Army imposters: diversification of army ant-mimicking beetles with their *Eciton* hosts

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Short running title: Diversification of myrmecophiles with hosts

Abstract

Colonies of neotropical army ants of the genus *Eciton* Latreille offer some of the most captivating examples of intricate interactions between species, with hundreds of associated species already described in colonies of *Eciton burchellii* Westwood. Among this plethora of species found with *Eciton* colonies, two genera of staphylinid beetles, *Ecitomorpha* Wasmann, and *Ecitophya* Wasmann, have evolved to mimic the appearance and parallel the colouration of the most abundant ant worker cast. Here, we study for the first time the association of these ant-mimicking beetles with their ant host in an evolutionary and population genetics framework. The central emphasis is on colonies of *E. burchellii*, the only *Eciton* species that harbours both genera of ant-mimicking beetles. Phylogenetic and population structure analyses using the same mtDNA COI region (802bp) for ants and beetles indicated that speciation patterns of the myrmecophiles were congruent with specialization to a particular *Eciton* (sub)species. Therefore, current taxonomic treatments of *Eciton* and its *Ecitomorpha* and *Ecitophya* associates need revision. Molecular clock analyses suggested that diversification of the *Eciton* hosts pre-date that of their guests, with a possible earlier association of *Ecitophya* (found with a large number of *Eciton* species) than with *Ecitomorpha* (found only with *E. burchellii* colonies). Population-level analyses revealed that patterns of diversification for the myrmecophiles are also consistent with specialisation to a particular host across broad geographical areas but not at small geographical scales, with gene flow within each species found between host colonies even across landscape features that are strong barriers for *Eciton* female-mediated gene flow.

Keywords

Gene flow; mimicry; mitochondrial DNA; myrmecophily; myrmecophory; population structure; speciation; taxonomy

Introduction

The study of associations between species is vital if we are to understand the evolution of biological diversity (Thompson, 2013). This was recognised by Darwin in the closing paragraph of *On the Origin of Species by Means of Natural Selection* (1859) when he used the term 'entangled bank' to refer to the interaction between species that form biological communities, as highlighted by Thompson (1994). Nature is full of examples of intricate and intimate associations between different species but it is in the world of ants where some of the most numerous and astonishing associations can be found (Hölldobler and Wilson, 1990). Among the ants, army ants harbour the most extensive array of species associations, with multiple vertebrate and invertebrate associate species exploiting the ants and the different environments and homeostatic conditions that their colony life creates (Gotwald Jr., 1995; Hughes et al., 2008). Of all army ants, those of the genus *Eciton*, inhabiting the tropics of the New World, are the ones exhibiting the most captivating display of associates (Gotwald Jr., 1995), with 557 species already recorded with *Eciton burchellii* and many more still to be described (Ivens et al., 2016; Rettenmeyer et al., 2011). Associates found with *Eciton* army ants include, among others, mites that feed on secretions and hemolymph of the ants; flies that feed on the middens' refuse; beetles that steal prey from the ants or predate on the ants or their brood; the iconic army-ant-following birds that feed on arthropod prey flushed out by the ants during their raids; and butterflies that feed on droppings from ant-following birds (Gotwald Jr., 1995; Kistner, 1979; Rettenmeyer et al., 2011; Schneirla, 1971)

As part of this plethora of associates, some have evolved to conceal their presence among the army ants through chemical, tactile and morphological mimicry (Gotwald Jr., 1995; Kistner, 1979). These 'imposters' have evolved different strategies to associate with army ants, with staphylinid beetles having mastered the art of blending in with the ants by evolving to resemble the appearance of their hosts (Hölldobler and Wilson, 1990; Kistner and Jacobson, 1990; Maruyama and Parker, 2017). Two genera of myrmecomorph (ant-like) staphylinid beetles, *Ecitomorpha* and *Ecitophya*, both in the subfamily Aleocharinae (tribe Athetini; Elven, Bachmann, & Gusarov, 2012), are found with neotropical army ants of the genus *Eciton*. These two genera of beetles are highly specialized to the epigaeic patterns and nomadic life of *Eciton* army ants and both mimic the most abundant worker cast in *Eciton* colonies (Seevers, 1965); the media workers (Franks, 1985). Both genera present similar morphological modifications that confer resemblance to their host: similarity in surface sculpturing, subpetiolate and ellipsoidal abdomens, slender heads and pronota, and long appendages (Seevers, 1965). Their appearance is considered moderately ant-like as they are not a perfect mimic of the ants, but their colour parallels that of the species or subspecies of *Eciton* with which they are associated (Akre and Rettenmeyer, 1966; Kistner and Jacobson, 1990; Seevers, 1965). The two genera are mainly distinguished by *Ecitophya* presenting a much slender body and longer appendages than *Ecitomorpha*, with a head more than twice as long as wide, a bilobed mentum, slender gula with sutures not converging in the front, longer antennae (more than six times as long as the head width) but with the terminal segments of the antennae not much wider than the preceding segments (terminal segments in *Ecitomorpha* are more club-shaped) (Seevers, 1965).

The mimicry of *Ecitomorpha* and *Ecitophya* to *Eciton* media workers is likely to be both an adaptive response to avoid predators such as ant-following birds (Batesian mimicry), as well as an adaptation for integration into the ant colony and avoidance of host aggression - Wasmannian mimicry (Parker, 2016). The presence of *Ecitomorpha* and *Ecitophya* in *Eciton* colonies is rare (and sometimes absent), with many colonies presenting less than one of these beetles per 1,000 worker ants. These two myrmecophile genera are considered hunting guests of *Eciton* army ants, as they are found running among ants in raiding columns where they feed on dropped prey or at booty caches (Kistner and Jacobson, 1990). These beetles are also found in emigration columns (Akre and Rettenmeyer, 1966; Kistner and Jacobson, 1990) when the conspicuously nomadic *Eciton* colonies move to another location to set up their new bivouac (temporary nest). During *Eciton* colonies emigration, *Ecitomorpha* and *Ecitophya* individuals run in the centre of the columns or ride on prey captured by the ants or on ant pupae (Kistner and Jacobson, 1990). Therefore, they are adapted to the movement and life cycle of their host (Akre and Rettenmeyer, 1966), as has been shown for other *Eciton* myrmecophiles (Berghoff et al., 2009; Von Beeren et al., 2016a, 2016b).

Taxonomically, the genus *Ecitophya* was initially divided into five species acknowledging the colour parallel between this myrmecophile and its *Eciton* hosts (Reichensperger, 1933). *Ecitophya rapaxae* Mann found associated with the ant *Eciton rapax* Smith, *Ecitophya consecta* Mann associated with *Eciton vagans* Olivier*,* *Ecitophya gracillima* Mann that is associated with *Eciton hamatum* Fabricius, and *Ecitophya simulans* Wasmann and *Ecitophya bicolor* Reichensperger associated with *E. burchellii*. The latter two species were later grouped into a single species, *E. simulans*, as it was considered that specimens of *Ecitophya* collected with *E. burchellii* colonies did not differ sufficiently to be considered as separate species (Kistner and Jacobson, 1990; Seevers, 1965). Another species found associated with *Eciton lucanoides* Emery was later described as *Ecitophya rettenmeyeri* (Kistner and Jacobson, 1990).

Ecitomorpha beetles have only been found with the army ant *Eciton burchellii*, with the first specimens of *Ecitomorpha* described by Wasmann in 1889 as *Ecitomorpha arachnoides* (Akre and Rettenmeyer, 1966). Reichensperger divided this genus into four species taking into account the colour polymorphism within *Eciton burchellii*: *Ecitomorpha arachnoides* Wasmann, *Ecitomorpha nevermanni* Reichensperger, *Ecitomorpha breviceps* Reichensperger and *Ecitomorpha melanotica* Mann (Reichensperger, 1935, 1933). However, due to the difficulty in finding consistent morphological characters (besides colouration) supporting the separation of these species, they were subsequently lumped back into a single species*, Em. arachnoides* (Kistner and Jacobson, 1990; Seevers, 1965).

In this study, we investigated for the first time in an evolutionary and population genetics framework the interaction of *Ecitomorpha* and *Ecitophya* ant-mimicking beetles with their *Eciton* hosts; in particular, *E. burchellii*, as it is the only *Eciton* species known to host both genera of beetles. Genetic analyses of ants and beetles collected in Panama, a geographical area where many different *Eciton* species have overlapping ranges (Watkins, 1976), were conducted to test the following hypotheses: (i) considering the strong level of association of *Ecitophya* and *Ecitomorpha* with *Eciton*, phylogenetic patterns of the myrmecophiles will mirror that of their host, (ii) due to the dependence of these two genera on *Eciton's* hunted prey and the pedestrian dispersal capability of the queen and workers, the level of specificity between host and myrmecophile will be observable at broad geographical scales, (iii) if myrmecophiles are truly host-specific and have evolved and diverged via increased specification on a particular *Eciton* host, molecular patterns should support earlier taxonomic classifications of *Ecitophya* and *Ecitomorpha* by Reichensperger (i.e. each *Eciton* species will host a particular *Ecitophya* and *Ecitomorpha* species).

Methods

Study area and sampling

Sampling for this study was targeted on colonies of *E. burchellii* ssp. *foreli* Mayr and *E. b.* ssp. *parvispinum* Forel, the two most-studied *E. burchellii* subspecies. These two subspecies are highly epigaeic and their distribution ranges overlap in Panama, Costa Rica, and Honduras (Watkins, 1976). Descriptions of these species highlight their morphological similarity (Borgmeier, 1955; Santschi, 1925), with main differences reported being the colouration of media workers, *E. b. foreli* having black head and mesosoma but reddish metasoma, and *E. b. parvispinum*'s media workers having complete black bodies. However, studies assessing the genetic differences between these two subspecies in view of their current taxonomic treatment have yet to be conducted. This study focused on sampling of colonies in Panama, an area where the geographical range of both subspecies partly overlaps. Three main areas of Panama were sampled (Fig. 1); in West Panama the Bosque Protector Palo Seco (BPPS) and the adjacent Reserva Forestal Fortuna (RFF), and in Central Panama the Área Protegida San Lorenzo and its buffer zone (APSL). As the Chagres River was found to be a barrier for *E. b. foreli* when gene flow was estimated with mtDNA markers (Pérez-Espona et al., 2012), this area was divided into two (APSLA and APSLB) to group colonies from each side of the Chagres River. In total, 13 colonies of *E. b. foreli* (4 in BPPS, 6 in APSLA and 3 in APSLB) and 12 colonies of *E. b. parvispinum* (all in RFF) for which we found associated *Ecitophya* and/or *Ecitomorpha* beetles were sampled (Fig. 1; Table 1). In addition, four colonies of *E. hamatum* and the associated *Ep. gracillima*: 1 colony in RFF, 2 colonies in BPPS, and 1 in Soberanía National Park (SOB), and one colony of *E. lucanoides* and its associated *Ep. rettenmeyeri* collected in RFF were opportunistically sampled. The number of *Ecitophya* and *Ecitomorpha* beetles sampled and sequenced from *E. b. foreli* and *E. b. parvispinum* colonies are summarized in Table 1. Although the number of beetles collected is not directly comparable between colonies, as effort spent searching and collecting the beetles was constrained by the time of the day a colony was encountered, the maximum number of beetles collected in a colony was 78 *Em. arachnoides* (colony E114 collected in RFF with *E. b. parvispinum*) and 40 *Ep. simulans* (colony E89 collected in BPPS with *E. b. foreli*).

The sampling protocol for our study consisted of walking all available trails and adjacent less accessible areas (off trails), through daily extensive walks (9 a.m. until dusk), to collect individuals from as many colonies of *E. b. foreli* and *E. b. parvispinum* as possible. Once an *E. burchellii* colony was encountered, workers from all castes were sampled from raid or emigration columns by removing them with the help of long forceps. Ant columns were then carefully observed at several points of the raid or emigration columns to sample *Ecitophya* and *Ecitomorpha* beetles with straight tube aspirators. Ant column observations per colony lasted several hours or until dusk, depending on the time of the day when a colony was encountered. Collections were simultaneously conducted by two people in order to maximise sampling of the beetles. All samples were preserved in 99% ethanol for further examination and subsequent genetic studies.

Laboratory procedures

DNA extraction

Abdomens of ants and beetles were carefully dissected before DNA extractions to avoid any contamination from consumed prey. To facilitate DNA extraction, the tissue samples were deposited in a 1.5mL Eppendorf tube and briefly immersed in liquid nitrogen prior to extraction procedures. Genomic DNA was extracted using the DNeasy tissue kit (QIAGEN) following the manufacturer's instructions.

Sequencing of the mitochondrial marker Cytochrome Oxidase subunit I

Ants and ant-mimicking beetles from both genera, *Ecitomorpha* and *Ecitophya*, were sequenced for the same region of the mitochondrial cytochrome oxidase subunit I gene (COI, *cox1*). Mitochondrial DNA markers are the most widely used genetic markers in species-level phylogenies and DNA barcoding studies. Due to their haploid nature, maternal inheritance, and smaller population size, sequences derived from mtDNA coalesce over a shorter time scale than those derived from nuclear DNA (Simon et al., 2006), with reciprocal monophyly at the species level reached faster after speciation in mtDNA phylogenies than in nuclear DNA phylogenies (Sunnucks, 2000). The faster mutation rate of mtDNA markers has been shown to offer more powerful resolutions of relationships between closely related taxa in phylogeographic and population-level studies (Avise, 2000; Zhang and Hewitt, 2003). In insects, mutation rates of mtDNA markers have been estimated to be 2 to 9 times faster than nuclear protein-coding genes (Moriyama and Powell, 1997) making them more suitable for the study of closely related species that have diverged recently (Lin and Danforth, 2004). COI fragments were amplified from the *Eciton* samples using a modified version of the primer pairs CI13/CI14 (Hasegawa et al., 2002) and Ben/Jerry (Simon et al., 1994). Details of the modified primers and Polymerase Chain Reactions (PCR) conditions can be found in Pérez-Espona *et al.* (2012). Fragments of COI were amplified from *Ecitomorpha* and *Ecitophya* using the primer pairs C1-J-1634/C1-N-2317 and C1-J-2216/C2-N-3431 (Maus et al., 2001). These beetle PCR amplifications were conducted in a total volume of 25µL, using 10-15ng of template DNA, 1X NH4 buffer, 2.5mM $MgCl_2$, 0.6µM of each primer, 1 unit of BIOTAQ polymerase (Bioline, London) and double processed tissue culture distilled water (Sigma-Aldrich, Buchs, Switzerland) to bring the volume up to 25µL. The PCR cycling protocol included an initial denaturation step of 94°C for 3 min, a three-step cycling consisting of a denaturing step of 94°C for 30 s, annealing at 51°C for 30 s and ramping at 0.3°C/s to an extension step of 72°C for 1 min. The cycle was repeated 29 times and was followed by a final extension of 72°C for 10 min. PCR products were run on a 1.5% agarose gel and visualised using ethidium bromide staining. Successful amplifications were purified using EXOSAP (GE Healthcare), and forward and reverse strands for each of the fragments sequenced in two reactions using 6µL of purified PCR product, 4µL of the reaction mix DYEnamic ET Terminator Cycle Sequence Kit (Amersham, GE

Healthcare) and 0.5μL of primer. Cycle sequencing consisted of 25 cycles including a denaturation step of 95°C for 20 s, an annealing step of 50°C for 15 s and an extension step of 60°C for 1 min. Sequences were run on a MegaBACE^{TM} 1000 capillary sequencer (Amersham GE Healthcare) at The University of Bristol. Electropherograms from the forward and reverse sequencing reads were edited and assembled into contigs using the software Geneious version 10 (Biomatters: http://www.geneious.com). The resulting consensus reads from each individual were sorted into unique haplotypes and subsequently manually aligned in Geneious, together with some additional sequences obtained from four individuals of *Eciton dulcium* Forel (collected in RFF and APSL) and sequences obtained from GenBank (Accession numbers: AY233691-4, AY233696, GQ980948). These additional sequences were selected as ingroup placeholders and outgroups based on the army ant phylogeny of Brady (2003) and the army ant-mimicking beetle phylogeny in Maruyama and Parker (2017). The resulting alignments were all trimmed to include the same region COI fragment (802bp). All unique sequences were submitted to DDBJ under the accession numbers LC258007-LC258019 for the *Eciton* sequences, and LC258020-LC258064 for the *Ecitophya* and *Ecitomorpha* sequences.

Phylogenetic and molecular clock analyses

Unique haplotype alignments for *Eciton* and *Ecitomorpha* with *Ecitophya* were initially evaluated for nucleotide compositional heterogeneity using the Chi-square test in PAUP version 4.0b10 (Swofford, 2002), and using tetrahedral plots and matched-pairs tests for symmetry implemented in SeqVis version 1.5 (Ho et al., 2006). The more conservative Chi-square test provided no significant evidence for compositional heterogeneity in either of the alignments. The more sensitive tetrahedral plots and matched-pairs tests for symmetry also provided no strong evidence for compositional heterogeneity in the *Ecitophya-Ecitomorpha* alignment; however, there was evidence for some heterogeneity in the *Eciton* alignment: tetrahedral plots contained co-dispersed clusters of data points and the number of the matched-pairs tests for symmetry was >5% at $P = 0.05$ and >1% at $P = 0.01$. To explore whether data re-coding could reduce the level of compositional heterogeneity in the *Eciton* alignment, C and T nucleotides were re-coded as Ys. SeqVis analysis of this re-coded DNA alignment revealed no significant evidence for heterogeneity. Therefore, in addition to analyzing the full *Eciton* DNA alignment, we also analyzed a data reduced AGY form of the *Eciton* alignment to account for artefacts that may arise from compositional heterogeneity.

Phylogenies were generated from the *Eciton* and *Ecitophya-Ecitomorpha* DNA alignments using Maximum Parsimony and Bayesian methods. For the Maximum Parsimony analysis, heuristic searches were conducted using PAUP, with the full *Eciton* and *Ecitophya-Ecitomorpha* alignments and the AGY re-coded *Eciton* alignment. Each heuristic search started from a random tree with 50 random addition replicates, one tree was held per step, saved trees set to a maximum of 10,000 and other settings left at default values. Confidence values were generated using non-parametric bootstrapping (10,000 replicates). For the Bayesian inference, the DNA alignments were analysed as a single partition using a mixed nucleotide substitution model and gamma corrected rate heterogeneity across sites with the software MrBayes version 3.2.6 (Ronquist et al., 2012). Parameter distributions were approximated using reversible jump Metropolis-coupled Markov chain Monte Carlo methods, with three chains of 10,000,000 generations, chain heating at 0.05, sampling frequency at 1,000 and other settings at default values. Posterior samples of parameter estimates were assessed using generation plots, distribution plots, the potential scale reduction convergence diagnostic and estimated sample sizes as recommended in the MrBayes manual. These revealed that the total number of generations and default burnin of 25% appeared to be sufficient to acquire final parameter estimates from a stationary distribution.

To analyse the *Eciton* alignment under AGY coding using Bayesian inference, we used the three-state 'AGY' model implemented in the software mcmcphase within the PHASE version 3.0 software package (https://github.com/james-monkeyshines/rna-phase-3). Heterogeneity across sites was modelled using a gamma correction, and a chain length of 10,000,000 iterations, sampling period at 1,000 and burnin of 25% were used, as in the MrBayes analyses. Perturbation proposal priorities were 10 for the tree and 1 for the substitution model. Within these tree and model components, the proposal priorities were 1 for topology changes, 10 for branch lengths (with an exponential (10) prior), 1 for frequencies, 1 for rate ratios, and 1 for the gamma parameter. Generation versus log probability plots, parameter distribution plots, and repeated analyses starting from different random seeds indicated that these settings generated final posterior estimates from a stationary distribution. Tree files generated using mcmcphase were analysed using the associated program mcmcsummarize and also Geneious.

Chronological estimates for key diversification events were obtained with additional Bayesian analyses conducted using MrBayes. For these analyses, we reduced the level of haplotype sampling in order to include only the most abundant haplotypes and key biogeographic placeholders. This was to ameliorate the impact of intra-specific differences in haplotype sampling between the *Eciton* and *Ecitomorpha-Ecitophya* datasets that could have a negative impact on the date estimation procedure. The molecular clock analyses used the same underlying model and chain settings as described for MrBayes above with an outgroup-ingroup division enforced as a strong prior topological constraint. Two different methods of clock calibration were explored: a standard 1% per million years' rate applied to both the *Eciton* and *Ecitomorpha-Ecitophya* datasets, and fixed date calibrations of 26 million years on the most recent common ancestor (MRCA) of *Eciton* following the results obtained by Brady (2003), and a date of 25 million years on the *Ecitomorpha-Ecitophya* divergence following the results in Maruyama and Parker (2017). Based on the data in Brady (2003) and Maruyama and Parker (2017), a uniform tree age prior of 10-100 million years was used. Clock model options were explored using stepping-stone sampling estimates of the marginal likelihood. The Thorne-Kishino 2002 'TK02' relaxed clock had the smallest log likelihood but this was less than 5 units better than the independent gamma rates 'IGR' relaxed clock and strict clock in both the *Eciton* and *Ecitophya-Ecitomorpha* datasets. This preliminary analysis thus provided no strong evidence in favor of either of these alternative clock models and, consequently, all three were used to generate date estimates. Posterior samples of parameter estimates were assessed for stationarity as described above.

Population-level analyses of E. burchellii and associates

Genetic diversity analyses for *E. b. foreli* and *E. b. parvispinum* colonies and their associated *Ecitophya* and *Ecitomorpha* beetles were conducted using the softwares Arlequin version 3.1 (Excoffier et al., 2005) and DnaSP version 5 (Librado & Rozas, 2009). Genetic diversity was estimated in terms of number of haplotypes, segregating sites (S) and average number of nucleotide differences (k). In order to compare divergence within and between species, S and *k* were also calculated for the different *Eciton*, *Ecitophya* and *Ecitomorpha* species included in our study. Population structure for the host and each of the associates was estimated using a hierarchical analysis of molecular variance (AMOVA) with the software popART (http://popart.otago.ac.nz). The partitioning of genetic variation was assessed within and among two main geographical areas, West Panama (BPPS) and Central Panama (APSLA, APSLB), and significance values obtained after 1,000 permutations. The software popART was also used to build Median Joining haplotype networks (epsilon = 0) to assess haplotype relationships and identify patterns of haplotype structure for *E. burchellii* and the myrmecophile beetles at different geographical scales.

Results

Diversification and species relationships

Phylogenetic and molecular clock analyses provided further insights into the evolution and diversification of the *Eciton* species and their *Ecitophya* and *Ecitomorpha* beetle associates (Figs. 2 & 3, Table 2 & 3). Analyses using the full and reduced datasets using different methods (i.e. standard and AGY coded, Parsimony and Bayesian analyses) resolved the same suites of well-supported haplotype relationships; this indicated that these were robust to the nucleotide compositional heterogeneity found in the *Eciton* DNA alignment. With the *Eciton* dataset, haplotypes of *E. dulcium* and *E. hamatum*, and the subspecies *E. b. foreli*, were grouped according to these taxonomic categories with strong statistical support (≥ 99% Parsimony bootstrap support, Bayesian posterior probabilities of 1.0). The subspecies *E. b. foreli* and *E. b. parvispinum* were also resolved as a single group but with lower support (≥ 60% bootstrap, ≥ 0.91 posterior probability). Other basal relationships between *Eciton* taxa included in this study were poorly supported in all phylogenetic analyses. In the *Ecitophya-Ecitomorpha* analyses, haplotypes assigned to *Ecitomorpha* and *Ecitophya* were clearly separated into these genera level categories (≥ 99% bootstrap, posterior probability of 1.0). The *Em. arachnoides* haplotypes formed two well-supported groups that corresponded to their *E. burchellii* host (≥ 98% bootstrap, posterior probability of 1.0); *E. b. foreli* and *E. b. parvispinum*. The *Ep. simulans* haplotypes also consisted of two well-supported groups following their *E. burchellii* host (100% bootstrap, posterior probabilities of 1.0). However, these two *Ep. simulans* groups formed a complex with two groups of *Ep. gracillima* (associated with *E. hamatum*). Within this complex, *Ep. simulans* (associated with *E. b. foreli*) was clearly grouped with *Ep. gracillima* (87% bootstrap, posterior probability of 0.95). *Ep. rettenmeyeri* (associated with *E. lucanoides*) was separated from the other *Ecitophya* species with 92% parsimony bootstrap and 0.78 posterior probability support. Lineage relationships within *Ecitophya*, therefore, did not mirror that of their *Eciton* host. The number of segregating sites (S) and the average pairwise nucleotide differences (*k*) between species revealed further insights into the taxonomy and species relationships of the myrmecophiles and their hosts (Table 3). Levels of divergence estimated as S and *k* between *E. b. foreli* and *E. b. parvispinum* were similar (or slightly higher) to those between taxa currently recognised as separate *Eciton* species. Although speciation patterns of *Ecitophya* did not mirror those of the hosts, a strong divergence was observed between *Ecitomorpha* or *Ecitophya* associated with each subspecies of *E. burchellii*. Divergence of *Ecitomorpha* was only assessed for two *E. burchellii* subspecies so further subspecies would need to be studied to elucidate further speciation patterns.

The molecular clock analyses recovered identical sets of *Eciton* and *Ecitophya-Ecitomorpha* haplotype relationships but with different associated date estimates depending on the clock model and calibration method used (Table 2). Analyses calibrated using fixed dates taken from the literature were consistently older than date estimates calibrated using a 1% rate, while differences arising from the use of different clock models were subtler. Regardless of the clock model or calibration method, the relative age differences between *Eciton* and *Ecitophya-Ecitomorpha* lineage divergences were similar in all molecular clock analyses (Table 2, Figure 3). Although the confidence intervals for the divergence estimates were wide and therefore we should be cautious with interpretations, median estimates of divergences indicated that the diversification of the genus *Eciton* is likely to be older than that of the associated myrmecophile genera *Ecitophya* and *Ecitomorpha*. This was the case when considering date estimates for the MRCA of *Eciton* species taken from literature (used as a fixed 26 MYA calibration date) and when considering independently obtained date estimates derived from the 1% calibration rate (Table 2, Fig 3). Median estimates for diversification of the main *Eciton* species (or subspecies) also appears to pre-date the diversification of the main myrmecophile lineages (Table 2, Fig 3). Poor branch support for most of the basal *Eciton* relationships (Fig 2, Fig 3) may, in part, be due to the rapid diversification of these primary *Eciton* lineages following the MRCA, as other studies using nuclear markers and phylogenomics have also failed to unambiguously resolve these species relationships (see Discussion). Key among the main *Eciton* diversification events was the MRCA of *E. b. parvispinum* and *E. b. foreli,* which pre-dated the diversification of the associated myrmecophiles *Em. arachnoides* and the *Ep. simulans – Ep. gracillima* complex (Table 2, Fig 2, Fig 3). Among the two genera of myrmecophiles, median estimates for the diversification of *Ecitophya*, which is found with more species of *Eciton*, appear to be older than those for *Ecitomorpha*, although confidence intervals did overlap for these estimates (Table 2, Fig 3). The genus *Ecitophya* has been reported in colonies of *E. rapax, E. vagans, E. burchellii, E. hamatum* and *E. lucanoides* while *Ecitomorpha* has only been reported for *E. burchellii.*

Population-level analyses of E. burchellii and its Ecitophya and Ecitomorpha associates

The diversification of the ant-mimicking beetles *Ecitophya* and *Ecitomorpha* with *E. burchellii* was further confirmed by the haplotype networks (Fig. 4). The networks clearly indicated a strong divergence between *E. b. foreli* and *E. b. parvispinum* haplotypes, with 90 segregating sites among haplotypes of both subspecies (Fig. 4). This divergence was mirrored in the associated beetles; however, the divergence between haplotypes in the beetles was smaller than that found for the host (55 segregating sites in *Ep. simulans* and 50 segregating sites in *Em. arachnoides*).

The haplotype networks confirmed that the diversification of *Ecitophya and Ecitomorpha* with *E. b. foreli* was also prevalent over broad geographical areas. Haplotypes of ants and beetles collected in West Panama (BPPS) markedly differed from those collected in Central Panama (APSLA and APSLB). Divergence time estimates for the separation of haplotype lineages between West and Central Panama were older for *E. b. foreli* than for the associate *Ep. simulans* (Fig. 3, Table 2). Although the haplotypes of *Em. arachnoides* found in the two main geographical study areas were clearly distinct (Fig. 4), with haplotypes derived from two distinct lineages found in West Panama (BPPS), the phylogenetic analyses did not support a strong grouping of haplotypes according to these geographical areas (Fig. 2). In the Central Panama study area (APSL) the Chagres River was a strong gene flow barrier for *E. b. foreli* females, with median estimates of divergence of *E. b. foreli* haplotypes either side of the river estimated at 0.4-1.6 MYA (Fig. 3, Table 2). The Chagres River, however, was not a gene flow barrier for either *Ecitomorpha* or *Ecitophya*, as haplotypes characteristic of a particular species of these myrmecophiles were shared between their *Eciton* host colonies either side of the river (Fig. 4).

Estimates of population structure were higher for *E. b. foreli* than for the associated *Ecitophya* and *Ecitomorpha* beetles (Table 4). In the host, genetic differentiation was found at all levels of analyses, with the majority of the genetic variation explained by differences among groups (76.31%), and hardly any variation explained due to differences within populations (0.20%). In *Ecitophya* and *Ecitomorpha*, most of the genetic variation was also explained by differences among groups (84.62% and 79.57%, respectively) but genetic variation due to differences within populations (15.51% and 22.03%, respectively) was much larger than that found for the host. Negative values in variation among populations within groups found in *Ecitophya* and *Ecitomorpha* indicate the large haplotype variation within populations and the sharing of haplotypes between these. No genetic variation was attributed to differences among populations collected on either side of the river within each of the beetle species, further corroborating that the Chagres River was not a gene flow barrier for these myrmecophiles.

Discussion

Taxonomy of Eciton and associated Ecitophya and Ecitomorpha

The phylogenetic analyses and haplotype networks generated for this study revealed that current taxonomic treatments of *E. burchellii* and of its *Ecitophya simulans* and *Ecitomorpha arachnoides* associates need further revision to take into account further speciation within these taxa. Morphological descriptions of *E. b. parvispinum* have highlighted its black colouration, reduced spines in the metanotum and barely protruding epinotum teeth

(Borgmeier, 1955; Santschi, 1925), but these characteristics were considered not to warrant species status for *E. b. parvispinum*. However, our results clearly indicate that genetic divergence between *E. b. foreli* and *E. b. parvispinum* is as high as (or higher than) between taxa currently considered separate *Eciton* species. Divergence estimates calculated in terms of S and *k* further suggest this speciation, as values between *E. b. foreli* and *E. b. parvispinum* were higher than the those obtained between *E. hamatum* – *E. dulcium* and *E. hamatum* – *E. lucanoides*; therefore, indicating that taxonomic treatments of *E. burchellii* need to be revised. Our results coupled with those from a recent study phylogeographic study of this species in Central America (Winston et al., 2017), provide strong evidence that speciation within this taxon has long been underestimated.

Although divergence between the *Eciton* species, and within *E. burchellii*, is clear from our genetic analyses, phylogenetic relationships between *Eciton* species were not fully resolved in our study, despite the suitability and widespread use of COI for resolving relationships between closely related insect taxa (Lin and Danforth, 2004; Sunnucks, 2000). Weak statistical support for relationships between *Eciton* species does not appear solely reflect our choice of COI as a molecular marker, because combined analyses of COI and nuclear markers (Brady, 2003), and even a recent phylogenomic approach (Winston et al., 2017), also failed to unambiguously resolve these *Eciton* species relationships. Rather, the lack of resolution in phylogenetic trees reported here is more likely indicative of a rapid diversification of lineages within *Eciton* (Whitfield and Lockhart, 2007).

Important insights into the taxonomy of *Ecitophya* and *Ecitomorpha* were also revealed by our genetic analyses. The taxonomy of these myrmecophiles has been challenging due to the subtle differences observed in morphological characters, besides colouration, within each of these genera (Kistner and Jacobson, 1990; Seevers, 1965). The *Ecitomorpha* species key by Reichensperger was based on head dimensions, thoracic plates sculpturing, length of the hind tibias and depressions behind the eyes (Reichensperger, 1933). Further differences between the species were highlighted in each of the species descriptions; these included different antennae formation, elytra sculpturing, and colouration in different parts of the body of the specimens. Species characterisation according to colouration was also indicated in previous descriptions such as that of *Em. melanotica* found with *E. b. parvispinum* (Mann, 1926). Kistner and Jacobson (1990), after analysing a large series of specimens, concluded that variation in colour was not consistent; however, they reported that black specimens of *Ecitomorpha* were more frequently found in geographical areas where *E. b. parvispinum* was present, and rarer in areas populated by *E. b. foreli*. In terms of other morphological features, Kistner and Jacobson (1990) did not find consistency either in the shape of the groove of the pronotum or in spermathecas in the male genitalia that would allow species differentiation. They, therefore, considered that early classifications of the *Ecitomorpha* species by Reichensperger could not be supported. For *Ecitophya*, early species classification by Reichensperger (1935, 1933) were based on the general appearance of specimens, measurements and characteristics of the head and legs, differences in antennal segments, elytra sculpturing, presence and characteristics of abdominal bristles, and the colouration of different body parts. In their analysis of *Ecitophya* specimens, that included dissections of male genitalia, Kistner and Jacobson (1990) concluded that only some characteristics of the abdominal bristles, the relative measurements of size of body parts and the relative length of antennal segments were reliable characters for species identification. This reduction in the number of reliable characters to distinguish species led Kistner and Jacobson (1990) to lump some of the earlier species described by Reichensperger into a single species, *E. simulans*.

Our study has shown the importance of the use of genetic studies for resolving taxonomic challenges and has provided strong evidence of speciation of *Ecitomorpha* and *Ecitophya* as a result of their specialisation to their *Eciton* hosts. The speciation patterns found in our study for both myrmecophile genera (i.e. specialisation to an *Eciton* host) would further support that *E. b. foreli* and *E. b. parvispinum* may be considered separate species. The diversification patterns obtained for *Ecitophya* and *Ecitomorpha* were more concordant with initial species classifications by Reichensperger (1935, 1933) . Divergence estimated as S and *k* further supported the speciation of *Ecitomorpha* and *Ecitophya* according to their host, with values between *Ep. simulans* found with *E. b. foreli* or *E. b. parvispinum* differing by a similar number of segregating sites than between *Ep. simulans* found with *E. b. foreli* and those found with *Ep. gracillima* (found with *E. hamatum*). Therefore, the current taxonomic treatment of these *Eciton burchellii* associates as single species, i.e. *Ep. simulans* and *Em. arachnoides*, merits revision to take into account the speciation patterns revealed by our genetic data. For *Ecitomorpha* beetles found associated with *E. b. parvispinum* we suggest the adoption of the previous name of *Em. melanotica* Mann. Given our results and those from Winston et al. (2017) supporting speciation of *E. burchellii* in Central America, we expect that further species of *E. burchellii* and its *Ecitomorpha* and *Ecitophya* associates will be reported, as morphological diversification of *E. burchellii* within its broad distributional range has long been acknowledged (Kistner and Jacobson, 1990; Reichensperger, 1935, 1933; Seevers, 1965).

The lack of consistent and reliable morphological characters to differentiate species within *Ecitomorpha* and *Ecitophya* reported by Kistner and Jacobson (1990) cannot be explained simply in terms of the recent and rapid diversification within these genera, as indicated by our genetic analyses. In myrmecophiles, it is expected that morphological variation between species may be reduced or absent due to strong selection on morphological, behavioural and physiological characters as adaptations to exploit their hosts (Schonrogge et al., 2002). Often, species are determined by visual inspection of morphological features; however, chemical and acoustic characters can be more important for species recognition in arthropods. Future studies of *Ecitophya* and *Ecitomorpha* diversification, therefore, will benefit from thorough analyses of chemical and acoustic characters, as these cues are likely to be very important for their adaptation to their *Eciton* hosts (Lenoir et al., 2001).

Diversification of Ecitophya and Ecitomorpha with Eciton species

Phylogenetic patterns of *Ecitophya* and *Ecitomorpha* indicated specialization of these myrmecophiles with their *Eciton* hosts, confirming previous observations of ant-resemblance and colouration parallels (Akre and Rettenmeyer, 1966; Kistner and Jacobson, 1990; Reichensperger, 1933), and behavioural observations such as the preference of following trails of the host species (Akre and Rettenmeyer, 1968). However, the patterns of speciation of the myrmecophiles did not mirror those of the host revealing that the beetles' phylogenies were not an 'evolutionary print' of the host (Thomas et al., 1996). Furthermore, phylogenetic analyses and estimates of S and *k* disagreed with previously reported evolutionary relationships between *Ecitophya* species based on morphological characters (Kistner and Jacobson, 1990). *Ep. simulans* found with colonies of *E. b. foreli* were more closely related to *Ep. gracillima* (associated with *E. hamatum*) than to the *Ep. simulans* found with *E. b. parvispinum*. Additionally, *Ep. rettenmeyeri* (associated with *E. lucanoides*) was not closely related to *Ep. gracillima* as previously inferred by the similar colouration between the ant hosts (Kistner and Jacobson, 1990); in fact, *Ep. rettenmeyeri* was sister to the other *Ecitophya*.

Confidence intervals for time divergence estimates overlapped slightly between the ants and the myrmecophiles and, therefore, we cannot discard with certainty potential coevolutionary processes between hosts and guests. However, median time divergence estimates indicated that diversification patterns of the ants probably pre-dates that of the associated beetles. We acknowledge that our divergence estimates are derived from a single genetic marker and therefore we focus our discussion on *Eciton* and myrmecophile divergences on relative (rather than absolute) date estimates. Comparisons of median time divergence estimates from the molecular clock analyses for both beetle genera suggest that diversification of *Ecitophya* with *Eciton* ants is likely to have occurred earlier than for *Ecitomorpha*. A possible earlier diversification of *Ecitophya*, and subsequently a longer time frame to fine-tune their interaction with their host, could explain the association of *Ecitophya* with a larger number of *Eciton* species.

Myrmecophily in Staphylinidae beetles is an ancient phenomenon, with a fossil of *Protoclaviger trichodens* gen. et sp. nov. (Clavigeritae) in amber being dated to the Early Eocene (c. 52 MYA; Parker and Grimaldi, 2014). This early association of Clavigeritae beetles with ants can explain the remarkable diversity of myrmecophilous species within this supertribe (Parker and Grimaldi, 2014). High species diversification due to myrmecophily has also been reported for ant-nest beetles of the genus *Paussus* L. (subfamily Paussinae; Moore and Robertson, 2014). Although new species of *Ecitophya* and *Ecitomorpha* are likely to be described, due to underestimated speciation within *E. burchellii* (and maybe in other *Eciton* species), the diversification of these two Aleocharinae genera is not as exceptional as that found for other myrmecophile Staphilinidae such as *Paussus*. This difference probably reflects the high specialisation of *Ecitophya* and *Ecitomorpha* to the genus *Eciton*, in contrast to *Paussus* beetles that are found associated with different, and sometimes distantly unrelated, ant genera (Moore and Robertson, 2014). Furthermore, our results suggest that contrary to other myrmecophile beetles found associated with *Eciton* colonies (e.g. *Vatesus*), *Ecitophya* and *Ecitomorpha* species have evolved more host-specific adaptations, probably as a result of stronger selection pressures because they are hunting guests of day-time raiding epigaegic army ants.

Geographical patterns of diversification of Ecitophya and Ecitomorpha with E. b. foreli

Broad phylogeographic patterns of *Ecitophya* and *Ecitomorpha* were concordant with their *E. b. foreli* host, with a clear separation of haplotypes between the West and Central Panama study areas. However, patterns of diversification of the host and these two myrmecophiles differed at smaller geographical scales, indicating that local differences might be the result of the spatial distribution of the host and the capability of dispersal of the myrmecophiles (Tack and Roslin, 2010; Thompson, 2005). The haplotype networks revealed that *Ecitophya* and *Ecitomorpha* beetles are not colony- or ant-mtDNA lineage-specific even though vertical transmission of myrmecophiles is likely to occur during colony fission (Schneirla, 1971). Ant colonies with different mtDNA haplotypes shared myrmecophile haplotypes, indicating that horizontal transmission of *Ecitophya* and *Ecitomorpha* mtDNA lineages occurs between colonies. Horizontal transmission of myrmecophiles between *Eciton* colonies has been previously reported for *Vatesus* (Akre and Torgerson, 1969), a beetle that does not mimic the physical appearance of the ants but most of its life cycle is tightly linked to that of its host (Von Beeren et al., 2016a). Horizontal transmission of *Ecitophya* and *Ecitomorpha* individuals, as a potential strategy to avoid inbreeding within a colony, might occur by dispersal through flight between colonies such as shown for *Vatesus* (Chatzimanolis et al., 2004; Von Beeren et al., 2016a), or whenever colony fusion - the aggregation of workers to another colony after losing their queen (Schneirla, 1940; Schneirla & Brown, 1950) - takes place (Kronauer et al., 2010). In our study, gene flow between the myrmecophile populations of each species was also found across the Chagres River despite this being a major gene flow barrier for *E. b. foreli* females (Pérez-Espona et al., 2012). Gene flow across the Chagres River, therefore, indicates a higher dispersal capability of the beetles in contrast to the obligate pedestrian dispersal of *Eciton* queen and worker ants. Reports of flight in these beetles have only been anecdotal and limited to observations of hovering of *Ep. consecta* over a colony of *E. vagans* when this colony was spreading to attack the observer (Mann, 1921), and an individual of *Ep. gracillima* found with a colony of *E. hamatum* (Pérez-Espona pers. obs.). In the latter case, hovering was observed when trying to aspirate one individual from a raiding column. This specimen hovered to seek refuge under some fallen leaves but after a few minutes tried to follow the ant trail (when it was successfully collected and included in this study). Fully developed wings with venation characteristic of staphylinids have been described for both *Ecitophya* and *Ecitomorpha* (Kistner and Jacobson, 1990). However, due to the close dependence of these beetles with their specific host and the relatively low density of colonies, at least for *E. burchellii* (Franks, 1982), dispersal of the myrmecophiles between colonies, in particular those located at further distance or separated by landscape features that act as barriers for *Eciton* female dispersal, might be challenging. In such scenarios, it is likely that dispersal between colonies is mediated by large and alate *Eciton* males when they leave their natal colony in search of conspecific colonies to find a queen for mating (Gotwald Jr., 1995; Schneirla, 1971). Males of *E. b. foreli* are produced in large numbers (c. 3,000) as part of sexual broods (including a small number of queens) when colony fission is imminent (Franks and Hölldobler, 1987; Gotwald Jr., 1995; Schneirla, 1971). Males have been shown to be strong fliers and able to disperse over 1km distances (Jaffé et al., 2009). They are therefore responsible for the majority of gene flow between colonies (Berghoff et al., 2008; Jaffé et al., 2009; Pérez-Espona et al., 2012; Soare et al., 2014). Dispersal of myrmecophiles by ant alate reproductives have been shown in *Atta* leaf-cutting ants, with *Attafila* cockroaches observed on ant queens departing for nuptial flights (Moser, 1967), and similar strategies have been suggested for the movement of myrmecophiles between *Eciton* colonies (Kronauer et al., 2010).

Ecological and evolutionary studies of myrmecophiles of army ants at the population-level are still in their infancy due to the difficulty of keeping the host and myrmecophiles in laboratory conditions (Kistner and Jacobson, 1990). However, as shown in this and recent studies in *Vatesus* and *Tetradonia* beetles (Von Beeren et al., 2016a, 2016b), genetic approaches can shed light on the evolution and levels of specificity of these army ant imposters. Our study was based on variation in mtDNA therefore future studies aiming to further elucidate the level of association of *Ecitomorpha* and *Ecitophya*, in particular at small geographical scales, would benefit through the use of highly variable markers such as microsatellites. Using a combination of mitochondrial and microsatellite data in previous studies has been demonstrated that deforestation has a major impact on the connectivity of *E. burchellii* populations (Pérez-Espona et al., 2012; Soare et al., 2014), threatening the long-term persistence not only of these top neotropical predators but also the multitudes of species associated with them. Further research on *Eciton* and their associates is crucial if we are to provide conservation solutions that would guarantee the maintenance of this manifestation of Darwin's 'entangled bank'.

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Figure legends

Figure 1. Map of Panama indicating the main study areas. RFF = Reserva Forestal de Fortuna, BPPS = Bosque Protector de Palo Seco, APSL = Area Protegida de San Lorenzo and its buffer zone; SOB = Soberanía National Park. Colonies collected in APSL and its buffer zone were grouped in the analyses as APSLA and APSLB to reflect colonies collected on either side of the Chagres River.

Figure 2. Majority-rule consensus phylograms for unique haplotypes derived from *Eciton* species (a) and the associated myrmecophiles *Ecitophya* and *Ecitomorpha* (b), generated with MrBayes using a mixed nucleotide model and gamma corrected rate heterogeneity. Support values are shown for nodes (marked by filled circles) found in ≥ 50% of parsimony bootstrap and posterior probability samples. Coloured bars indicate differences in abdomen (or whole body) colour of different taxa.

Figure 3. All compatible groups consensus chronograms for the selected *Eciton* (a) and associated myrmecophiles *Ecitophya* and *Ecitomorpha* (b) haplotypes, generated with MrBayes using a mixed nucleotide model, gamma corrected rate heterogeneity, tk02 relaxed clock and fixed node calibration of 26 (*Eciton*) or 25 (associated myrmecophiles) MYA (indicated by a star). Nodes are scaled to median date estimates with the 95% Highest Posterior Density indicated by a translucent blue bar. Nodes with date estimates are labelled with roman numerals and correspond to values shown in Table 2. Node support values are given for the presented chronograms, filled circles indicate nodes found in ≥ 50% of parsimony bootstrap and posterior probability samples in the main phylogenetic analyses. Coloured bars indicate the abdomen (or whole body)'s colour of different taxa.

Figure 4. Haplotype networks of *Eciton burchellii* (a) and associated *Ecitophya* (b) and *Ecitomorpha* (c) in the main study areas in Panama (RFF, BPPS, APSLA, APSLB) constructed using a median-joining approach. Study areas are indicated with different colours. The size of the circles is proportional to the number of individuals representing a particular haplotype. Missing intermediated haplotypes are indicated with black dots, nucleotide substitutions between haplotypes are indicated by small lines over the haplotype connecting branches.

Table 1. Details of collection and sequencing of *Ecitophya* and *Ecitomorpha* myrmecophiles found with different *Eciton* army ant species in the study areas in Panama

			Individuals collected		**Individuals sequenced**	
Ant species	**Location**	**Ant colony**	***Ecitomorpha***	***Ecitophya***	***Ecitomorpha***	***Ecitophya***
E. b. parvispinum	RFF	E85	6	2	3	2
E. b. parvispinum	RFF	E86	4	12	2	0
E. b. parvispinum	RFF	E87	1	0	1	0
E. b. parvispinum	RFF	E94	3	8	3	4
E. b. parvispinum	RFF	E95	23	8	4	2
E. b. parvispinum	RFF	E96	2	0	2	0
E. b. parvispinum	RFF	E97	12	5	3	2
E. b. parvispinum	RFF	E99	8	20	2	6
E. b. parvispinum	RFF	E100	13	10	4	6
E. b. parvispinum	RFF	E108	15	4	2	1
E. b. parvispinum	RFF	E109	58	28	1	5
E. b. parvispinum	RFF	E114	78	32	8	8
E. b. foreli	BPPS	E89	15	40	4	2
E. b. foreli	BPPS	E101	21	2	2	2
E. b. foreli	BPPS	E103	5	13	2	2
E. b. foreli	BPPS	E104	7	12	2	2
E. b. foreli	APSLA	E126	8	6	6	2
E. b. foreli	APSLA	E127	9	3	2	2
E. b. foreli	APSLA	E132	28	17	14	8
E. b. foreli	APSLA	E143	14	6	13	5
E. b. foreli	APSLA	E154	4	0	2	2
E. b. foreli	APSLA	E156	8	4	2	1
E. b. foreli	APSLB	E162	3	5	2	3
E. b. foreli	APSLB	E165	2	3	2	2
E. b. foreli	APSLB	E166	8	4	2	2
E. hamatum	BPPS	E88	0	69	0	6
E. hamatum	RFF	E105	0	13	0	3
E. hamatum	BPPS	E110	0	8	0	2
E. hamatum	SOB	E169	0	1	0	1
E. lucanoides	RFF	E107	0	6	0	1

Table 2. Date estimates for selected nodes in the *Ecitophya* and myrmecophiles' chronograms generated using MrBayes with TK02, IGR and strict clock models and either a fixed date calibration according to Brady (2003) and Maruyama and Parker (2017) or 1% rate calibration.

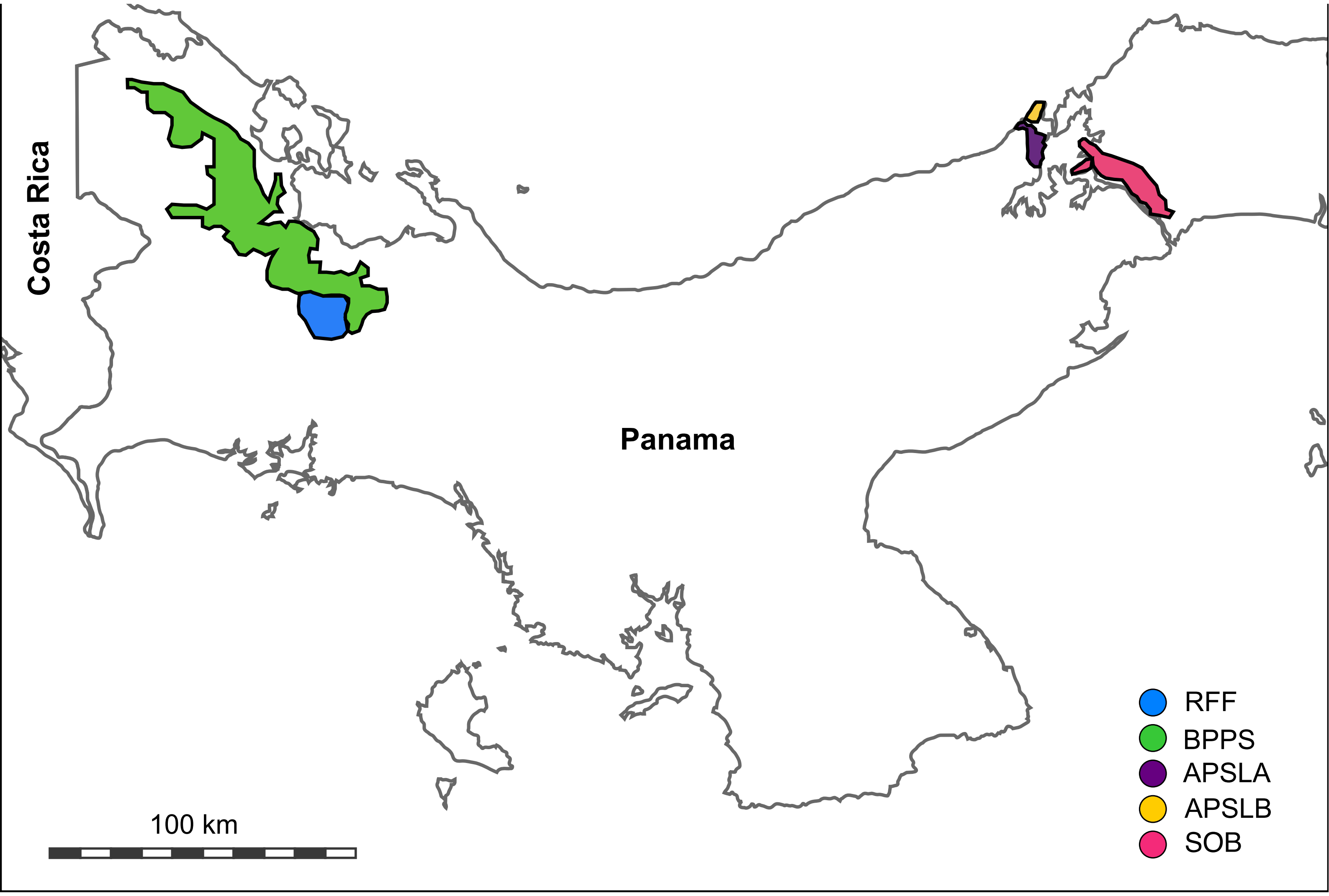
Code	Dataset	Method	Median	95% Lower	95% Upper
I	Eciton	TK02 clock, 26 MYA date calibration	26.0	n.a.	n.a.
I	Eciton	TK02 clock, 1% rate calibration	13.7	8.8	21.9
I	Eciton	IGR clock, 26 MYA date calibration	26.0	n.a.	n.a.
I	Eciton	IGR clock, 1% rate calibration	12.1	8.9	16.7
I	Eciton	Strict clock, 26 MYA date calibration	26.0	n.a.	n.a.
I	Eciton	Strict clock, 1% rate calibration	11.5	9.2	14.5
II	Eciton	TK02 clock, 26 MYA date calibration	18.0	12.3	24.0
II	Eciton	TK02 clock, 1% rate calibration	9.5	5.0	16.4
II	Eciton	IGR clock, 26 MYA date calibration	16.8	10.2	23.6
II	Eciton	IGR clock, 1% rate calibration	7.8	4.3	11.8
II	Eciton	Strict clock, 26 MYA date calibration	17.9	13.4	22.8
II	Eciton	Strict clock, 1% rate calibration	7.8	5.7	10.3
III	Eciton	TK02 clock, 26 MYA date calibration	3.5	1.3	7.5
III	Eciton	TK02 clock, 1% rate calibration	1.8	0.6	5.3
III	Eciton	IGR clock, 26 MYA date calibration	3.9	1.2	8.3
III	Eciton	IGR clock, 1% rate calibration	1.8	0.5	4.0
III	Eciton	Strict clock, 26 MYA date calibration	2.4	1.3	3.8
III	Eciton	Strict clock, 1% rate calibration	1.1	0.6	1.6
IV	Eciton	TK02 clock, 26 MYA date calibration	1.3	0.3	3.5
IV	Eciton	TK02 clock, 1% rate calibration	0.7	0.1	2.3
IV	Eciton	IGR clock, 26 MYA date calibration	1.6	0.2	4.1
IV	Eciton	IGR clock, 1% rate calibration	0.7	0.1	1.9
IV	Eciton	Strict clock, 26 MYA date calibration	0.9	0.4	1.7
IV	Eciton	Strict clock, 1% rate calibration	0.4	0.2	0.7
IX	Myrmecophiles	TK02 clock, 25 MYA date calibration	0.8	0.2	2.0
IX	Myrmecophiles	TK02 clock, 1% rate calibration	0.5	0.1	1.4
IX	Myrmecophiles	IGR clock, 25 MYA date calibration	1.1	0.1	2.9
IX	Myrmecophiles	IGR clock, 1% rate calibration	0.5	0.1	1.2
IX	Myrmecophiles	Strict clock, 25 MYA date calibration	0.7	0.2	1.4
IX	Myrmecophiles	Strict clock, 1% rate calibration	0.4	0.1	0.7
V	Myrmecophiles	TK02 clock, 25 MYA date calibration	25.0	n.a.	n.a.
V	Myrmecophiles	TK02 clock, 1% rate calibration	14.3	9.5	23.1
V	Myrmecophiles	IGR clock, 25 MYA date calibration	25.0	n.a.	n.a.
V	Myrmecophiles	IGR clock, 1% rate calibration	12.3	9.3	16.1
V	Myrmecophiles	Strict clock, 25 MYA date calibration	25.0	n.a.	n.a.
V	Myrmecophiles	Strict clock, 1% rate calibration	12.3	9.8	15.4
VI	Myrmecophiles	TK02 clock, 25 MYA date calibration	8.0	4.2	14.1
VI	Myrmecophiles	TK02 clock, 1% rate calibration	4.5	1.9	11.2
VI	Myrmecophiles	IGR clock, 25 MYA date calibration	6.7	3.4	11.7
VI	Myrmecophiles	IGR clock, 1% rate calibration	3.2	1.7	5.2
VI	Myrmecophiles	Strict clock, 25 MYA date calibration	5.9	3.8	8.1
VI	Myrmecophiles	Strict clock, 1% rate calibration	2.9	2.0	4.0
VII	Myrmecophiles	TK02 clock, 25 MYA date calibration	13.8	9.9	18.0
VII	Myrmecophiles	TK02 clock, 1% rate calibration	7.8	4.4	14.2
VII	Myrmecophiles	IGR clock, 25 MYA date calibration	14.7	10.4	19.9
VII	Myrmecophiles	IGR clock, 1% rate calibration	7.1	4.8	9.7
VII	Myrmecophiles	Strict clock, 25 MYA date calibration	14.2	11.2	17.4
VII	Myrmecophiles	Strict clock, 1% rate calibration	6.9	5.3	8.8
VIII	Myrmecophiles	TK02 clock, 25 MYA date calibration	7.8	4.6	12.0
VIII	Myrmecophiles	TK02 clock, 1% rate calibration	4.4	2.1	9.5
VIII	Myrmecophiles	IGR clock, 25 MYA date calibration	8.4	5.1	13.3
VIII	Myrmecophiles	IGR clock, 1% rate calibration	4.0	2.6	6.0
VIII	Myrmecophiles	Strict clock, 25 MYA date calibration	7.4	5.4	9.6
VIII	Myrmecophiles	Strict clock, 1% rate calibration	3.7	2.7	4.8

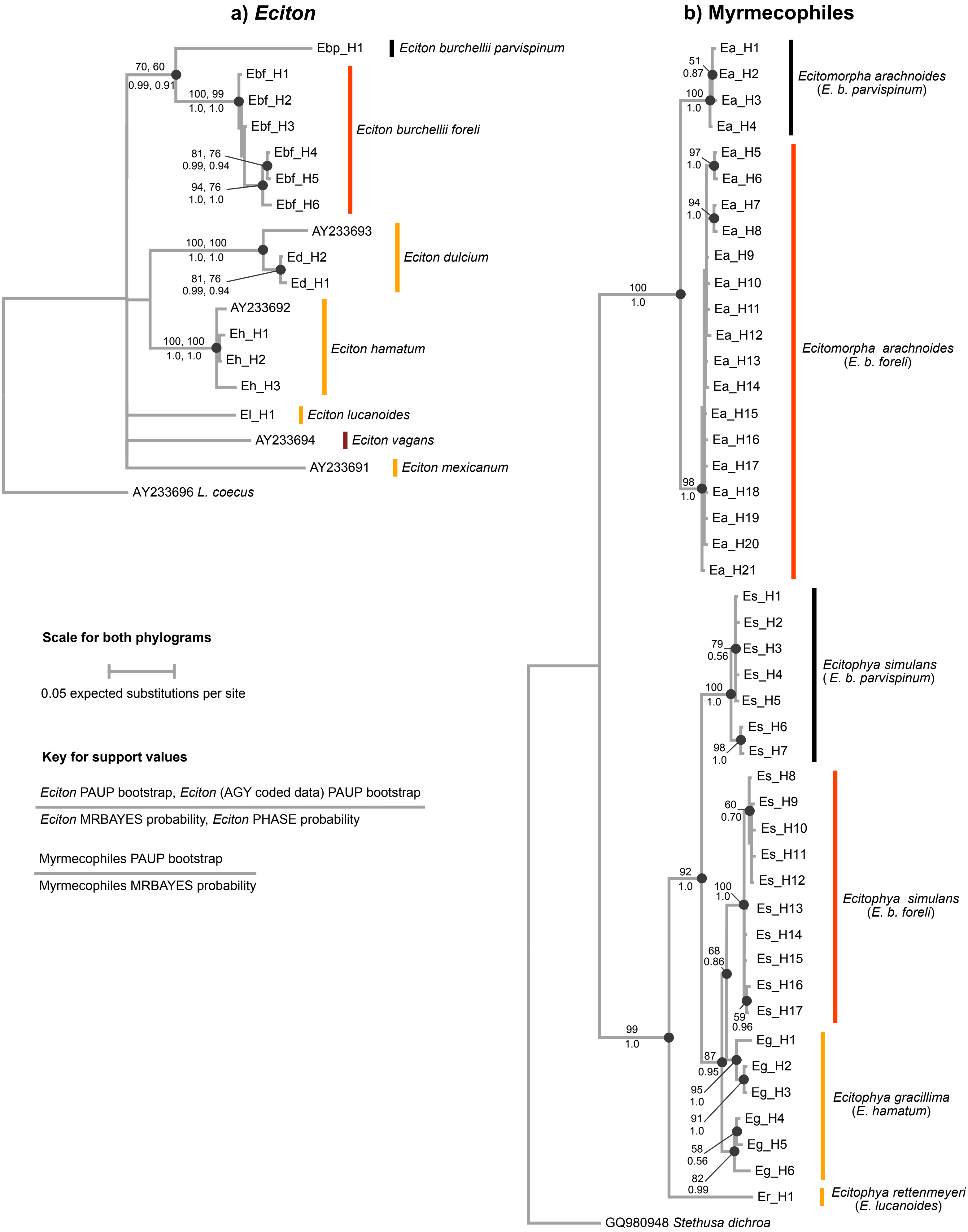
Table 3. Number of segregating sites (S) and average number of nucleotide differences (*k*) between *Eciton* species, between the *Ecitophya* and *Ecitomorpha*, and between species within each of these genera; based on unique haplotypes (802bp mtDNA COI).

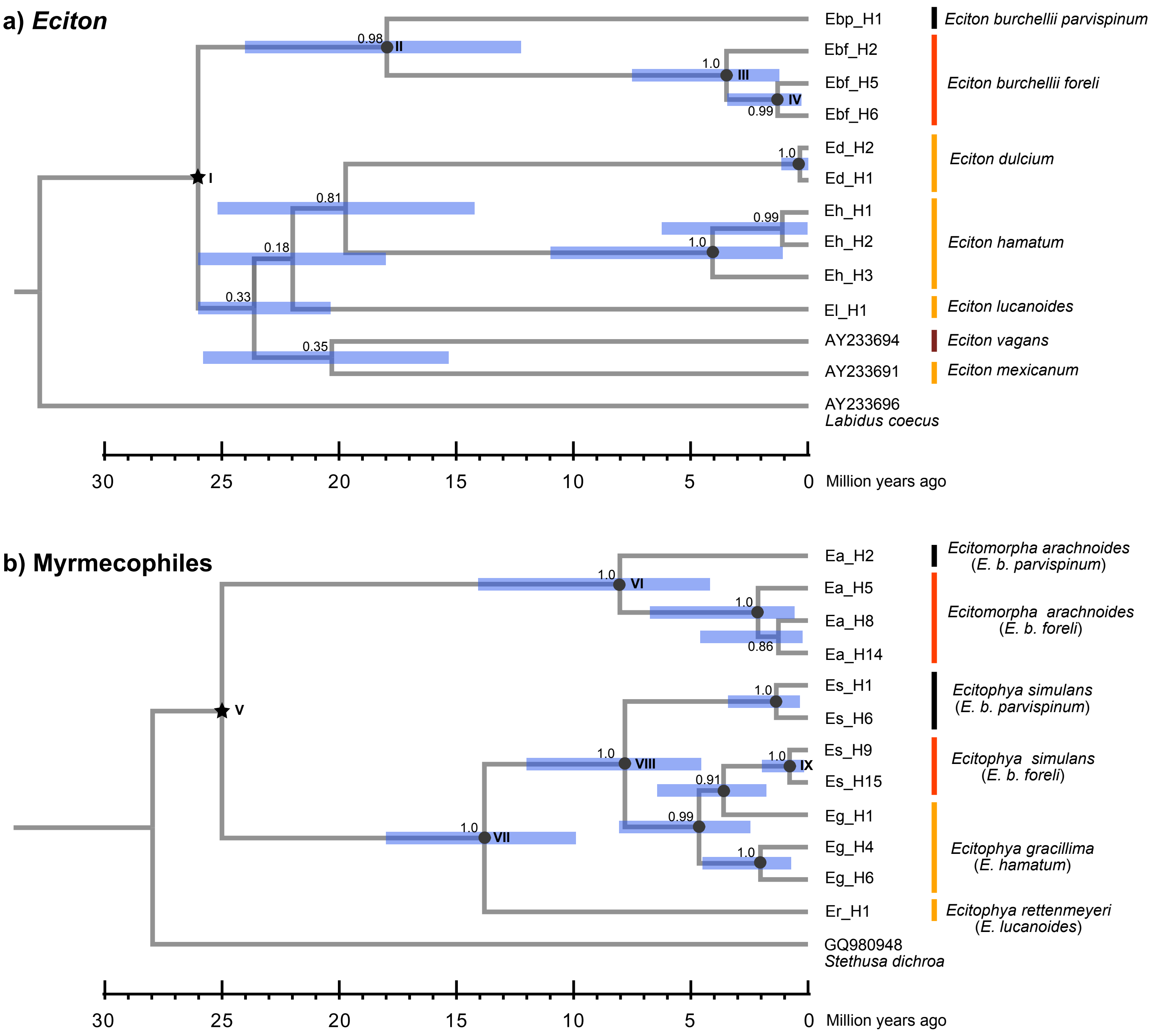
	Comparison	**S**	***k***
Ants			
Eciton	*b. parvispinum – dulcium*	104	103.5
	b. parvispinum – hamatum	100	94.33
	b. foreli – dulcium	100	91.67
	b. parvispinum – lucanoides	88	88.00
	lucanoides – dulcium	86	85.50
	b. foreli – lucanoides	90	81.83
	b. foreli – b. parvispinum	90	81.00
	b. foreli – hamatum	92	79.28
	hamatum - dulcium	81	74.33
	hamatum – lucanoides	80	73.33
Beetles	*Ecitomorpha – Ecitophya*	185	103.98
Ecitomorpha	*arachnoides* (*foreli*) *– arachnoides* (*parvispinum*)	19	33.90
Ecitophya	*simulans* (*foreli) – rettenmeyeri*	82	77.30
	gracillima – rettenmeyeri	97	74.67
	simulans (*parvispinum*) *– rettenmeyeri*	79	73.71
	simulans (*parvispinum*) *– gracillima*	72	42.43
	simulans (*foreli*) *– simulans* (*parvispinum*)	54	41.81
	simulans (*foreli*) *– gracillima*	55	24.83

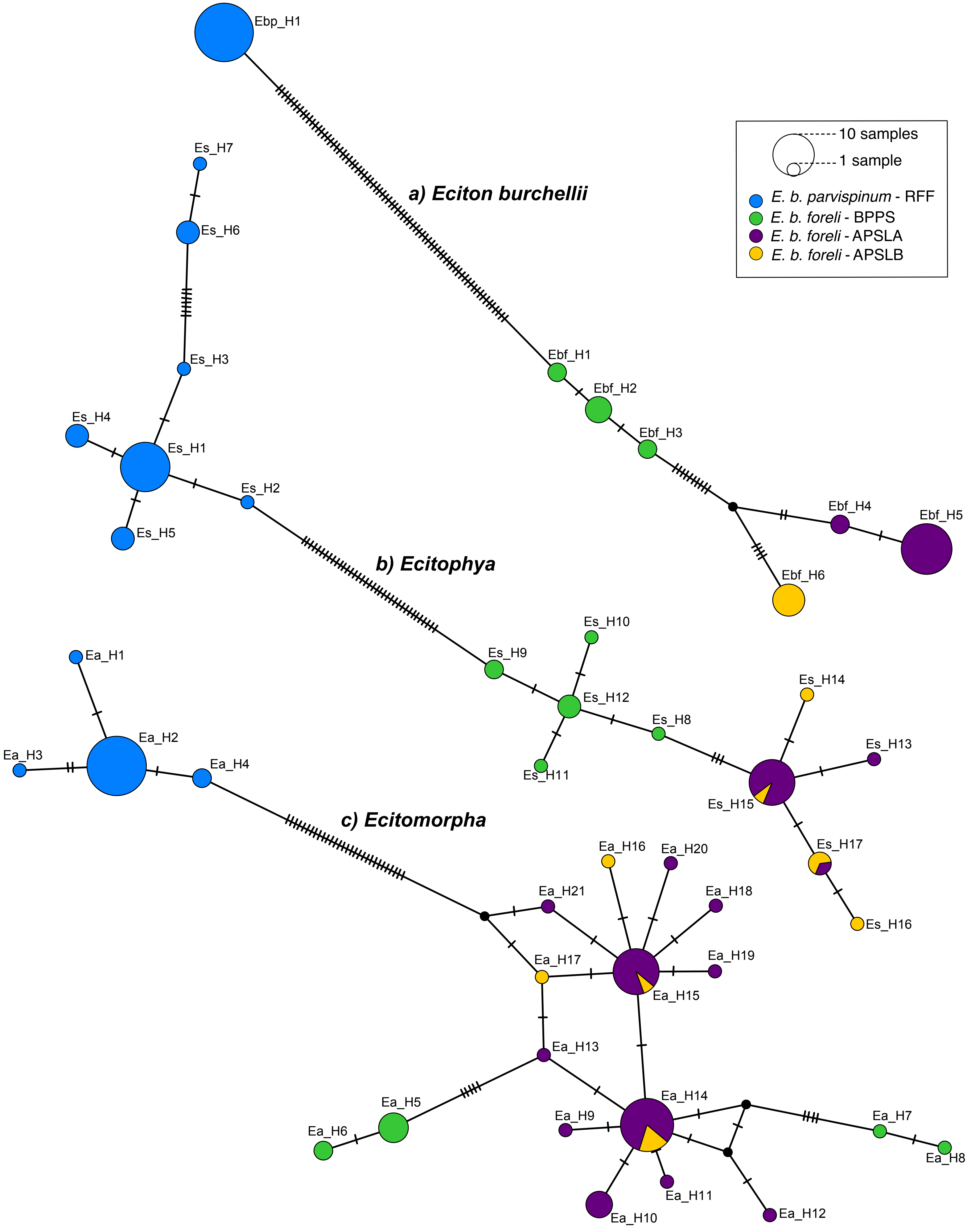
Table 4. Population structure estimates derived from haplotypic data from *E. burchelli foreli* and their associated populations of *Ecitophya* and *Ecitomorpha* myrmecophile beetles between the areas studied in West (BPPS) and Central Panama (APSLA, APSLB). * P <0.001, NS non-significant

Source of variation	**df**	**SSQ**	**Variance**	**% variation**	**Fixation index**
Eciton burchellii foreli					
Among groups	1	1139.658	76.888	76.31	$\Phi_{ST} = 0.9979$*
Among populations within groups	1	210.061	23.660	23.48	$\Phi_{SC} = 0.9914$*
Within populations	28	5.765	0.206	0.20	$\Phi_{CT} = 0.7631$
Total	30	1355.484	100.574		
Ecitophya simulans					
Among groups	1	107.796	8.859	84.62	$\Phi_{ST} =0.8449$*
Among populations within groups	1	1.522	-0.014	-0.13	$\Phi_{SC} =-0.0088$NS
Within populations	24	38.978	1.624	15.51	$\Phi_{CT} =0.8462$*
Total	26	148.296	10.469		
Ecitomorpha arachnoides					
Among groups	1	218.331	14.713	79.57	$\Phi_{ST} =0.7797$*
Among populations within groups	1	1.032	-0.296	-1.59	$\Phi_{SC} =-0.0783$NS
Within populations	48	195.5	4.073	22.03	$\Phi_{CT} =0.7957$*
Total	50	414.863	18.491		


Costa Rica
Panama
100 km
RFF
BPPS
APSLA
APSLB
SOB


a) Eciton
70, 60
0.99, 0.91
Ebp_H1
Eciton burchellii parvispinum
Ebf_H1
100, 99
1.0, 1.0
Ebf_H2
Ebf_H3
Eciton burchellii foreli
81, 76
0.99, 0.94
Ebf_H4
Ebf_H5
94, 76
1.0, 1.0
Ebf_H6
100, 100
1.0, 1.0
AY233693
Ed_H2
Eciton dulcium
81, 76
0.99, 0.94
Ed_H1
AY233692
Eh_H1
100, 100
1.0, 1.0
Eh_H2
Eciton hamatum
Eh_H3
El_H1
Eciton lucanoides
AY233694
Eciton vagans
AY233691
Eciton mexicanum
AY233696 L. coecus
Scale for both phylograms
0.05 expected substitutions per site
Key for support values
Eciton PAUP bootstrap, Eciton (AGY coded data) PAUP bootstrap
Eciton MRBAYES probability, Eciton PHASE probability
Myrmecophiles PAUP bootstrap
Myrmecophiles MRBAYES probability
b) Myrmecophiles
Ea_H1
51
0.87
Ea_H2
100
1.0
Ea_H3
Ea_H4
Ecitomorpha arachnoides (E. b. parvispinum)
97
1.0
Ea_H5
Ea_H6
94
1.0
Ea_H7
Ea_H8
Ea_H9
100
1.0
Ea_H10
Ea_H11
Ea_H12
Ecitomorpha arachnoides (E. b. foreli)
Ea_H13
Ea_H14
Ea_H15
Ea_H16
Ea_H17
98
1.0
Ea_H18
Ea_H19
Ea_H20
Ea_H21
Es_H1
Es_H2
79
0.56
Es_H3
Ecitophya simulans (E. b. parvispinum)
Es_H4
100
1.0
Es_H5
Es_H6
98
1.0
Es_H7
Es_H8
60
0.70
Es_H9
Es_H10
Es_H11
Es_H12
92
1.0
100
1.0
Es_H13
Ecitophya simulans (E. b. foreli)
Es_H14
68
0.86
Es_H15
Es_H16
59
0.96
Es_H17
99
1.0
Eg_H1
87
0.95
Eg_H2
95
1.0
Eg_H3
Ecitophya gracillima (E. hamatum)
91
1.0
Eg_H4
58
0.56
Eg_H5
82
0.99
Eg_H6
Er_H1
Ecitophya rettenmeyeri (E. lucanoides)
GQ980948 Stethusa dichroa


a) Eciton
0.98
II
1.0
III
IV
0.99
1.0
I
0.81
0.99
0.18
1.0
0.33
0.35
Ebp_H1
Ebf_H2
Ebf_H5
Ebf_H6
Ed_H2
Ed_H1
Eh_H1
Eh_H2
Eh_H3
El_H1
AY233694
AY233691
AY233696
Labidus coecus
Eciton burchellii parvispinum
Eciton burchellii foreli
Eciton dulcium
Eciton hamatum
Eciton lucanoides
Eciton vagans
Eciton mexicanum
30
25
20
15
10
5
0
Million years ago
b) Myrmecophiles
1.0
VI
1.0
0.86
V
1.0
1.0
VIII
1.0
IX
0.91
0.99
1.0
VII
1.0
Ea_H2
Ea_H5
Ea_H8
Ea_H14
Es_H1
Es_H6
Es_H9
Es_H15
Eg_H1
Eg_H4
Eg_H6
Er_H1
GQ980948
Stethusa dichroa
Ecitomorpha arachnoides (E. b. parvispinum)
Ecitomorpha arachnoides (E. b. foreli)
Ecitophya simulans (E. b. parvispinum)
Ecitophya simulans (E. b. foreli)
Ecitophya gracillima (E. hamatum)
Ecitophya rettenmeyeri (E. lucanoides)
30
25
20
15
10
5
0
Million years ago


10 samples
1 sample
E. b. parvispinum - RFF
E. b. foreli - BPPS
E. b. foreli - APSLA
E. b. foreli - APSLB
a) Eciton burchellii
Ebp_H1
Ebf_H1
Ebf_H2
Ebf_H3
Ebf_H4
Ebf_H5
Ebf_H6
b) Ecitophya
Es_H7
Es_H6
Es_H3
Es_H4
Es_H1
Es_H2
Es_H5
Es_H10
Es_H9
Es_H12
Es_H8
Es_H11
Es_H14
Es_H13
Es_H15
Es_H17
Es_H16
c) Ecitomorpha
Ea_H1
Ea_H2
Ea_H3
Ea_H4
Ea_H16
Ea_H20
Ea_H21
Ea_H18
Ea_H17
Ea_H19
Ea_H15
Ea_H13
Ea_H5
Ea_H14
Ea_H6
Ea_H9
Ea_H7
Ea_H8
Ea_H11
Ea_H10
Ea_H12