and JSLS) and northwestern (SXYP) regions. Finally, clade 4, the most widespread clade, comprised 10 haplotypes found among 27 individuals from 10 sampling sites (JSGC, JSBH, JSXS, JSLS, JSNJ, AHQJ, AHHX, GDQY, HNCS, and SXYP). Moreover, the haplotype network (Figure 4) suggested that there were 4 mutation steps between HAP-3 and HAP-8 and 3 steps between HAP-4 and HAP-5, indicating significant genetic divergence between the southwestern regions and east+south+central regions.

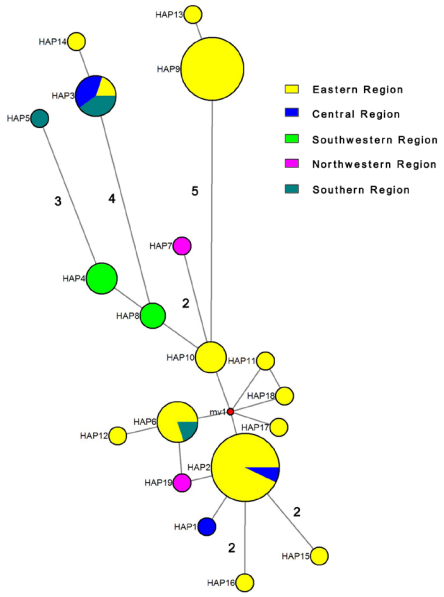

Figure 4. Ninety-five percent parsimony network of 19 haplotypes generated based on sequences of the 965-bp mtDNA COI fragment from 56 *Placusa pinearum* individuals. The node size is proportional to the number of individuals with the same haplotype. Red nodes represent median vectors (mv1). The numbers on branches indicate the number of mutations between 2 nearest nodes, and only those with larger than 1 mutation step are marked.

The demographic history of *P. pinearum* was also inferred on the basis of the Bayesian skyline plot, without any assumption of a particular demographic model (Drummond, 2005). In the absence of a suitable intrinsic calibration, we could apply the approximate 2% per million years divergence rate for insect mtDNA (Brower, 1994). The Bayesian skyline analysis demonstrated that the *P. pinearum* populations in China had undergone a major sudden population expansion starting from approximately around 20,000 years ago (0.02 Mya) (Figure 5), which was also confirmed by the Fu and Li D test (-2.45378, $P < 0.05$; data not shown).

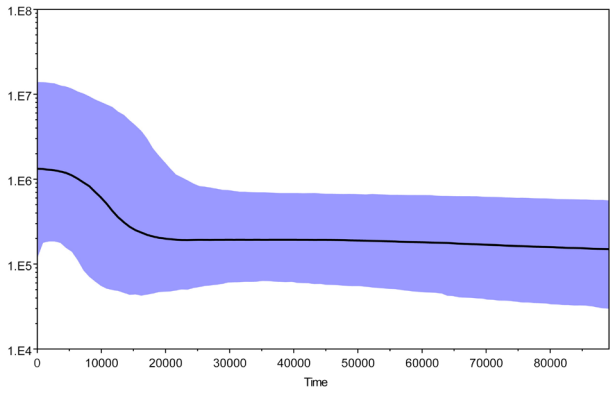

Figure 5. Bayesian skyline plot for *Placusa pinearum*. The x-axis measures time in years before the present and the y-axis is the scaled effective population size (units = $N_e\tau$, the product of effective population size and generation length in years). The 95% confidence interval is indicated in blue.

DISCUSSION

Genetic diversity

The subfamily Aleocharinae, one of the largest lineages in the family Staphylinidae, is by far the most successful group of inquilines in the nests of social insects, especially in those of ants and termites (Seevers, 1965). However, little research has focused on the genetic structure and genetic divergence of the beetles in this subfamily, other than some studies on the phylogeny of a higher taxonomic unit of the family Staphylinidae (Maus et al., 2001; Chatzimanolis et al., 2010). Previously, Thomas (2009) reported a tribal-level phylogeny of the subfamily Aleocharinae by using partial sequences of the 12S and 16S mitochondrial genes, and Ahn et al. (2009) reported a phylogeny of 21 species in the tribe Liparocephalini by using partial sequences of mtDNA COI and COII as well as 12S and 18S rDNA. Notably, several studies have suggested that mtDNA COI is an ideal marker for examining interspecific or intraspecific phylogenetic relationships among insects (Sole et al., 2008; Ahmed et al., 2009). However, to date, only 1 mtDNA COI gene sequence of *Placusa* sp can be found in GenBank (accession No. GQ980883; Elven et al., 2010), and the intraspecific phylogenetic relationship in the family Staphylinidae has not been previously reported.

Our recent study (to be published) showed that the gene sequences of either mitochondrial 18S rRNA or ITS2 in *P. pinearum* are not suitable for intraspecific phylogenetic analysis due to the lack of sufficient polymorphic sites. In contrast, mtDNA COI was found to have more polymorphic sites compared to 16S mitochondrial genes in *P. pinearum*. Therefore, mtDNA COI was used as a molecular marker in this study to investigate the intraspecific genetic variation of *P. pinearum* collected from different geographical regions in China.

The analysis of 56 mtDNA COI sequences obtained from different *P. pinearum* individuals showed that the sequences are predominantly AT-rich (68%), which is a common feature of mitochondrial DNA in other coleopteran insects (Giannoulis et al., 2011). The average values of h and π were also calculated as 0.8784 and 0.0049, respectively. Overall, the entire data set of *P. pinearum* with relatively low π and high h was similar to that of other coleopteran insects (Anducho-Reyes et al., 2008).

Morphological and genetic divergence

As shown in Figure 2, all populations from the southern and northwestern region and the majority of populations from the eastern regions were clustered into Group 1, whereas the populations from the southwestern regions were clustered into Groups 2 and 3. This result showed that there was a morphological divergence of the populations in *P. pinearum* between the southwestern and the other 3 geographic regions (central, northwestern, and eastern regions). The median-joining network analysis indicated the absence of shared haplotype between the southwestern and the other geographical regions. The pairwise F_{ST} value between the southwestern and the others was significantly high, indicative of weak gene flow between these regions. This suggests that morphological divergence is generally in congruence with genetic divergence in *P. pinearum* populations. Moreover, Group 2 contained populations from the southwestern (SCLS), southern (GDQY), and eastern (JSSZ) regions (Figure 1), which was also partially in congruence with the molecular-based tree because the HAP-5

haplotype from the southern region (GDQY) and the HAP-4 haplotype from the southwestern region (SCLS) were closely clustered together in clade 2 (88% node-supported) (Figure 3).

The molecular-based tree showed that the HAP-9 and HAP-13 haplotypes from the eastern regions were clustered into clade 1, the most divergent group in the phylogenetic tree (Figure 3). Significant genetic divergence within the populations from eastern China was found partially congruent with morphological divergence. The HAP-9 haplotype was shared with an individual from JSSZ, whereas the unique HAP-13 haplotype corresponded geographically to JSGC. Moreover, the morphology-based tree clearly showed morphological divergence between the populations from JSSZ or JSGC and the other regions in eastern China. All the evidence mentioned above suggests that morphological divergence is generally in congruence with genetic divergence in *P. pinearum* populations.

Several studies have also suggested that geographical isolation or host specialization may be important factors that influence genetic structure and differentiation in insects (Abreu and Solferini, 2008; Craft et al., 2010). Therefore, it is possible that the current high level of genetic divergence between *P. pinearum* populations from the southwestern and other regions might have resulted from geographical distance isolation or host preference. However, AMOVA results from our study showed that there was no distinct geographical structure of genetic variation among different *P. pinearum* groups. The results of the Mantel test showed that there was no significant correlation between the F_{ST} and geographic distance (km), even though the geographical distances between sampling sites in the southwestern regions and the other 4 main geographical regions were often greater than 1000 km. Therefore, we infer that genetic divergence between the southwestern and other region populations may be explained by the consequence of topographic barriers; that is, the southwestern sample sites (SCLS and YNKM) were in the Hengduan Mountains, the second largest mountain in China, in contrast to the hills or plateaus in the eastern, central, or southern regions.

The Bayesian skyline plot analysis suggested that the *P. pinearum* populations in China had undergone a major population expansion, starting approximately at the peak of the last glacial maximum (approximately 0.02 Mya). During the period between 0.018 and 0.02 Mya, a large number of insects expanded northward from the southern refuges in China due to gradually increased temperature (Jia, 2010). From a historical perspective, the higher genetic diversity in populations suggests that they could be ancestral populations, and the lower genetic diversity suggests that they were colonized more recently (Kodandaramaiah et al., 2012) or underwent recent population declines resulting in population bottlenecks (Kim and Sappington, 2006). In this study, π and h were significantly higher in *P. pinearum* populations from the eastern, southern, central, and northwestern regions than in those from the southwestern regions. Moreover, the HAP-2 and HAP-10 haplotypes, corresponding to the individuals mostly from the eastern and south+central regions in China, were located in the central position in the haplotype network, suggesting that HAP-2 and HAP-10 were most likely the ancestral haplotypes. Meanwhile, the *P. pinearum* haplotypes from eastern China scattered among different clades, suggesting that the eastern region was not only the main geographical distribution area but also the center of genetic diversity. In contrast, π and h were significantly lower in the southwestern populations than in other geographical populations. Specifically, the lowest value of π was found in the southwestern populations (0.00062), which was only approximately one-seventh of that (0.004508) in the eastern populations, approximately one-ninth of that (0.005354) in central population and approximately one-tenth of that (0.006390)

in the southern population. The low genetic diversity of the southwestern populations suggests that they were colonized more recently and might have become isolated either through dispersal from the eastern or south+central regions or by undergoing bottlenecks due to population size fluctuations.

Since we obtained only small numbers of samples from the southwestern region (N = 5) in this study, it is necessary to examine more samples to draw reliable conclusions in further studies.

Implications of genetic divergence

Overall, this study provides evidence that morphological divergence in *P. pinearum* is basically in congruence with its genetic divergence. These findings also suggest that phenotypic traits could serve as a valid criterion for selection of a suitable source colony from field-collected materials, especially when molecular data are not available (García et al., 2008).

To date, a number of studies have reported the use of some species of the subfamily Aleocharinae as biocontrol agents. For example, Hagen et al. (1999) documented that several species of *Aleochara* introduced into North America from Europe were mass cultured and periodically released against dipteran rootworms. As a closely related species to those from the genus *Aleochara*, *P. pinearum* could potentially be used as a carrier to deliver microbial control agents against the pine shoot moth because of their high degree of niche overlap. The results presented here regarding the morphological and genetic divergence in *P. pinearum* can be helpful for selection of a suitable source colony in the species gene pool in future biological control programs.

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REFERENCES

Abreu AG and Solferini VN (2008). Genetic population structure and hybridization in two sibling species, *Tomoplagia reticulata* and *Tomoplagia pallens* (Diptera: Tephritidae). *Genet. Mol. Res.* 7: 1298-1311.

Ahmed MZ, Shatters RG, Ren SX, Jin GH, et al. (2009). Genetic distinctions among the Mediterranean and Chinese populations of *Bemisia tabaci* Q biotype and their endosymbiont *Wolbachia* populations. *J. Appl. Entomol.* 133: 733-741.

Ahn KJ, Jeon MJ and Branham MA (2009). Phylogeny, biogeography and the stepwise evolutionary colonization of intertidal habitat in the Liparocephalini based on morphological and molecular characters (Coleoptera: Staphylinidae: Aleocharinae). *Cladistics* 26: 344-358.

Anducho-Reyes MA, Cognato AI, Hayes JL and Zúñiga G (2008). Phylogeography of the bark beetle *Dendroctonus mexicanus* Hopkins (Coleoptera: Curculionidae: Scolytinae). *Mol. Phylogenet. Evol*. 49: 930-940.

Audisio P, Belfiore C, De Biase A and Antonini G (2001). Identification of *Meligethes matronalis* and *M. subaeneus* based on morphometric and ecological characters (Coleoptera: Nitidulidae). *Eur. J. Entomol.* 98: 87-97.

Bandelt HJ, Forster P and Rohl A (1999). Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16: 37-48.

Brower AV (1994). Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc. Natl. Acad. Sci. U. S. A*. 91: 6491-6495.

Chatzimanolis S, Cohen IM, Schomann A and Solodovnikov A (2010). Molecular phylogeny of the mega-diverse rove beetle tribe Staphylinini (Insecta, Coleoptera, Staphylinidae). *Zool. Scr.* 39: 436-449.

Craft KJ, Pauls SU, Darrow K, Miller SE, et al. (2010). Population genetics of ecological communities with DNA barcodes: An example from New Guinea Lepidoptera. *Proc. Natl. Acad. Sci. U. S. A*. 107: 5041-5046.

Drummond AJ, Rambaut A, Shapiro B and Pybus OG (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol. Biol. Evol.* 22: 1185-1192.

Drummond AJ and Rambaut A (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7: 214.

Elven H, Bachmann L and Gusarov VI (2010). Phylogeny of the tribe Athetini (Coleoptera: Staphylinidae) inferred from mitochondrial and nuclear sequence data. *Mol. Phylogenet. Evol.* 57: 84-100.

Excoffier L, Smouse PE and Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.

Gao JY, Ji BZ and Liu SW (2011). A new species of *Placusa* Erichson (Coleoptera, Staphylinidae, Aleocharinae) from China. *Zootaxa* 3094: 43-51.

Gao JY, Ji BZ, Liu SW, Wang GX, et al. (2010). Population composition and niche of arthropod community in pine shoot tunnel bored by *Dioryctria rubella*. *Chin. J. Ecol.* 29: 363-369.

García JT, Suarez F, Garza V, Calero-Riestra M, et al. (2008). Genetic and phenotypic variation among geographically isolated populations of the globally threatened Dupont's lark *Chersophilus duponti*. *Mol. Phylogenet. Evol.* 46: 237-251.

Giannoulis T, Dutrillaux AM, Mamuris Z, Montreuil O, et al. (2011). Evolution of European cockchafers (Melolonthinae: Scarabaeidae: Coleoptera): a morphological, molecular and chromosomal study of intra- and inter-specific variations. *Bull. Entomol. Res.* 101: 345-352.

Gross HR, Hamm JJ and Carpenter JE (1994). Design and application of a hive-mounted device that uses honey bees (Hymenoptera: Apidae) to disseminate *Heliothis* nuclear polyhedrosis virus. *Entomology* 23: 492-501.

Hagen KS, Mills NJ, Gordh G and McMurtry JA (1999). Principles and Applications of Biological Control. In: Terrestrial Arthropod Predators of Insect and Mite Pests (Bellows TS and Fisher TW, eds.). Academic Press, San Diego, 448-450.

Heard SB (2012). Use of host-plant trait space by phytophagous insects during host-associated differentiation: The gape-and-pinch model. *Int. J. Ecol.* 2012: Article ID 192345.

Ji BZ, Liu SW, Gao JY, Kuang MH, et al. (2008). Advances in pine tip moths. *Entomol. J. East China.* 17: 149-155.

Jia FL (2010). Impaction of quaternary glacier to insect fauna of China. *Acta Sci. Nat. Univ.* 49: 79-85.

Kim KS and Sappington TW (2006). Molecular genetic variation of boll weevil populations in North America estimated with microsatellites: implications for patterns of dispersal. *Genetica* 127: 143-161.

Klimaszewski J, Godin B, Pelletier G and Savard K (2008). Six new species and records of *Aleocharine* beetles from the Yukon and Alaska (Coleoptera: Staphylinidae: Aleocharinae). *Can. Entomol.* 140: 265-291.

Kodandaramaiah U, Konvicka M, Tammar T, Wahlberg N, et al. (2012). Phylogeography of the threatened butterfly, the woodland brown *Lopinga achine* (Nymphalidae: Satyrinae): implications for conservation. *J. Insect Conserv*. 16: 305-313.

Krafsur ES, Obrycki JJ and Harwood JD (2005). Comparative genetic studies of native and introduced Coccinellidae in North America. *Eur. J. Entomol.* 102: 469-474.

Mackauer M (1980). Some Aspects of Quality and Quality Control of Biological Control Agents During Insectary Propagation. International Symposium on Biological Control of Weeds, Brisbane, 207-220.

Magalhães S, Forbes MR, Skoracka A, Osakabe M, et al. (2007). Host race formation in the Acari. *Exp. Appl. Acarol.* 42: 225-238.

Maus C, Peschke K and Dobler S (2001). Phylogeny of the genus *Aleochara* inferred from mitochondrial cytochrome oxidase sequences (Coleoptera: Staphylinidae). *Mol. Phylogenet. Evol.* 18: 202-216.

Nei M (1987). Molecular Evolutionary Genetics. Columbia University Press, New York.

Newton AF and Thayer MK (2005). Catalog of Higher Taxa of Staphyliniformia and Genera and Subgenera of Staphylinoidea. Available at [http://www.fieldmuseum.org/peet_staph/db_1a.html]. Accessed January 16, 2011.

Peng HY, Chen XW, Jiang Y, Shen RJ, et al. (1998). Controlling *Dendrolimus punctatus* with *Trichogramma dendrolimi* carrying cytoplasmic polyhedrosis virus. *Chin. J. Biol. Control.* 14: 111-114.

Ribera I and Nilsson AN (1995). Morphometric patterns among diving beetles (Coleoptera: Noteridae, Hygrobiidae). *Can. J. Zool.* 73: 2343-2360.

Rozas J, Sanchez-DelBarrio JC, Messeguer X and Rozas R (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19: 2496-2497.

Rudh A, Rogell B and Höglund J (2007). Non-gradual variation in colour morphs of the strawberry poison frog *Dendrobates pumilio*: genetic and geographical isolation suggest a role for selection in maintaining polymorphism. *Mol. Ecol.* 16: 4284-4294.

Schneider S, Kueffer JM, Roessli D and Excoffier L (1997). Arlequin ver.1.1: A Software for Population Genetic Data Analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva.

Schneider S, Roessli D and Excoffier L (2000). ARLEQUIN Version 2.000: A Software for Population Genetics Analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva.

Seevers CH (1965). The systematic, evolution and zoogeography of staphylinid beetles associated with army ants (Coleoptera: Staphylinidae). Chicago Natural History Museum, Chicago. *Fieldiana*: *Zool.* 47: 139-351.

Sole CL, Bastos AD and Scholtz CH (2008). Intraspecific patterns of mitochondrial variation in natural population fragments of a localized desert dung beetle species, *Pachysoma gariepinum* (Coleoptera: Scarabaeidae). *J. Hered.* 99: 464-475.

Sota T, Hayashi M and Yagi T (2007). Geographic variation in body and ovipositor sizes in the leaf beetle *Plateumaris constricticollis* (Coleoptera: Chrysomelidae) and its association with climatic conditions and host plants. *Eur. J. Entomol.* 104: 165-172.

Stapel H, Misof B and Wagner T (2008). A molecular and morphological phylogenetic analysis of afrotropical *Monolepta* species and related Galerucinae (Coleoptera: Chrysomelidae). *Arthropod Syst. Phylogeny* 66: 3-17.

Thomas JC (2009). A preliminary molecular investigation of Aleocharine phylogeny (Coleoptera: Staphylinidae). *Ann. Entomol. Soc. Am.* 102: 189-195.

Thompson JD, Higgins DG and Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.