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Phylogeny and population genetic structure of the ant genus *Acropyga* (Hymenoptera : Formicidae) in Papua New Guinea

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Abstract. Spatial isolation and geological history are important factors in the diversification and population differentiation of species. Here we describe distributional patterns of ants in the genus *Acropyga* across Papua New Guinea (PNG), a highly biodiverse but little-studied region. We estimate phylogenetic relationships among currently recognised species of *Acropyga* and assess population genetic structure of the widespread species, *A. acutiventris*, across lowland areas of the island. We find that species of *Acropyga* present in PNG diversified during the Pliocene, between six and two million years ago. Most species now exhibit a patchy distribution that does not show a strong signal of geological history. However, the population genetic structure of the widespread species *A. acutiventris* has been influenced by geography, habitat association and, possibly, historical habitat fragmentation. There is a significant effect of isolation-by-distance within continuous lowland forest, and proximity to Australia has had a larger impact in structuring populations of *A. acutiventris* in PNG than has the Central Papuan Cordillera. This study is the first to describe population genetic patterns of an ant species in Papua New Guinea.

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Introduction

Tropical environments contain an extraordinary diversity of insect species, but the ecological and evolutionary factors maintaining such diversity are not well understood. Two major patterns are generally observed: high species richness of insects across tropical habitats and, simultaneously, low species turnover of insect guilds across large areas of lowland rainforests (Dyer *et al*. 2007; Novotny *et al*. 2007). This could be due to the presence of undetected cryptic species that bias estimates of species richness of lowland communities. However, if the low diversity turnover is genuine, the high gamma-diversity is likely to be generated in mountain ranges and along altitudinal gradients that are not as commonly sampled as lowland regions.

Molecular genetic comparisons across taxa that could reveal the cryptic diversity or assess levels of dispersal and vicariance are largely absent for insects inhabiting lowland tropical forests. Furthermore, population-level processes that structure local community compositions cannot be directly observed from species-level distributional data generated by most ecological studies. Thus a comparative phylogeographic and population genetic approach covering various temporal and spatial scales can help illuminate the ecological and evolutionary processes driving the origin and maintenance of species diversity (Stireman *et al*. 2005).

In this study we investigate the distribution, phylogeny and population genetic patterns of ants in the genus *Acropyga* (Roger, 1862) (Hymenoptera : Formicidae) in tropical lowland habitats of Papua New Guinea (PNG). Ants are excellent models for studying how ecological and evolutionary processes affect species distribution and population structure in the tropics. Ants are an omnipresent and ecologically dominant group of insects in most terrestrial ecosystems. They are important predators, omnivores, mutualists and decomposers, and they include species with sedentary life strategies as well as species with broad dispersal abilities. The rarely studied but well-described Formicinae genus *Acropyga* contains 41 described species primarily living in leaf litter and soil throughout the tropics and subtropics (LaPolla 2004). Despite a relatively cryptic lifestyle, some species appear to be locally abundant

and relatively widespread. Of New Guinea's nearly 900 currently described ant species (Janda *et al*. 2014), several *Acropyga* species are frequently encountered in local ant assemblages from different habitats. This makes them good candidates for phylogeographic and genetic studies of connectivity and gene flow among habitats or geographic areas.

One of the striking features of the ecology of *Acropyga* is that most, if not all, species have a mutualistic relationship with rhizoecid mealybugs (Hemiptera : Coccoidea : Rhizoecidae), which contribute to the nutrient supply of the ant colony and are transported by the queen during the establishment of a new nest (Williams 1998; LaPolla *et al*. 2002; Schneider and LaPolla 2011). Several species of *Acropyga* are widely distributed, which suggests that: (i) this could be an old group that has had time to disperse widely, (ii) early claims that the genus is composed of poor dispersers are incorrect (Bünzli 1935), or (iii) a large number of cryptic species might be present. The fact that *A. acutiventris* occurs from continental Asia to Australia, and is found on some Melanesian islands as well as on small reef islands off the shore of New Guinea, would suggest that at least this species might disperse relatively easily.

New Guinea is a centre of Indo-Australian biodiversity (Gressitt 1982*a*) and one of the last biogeographic realms with vast areas of undisturbed lowland rainforest. The northern part of the island is formed by more than 30 terrains of various origins that collided with the southern part of the island, which is of Australian origin ('Sahul'), ~10 Ma (million years ago; Hall 1998). The vast northern and southern lowlands are separated by the Central Cordillera (which uplifted 4.7–5.8 Ma; Hill and Gleadow 1989; Haig and Medd 1996), providing opportunities to study the effect of geographic distance and different types of barriers on species' distributions, population structure and diversification. Indeed, the extent to which the geological history of New Guinea has shaped the current distributional patterns of its biota is still a matter of vigorous debate (Gressitt 1982*b*; Heads 2006; Craft *et al*. 2010; Toussaint *et al*. 2014). Several studies have found a strong influence of geological history and vicariance on distributional patterns of New Guinean species (Heads 2001, 2002). On the other hand, recent evidence from analyses of multiple Lepidoptera species inhabiting New Guinea lowlands indicate low regional differentiation and limited effects of geography and distance on genetic differentiation and population structure (Craft *et al*. 2010).

Here, we compare diversity and distribution patterns of species of *Acropyga* collected during systematic surveys of the fauna of PNG. We use molecular data to establish species limits among morphologically variable lineages and to reconstruct phylogenetic relationships of New Guinean species of *Acropyga*. We then analyse population genetic patterns of the widespread species *A. acutiventris* across lowland areas of PNG. We assess the relationships among ant populations distributed in continuous forest habitat and examine the effect of distance and geographic barriers on gene flow.

Materials and methods

Collection of samples and study area

We surveyed ant communities at 13 sites across PNG between 2002 and 2012 (Fig. 1). For the Muller Range, Mendi and Port

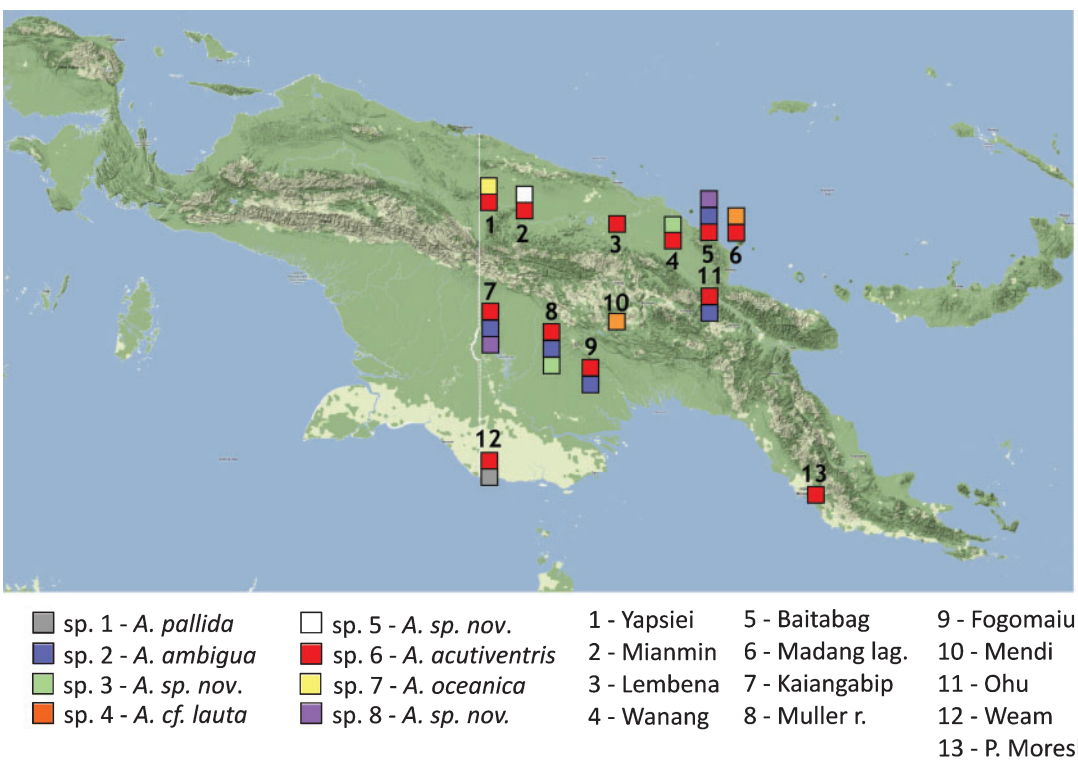

Fig. 1. Map of collecting sites and distribution of eight *Acropyga* species recorded during this study across Papua New Guinea. Each coloured square corresponds to a different species and collecting sites are numbered 1–13.

Moresby (sites 8, 10 and 13 respectively; Fig. 1), ants were sampled by general hand collecting without a plot design due to time limitations. At all other sites, samples were collected by hand and by leaf litter extraction (e.g. Agosti *et al.* 2000) in a system of 20 m × 20 m plots, with two to 12 plots surveyed at each site. The sampling effort for each plot included nine samples of 1 m^2 leaf litter to the depth that was removable without including soil, extraction for 48–72 h and at least four person-hours of direct (hand) collecting. The total area at each site across which the surveyed plots were distributed ranged from 20 to 25 km^2. For this study, we selected between one and three samples of each *Acropyga* species from each collecting plot where they were recorded. At three sites (Baitabag, Ohu and Madang Lagoon), we increased population sampling by targeted collecting at colonies of *Acropyga* outside the survey plots.

Seven sites were located in lowland primary rainforest (0–400 m above sea level, masl), two were in mid-elevation rainforest (500–1000 masl), one was in mountain forest (Mendi, 1800 masl) and one was in coastal habitat that included three small adjacent islands (Madang Lagoon, 1 masl) (Fig. 1; Table S1). The two southernmost sites (Weam and Port Moresby) were situated in mosaics of savannah habitats and drier rainforest. They likely represent environments with different histories as they are separated from the other sites by large patches of grasslands, while all other localities are connected by continuous forest. Savannah-like environment and dry forest areas have persisted in south PNG for, probably, the last 40 thousand years (ka; Torgersen *et al.* 1988); however, there is only limited knowledge about the history and past extent of these habitats (McNiven *et al.* 2012).

For the population-level study of *A. acutiventris*, we used samples from eight sites that yielded samples of sufficient DNA quality. Seven of these sites were located in rainforest (sites 4, 5, 7, 9, 11, 12, 13) and one (site 6) in coastal habitat (Fig. 1). At two of the sites (12, 13) the rainforest was drier and separated from the adjacent forest regions by savannah. Half the sites were located in the northern part of PNG and half in the southern part, thus representing areas with different geological histories and separated by the Central Cordillera (Fig. 1). For initial comparisons, we also included one population of *A. acutiventris* from Bornean rainforest (Kalimantan) and one Australian sample obtained from GenBank. The distances among sites in New Guinea covered multiple degrees of spatial separation, with the closest localities separated by 7 km (Madang Lagoon and Baitabag), and the most remote ones separated by 770 km (Port Moresby and Kaiangabip). The pair-wise geographic distances among sites ranged from 7 to 770 km between any two localities. Our design thus allowed assessment of the effect of spatial distance in continuous connected habitat as well as the effect of a major geographical barrier (a mountain range) on the genetic structure of ant populations.

Ants were stored in 99% ethanol and their morphology was investigated under an Olympus SZ50 microscope. Species identifications were based on comparison with specimens deposited at the Harvard Museum of Comparative Zoology and on a study by LaPolla (2004), who also confirmed the identifications and status of undescribed species (J. LaPolla, pers. comm.). Voucher specimens are stored in the Melanesian Ant Collection at the Biology Centre, Czech Academy of Sciences, with duplicates deposited at the Harvard Museum of Comparative Zoology and Smithsonian Museum of Natural History.

DNA extraction, sequencing and alignment

Total genomic DNA was extracted using the Genomic DNA Mini Kit Tissue (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturer's protocol. Fragments of the nuclear protein-coding genes elongation factor 1-α F1 copy (EF1αF1) and F2 copy (EF1αF2), rudimentary (CAD) and the mitochondrial cytochrome *c* oxidase I (COI) were amplified using published primers and polymerase chain reaction conditions (Table S2). We primarily used the LCO/HCO pair for amplifying COI, but for several samples that did not amplify well we used LCO1490 instead of LCO. In addition, COI sequences for 15 samples were generated by Barcodes of Life Initiative (BOLD; Ratnasingham and Hebert 2007) using their standard protocols and primers LF1 and LR1 (Smith *et al.* 2005). DNA sequences were assembled, edited and aligned in Geneious version 6.1 (http://www.geneious.com, accessed 1 July 2014; Kearse *et al.* 2012). Sequence alignment was performed with MAFFT v. 7 (Katoh *et al.* 2002) and coding regions were checked for consistency with a reading frame.

Phylogenetic analyses

To assess the species limits and estimate the phylogeny of *Acropyga* from PNG, we expanded our dataset to include DNA sequences of three non-Indo-Australian species of *Acropyga* and six species of formicine outgroups available in GenBank: *Lasius californicus* (W. M. Wheeler, 1917), *Myrmecocystus flaviceps* (W. M. Wheeler, 1912), *Brachymyrmex depilis* (Emery, 1893), *Nylanderia nuggeti* (Donisthorpe, 1941), *Pseudolasius australis* (Forel, 1915) and *Anoplolepis gracilipes* (F. Smith, 1857) (Table S1). For the final combined analysis, we retained only *A. gracilipes*, which was consistently recovered in all primary analyses as most closely related to *Acropyga*.

A dataset with all COI sequences was analysed in MrBayes v. 3.2.1 (Ronquist *et al.* 2012). The dataset was partitioned by codon position and run two independent times each for 10 million generations, sampling trees every 1000th generation and using four simultaneous chains, one cold and three heated. The HKY + G model of evolution was applied to each partition, as recovered by jModelTest 0.1.1 (Posada 2008). The following additional datasets were analysed: (i) three separate individual gene alignments (EF1αF1, EF1αF2, CAD), (ii) concatenation of all nuclear genes, and (iii) concatenation of sequences from individuals for which all four genes were sequenced to maximise gene coverage. These datasets were run in MrBayes v. 3.2.1 on the CIPRES Portal v. 3.1 (Miller *et al.* 2010) for 50 million generations with sampling of trees every 5000 generations and using four chains, one cold and three heated, for two independent runs. Parameters and model of evolution (mixed model) were unlinked across partitions. Convergence was evaluated by the mixing of chains and stationary distribution of log-likelihoods after discarding the first 25% of sampled trees. The final average standard deviation of split frequencies was below 0.01 and the potential scale reduction factor for each estimated parameter was close to 1.

Individual gene trees (Fig. S1) were compared to assess incongruence among data partitions, which could indicate

incomplete lineage sorting or introgression. Reciprocal monophyly across multiple independent loci increases confidence in species limits. All sequences have been deposited in GenBank and BOLD database (accession numbers listed in Table S1). The estimation of species' relationships was based on a matrix of individuals from 25 colonies for which all four genes were sequenced and combined with data from three non-Indo-Australian species for which comparable data were obtained from GenBank.

In addition to Bayesian inference (BI), we performed maximum likelihood (ML) analysis with PhyML software (Guindon *et al.* 2010) of the nuclear, mitochondrial and concatenated data matrices. PhyML analyses were performed using the GTR model with six substitution rate categories, the 'BEST' topology search algorithm and estimating the proportion of invariable sites and the α parameter for the gamma distribution. Robustness was assessed using 500 bootstrap pseudoreplicates.

Divergence time and calibration analysis

We estimated the divergence times among Papua New Guinean *Acropyga* using a secondary calibration point based on a fossil-calibrated ant phylogeny (Moreau and Bell 2013). The age of the split between *Acropyga* and the closely related genus *Anoplolepis* was constrained to a normal distribution with mean = 45 Ma and s.d. = 3 Ma. The crown age of *Acropyga* was set to a minimum age of the genus (15 Ma) following a lognormal distribution with mean = 1, s.d. = 1 and offset = 15, in which the 5% and 95% distribution encompasses 15.5–29.1 Ma, corresponding to the age of the documented Dominican amber fossil of *A. glaesaria* (LaPolla 2005). The analyses were carried out in BEAST v. 1.7.5 (Drummond *et al.* 2012) through the CIPRES Portal. We used an uncorrelated lognormal relaxed clock model (UCLN), and the HKY + G model of nucleotide substitutions for the COI partition and the K80 + G model for the nuclear gene partitions, as recommended by jModelTest. Priors were left as default except for the tree prior, which was set to the birth–death speciation process. The analyses were run four independent times for 50 million generations each, sampling trees every 5000th generation. We combined the results of the four runs after checking for convergence and that effective sample sizes (ESS) were higher than 200 using LogCombiner v. 1.7.5. The maximum clade credibility tree was determined and annotated in TreeAnnotator v. 1.7.5.

Population genetic analyses of A. acutiventris

Population genetic analyses were based on 96 workers, each representing one individual colony of *A. acutiventris*, which was the most abundant species in our surveys. In addition to 94 samples from New Guinea, two colony samples from Borneo (Kalimantan) were included to assess the level of divergence among distant populations. We used 595 bp of COI, which was 64 bp shorter than the one used for phylogenetic and divergence analyses because the alignment was cropped to match some shorter sequences available in BOLD. None of the nuclear genes provided sufficient variability for population-level comparisons (percentage of variable sites EF1αF1 = 0.6%, EF1αF2 = 0.5%, CAD = 0%).

The haplotypes were collapsed using the PHASE algorithm (Stephens *et al.* 2001) implemented in the software package DNASP v. 5 (Librado and Rozas 2009). Programs ARLEQUIN v. 3.5 (Excoffier and Lischer 2010), GenAlEx v. 6 (Peakall and Smouse 2012) and DNASP v. 5 were used for estimating population-genetic parameters, including gene diversity, nucleotide diversity, population differentiation (Φ_{ST}, Φ_{PT}), molecular variance (AMOVA) and departure from neutrality. Due to small samples sizes, we preferred Ramos-Onsins and Rozas' R_2 test (Ramos-Onsins and Rozas 2002). Significance of R_2 was evaluated by 10 000 replicates, using the empirical population sample size and observed number of segregating sites implemented by DNASP v. 5.

We examined relationships among haplotypes by reconstructing a network based on the minimum spanning tree output from ARLEQUIN v. 3.5 visualised in Hapstar 0.7 (Teacher and Griffiths 2011). To test for isolation by distance (IBD), we performed a Mantel test (1000 randomisations) between Φ_{PT} values among populations and geographic distances, using the Isolation By Distance Web Service (Jensen *et al.* 2005). A matrix of geographic distances was created using the Geographic Distance Matrix Generator software, available at the American Museum of Natural History website (http://biodiversityinformatics.amnh.org/open_source/gdmg/documentation.php).

To assess population structure in a geographic context, we analysed our dataset with spatial analysis of molecular variance (SAMOVA) and Bayesian analysis of population structure (BAPS). We used SAMOVA (Dupanloup *et al.* 2002) to detect the most probable number of genetically homogeneous groups of populations by combining molecular and geographic sample information. The populations were randomly partitioned into 2–10 clusters (K) for 100 simulated annealing processes. The largest increase of F_{CT} index (among-group variance component) together with the largest decrease of F_{SC} index (within-group variance component) were assessed to identify the most realistic number of groups defining spatial population structure.

The spatial-genetic structure of *A. acutiventris* populations was inferred using Bayesian genetic mixture analysis in BAPS 5 (Corander *et al.* 2008). The program considers both the frequencies of the haplotypes and the number of genetically diverged groups as random variables and uses stochastic optimisation to infer the mode of the posterior distribution. Using COI sequences and geographical position information, we applied 'spatial clustering' analysis to divide the population landscape into a coloured Voronoi tessellation. A cell of the tessellation corresponds to the area of an observed datum point, and is coloured according to the cluster membership. Bayesian analysis of population structure was run with the maximal number of groups (K) set to 2–10, and each run was repeated four times to test for congruence between runs. The number of optimal partitions was assessed by Log (marginal likelihood). Following the results obtained from SAMOVA and BAPS, 96 samples of *A. acutiventris* were divided into: (i) eight groups based on their locality; (ii) five groups based on their clustering in SAMOVA; and (iii) four groups on the basis of their clustering in BAPS 5. To assess the structure among the sampled populations and their putative groupings, we conducted an analysis of molecular variance (AMOVA) using pairwise differences as a distance method and 1000 permutations. Analysis of molecular variance was first performed without grouping of populations into regions and second

with grouping of populations into five regions suggested by SAMOVA.

The program Barrier 2.2 (Manni *et al*. 2004) was used to identify the sequence of genetic discontinuities within the IBD context. The program uses Delaunay triangulation to calculate a geometric network of populations connected by genetic distance values (Φ_{ST}). A Monmonier algorithm is then used to identify the edges with the largest distance values, given the geographic distance.

Divergence times of populations of A. acutiventris

We compared the outcome of our species-level tree calibrated using secondary constraints and population-level divergence dates that were calculated using standard insect mitochondrial mutation rates and mutation rates estimated previously for ants. These ranged from a strict clock of 1.5% (Quek *et al*. 2004) to 2.0% (DeSalle *et al*. 1987; Van Zandt Brower 1994) and 5.0% (Leppänen *et al*. 2011) divergence per million years. Combining within-species substitution rates and rates of species divergence can lead to bias in molecular dating; however, information for assessing the intraspecific mutation rates for ants is rather limited. We used the coalescent constant size tree model as implemented in BEAST and ran the program for 50 million generations sampling parameters every 5000th generation and evaluated its convergence in Tracer (ESS > 200) after discarding the first 25% of sampled trees.

In addition, we generated a Bayesian skyline plot (BSP) in BEAST using the coalescent Bayesian skyline tree model with the number of steps set to five and the skyline model to piecewise-constant. We used a strict clock and the 2.0% divergence per million years mitochondrial rate consistent with standard insect mitochondrial mutation rates (DeSalle *et al*. 1987). We ran analyses for 10 million generations and sampled parameters every 1000th generation. The substitution model for COI was set to HKY + G as recovered by jModelTest. In order to provide a sufficient sample size, all Papua New Guinean haplotypes were combined into one group. We pooled PNG populations, aware that the data might depart from neutrality, which in turn may affect BSP performance. However, the common cause of inaccurate reconstructions by BSP when pooling highly divergent populations is punctuated episodes of population growth (Wakeley and Aliacar 2001), which was not the case in our data. For all the divergence analyses we used uncropped COI alignment identical in length with the interspecific analysis (659 bp) as it provided more data. BEAST analysis allows for use of partially missing data, but for the other population-level analyses the alignment was cropped to exclude missing bases that could influence the statistical estimates.

Results

Species diversity and distribution

We collected specimens from 149 colonies of *Acropyga* that were assigned to eight species based on their morphological and molecular variation. Five of these species have been described previously. Three putative species did not match descriptions of any valid species and are considered newly discovered: their formal taxonomic description will be the subject of a separate study. Four of the species recorded during this study (*A. acutiventris*, *A. ambigua*, *A. oceanica*, *A. pallida*) have an Indo-Australian distribution. One species, *A. lauta* (Mann, 1919), had been known only from the Solomon Islands and represents a new species record for PNG. We did not find the only other species of *Acropyga* previously described from New Guinea, *A. major* (Donisthorpe, 1949), which is considered a *nomen dubium* as the only surviving material of the species is a damaged male specimen (LaPolla 2004).

We found one to three species of *Acropyga* at each of the 13 study sites with an average species richness per site of 1.92 (s. d. = 0.70). Even at sites with high sampling effort (102 leaf litter samples, over 150 person-hours of hand collecting; Table S2), we recorded no more than three species. The most frequently collected species were *A. acutiventris* and *A. ambigua*, occurring at 12 and five sites respectively, and in both northern and southern geological regions of PNG. Each of the other six species occurred at only one or two sites. The distributions of the species that occurred at only two sites (*A.* sp. 3, *A.* sp. 4, *A.* sp. 8) were disjunct across the Central Cordillera in each case (Fig. 1). Three species were recorded only at one site: *A. oceanica* and *A.* sp. 5 in the northern region, and *A. pallida* in the southern savannah region.

The species were found nesting in two main microhabitats: (i) inside dead wood on the ground, and (ii) in soil chambers beneath leaf litter, stones or wood. *Acropyga acutiventris*, *A. ambigua*, *A. pallida* and *A.* sp. 5 nested in both microhabitats, whereas each of the other species was found in only one or the other microhabitat.

In terms of habitat preferences, most of the species were found in primary rainforest, where our sampling effort was also greatest. The lowland forest had the highest species richness per site. Of the five species found at two or more sites, only two occupied more than one type of habitat. Of these species, *A. lauta* was found both in coastal habitat and in high-elevation forest, whereas *A. acutiventris* was recorded in all habitats sampled and at different altitudes (lowland forest, mid-elevation forest, savannah, coastal area; Table S1). In contrast, the second most frequently collected species, *A. ambigua*, was found only in primary lowland forest. *Acropyga pallida*, which is also widely distributed in Australia, was found exclusively in savannah habitat (Table S1). Photographic and distributional information for all recorded species, including their morphospecies codes, are available online at the Ants of New Guinea database (www.newguineants.org).

Phylogenetic relationships of Papua New Guinean Acropyga *species*

The combined matrix consisted of 2052 bp, of which 216 bp were phylogenetically informative (169 bp from COI, 6 bp from Ef1αF1, 21 bp from Ef1αF2, 20 bp from CAD). For *A. oceanica*, we were unable to obtain molecular data due to insufficient DNA preservation and the species was not included in phylogenetic estimations.

The seven currently recognised species and the three undescribed species were each recovered as monophyletic clades with high support in both BI and ML analyses. The topologies resulting from ML analyses were identical to BI (Fig. 2; ML not shown). There was some conflict between individual gene trees and those from analyses of the concatenated data (Fig. S1). The African species (*A. arnoldi*)

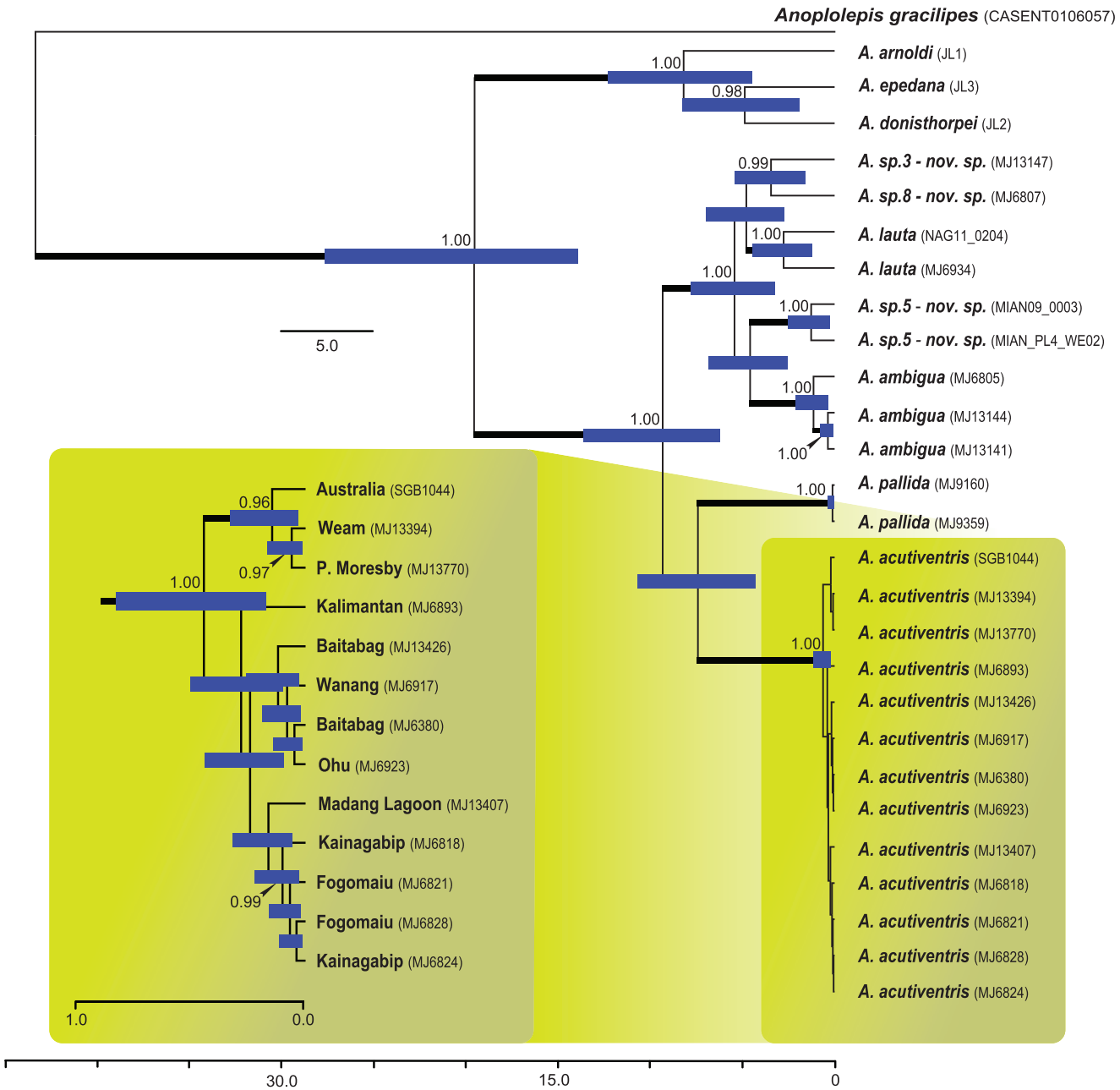

Fig. 2. Estimates of phylogenetic relationship and divergence times for *Acropyga* species based on a concatenated dataset of four genes (COI, EF1αF1, EF1αF2 and CAD). The tree is a chronogram (relaxed molecular clock) based on fossil-calibrated BEAST analysis. Blue bars represent 95% highest posterior density for node age estimates. The branches with bootstrap support above 50 (1000 replicates) are marked in bold and support values corresponding to posterior probability are shown above them. The time scale is in million years ago. The relationship among lineages of *A. acutiventris* is enlarged on the left. The branches are labelled according to corresponding localities of the corresponding samples.

was recovered as sister to the clade of Indo-Australian species with posterior probability (PP) = 1.0 in analyses of Ef1αF1, but formed a well-supported clade with the New World species (*A. donisthorpei*, *A. epedana*) (PP = 1.0) in analyses of Ef1αF2 and combined nuclear data (Fig. S1). The topology from the BI analysis of the concatenated four-gene dataset (Fig. 2) reflects the latter, and this is probably because Ef1αF2 provided more informative sites (21 sites) than Ef1αF1 (6 sites). Three major clades were recovered within the Indo-Australian species: *A. acutiventris*, *A. pallida* and a third comprising *A. lauta*, *A. ambigua*, *A.* sp. 3, *A.* sp. 5 and *A.* sp. 8 (Fig. 2). There was no supported resolution among these three clades.

The COI pairwise divergence between species, expressed as the number of base substitutions per site under a Kimura 2-parameter model (Tamura *et al.* 2011), ranged from 0.156 between *A.* sp. 3 and *A.* sp. 8 to 0.264 between *A. ambigua* and *A. acutiventris*.

Divergence times

The results from our relaxed molecular clock analysis in BEAST indicated that the split between Indo-Australian and the other lineages of *Acropyga* occurred during the early Miocene ~20 Ma (95% highest posterior density – HPD 15.1–28.1 Ma; Fig. 2). The crown age of the Indo-Australian lineage occurred ~9 ± 4 Ma. The stem age of the widespread *A. acutiventris* was dated to be between 3 Ma and 9 Ma. Diversification of the remaining species related to *A. ambigua* and *A. lauta* was estimated to have occurred 2–6 Ma.

There was an older divergence between the two samples of *A. lauta* (~2.5 Ma; Fig. 2) than within any other species, but there were no morphological differences that allowed us to consider the two samples of *A. lauta* as being two separate species.

Population genetics of Acropyga acutiventris

We obtained COI sequences from 101 colonies of *A. acutiventris* from PNG and Borneo (Fig. S2; Table S1), but only those from sites with two or more colonies (nine sites) and with sequence length ≥595 bp were included in population-level analyses (96 colonies). A minimum required number of two colonies per site excluded the possibility of using the single Australian sample for most of the population-level analyses. Five of the sites (Madang Lagoon, Baitabag, Ohu, Fogomaiu, Kaiangabip) provided eight or more colonies for detailed analysis. Twenty-three unique haplotypes were recovered from the 96 COI sequences (595 bp).

Haplotype (gene) diversity per site ranged from 0.42 to 0.78, nucleotide diversity from 0.11% to 0.35%, and percentage of polymorphic loci per population was between 8.8% and 17.6% (Table 1). None of the populations exhibited more than four COI haplotypes. When considering only populations with at least two haplotypes, the average Φ_{ST} was 0.73, while pairwise Φ_{ST} values ranged from 0.11 between Ohu and Baitabag to 0.94 between Kalimantan (Borneo) and Fogomaiu.

The Ramos-Onsins and Rozas's R_2 test did not yield a significant signal for departure from population neutrality at any site except Kaiangabip (Table 1). Spatial analysis of molecular variance indicated five groups as the most probable genetic structure (delta $F_{CT} = 0.046$, delta $F_{SC} = -0.19$): group 1 – Baitabag, Ohu, Wanang; group 2 – Madang Lagoon; group 3 – Weam, Port Moresby; group 4 – Fogomaiu, Kaiangabip; group 5 – Kalimantan. However, four groups also received favourable parameters in SAMOVA ($F_{CT} = 0.037$, $F_{SC} = -0.23$). In the latter case, Madang Lagoon was included as a member of group 1. Analysis of molecular variance revealed that most of the variance was found among the five groups (75%, d.f. = 4), with 6.4% of the variation explained by variance among populations within regional groups (d.f. = 4) and 18.5% of the variation explained by within-population variance (d.f. = 87). Similarly, AMOVA without grouping populations into the five regions detected most of the variance among populations (75%, d.f. = 7) and only 25% within populations (d.f. = 86) (for details see Table 2).

To examine regional patterns in more detail, we excluded the Bornean population (Kalimantan) from further analyses, and focused on assessment of genetic structure among PNG populations (Fig. 3). Spatial clustering analysis in BAPS revealed structure congruent with SAMOVA, but with more finely divided populations in north-east PNG. Four optimal clusters were identified (Log (marginal likelihood) = –393.43): (1) southern I (Weam, Port Moresby); (2) southern II (Kaiangabip, Fogomaiu); (3) north-east I (part of Ohu, part of Baitabag, Wanang); (4) north-east II (Madang Lagoon, rest of Baitabag, rest of Ohu) (Fig. 3*B*). The identity of samples in population clusters based on individual mixture analysis is presented in Table S1.

The four most significant spatial discontinuities among PNG populations indicated by analyses using Barrier were: (1) Port Moresby vs rest of PNG; (2) Weam vs rest of PNG; (3) southern populations Weam, Fogomaiu and Kaiangabip vs northern populations; and (4) Madang Lagoon vs Ohu, Baitabag and Wanang.

The haplotype network (Fig. 3*C*) revealed relationships of haplotypes that mainly correspond with clusters recovered by SAMOVA and BAPS (Fig. 3*B*). The southern populations form two separate clusters, with Weam and Port Moresby haplotypes more similar to each other than to geographically closer populations of Kaiangabip and Fogomaiu. Haplotypes from these latter two sites clustered together. The haplotypes of the two southernmost sites (Port Moresby and Weam) appear to be almost as distant from the most common haplotype as the one from Borneo (9 vs 11 steps; Fig. 3*C*). The three north-east sites

Table 1. Summary statistics for sampled populations of *Acropyga acutiventris* based on mtDNA

N, number of genotyped colonies; S, number of haplotypes detected; h, gene diversity; π, nucleotide diversity; PPB, percentage of polymorphic loci. Significant values for Ramos-Onsins and Rozas' R (R_2) indicated by bolding; BAPS, Bayesian analysis of population structure

Population	N	S	h	π	PPB	*R2*	BAPS cluster
Baitabag	51	4	0.640	0.00195	11.76%	0.14	3,4
Ohu	12	4	0.651	0.00354	8.82%	0.16	3,4
Madang Lagoon	8	4	0.785	0.00216	8.82%	0.22	4
Fogomaiu	9	3	0.416	0.00112	11.76%	0.22	2
Kaiangabib	8	3	0.607	0.00186	17.64%	**0.19**	2
Port Moresby	2	1	–				1
Weam	2	1	–				1
Wanang	2	1	–				3
Kalimantan	2	2	–	0.00168		0.43	–

Table 2. Analysis of molecular variance (AMOVA) among *Acropyga acutiventris* populations using mtDNA
The regional grouping is based on BAPS results and combines populations into the following groups: 1 – Baitabag, Ohu and Wanang; 2 – Madang Lagoon; 3 – Weam and Port Moresby; 4 – Fogomaiu and Kaiangabip; 5 – Kalimantan

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation %
With regional grouping				
Among groups	4	125.81	2.43	75.03
Among populations within groups	4	9.31	0.21	6.40
Within populations	87	52.15	0.59	18.50
Without regional grouping				
Among populations	7	113.78	16.25	74.50
Within populations	86	51.65	0.60	25.50

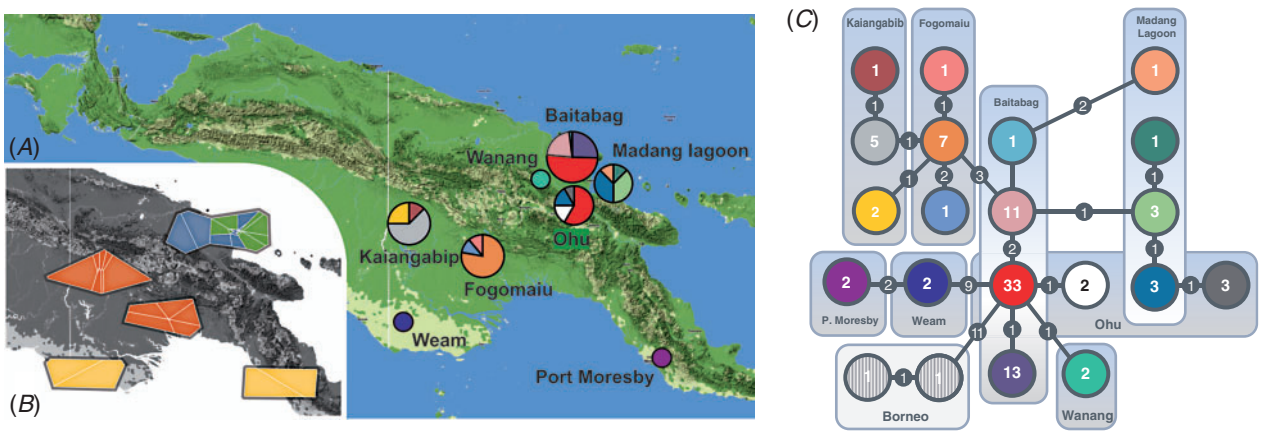

Fig. 3. (*A*) Distribution of COI haplotypes of *Acropyga acutiventris* across eight sites in Papua New Guinea. Each colour corresponds to a different haplotype and the pie graphs are scaled according to number of samples with smallest $N = 2$ and largest $N = 51$. (*B*) The four optimal clusters for *A. acutiventris* inferred using Bayesian analysis of population structure of spatial genetic population clustering of individuals based on geographic position of sample site (Log (marginal likelihood) = –393.43). Each group of polygons corresponds to a sampling site and populations clustering together are indicated by the same colour. (*C*) Minimum spanning network between COI haplotypes of *A. acutiventris*. Each circle and colour represents a different haplotype with colour-coding as per Fig. 3*A*. The frequency of each haplotype is indicated by the number in each circle. The numbers on connecting lines between haplotypes correspond to the number of mutational steps. The locality identity is visualised by rectangles around haplotypes.

(Madang Lagoon, Baitabag and Ohu) share multiple haplotypes, but also each have unique haplotypes. Baitabag and Ohu share the most abundant haplotype, but the latter site hosts haplotypes from two separate lineages. Similarly, two different haplotype lineages are found in Madang Lagoon.

The Mantel test for IBD revealed a strong positive relationship between geographical and genetic distances for the entire PNG region ($r = 0.76$, $P = 0.001$) (Fig. S3).

There were fewer recovered relationships among haplotypes estimated using BI or BEAST, but there was good support (PP = 0.99) for the haplotypes from Port Moresby and Weam being sister to the one sampled from Australia (Fig. S2*B*). In the BEAST analysis, this clade is sister to the cluster of other haplotypes from PNG, but there is low support for this relationship (PP = 0.58).

Using a mutation rate of 2.0% per Myr for COI in BEAST, we estimated the following population divergence times: southernmost populations (Weam and Port Moresby) and the rest of PNG = 580 ka (thousand years ago); Weam and Port Moresby = 60 ka; southern (Fogomaiu and Kaiangabip) and northern populations (Madang area) = 390 ka; Fogomaiu and Kainagabip = 60 ka (Fig. S2*B*). The Bayesian skyline analyses did not indicate any significant fluctuations in effective population size of *A. acutiventris* across PNG (Fig. S4).

Discussion

Species distribution and habitat preferences

Two main patterns emerged from distribution of *Acropyga* at the species level: (1) most species were collected at only one or two sites whereas two species were more widespread (*A. acutiventris* at 12/13 sites and *A. ambigua* at 5/13 sites), and (2) no more than three species of *Acropyga* co-occurred at any one site. Four of the species that occurred at two or more sites were found both north and south of the Central Cordillera (Fig. 1) suggesting that, although they may be locally rare, they are not restricted to a single geological region.

Many of the *Acropyga* species were recorded in one type of habitat (Table S1), which suggests that their habitat preferences might be conserved, at least in PNG. On the other

hand, the wide-ranging *A. acutiventris* was found across all types of habitats and altitudes (up to mid-elevation forest). Such habitat-association flexibility could be one of the key reasons for its wide distribution across Indo-Australia and South East Asia, as predicted by the concept of taxon cycles (e.g. Economo *et al.* 2015) or as documented recently in *Nylanderia* ants in the Indo-Pacific (P. Matos-Maravi, R. M. Clouse, E. M. Sarnat, E. P. Economo, J. S. LaPolla, M. Borovanska, C. Rabeling, J. Czekanski-Moir, F. Latumahina and M. Janda, unpubl. data).

The habitat preferences of A. pallida have been also described as more variable than was found in our study. In Australia, the species mostly inhabits sclerophyll and savannah forests while Asian records are from rainforests (LaPolla 2004; www.antwiki.org, accessed 1 July 2014). The three other records of *A. pallida* in PNG come from mid-elevation and lowland rainforests (LaPolla 2004). The fact that we did not record *A. pallida* in these habitats suggests insufficient sampling and/or a patchy distribution across PNG. Even less variable than the habitat associations were the nesting preferences of the *Acropyga* species recorded in PNG. All species exhibited very similar nesting requirements in soil chambers and inside decaying wood, suggesting that this trait might be conserved across the species.

The overall distributional patterns show that many *Acropyga* species in PNG either have a disjunct distribution or are quite rare (Table S3). This is not likely to be an artefact as our sampling of local assemblages was quite comprehensive and seemed to capture the majority of locally present *Acropyga* species, despite their cryptic lifestyle (Table S3). However, it is possible that rare species are present at other sites in the intervening areas and that further surveys will contribute additional records that might prove these distributions to be less disjunct than noted here.

In all cases, species recorded at two or more sites were found >300 km apart and separated by the Central Cordillera (Fig. 1), i.e. in regions of different geological origin. This suggests that, at the species level, the Central Cordillera is not an impenetrable barrier for *Acropyga* ants. Similarly, these ants are able to disperse well across water as evident from their occurrence on some islands outside PNG.

Phylogenetic relationship and divergence

LaPolla (2004) hypothesised phylogenetic relationships among 25 *Acropyga* species based on morphological characters, and included five species in common with our dataset. Some of our DNA-based results are not congruent with LaPolla's (2004) reconstruction, such as the monophyly of the lineage containing *A. epedana* and *A. arnoldi* recovered in our phylogeny. A comprehensive phylogenetic reconstruction of the whole genus, including the newly recorded species from this study, is needed to resolve these discrepancies.

Acropyga ambigua has been noted as morphologically variable (LaPolla 2004), raising questions about its species status, but here we recovered it as monophyletic (Fig. 2) and with relatively small genetic divergences among samples from distant localities (north-east PNG vs south-west PNG), suggesting that our samples belong to a single species. On the other hand, the high genetic divergence between samples of *A. lauta* might indicate the presence of cryptic species. However, we were unable to identify any consistent morphological differences between our two divergent collections. A detailed investigation of more material from the whole distributional range of this rare species will be necessary for proper species delimitation.

Our molecular dating indicates that the oldest split among the sampled species of *Acropyga* from PNG occurred during the late Miocene (13–5 Ma), when most of the regional terrains were arranged in their current positions (Hall 1998). This also corresponds with the period during which a group of aquatic beetles colonised and subsequently radiated across New Guinea (Balke *et al.* 2004; Toussaint *et al.* 2014). However, it is more likely that the initial split among the main lineages of *Acropyga* occurred outside the island, as most New Guinean lowlands emerged above water rather late, during the late Pliocene or early Pleistocene (Hall 1998, 2001; Deiner *et al.* 2011; Toussaint *et al.* 2014). Between 6 and 2 Ma, the remaining *Acropyga* lineages appear to have become established across New Guinea. Although the timing coincides with the emergence of more extensive lowlands, the fact that most of these species also occur outside PNG suggests that the species could have colonised the lowlands from elsewhere and/or expanded their ranges from higher, unsubmerged parts of older New Guinea terrains.

Population structure of A. acutiventris

Analyses of mitochondrial data revealed that the population genetic structure of *A. acutiventris* is influenced by geographic patterns as well as contemporary habitat associations. Population structure is partially explained by IBD and by inferred barriers to gene flow at the landscape level. The major source of molecular variation was detected among populations and among groups of populations defined by geographic areas. The strong population structure was demonstrated by a high average Φ_{ST} (>0.7) and by the fact that populations separated by more than 30 km shared no haplotypes (Fig. 3*B*, *C*). However, in many cases, the groups of haplotypes formed regional clusters with well-resolved relationships among them (Fig. 3*C*).

The fact that no more than four mitochondrial haplotypes were detected in any one population, despite sampling up to 51 individuals (Baitabag; Table 1), suggests that our coverage of the local genetic diversity might be representative. The Bayesian coalescent analysis of the combined set of Papua New Guinean haplotypes did not detect any significant population changes, although some population expansion might have recently occurred (Fig. S4). However, the non-significant signature of population expansion is more likely a consequence of our analysis in which interspecific rates of divergence were used, which can lead to 'pull to the present' phenomena (Nee *et al.* 1994). A combination of several unlinked loci would be needed for a reliable assessment of past population changes (Trucchi *et al.* 2014).

The effect of IBD observed in *A. acutiventris* is stronger than that detected previously in other species from the Papua New Guinean lowlands. In little shrikethrush (*Colluricincla megarhyncha*; Aves : Passerinae), the amount of genetic differentiation explained by geographic distance was only 16% (compared with 43% in *A. acutiventris*) and was even lower when only populations within one lowland basin were considered (Deiner *et al.* 2011). Similarly, Craft *et al.* (2010) found effects of IBD in populations of only five out of 27 species of Lepidoptera, mostly structured across the east–west

axis of the lowland basin located north of the Papua New Guinean Central Cordillera. The stronger effect of IBD in *Acropyga* is likely a consequence of the restricted distance that the queens are able to disperse per generation compared with Lepidoptera, which are mostly good dispersers and are able to fly longer distances than ants (Novotny *et al*. 2007).

One of the noticeable patterns from the spatial genetic analyses is the close relationship among the two southernmost Papua New Guinean populations and their large differentiation from other PNG populations (Figs 3*C*, S2). Given that the haplotypes from Port Moresby and Weam fell in a well-supported clade with the haplotypes sampled from Australia (Fig. S2*B*), we hypothesise that they represent a lineage that occurred across the Austro-Papuan Sahul region that is now disjunct, or a lineage that dispersed from Australia when both areas were connected during the Pleistocene and Pliocene. During that period, sea levels were between 75 m and 120 m below present levels and land bridges allowed numerous opportunities for terrestrial species to move between both areas (Voris 2000). This split between the southern lineages and the rest of the PNG populations is estimated to have occurred around 580 ka, coinciding with the emergence of the Trans-Fly lowlands, where the Weam mitochondrial lineage occurs, ~500 ka.

The evidence for genetic connectivity between north Australia and south New Guinea is well documented but depends in part on which taxon is sampled. While some species appear to have had occasional gene flow between the two areas throughout the Pleistocene, e.g. the palm cockatoo, *Probosciger aterrimus* (Murphy *et al*. 2007), or eastern brown snake, *Pseudonaja textilis* (Williams *et al*. 2008), others have had little or no connectivity, e.g. marsupials (Macqueen *et al*. 2011) and black butcherbird (*Cracticus quoyi*) (Kearns *et al*. 2011). A lineage of *Camponotus* ants colonised New Guinea from northern Australia and then diversified further on the island. However, this colonisation occurred before the Pleistocene (Clouse *et al*. 2014).

Habitat isolation also might have played some role in the divergence, or lack of admixture, of the two southernmost populations of *A. acutiventris* from those to the north. The Trans-Fly lowlands and parts of the Papuan Peninsula harbour dry savannahs that surround patches of drier forest in which both populations were collected. The savannah could represent an ecological barrier to local dispersal and restrict gene flow of populations living in forest patches. Unfortunately, our dataset does not include sufficient sampling of Australian *A. acutiventris* to provide more detailed estimates of divergence and gene flow.

Another prominent division among *A. acutiventris* populations appears to be the Central Cordillera, which separates populations from the southern lowland forests (Fogomaiu and Kaiangabip) from those in the same habitat types in the north-east (Fig. 3*B*). The effect of this mountain barrier on divergence in other species is well documented (reviewed in Kearns *et al*. 2011). However, the estimated divergence of these disjunct populations of *A. acutiventris* occurred ~390 ka, well after the emergence of the Central Cordillera between 8 and 4 Ma (Michaux 1994; Cloos *et al*. 2005). These findings are in agreement with the pattern observed at the species level, where we documented that most of other *Acropyga* species also occur across the Central Cordillera (Fig. 1). Although this barrier has a demonstrable effect on population-level structuring of *A. acutiventris* by restricting gene flow, over longer time periods it is permeable, possibly via long distance dispersal. Occasional long distance dispersal is also the best explanation for the documented presence of *A. acutiventris* on relatively remote oceanic islands and archipelagos, such as the Solomon Islands, Nicobar Islands or Krakatau Islands (www.antwiki.org).

There is also strong population structuring of *A. acutiventris* among the three sites in north-east PNG (Baitabag, Ohu and Madang Lagoon). Although separated by less than 30 km, each population shares only one COI haplotype (Fig. 3*C*). At this scale, sharing of haplotypes does not seem to reflect the geographic proximity, and the coastal population shares a haplotype with the more distant site (Ohu). The low sharing of haplotypes across sites and the occurrence of many haplotypes unique to each site indicate relatively restricted female dispersal in *A. acutiventris*, even at distances less than 30 km.

The results of our molecular dating should be treated with caution, and those for *A. acutiventris* could be overestimated. The divergence dates were estimated using interspecific divergence rates and multiple representatives of each species, which can lead to biased estimates of diversification rates (Nee *et al.* 1994). It is also well documented that analyses of species and population-level divergences based on single genetic markers can provide inaccurate estimates (e.g. Carstens and Knowles 2007) due to limitations such as introgression and ancestral polymorphism (Godinho *et al*. 2008; Rodriguez *et al*. 2010).

Conclusions

The ant diversity of New Guinea is still largely unknown, as indicated by detection of several undescribed species of *Acropyga*, a genus that has recently been revised on a global scale (LaPolla 2004). Most of the PNG species of *Acropyga* appear to be rather rare with patchy distributions, whereas two species are much more widespread. Whether this is a consequence of particular life history traits, species' evolutionary histories, or other biological factors remains to be determined.

At the species level, major geographic barriers such as the Central Cordillera do not seem to limit the distribution of most *Acropyga* species. However, we also found that population-level genetic patterns of the widespread species *A. acutiventris* appear to be structured by geological history and, to some extent, by habitat associations. In continuous lowland forest, *A. acutiventris* has restricted female gene flow, and high differentiation was observed also between geographically proximate populations living in habitats with different history and/or climate. The largest source of molecular variation was among areas defined by common geological origin, but the more prominent division was between the southernmost populations of *A. acutiventris* living at the edge of savannah rather than between populations separated by the Central Cordillera. The maintenance of long-term genetic connectivity across or around the Central Cordillera has been shown in several vertebrate species (Kearns *et al*. 2011), and our study is one of the first to find evidence for such population-level connectivity in ants.

Further genetic studies based on comparative sampling of multiple species and populations distributed between New Guinea and northern Australia could facilitate better comparisons

of how ecological specialisation interacts with short- and long-term climate change to shape population genetic patterns of species that straddle this boundary. For example, *A. pallida* is associated with dry forests and savannahs in Australia and southern PNG, but is also reported from humid forest in northern PNG, and could be a good candidate for detailed assessment of how habitat associations affect the population genetic history of ants. Northern Australia also appears to have played an important role as a source for colonisation of New Guinea by several ant lineages (Clouse *et al.* 2014); however, the extent to which these patterns are repeated at the population level and influenced by the climatic changes of late Pleistocene are largely unknown.

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