



# Hopping out of Mindanao: Miocene–Pliocene geological processes and cross-island dispersal as major drivers of diversity for Philippine treehoppers

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## ABSTRACT

**Aim** We investigated the biogeographical history of an endemic Philippine treehopper, *Pyrgonota bifoliata* (Membracidae), to test the effect of Neogene geological events and Pleistocene climate change in generating speciation within the Philippines.

**Location** The Philippine archipelago.

**Methods** Phylogenies were reconstructed based on the mitochondrial cytochrome *c* oxidase I subunit (*cox1*) and nuclear wingless (*wg*) genes using maximum parsimony, maximum likelihood and Bayesian criteria. Divergence times were estimated in BEAST based on a range of mutation rates. Ancestral ranges were reconstructed using RASP (Reconstruct Ancestral State in Phylogenies) and Bayesian stochastic search variable selection (BSSVS). Species boundaries were tested using the Bayesian general mixed Yule coalescent (bGMYC) and Bayesian phylogenetic and phylogeography (BPP) methods.

**Results** *Pyrgonota bifoliata* showed one of the lowest levels of between-Pleistocene island genetic variation among all the taxa examined. Most Pleistocene aggregate island complex (PAIC) lineages and extant *P. bifoliata* originated before the onset of the first extensive Pleistocene glacial cycle. Ancestral *P. bifoliata* originated in the late Miocene from Mindanao, followed by a sequential northwards colonization of the Visayan Islands and Luzon in the Pliocene–early Pleistocene, which coincided with the spatial reorganization of the Philippines. The statistically delimited nine provisional species of what we now view as the *P. bifoliata* species complex correspond to current island boundaries and specialized host plants.

**Main conclusions** The temporally calibrated phylogeny of *P. bifoliata* represents the first invertebrate study to support the hypothesis that Miocene–Pliocene geological events and consequent cross-island dispersal were pivotal promoters of species diversity in the Philippines. We hypothesize that ecological adaptation to specialized host plants, together with either local selection or genetic drift through within-island allopatric isolation, present a plausible *in situ* speciation mechanism for the origin of these herbivorous insects.

## Keywords

Colonization, cryptic species, host-plant adaptation, island biogeography, long-distance dispersal, Membracidae, Pleistocene islands, *Pyrgonota bifoliata*, The Philippines.

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## INTRODUCTION

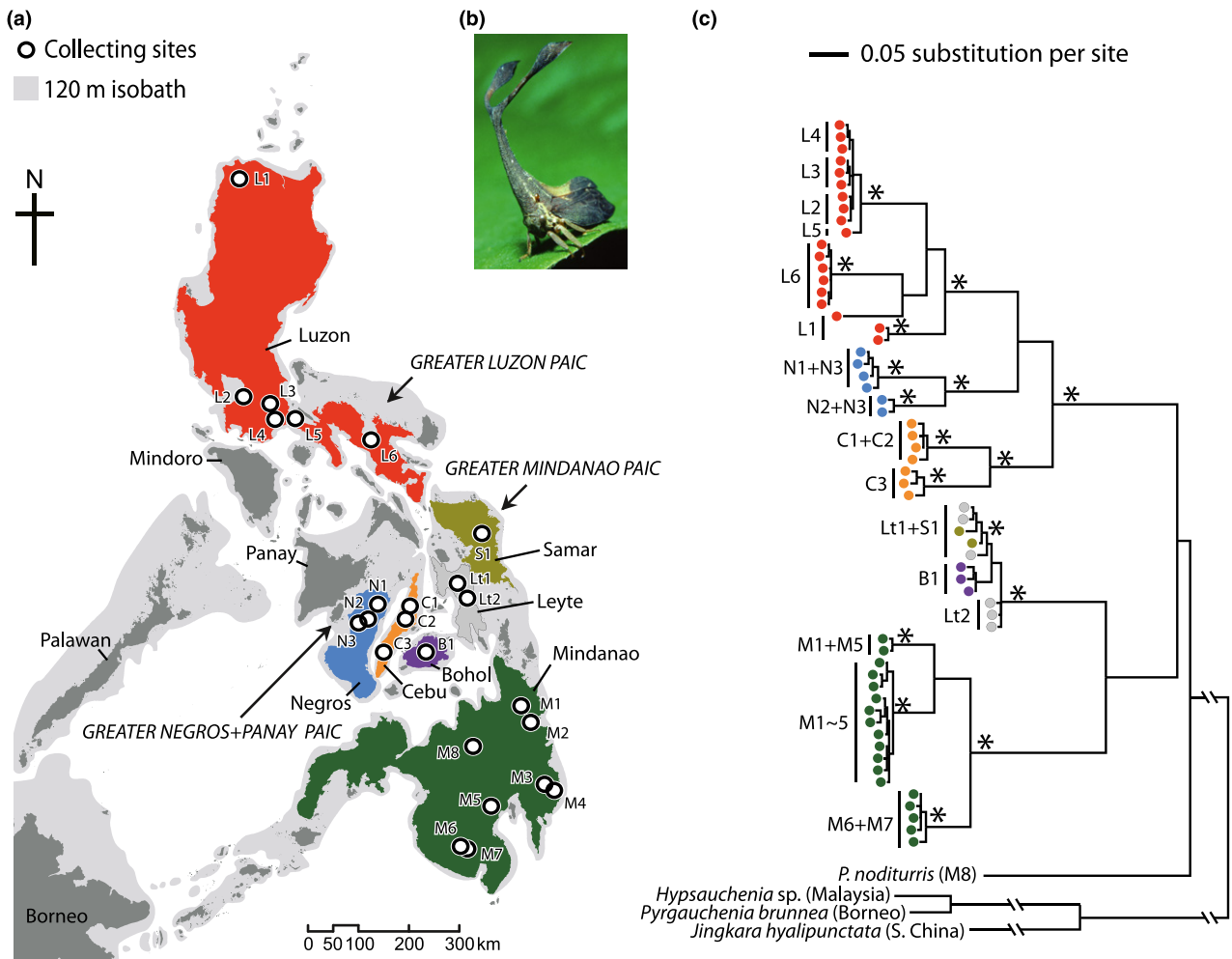
The Philippine archipelago, which includes more than 7000 oceanic islands and is situated between the Oriental and

Australasian faunal regions, presents a unique geographical setting and exhibits one of the world's most remarkable arrays of tropical biodiversity (Myers *et al.*, 2000; Catibog-Sinha & Heaney, 2006; Brown & Diesmos, 2009). This

archipelago originated as a set of *de novo* oceanic islands beginning in the early Oligocene, and most of the extant islands have emerged from the ocean floor since the late Miocene and Pliocene, without dry-land connections to Southeast Asia (Hall, 1998). During periods of lower sea levels (averaging 120 m below current levels) within the Pleistocene glacial cycles, land bridges connected neighbouring islands into four major larger islands known as the Pleistocene aggregate island complexes (PAICs) (Heaney, 1985, 1986; Brown & Diesmos, 2002): Greater Luzon, Greater Mindanao, Greater Negros + Panay and Greater Palawan (Fig. 1a). Miocene–Pliocene geographical rearrangement and Pleistocene climatic oscillations represent two important, but not mutually exclusive, geological and climatic processes driving the diversification of tropical biota (Hewitt, 2000; Rull, 2011). These historical events may have provided ample

opportunities for colonization, allopatric isolation, local adaptation, secondary contact and consequent gene flow between tropical organisms.

The repeated formation and breakup of the PAICs represents a simplified explanatory model for understanding the patterns of diversification in the Philippine archipelago (Heaney, 1985, 1986; Brown & Diesmos, 2002). This PAIC model of speciation proposes that during late Pleistocene sea-level fluctuations, the cyclical fragmentation and reformation of the PAICs generated and maintained the extant species diversity. Earlier studies on vertebrates have shown patterns of faunal affinity between islands within the limits of these Pleistocene islands (e.g. Heaney, 1986; McGuire & Alcala, 2000; Esselstyn *et al.*, 2004). More recent phylogenetic studies have revealed concordance of some phylogroups of extant species with PAIC boundaries (Evans *et al.*, 2003;



**Figure 1** Sampling sites and phylogeny for *Pyrgonota bifoliata*. (a) Map of the Philippine archipelago. The 120-m isobath contours of the Pleistocene aggregate island complexes (PAICs) are shown in grey. Open circles indicate the collection sites using the locality codes listed in Appendix S1. (b) A female of *P. bifoliata* collected from Mt Makiling, Laguna, Philippines, on 6 July 2000 (photo by C.-P. Lin). (c) The phylogeny of *P. bifoliata* derived from Bayesian analyses of the combined *cox1* and *wg* data sets. *Jingkara hyalipunctata*, *Hypsauchenia* sp., *Pyrgauchenia brunnea* and *Pyrgonota noditurris* were used as outgroups. An asterisk above a tree branch indicates a branch with a Bayesian posterior probability greater than 0.9, and maximum likelihood (ML) and maximum parsimony (MP) bootstrapping values greater than 70%.

Esselstyn & Brown, 2009; Sánchez-González & Moyle, 2011) and the PAIC-structured genetic variation of widely distributed species (Peterson & Heaney, 1993; Heaney *et al.*, 2005; Esselstyn & Brown, 2009; Sánchez-González & Moyle, 2011). These phylogenetic studies suggest that the extent of the Pleistocene islands might have played a role in limiting gene flow among PAICs and homogenizing gene pools of island populations within PAICs. Nevertheless, many earlier empirical studies have found phylogeographical patterns contradicting the topological predictions derived from a strict PAIC model (Steppan *et al.*, 2003; Jones & Kennedy, 2008; Linkem *et al.*, 2010; Oliveros & Moyle, 2010; Siler *et al.*, 2010). These and other studies (Table 1) explicitly reject the role of PAICs in generating speciation, and emphasize pre-Pleistocene geological history, inter-island colonization and island topography/ecology as major drivers of species diversity in the Philippines. Recent studies involving the estimation of divergence time have shown that the majority of speciation events in the Philippines substantially pre-date the Pleistocene and occurred in the Neogene (Steppan *et al.*, 2003; Jansa *et al.*, 2006; Roberts, 2006b; Esselstyn & Brown, 2009; Siler *et al.*, 2012), thus strongly supporting the roles of Miocene–Pliocene geological processes and cross-island colonization in shaping the biodiversity of the Philippine archipelago.

Previous studies of the patterns and processes of Philippine diversification have focused predominantly on vertebrate fauna. Performing a rigorous evaluation of the speciation model for *non-vertebrate* species is therefore timely and important, especially for the more diverse and abundant invertebrates, such as insects, which frequently show varied dispersal abilities and life-history characteristics. Here, we present the first invertebrate case study aimed specifically at testing predictions derived from the Pleistocene island speciation model in an endemic Philippine treehopper, *Pyrgonota bifoliata* (Westwood, 1841) (Insecta: Hemiptera: Membracidae). Treehoppers are phytophagous insects with limited ability for long-distance dispersal (Wood, 1993a). Their geographical distributions are often associated with latitudinal gradients, host-plant specialization, subsocial behaviour and ant mutualism (Wood, 1993a). *Pyrgonota bifoliata* shows a high degree of host-plant specialization by feeding on *Bridelia* (Phyllanthaceae) and *Rubus* (Rosaceae) species, and occurs in pristine habitats within primary or mature second-growth lowland forests (Lin, 2003, 2006). Data from previous publications and museum collections suggest that *P. bifoliata* is restricted to Luzon and Panay in the Oceanic Philippines (excluding Palawan) (Funkhouser, 1915). However, our collection records indicate a much wider distribution, including at least six additional major islands (Negros, Cebu, Bohol, Samar, Leyte and Mindanao) across the archipelago. Higher level phylogenetic analysis has indicated that *Pyrgonota* is nested within two oceanic Philippine genera of apparent Mindanao origins: *Bulbauchenia* and *Funkhouserella* (Wallace & Deitz, 2004, 2006), suggesting that the genus has experienced an *in situ* diversification within the archipelago.

This study examined the genetic variation, time-calibrated phylogenies and biogeography of *P. bifoliata* derived from mitochondrial and nuclear genes to test the following specific predictions of a strict PAIC model of diversification: that there should be (1) monophyletic island populations within individual PAICs; (2) greater genetic divergence between PAICs than within PAICs; and (3) an association between the population divergence time among PAICs and the Pleistocene glacial maximum (*c.* 1–0.018 Ma). The ancestral range and dispersal pattern of *P. bifoliata* were reconstructed to examine the congruence between the pattern of colonization and the temporal formation of palaeo-islands during the Miocene–Pliocene. Being decorated with a conspicuous, highly elevated and bifurcated ‘horn’-like structure (the pronotum) (Fig. 1b), *P. bifoliata* is one of the most charismatic insects not only in the Philippines but throughout the Old World tropics, representing a well-established treehopper species that was described more than 150 years ago (Westwood, 1841). However, a cursory examination of available *P. bifoliata* specimens has revealed extensive morphological differences characterizing regional populations, giving rise to uncertainty regarding species boundaries and the number of cryptic species throughout the spatio-temporal range of *P. bifoliata*. We have therefore used recently developed coalescent-based species delimitation methods (Fujita & Leaché, 2011) to test species boundaries within *P. bifoliata*.

## MATERIALS AND METHODS

### Insect sampling

We collected 61 *P. bifoliata* specimens from 23 sites on seven major islands: Luzon, Negros, Cebu, Bohol, Samar, Leyte and Mindanao (Fig. 1a). These samples were adequate to reveal the major patterns of diversification in *P. bifoliata*. However, we recognize that a larger sample size might identify additional lineages and colonization events.

Field-collected specimens were immediately preserved in 95% ethanol, followed by long-term storage in a –80 °C freezer at the insect collection of Tunghai University (Taichung, Taiwan). Specimens of *Jingkara hyalipunctata*, *Hypsauchenia* sp., *Pyrgauchenia brunnea* and *Pyrgonota noditurris* were collected and used as outgroups for the phylogenetic analyses. More closely related *Pyrgonota* species are required in follow-up studies to shed greater light on the biogeography of *P. bifoliata* within the Philippines. Information on the collection of specimens is listed in Appendix S1 in Supporting Information.

### DNA extraction and sequencing

Genomic DNA was extracted from the leg or thoracic muscle of each specimen using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA fragments consisting of 836 bp of the mitochondrial cytochrome *c* oxidase I subunit (*cox1*) gene were amplified using previously reported primers [Ron, C1-J-1751 (Simons *et al.*, 1994) and Calvin, C1-N-2725 (Lin

**Table 1** Analyses of molecular variation (AMOVA), Pleistocene aggregate island complex (PAICs) hypothesis and the pattern of diversification in *Pyrgonota bifoliata* and selected vertebrate taxa\*.

Taxa	AMOVA (%)†				Oceanic dispersal	PAICs	Origin	Dispersal route	Monophyly of all PAICs	Age of Philippine lineage (Ma)	Strict PAIC model	Diversification drivers other than PAIC	Source(s)	Data sets
	Between PAICs	Between islands	Within islands	Within islands										
<i>Pyrgonota</i> Treehopper	19	52	29	29	Very poor	Luzon, Negros + Panay, Mindanao	Mindanao	Mindanao to Luzon	No	10	Rejected	Colonization	This study	Original
<i>Rhipidura</i> Finch	23	48	29	29	Moderate	Luzon, Negros + Panay, Tablas, Mindanao	NA	Mindanao to Luzon	Yes	>1‡	Accepted	Geological history Isolation by distance Local adaptation Colonization Island topography Multiple invasions	Sánchez-González & Moyle (2011)	Clade A, B & C
<i>Pinoyascincus</i> Skink	26	62	12	12	Poor	Luzon, Negros + Panay, Mindanao	NA	Multiple invasion	No	NA	Rejected	Clade age Geological history Island topography Isolation by distance	Linkem <i>et al.</i> (2010), Oaks <i>et al.</i> (2013)	<i>P. jagori</i> clade
<i>Crocodyra</i> Shrew	29	48	23	23	Poor	Luzon, Negros + Panay, Mindanao, Palawan	Malaysia	Multiple invasion	Yes‡	2.5	Partially accepted	Colonization	Esselstyn & Brown (2009)	Original
<i>Cyrtodactylus</i> Gecko	32	28	40	40	Poor	Luzon, Negros + Panay, Borneo	Sulawesi, Borneo	NA	No	NA	Rejected	Island area Isolation by distance NA	Siler <i>et al.</i> (2010)	Original
<i>Limnonectes</i> Frog	59	6	35	35	Poor	Luzon, Negros + Panay, Mindanao	Sulawesi	Single invasion	No	~1	NA	NA	Evans <i>et al.</i> (2003)	Clade E
<i>Haplonycteris</i> Fruit bat	78	12	10	10	Moderate	Luzon, Negros + Panay, Mindoro, Sibuyan, Mindanao	NA	NA	Yes	6	Rejected	Colonization Island topography	Roberts (2006b)	Original

\*The vertebrate data sets were selected only if the distribution of the clade(s) included multiple PAICs and multiple islands within PAICs of the oceanic Philippines.

†The three-level AMOVA for the same PAIC/between island/within island partitions was statistically significant for all data sets.

‡Small island samples not belonging to any PAICs were nested within PAIC lineages.

§Pre-late Pleistocene age inferred by the authors.

NA, not available.

& Wood, 2002)]. The combination of the *wg1a* and *wg2a* primers (Brower & DeSalle, 1998) produced a 324-bp polymerase chain reaction (PCR) product from the nuclear wingless (*wg*) gene. The PCR amplification methods followed the profile detailed in Lin *et al.* (2004). The amplified PCR products were either sequenced directly from both directions or gel-purified and cloned into competent cells and then sequenced in an ABI PRISM™ 377 automatic sequencer (Perkin Elmer, Waltham, MA, USA). The DNA sequences (GenBank accessions KC848058–KC848179) were edited and assembled manually, and then aligned using CLUSTALW (DNASTAR, Madison, WI, USA). The heterozygous sites were coded using standard International Union of Pure and Applied Chemistry (IUPAC) codes.

### Genetic divergence and phylogenetic analyses

Analyses of molecular variance (AMOVA) (Excoffier *et al.*, 1992) with three hierarchical levels (sites, islands and PAICs) and 10,000 permutations were computed in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Phylogenetic relationships were reconstructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian criteria (TreeBASE accession number S14090). Equally weighted parsimony analyses were performed in PAUP\* 4b10 (Swofford, 2002) through a heuristic search with trees obtained via 1000 random taxon additions. We followed the parsimony ratchet procedure (Nixon, 1999) to calculate bootstrap support for the nodes in PAUPRAT (Sikes & Lewis, 2001). For the ML and Bayesian analyses, we employed jMODELTEST 2.1.3 (Posada, 2008) to select the best-fitting nucleotide substitution model (HKY+I+G for first positions in *cox1* and *wg*; F81+I+G for second positions in *wg*; GTR+G for second positions in *cox1* and third positions in *cox1* and *wg*) using the Akaike information criterion (AIC). ML analyses were conducted using GARLI 2.0 (Zwickl, 2006), with branch support calculated from 1000 bootstraps. Bayesian phylogenetic analyses were performed using two independent Markov chain Monte Carlo (MCMC) chains of  $6 \times 10^8$  generations in MRBAYES 3.2.1 (Ronquist & Huelsenbeck, 2003). The convergence of the runs was assessed based on the effective sample size (ESS) values of the parameters ( $> 200$ ) from likelihood scores obtained from time plots using TRACER 1.5 (Rambaut & Drummond, 2009) and by examining the correlation of the posterior split frequencies between runs in AWTY (Nylander *et al.*, 2008). The first 25% of the sampled trees were discarded as burn-in, and the posterior probability of the internal nodes was calculated by constructing a 50% majority consensus tree.

### Divergence time estimation

Divergence times were estimated in BEAST 1.7.4 (Drummond *et al.*, 2012) based on a range of mutation rates between the standard (0.0115 mutations site<sup>-1</sup> Myr<sup>-1</sup> or 2.3% Myr<sup>-1</sup>; Brower & DeSalle, 1998) and a revised (0.0177 mutations

site<sup>-1</sup> Myr<sup>-1</sup>, Papadopoulou *et al.*, 2010) molecular clock for *cox1*. For *wg*, a range of mutation rates between 50% of the *cox1* substitution rate (0.0058 mutations site<sup>-1</sup> Myr<sup>-1</sup>, based on the uncorrected average pairwise sequence divergence of *cox1* and *wg*) and the synonymous substitution rate for nuclear *Arrestin* (*arrB*) (0.012 mutations site<sup>-1</sup> Myr<sup>-1</sup>; Moriyama & Gojobori, 1992) was applied. The substitution rates for *cox1* and *wg* were set as unlinked. A lognormal relaxed clock model of the mean, equal to the specified rates, and its standard deviation (uclid.stdev), set to 0.001, were applied to both genes. A Yule speciation (Gernhard, 2008) tree prior was specified for the analyses. Four independent MCMC analyses were run for  $6 \times 10^8$  generations, with a sampling frequency of 1000 iterations and 25% burn-in.

### Species delimitation

The species boundaries of *P. bifoliata* were tested using the Bayesian general mixed Yule coalescent (bGMYC; Reid & Carstens, 2012) and Bayesian phylogenetic and phylogeography (BPP; Yang & Rannala, 2010) methods. Specifically, we assessed whether speciation events were associated with sites, islands or PAICs. The general mixed Yule coalescent (GMYC) method simulates the transition of the branching rate of the phylogeny between cladogenesis and coalescence, under the assumption that species-level processes (speciation and extinction) will occur at a slower rate than population-level processes (allele coalescence). bGMYC 1.0 (Reid & Carstens, 2012) is a version of the GMYC that accounts for the uncertainty of phylogenetic tree estimates using a Bayesian methodology. The BPP methodology employs a reversible-jump MCMC to search the parameter space and estimate the distribution of gene trees from multiple loci using user-specified guide trees. The program then computes the Bayesian posterior probability of the proposed nodes of the species phylogeny. For the bGMYC analyses (Pons *et al.*, 2006), we followed Reid & Carstens (2012) and obtained a subsample of 100 trees from the posterior distribution of ultrametric trees from the combined data set generated with a phylogenetic clock model in BEAST 1.7.4. Under each tree topology,  $5 \times 10^4$  MCMC generations were run, with a sampling frequency of 100 and a burn-in of 400 trees.

For the BPP analyses, external morphology (Fig. 2) was employed first to reconstruct the higher level relationships of a user-specified guide tree, and then combined with the tree topology derived from MRBAYES analyses to build the relationships of the lineages between islands. The relatedness between collection sites within islands was constructed using geographical distances. This process resulted in a 20-species guide tree by treating sites with geographical distances greater than 20 km as proposed species (M6 + M7 in Mindanao, M3 + M4 in Mindanao, and N2 + N3 in Negros, were each grouped into a single species; Fig. 1). BPP 2.2 (Yang & Rannala, 2010) specifies a gamma (G) prior for the population size ( $\theta$ ) and root age of the tree ( $\tau_0$ ) parameters, while the other divergence time parameters are assigned with



the Dirichlet prior (Yang & Rannala, 2010). Two different combinations of the prior distributions for  $\theta$  and  $\tau_0$  were employed to estimate the posterior probabilities of the models: (1)  $\theta \sim G(1, 10)$  and  $\tau_0 \sim G(2, 2000)$ , representing small population sizes and deep divergence for models with more species; and (2)  $\theta \sim G(2, 2000)$  and  $\tau_0 \sim G(1, 10)$ , representing large population sizes and shallow divergence for models with fewer species. The BPP analyses were run multiple times to confirm consistency using algorithm1 and algorithm0 with automatically adjusted fine-tuning parameters. Each run was performed for  $1 \times 10^6$  generations, with a sampling frequency of five and a burn-in of  $2 \times 10^4$  generations.

### Ancestral range and historical dispersal pattern

Potential ancestral ranges were first reconstructed using the Bayesian binary MCMC (BBM) methods of RASP 2.1 (Reconstruct Ancestral State in Phylogenies; Yu *et al.*, 2012). The analyses were performed using the final trees (Fig. 1c) generated from MRBAYES phylogenetic analyses, with the range of the specimen coded as the island of origin. The MCMC chains of two independent runs were conducted for  $5 \times 10^5$  generations, with a sampling frequency of 1000 and a burn-in of 100 trees. Reconstruction of the ancestral range was also performed using a Markov discrete phylogeographical model with Bayesian stochastic search variable selection (BSSVS; Lemey *et al.*, 2009) implemented in BEAST 1.7.4. Seven biogeographical areas based on current island boundaries were considered, with additional specification of an asymmetrical discrete trait substitution model for areas. Dispersal routes with significant non-zero migration rates were identified using Bayes factors in SPREAD (Bielejec *et al.*, 2011).

## RESULTS

### Phylogeny, genetic divergence and species status

The MP, ML and Bayesian reconstruction of the two gene trees were largely consistent, with weakly supported topological conflicts of the *wg* tree (Appendix S2). The *cox1* gene tree was well resolved, whereas the *wg* tree showed limited phylogenetic resolution and supported only three major clades (Mindanao, Luzon and Greater Negros + Panay + Luzon PAICs). The phylogeny based on combined data showed prominent phylogenetic gaps distinguishing deep allopatric *P. bifoliata* lineages (Fig. 1c). This tree recovered five reciprocally monophyletic lineages grouped by current island boundaries (Luzon, Negros, Cebu, Bohol and Mindanao). The Greater Mindanao (Mindanao + Leyte + Samar + Bohol) and Greater Luzon PAICs each formed a monophyletic lineage, with the exception of the insects from Negros and Cebu in the Greater Negros + Panay PAIC, which were found to be paraphyletic. Although the AMOVA results showed that a significant ( $P < 0.01$ ) proportion of genetic

variation was explained by each level of the AMOVA (Table 1), the between-islands-within-PAIC category explained the greatest proportion of genetic variation. In comparison with selected studies of endemic Philippine vertebrates (Brown & Guttman, 2002; Esselstyn & Brown, 2009; Siler *et al.*, 2010; Sánchez-González & Moyle, 2011; Oaks *et al.*, 2013), the between-PAIC category explained the smallest proportion of genetic diversity for *P. bifoliata* (Table 1).

The bGMYC species delimitation suggested a total of 14 species, whereas the BPP species delimitation provided a relatively conservative estimate of nine species among the *P. bifoliata* samples (Fig. 2). We recognize the nine BPP-delimited species that are congruent between the two methods, and refer to them as different 'operational' *P. bifoliata* species. The results of both species delimitation methods indicate the presence of multiple species within the Greater Negros + Panay PAIC (two to four species) and the Greater Mindanao PAIC (four to six species). At least one species was indicated for the smaller islands (Negros, Cebu, Bohol) and island groups (Samar + Leyte) and more than one species for larger islands (Luzon, three species; Mindanao, two to three species). The statistically inferred species boundaries within Luzon and Mindanao were associated with the geographical distribution of the samples (Fig. 1a) and the observed morphological characteristics (Fig. 2), with the *P. bifoliata* specimens from northern, central and southern Luzon and those from eastern and southern Mindanao each being identified as a separate species.

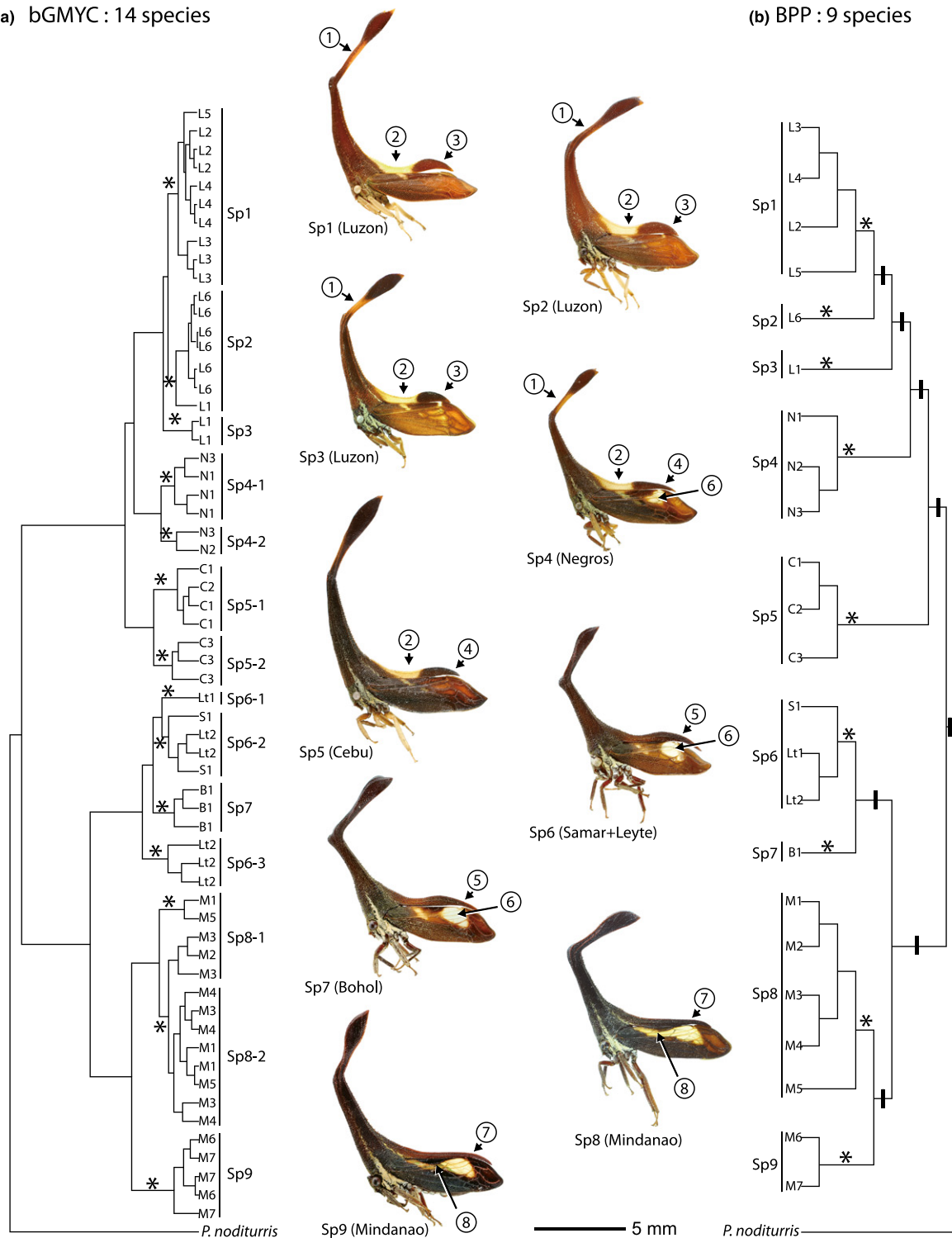
### Divergence time and colonization history

Divergence time estimates based on a range of mutation rates provided similar time frames for the diversification of *P. bifoliata* (Fig. 3, Appendix S3). We defined the 'species age' as the most recent common ancestors (MRCA) of the extant lineages. The results based on the standard mitochondrial clock for insects (Fig. 3) provided a mean estimate of nearly 9.5 Ma [confidence interval (CI): 6.8–12.8] for the origin of *P. bifoliata*. The origins of the PAIC lineages were dated to between approximately 5.8 Ma (CI: 3.9–8.2) in the late Miocene and 3.9 Ma (CI: 2.6–5.5) in the middle Pliocene. The nine extant *P. bifoliata* species (a consensus of the two delimitation methods; sp1–9 identified in BPP; Fig. 2b) originated between the early [2.4 Ma (CI: 1.3–3.7) for sp5 in Cebu] and middle [1.0 Ma (CI: 0.5–1.7) for sp1 in Luzon] Pleistocene, with the exception of the late Pleistocene origin indicated for the youngest species, sp3, from northern Luzon (0.3 Ma, CI: 0.02–1.03). Most of the between-island-within-PAIC splits observed in the *P. bifoliata* lineage arose from the Pliocene to 2.4 Ma (in the early Pleistocene) (between Samar + Leyte and Bohol), whereas the majority of intraspecific lineage splits within islands occurred after the onset of the earliest glaciation maximum (*c.* 1.0 Ma) in the middle Pleistocene (Fig. 3).

The two ancestral range reconstruction methods yielded comparable results and suggested that Mindanao

(a) bGMYC : 14 species

(b) BPP : 9 species



**Figure 2** The Bayesian species delimitation and morphological divergence of *Pyrgonota bifoliata* using *P. noditurrus* as an outgroup. (a) The Bayesian general mixed Yule coalescent (bGMYC) species delimitation resulted in 14 species. An asterisk indicates that the Bayesian posterior probability that all members within a lineage belong to one species is greater than 0.95. (b) The Bayesian phylogenetic and phylogeography (BPP) species delimitation indicated nine species. The results shown here were derived using the 20 species/locality guide tree, with the population size parameter,  $\theta$ , set to  $\sim G(2, 2000)$  and the root age of the tree parameter,  $\tau_0$ , set to  $\sim G(1, 10)$ , representing a more conservative estimate of species numbers. A vertical bar on a branch indicates that the Bayesian posterior probability of a speciation event at a given node is greater than 0.95 (i.e. there were two descendant species following the split). The central images are representative *P. bifoliata* specimens from each species inferred statistically through BPP, with diagnostic morphological features indicated with numbers.

represented the most probable ancestral range for *P. bifoliata* (Fig. 3). Mindanao and Leyte, respectively, were reconstructed as the ancestral ranges with the highest posterior probabilities for the Greater Mindanao PAIC and Leyte + Samar + Bohol. The BSSVS reconstruction estimated that Cebu and Negros, respectively, were the most likely ancestral ranges for the Greater Negros + Panay PAIC + Luzon and Negros + Luzon. The dispersal routes showing higher Bayes factors values (Fig. 4) generally suggested a continuous northern trend of cross-island colonization over approximately the past 5 Myr, although a few reverse dispersals were equally possible. The ancestral *P. bifoliata* treehoppers probably originated in Mindanao *c.* 7–13 Ma in the late Miocene (Fig. 4a) and began to disperse out of Mindanao during the early Pliocene (4–8 Ma; Fig. 4b), followed by the first cross-island colonization to Cebu in the middle Pliocene (3–5 Ma, sp5; Fig. 4c). Subsequently, the ancestral *P. bifoliata* colonized Negros from Cebu (sp4), and one lineage then colonized Leyte from Mindanao (sp6) in the late Pliocene and early Pleistocene (2–4 Ma; Fig. 4d). By the early Pleistocene, the ancestral *P. bifoliata* had migrated from Leyte into first Samar (sp7) and then Bohol, and one lineage arrived in southern Luzon via Negros (1–3 Ma, sp2; Fig. 4e) and subsequently radiated to reach central (sp1) and northern Luzon (sp3) and southern regions of Mindanao (sp9) during the middle to late Pleistocene (0.5–2.5 Ma; Fig. 4f).

## DISCUSSION

### Ancient geological and dispersal processes as major drivers of diversity

This study indicates that the mitochondrial *cox1* gene provides the majority of informative characters in resolving major clades of *P. bifoliata*, while the nuclear *wg* gene tree has little resolution. The spatio-temporal reconstruction of the evolutionary history of *P. bifoliata* based on combined data supports the hypothesis that Miocene–Pliocene geological events and cross-island dispersal were major drivers of the diversification of this species complex within the Philippine archipelago. The ancestral *P. bifoliata* treehoppers originated in Mindanao *c.* 7–13 Ma during the late Miocene, followed by the splitting of most island lineages *c.* 2–6 Ma, in the Pliocene–early Pleistocene. *Pyrgonota bifoliata* therefore shows clear evidence of an ancient lineage that has persisted for millions of years after its origin on these volcanic islands. This further implies that the species turnover rate of *P. bifoliata* is relatively slow, which contrasts with the emphasis on rapid species turnover envisioned in the classic equilibrium model of island biogeography (MacArthur & Wilson, 1967). The equilibrium model emphasizes frequent colonization and extinction, rather than persistence and diversification, and the latter processes have often been found to be important on ancient, large and highly isolated island archipelagos such as the Philippines, where speciation

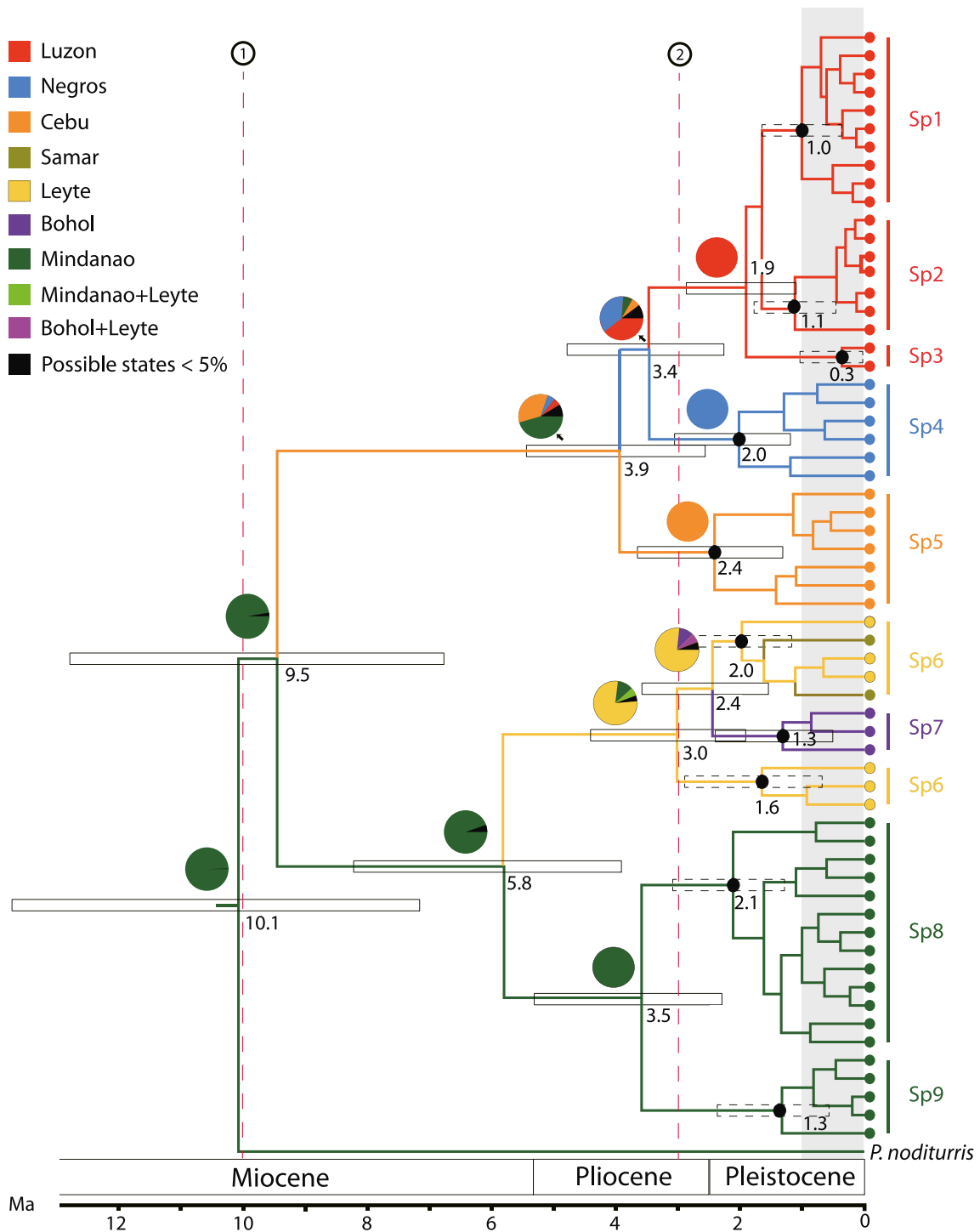
over time and with local adaptation is more common (Heaney, 1986, 2000; Whittaker & Fernández-Palacios, 2007). The general dynamic model of oceanic island biogeography (Whittaker *et al.*, 2007, 2008), by incorporating the ontogeny of oceanic islands with evolutionary time-scales and a significant role for *in situ* speciation, is more appropriate for explaining the complex history of Philippine's diversification (Heaney, 2007; Whittaker *et al.*, 2008). Our phylogeographical analyses of *P. bifoliata* provide evidence for a dominant pattern of colonization events from southern to northern islands within the Philippine archipelago, with speciation arising on newly colonized islands. This pattern is consistent with the predictions derived from the general dynamic model (prediction numbers 8 and 10; Whittaker *et al.*, 2008) and evidence from several recent vertebrate studies of the Philippines (e.g. Esselstyn *et al.*, 2009; Siler *et al.*, 2012; Blackburn *et al.*, 2013). The extant *P. bifoliata* species on each island are monophyletic, which indicates that they typically are descendants of single colonization events. Single-island endemic members of the *P. bifoliata* species group were derived from a series of ancient cross-island dispersals and subsequent divergence events, which mainly coincided with the spatial reorganization and temporal sequence of dry-land formation in the oceanic Philippines.

The finding of sequential cross-island colonization events for *P. bifoliata* provides strong empirical evidence of long-distance dispersal as a regular phenomenon among terrestrial organisms on islands, allowing clades to overcome oceanic water barriers during geological time spans of millions of years (de Queiroz, 2005; Heaney, 2007; Whittaker *et al.*, 2008). It also suggests that ancient oceanic dispersal was a more important contributor to the diversification of *P. bifoliata* than tectonic vicariance processes or Pleistocene land bridges, as no recent colonization between PAICs is indicated by our data. Nevertheless, the mechanism of effective long-distance dispersal across oceanic barriers is unknown for treehoppers. Because they are endowed with an enormous complex pronotal structure on the upper part of their bodies, *P. bifoliata* probably have a reduced ability to fly and limited capacity for long-distance dispersal using their wings compared with treehoppers with a compact pronota (Lin, 2003). The historical dispersal of winged *P. bifoliata* adults into neighbouring islands may have been facilitated by prevailing monsoon winds or strong typhoon winds, as has been observed among the migratory planthoppers (Kisimoto & Sogawa, 1995; Anderson *et al.*, 2010). Another potential mechanism of cross-island dispersal for *P. bifoliata* is via drifting floating vegetation of host plants on oceanic currents, resulting in the passive transport of adults, wingless nymphs and/or eggs inserted in host-plant tissues.

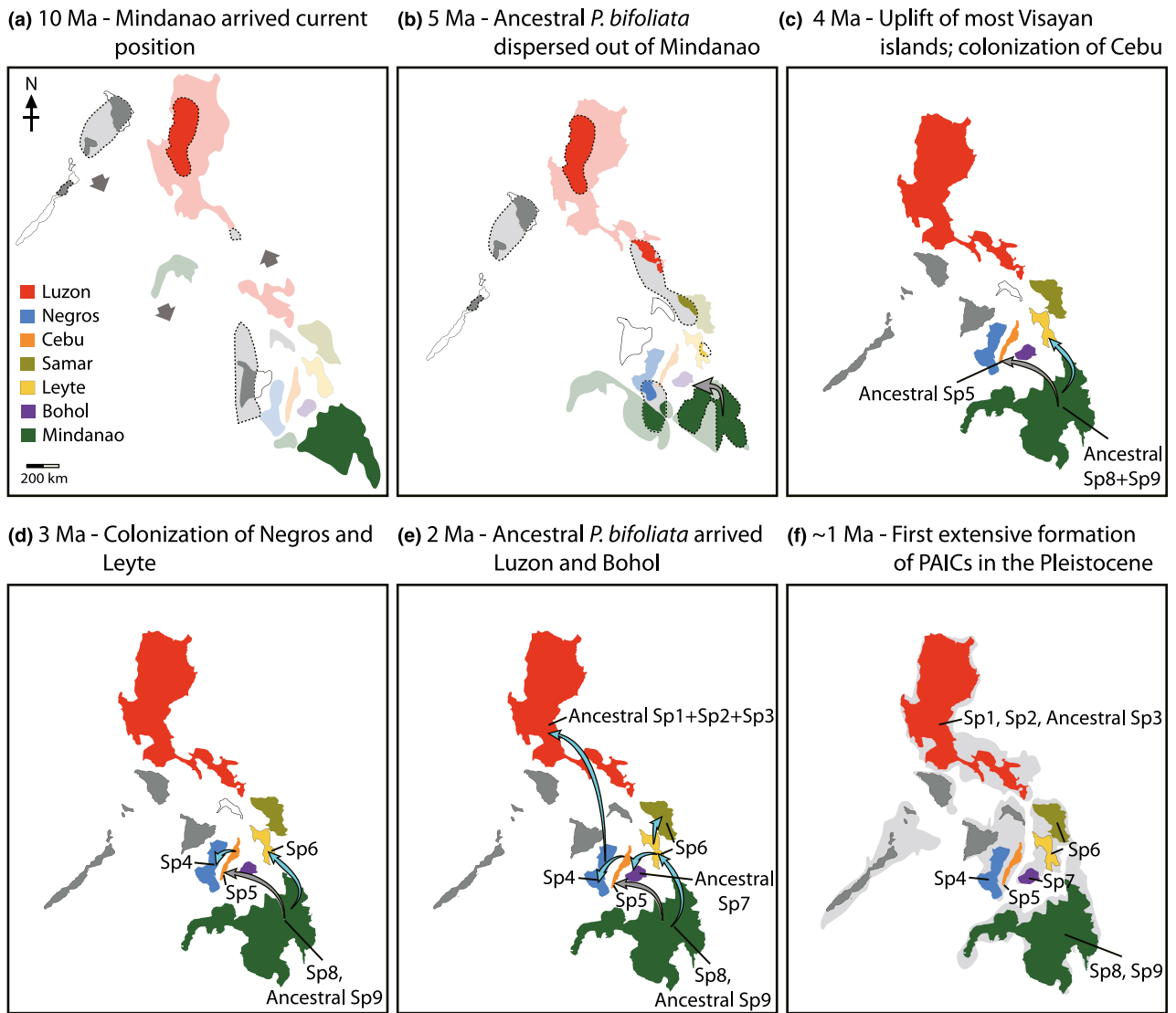
### Testing the strict PAIC speciation hypothesis

By testing specific predictions derived from a strict PAIC model as a vicariant hypothesis, our analyses have demonstrated that all the *P. bifoliata* lineages from the same PAIC





**Figure 3** The spatio-temporal reconstruction of diversification of *Pyrgonota bifoliata* using *P. noditurris* as an outgroup. Divergence time estimation was made using the standard arthropod *cox1* and nuclear *wg* molecular clocks, and the Bayesian reconstructions of ancestral ranges using Bayesian stochastic search variable selection (BSSVS; Lemey *et al.*, 2009) and Bayesian binary Markov chain Monte Carlo (BBM; Yu *et al.*, 2012) models. The maximum clade credibility tree is shown. The colour of a branch indicates the reconstructed ancestral range that received the highest posterior probability in the BSSVS model. The pie charts show the relative ancestral range probability obtained using the BBM model, with arrows indicating the highest posterior probability of the reconstructed area (state). The circled 1 denotes the time when the Greater Mindanao Pleistocene aggregate island complex (PAIC) arrived at its current position (Hall, 1998). The circled 2 refers to the period of uplift of Leyte and neighbouring islands (Sajona *et al.*, 1997). The vertical grey bar indicates the time frame of the late Pleistocene glacial maximum (c. 1–0.018 Ma). The horizontal empty bar at the internode indicates the 95% probability of the highest density of branch height. The black dot indicates the most recent common ancestor (MRCA) of the species inferred statistically through BPP analysis.



**Figure 4** Palaeogeological rearrangement of the Philippine islands (Hall, 1998) and the inferred ancestral dispersal routes for *Pyrgonota bifoliata*. (a) 10 Ma: eastern and central Mindanao arrived at approximately their current positions, while only portions of northern Luzon, Mindoro, Palawan, Panay and Mindanao were above sea level. Arrows denote the direction of plate tectonic movement. (b) 5 Ma: most of the major islands were nearly at their present positions, but the majority of the extant Visayan Islands were still submerged, except for parts of Negros and Samar. (c) 4 Ma: the emergence of most of Luzon (the Visayan Islands) occurred. (d) 3 Ma: the colonization of Negros and Leyte by ancestral *P. bifoliata* occurred. (e) 2 Ma: the emergence of Masbate took place, and all of the islands were at their current positions. (f) 1 Ma: the first extensive formation of Pleistocene aggregate island complexes (PAICs) occurred (in the late Pleistocene). The solid grey and coloured areas indicate the landmass that was above sea level at the given time. The tinted colour areas indicate current island positions and boundaries. The dashed lines represent areas of ambiguous dry-land boundaries. The dispersal route shown in blue corresponds to the route with significant non-zero rates (Bayes factor > 3). The dispersal route presented in grey corresponds to the route reconstructed using the Bayesian stochastic search variable selection (BSSVS) model with non-significant rates (Bayes factor < 3). The species number corresponds to the species inferred statistically through Bayesian phylogenetic and phylogeography (BPP) analysis.

are monophyletic, with the exception of lineages from Negros and Cebu (the Greater Negros + Panay PAIC). This result appears to be congruent with a strict PAIC model, which predicts monophyly of the lineages from a given PAIC. However, the AMOVA results suggest that within-PAIC, rather than between-PAIC, partitioning explains the greatest fraction of the genetic diversity found in *P. bifoliata* (Table 1). Compared with the majority of previous studies

performed on vertebrates, *P. bifoliata* shows lower between-PAIC genetic variation, representing one of the strongest pieces of empirical evidence against a strict PAIC model (see also Roberts, 2006a). In addition, our divergence time estimates show that most PAIC lineages and extant *P. bifoliata* lineages originated before the onset of the first extensive Pleistocene glacial cycle and were not associated with the late Pleistocene glacial maximum (c. 1–0.018 Ma). These

results are highly inconsistent with a strict PAIC speciation model. Our analyses of the invertebrate *P. bifoliata* therefore contribute to a growing number of vertebrate studies that support the importance of the Neogene speciation and highlight the roles of cross-island colonization and geological changes in generating speciation within the Philippine fauna (Steppan *et al.*, 2003; Jansa *et al.*, 2006; Roberts, 2006b; Jones & Kennedy, 2008; Esselstyn & Brown, 2009; Linkem *et al.*, 2010; Oliveros & Moyle, 2010; Siler *et al.*, 2010, 2012).

Our results emphasize that findings of discordance or concordance with PAIC-derived topological predictions need to be interpreted with caution. For example, the topological prediction of monophyletic lineages derived from the same PAICs is not a unique outcome of PAIC-driven speciation patterns. Instead, these phylogenetic relationships can often be equally well explained by non-PAIC scenarios, such as those related to colonization history. In the present study on *P. bifoliata*, the prediction of a monophyletic Greater Mindanao PAIC could itself have resulted from sequential northwards colonization from Mindanao into neighbouring Leyte, Samar and Bohol, without explicitly assuming a strict PAIC model. Furthermore, the strict PAIC model did not explicitly state the between-PAIC relationships, making testing this model difficult based merely on the topology of the phylogenies. We suggest that the empirical evidence necessary for fully examining the effect of PAICs should be drawn not only from the topology of phylogenies but also from the divergence time, origin, colonization routes and life-history characteristics of the target species (Table 1).

### Cryptic diversity and the potential role of host-plant adaptation

Cryptic species present unique opportunities for studying mechanisms of speciation, mate recognition and effects of conservation management that were previously undetected because of morphologically indistinguishable taxa (Bickford *et al.*, 2007). In contrast to earlier taxonomic arrangements of this widely distributed species (Funkhouser, 1915), we have found evidence of at least nine divergent, monophyletic, cryptic species of *P. bifoliata*, as delimited through the statistical inference of genetic data. The species boundaries of four of the nine cryptic species correspond to current island boundaries (Negros, Cebu, Bohol and Samar + Leyte), suggesting a strong role of allopatric isolation as a result of oceanic barriers in preserving the genetic cohesion of these species. Upon closer inspection of specimens from distinct phylogenetic lineages, these species showed apparent differences in several diagnostic morphological characteristics. We propose that all nine *P. bifoliata* identified in this study should be recognized as candidate species based on the statistical species limits and the monophyletic status of each species lineage, in combination with their allopatric distributions and morphological variations. A formal taxonomic revision based on morphological and molecular traits in this

complex is underway, with the probability of finding additional species being recognized from examining larger geographical samples.

Plant-feeding insects such as treehoppers represent potential warehouses of tremendous cryptic diversity because many of these organisms have specialized interspecific interactions with host plants in local habitats (Berlocher & Feder, 2002). Treehoppers exhibit species recognition and mate choice systems that rely heavily on non-morphological characteristics, including substrate-borne vibrational mating signals (Wood, 1993b), host-plant cues (Wood, 1993b) and species-specific pheromones (Nault *et al.*, 1974). For example, the North American *Enchenopa binotata* species complex constitutes a good model for investigating the cryptic diversity of treehoppers, among which speciation has originated from shifts to novel host plants, without measurable changes in morphology (Cocroft *et al.*, 2008). The *E. binotata* complex is associated with 11 different host-plant species, and its members display indistinguishable male genitalia and can only be recognized through subtle differences in female pronotal shape, nymphal characters, allozymes (Wood, 1993b) and mitochondrial haplotypes (Lin & Wood, 2002). Nevertheless, the 11 *E. binotata* species differ substantially in both sexual (male advertisement signals and female preference; Cocroft *et al.*, 2008) and ecological (host-plant phenology and life history; Wood, 1993b) traits that are important in reproductive isolation and speciation, making them valid species. Here, the five cryptic *P. bifoliata* species identified from Luzon and Mindanao correspond to separate biogeographical regions within an island, indicating that isolation by distance and/or ecological adaptation to specialized host plants may have played important roles in reducing gene flow and maintaining genetic differentiation among extant species. Given the frequent speciation as a result of host shifting observed in plant-feeding insects (Berlocher & Feder, 2002), ecological adaptation to specialized host plants, together with either local selection or genetic drift through allopatric isolation, present a more likely mechanism of ecological speciation (Rundle & Nosil, 2005) for the origin of *P. bifoliata* species. Our preliminary field observations suggest that *P. bifoliata* treehoppers exploit a range of distantly related host plants, with species from northern Luzon (sp3, L1) feeding on woody trees (*Bridelia*, Phyllanthaceae) and species from southern Mindanao (sp8, M5) feeding on raspberries (*Rubus*, Rosaceae). Evidently, the hypothesis of ecological speciation proposed for *P. bifoliata* will need to be tested through confirmation of host-plant adaptation and detailed experimental studies of divergence-related sexual and ecological traits across the geographical range of these insects within the Philippine archipelago.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Localities, collection information and GenBank accession numbers of *Pyrgonota bifoliata* and outgroup species.

**Appendix S2** *cox1*, *wg* and combined phylogenies of *Pyrgonota bifoliata*.

**Appendix S3** Divergence time estimates for *Pyrgonota bifoliata* based on a revised insect molecular clock.

## BIOSKETCH

**Chung-Ping Lin** is a systematist and evolutionary biologist. The research interests of his laboratory include molecular phylogenetics, character evolution, island diversification and speciation of insects (laboratory webpage <http://web.ntnu.edu.tw/~treehopper>).

Author contributions: C.-P.L. designed the study; C.-P.L., R.J.T.V. and O.M.N. carried out the fieldwork; J.-F.W. and Y.-C.S. generated the molecular data; Y.-C.S. and J.-F.W. conducted phylogenetic and statistical analyses with input from C.-P.L.; and C.-P.L. led the writing.

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