Renewed diversification is associated with new ecological opportunity in the Neotropical turtle ants

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Abstract

Ecological opportunity, defined as access to new resources free from competitors, is thought to be a catalyst for the process of adaptive radiation. Much of what we know about ecological opportunity, and the larger process of adaptive radiation, is derived from vertebrate diversification on islands. Here, we examine lineage diversification in the turtle ants (*Cephalotes*), a species-rich group of ants that has diversified throughout the Neotropics. We show that crown group turtle ants originated during the Eocene (around 46 mya), coincident with global warming and the origin of many other clades. We also show a marked lineage-wide slowdown in diversification rates in the Miocene. Contrasting this overall pattern, a species group associated with the young and seasonally harsh Chacoan biogeographic region underwent a recent burst of diversification. Subsequent analyses also indicated that there is significant phylogenetic clustering within the Chacoan region and that speciation rates are highest there. Together, these findings suggest that recent ecological opportunity, from successful colonization of novel habitat, may have facilitated renewed turtle ant diversification. Our findings highlight a central role of ecological opportunity within a successful continental radiation.

Introduction

Adaptive radiation, the evolution of ecologically and phenotypically divergent species within a multiplying lineage, is a key process in the generation of biodiversity (Simpson, 1944, 1953; Schluter, 2000). It is widely thought that adaptive radiation is spurred by ecological opportunity (Schluter, 2000; Losos & Mahler, 2010; Yoder *et al.*, 2010), defined as an abundance of evolutionarily accessible resources little used by competitors. Ecological opportunity may result from several scenarios: the emergence of new resources,

the evolution of a trait that allows for novel exploitation of existing resources, the extinction of species competing for the same resources or the colonization of an area with unused resources (Simpson, 1953). Completely or partially freed from competitive interactions, a lineage can multiply rapidly to fill the newly available niche space, but will ultimately experience a slowdown in the diversification rate as niches become filled by the new species. The signature of this process is a pattern of slowing species accumulation over time, known as 'diversity dependence' (e.g. Harmon *et al.*, 2003; Weir, 2006; McPeek *et al.*, 2008; Phillimore & Price, 2008; Rabosky & Lovette, 2008). This ecological process can be retriggered whenever new ecological opportunity is available. A decline in the lineage-wide diversification rate may therefore become decoupled from a renewed burst of diversification

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within any subclade that gains access to new resources (Etienne & Haegeman, 2012). Studies that capture these kinds of complex patterns of diversification, associated with differences in ecological conditions, provide compelling support for the key role of ecological opportunity in the process of diversification (e.g. Jetz *et al.*, 2012; Jønsson *et al.*, 2012; Near *et al.*, 2013).

Many of the critical insights into how ecological opportunity drives diversification have been generated from a relatively small number of well-studied vertebrate lineages on islands, including the iconic *Anolis* lizards of the Caribbean (Losos, 2009), Galapagos finches (Grant & Grant, 2011) and the Hawaiian honeycreepers (Lovette *et al.*, 2002). Islands provide a discrete and tractable spatial structure for studying the ecological processes that drive diversification. Nevertheless, it remains to be seen whether patterns of diversification on islands, and the resulting inferences, can be generalized to continents. For instance, continents usually have a larger spatial and longer temporal context for diversification (Gavrilets & Losos, 2009; Derryberry *et al.*, 2011; Day *et al.*, 2013).

To date, only a handful of studies have addressed how diversification rates in continental lineages might be shaped by ecological opportunity, and they achieve little consensus. Results from these studies include detection of lineage-wide diversity-dependent patterns indicative of niche filling (e.g. Burbrink & Pyron, 2009; Barker *et al.*, 2013), increases in diversification rates in clades within lineages that may (e.g. Drummond *et al.*, 2012; Schenk *et al.*, 2013) or may not (e.g. Claramunt *et al.*, 2012) be attributed to ecological opportunity and constant rates of lineage diversification suggestive of a lack of ecological limits in these lineages (e.g. Derryberry *et al.*, 2011; Day *et al.*, 2013). Beyond the focus on island diversification, the emphasis on vertebrate lineages may have introduced a systematic bias to our understanding of the general patterns of diversification. The motifs and mechanisms of diversification in the hyperdiverse insects, for example, have been little studied (but see Mitter *et al.*, 1988; Farrell, 1998; Fordyce, 2010; Litman *et al.*, 2013), and it is unclear how they may mirror or contrast those seen in vertebrates. All considered, much may be learned by asking whether continental lineages of important insect groups show the signature of diversification shaped by ecological opportunity.

The remarkably diverse Neotropical ecozone has featured prominently in research on continental diversification. The primary focus has been on the role of time and region-specific geologic and climatic events in generating taxonomic diversity (e.g. Prothero, 1994; Coates & Obando, 1996; Colinvaux *et al.*, 2000; Gregory-Wodzicki, 2000; Moritz *et al.*, 2000; Haffer & Prance, 2001; Knapp & Mallet, 2003; Haffer, 2008; Räsänen *et al.*, 1995). However, recent studies indicate

that the mechanisms driving diversification are more complex than can be attributed to one event or time period (Bush, 1994; Zink *et al.*, 2004; Bush & Oliveira, 2006; Rull, 2008; Hoorn *et al.*, 2010; Rull, 2011). Consequently, new studies addressing how ecological processes may drive diversification are likely to be of considerable value in understanding Neotropical diversity (e.g. Hughes & Eastwood, 2006; Derryberry *et al.*, 2011). Work focusing on the role of ecological opportunity in the diversification of poorly studied but highly successful Neotropical insect lineages would provide direct and much needed tests of the potentially central role of ecological opportunity in diversification. Crucially, they would also expand the taxonomic and spatial scope of study systems used to test this role.

Ants are a particularly important group within the Neotropics, with nearly 4000 described species from the region (AntWeb, 2013) and far reaching ecological impacts via their rich trophic interactions (reviewed in Lach *et al.*, 2010). The arboreal and highly distinctive genus *Cephalotes*, known commonly as turtle ants because of their shell-like armour (Fig. 1 insert), is an especially appealing group for studying ecological drivers of Neotropical diversification. First, the turtle ants are one of the most diverse ant genera confined to the New World (Bolton, 2012), and most of the 115 described species are limited to the Neotropics (Fig. 2; de Andrade & Baroni-Urbani, 1999). They therefore represent a highly successful Neotropical radiation that is still quite tractable. Second, we have unusually good specimen availability and distribution data for the turtle ants (de Andrade & Baroni-Urbani, 1999), largely because they are often well represented in canopy arthropod samples. Complete taxon sampling remains a challenge, as it does for any diverse Neotropical lineage. Nevertheless, the turtle ants offer a best-case scenario for broad phylogenetic coverage of a diverse ant lineage, and thus allow for the robust application of modern analyses that account for missing taxa. Third, they inhabit several ecologically distinct biogeographic regions of the New World, ranging from deserts, to wet forests, to seasonal savannahs (de Andrade & Baroni-Urbani, 1999). The potential for a signature of ecological drivers of diversification is therefore considerable. And finally, turtle ants have a rich amber fossil record (de Andrade & Baroni-Urbani, 1999), allowing for time calibration of the phylogeny and insight into trait evolution (Slater *et al.*, 2012).

In this study, we take a critical first step in studying diversification in the turtle ant system. More specifically, we use an integrative approach to ask whether turtle ant diversification has been shaped by ecological opportunity in the Neotropics. Although the general ecology of turtle ants is known, the evolutionary history and the timing of diversification of the
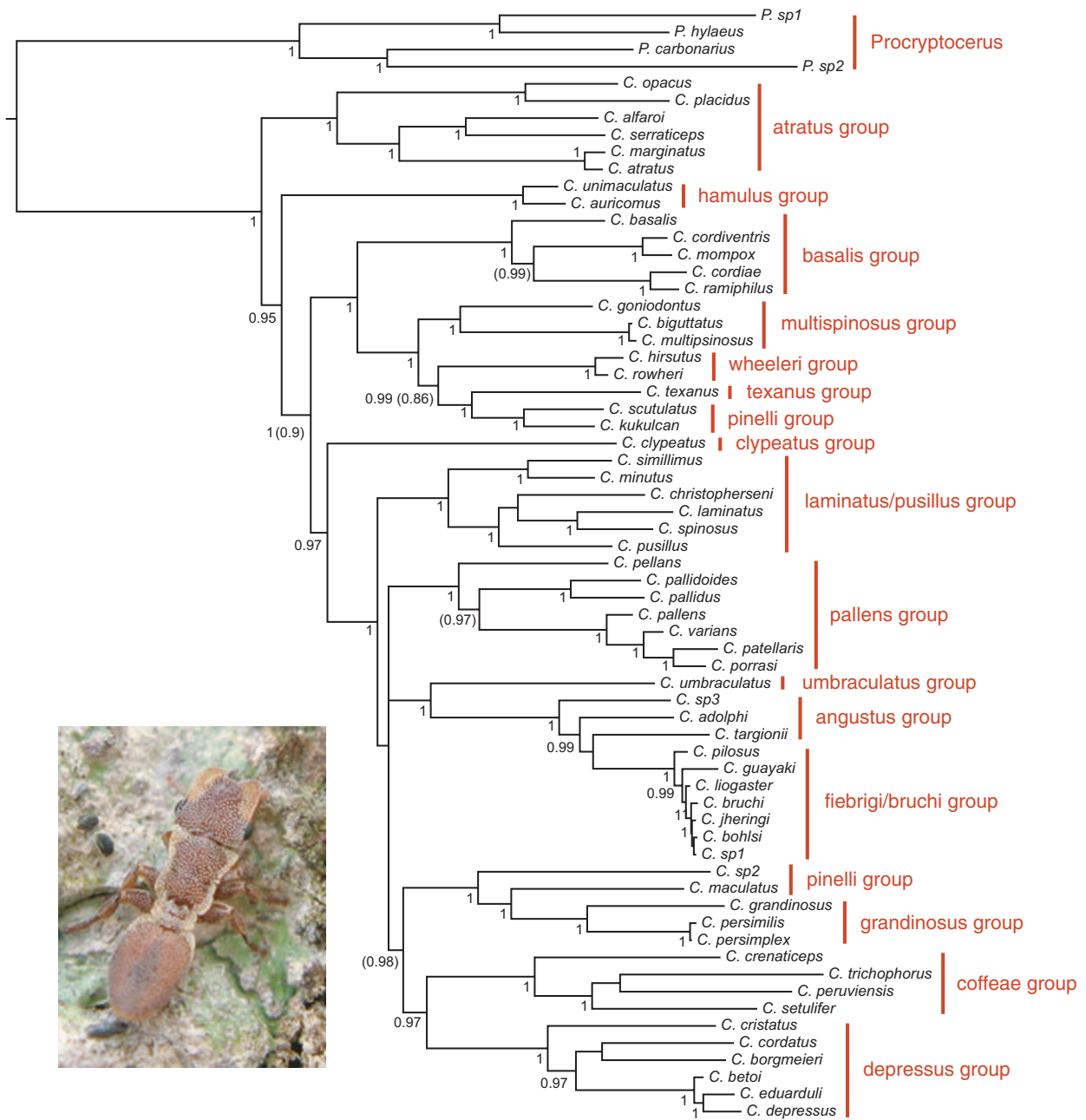
Fig. 1 A Bayesian consensus phylogram of *Cephalotes* based on both the combined morphological and molecular data set and only the molecular data set. PP values ≥ 0.95 for the combined data set are given. PP values for the molecular data set that are different from the combined data set by ≥ 0.05 are given in parentheses. Values only in parentheses indicate that support for the combined data set was < 0.95; therefore, values for only the molecular data set are given in parentheses. The names of previously defined species groups based on morphological characters (de Andrade & Baroni-Urbani, 1999) are shown in red. The photograph insert is of a *Cephalotes persimilis* worker (photo by S. Powell). PP, posterior probability.

genus are not. To that end, we first use a combined molecular and morphological phylogenetic approach to produce the first time-calibrated phylogeny of the turtle ants. We then explore factors influencing turtle ant diversification by testing for topological and temporal shifts in rates of diversification. These analyses are complemented by studies of community phylogenetics and geographic state-dependent speciation
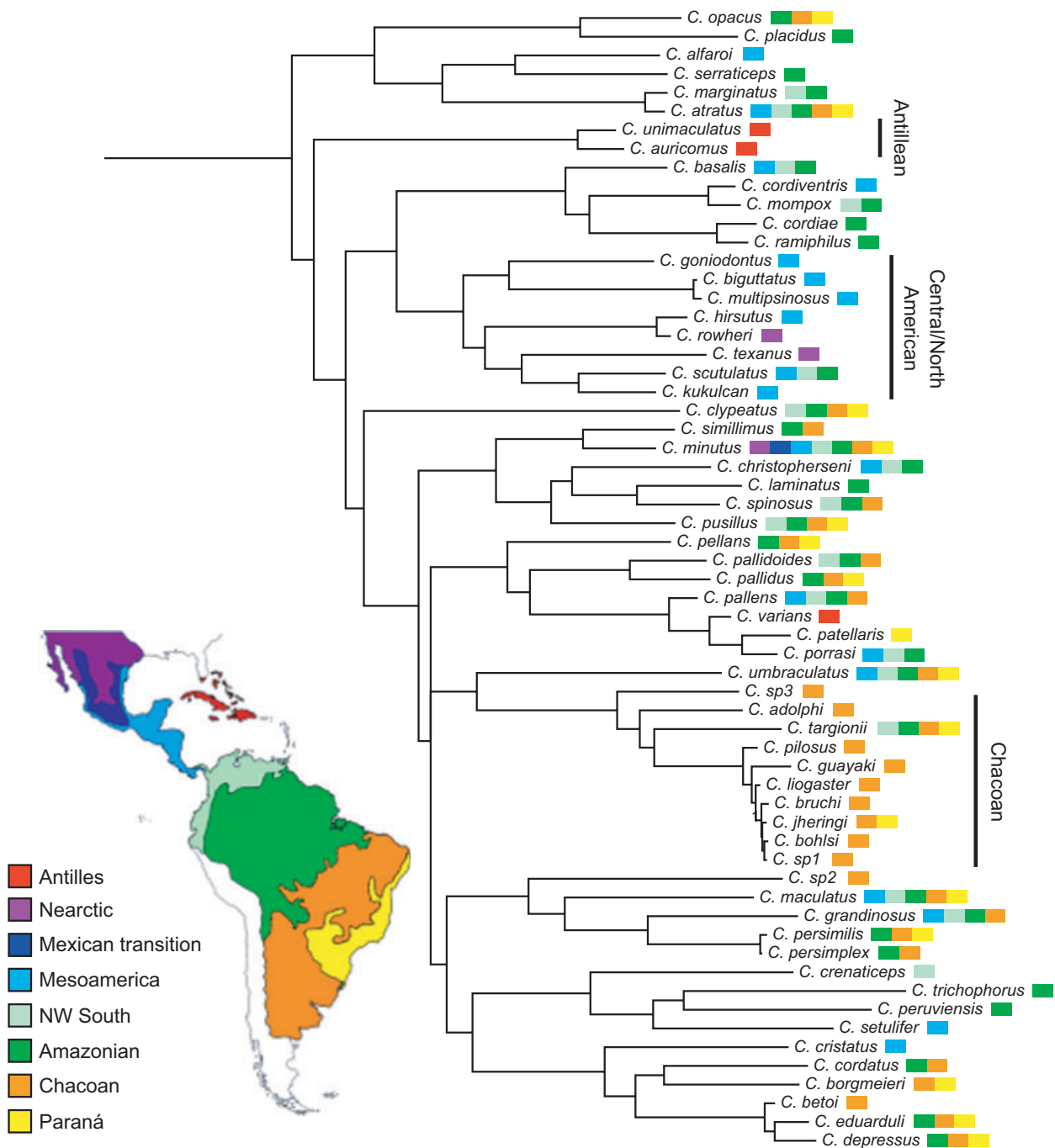
Fig. 2 The biogeographic regions inhabited by turtle ant species, mapped onto a Bayesian consensus phylogram. Colour coding on the tips corresponds to the separate coloured areas on the map, which represent eight biogeographic regions of insect endemism (adapted from Morrone, 2006). The combined area covered by the coloured regions on the distribution map represents the full geographic distribution of the turtle ants. The labelled clades indicate the taxa primarily occurring in three areas: The Antilles, Central and North America and the Chacoan biogeographic region.

and extinction models to understand how turtle ants evolved with respect to ecologically distinct biogeographic regions of the New World. Our analyses reveal the extent to which ecological opportunity has influenced diversification in this species-rich ant lineage.

Materials and methods

Molecular and morphological data

The turtle ants (*Cephalotes*) are highly distinctive and heavily collected, and their taxonomy has been studied extensively over the past 200 years (e.g. Latreille, 1802; Emery, 1906, 1922; Kempf, 1951; Wilson, 1985; de Andrade & Baroni-Urbani, 1999). The most recent complete taxonomic revision provided strong morphological evidence for the long-proposed monophyly of the lineage and further identified a number of distinct morphological groups within the genus. We sampled 58 described and three undescribed turtle ant species, covering approximately half of the known 115 species (Table S1). Our sampling spans 19 of the 24 previously defined morphological groups; unsampled groups are monotypic or bitypic, and within group coverage averaged 46% (Table S2). We included four species of *Procryptocerus*, the well-established sister genus of *Cephalotes* (de Andrade & Baroni-Urbani, 1999; Moreau *et al.*, 2006; Moreau & Bell, 2013), as outgroups. Preliminary analyses with 2–4 individuals of each of 12 widespread species were used to confirm species monophyly, which was supported in all but two cases (see Materials and methods and Results in Supporting information; Fig. S1).

DNA was isolated from whole ants preserved in ethanol or nondestructively extracted from pinned museum specimens using the DNeasy Kit (Qiagen, Valencia, CA, USA). Segments were sequenced for three protein-coding nuclear [*long wavelength rhodopsin* (*LR*), 456 base pairs (bp); *elongation factor 1α F2* (*EF1αF2*), 517 bp; *wingless* (*WG*), 484 bp] and three mitochondrial [*cytochrome oxidase I* (*COI*), 1066 bp; *cytochrome oxidase II* (*COII*), 523 bp; *cytochrome b* (*Cytb*), 433 bp] genes that are commonly used in ant phylogenetics (e.g. Ward & Downie, 2005; Brady *et al.*, 2006; Moreau *et al.*, 2006; see Methods in Supporting information; Table S3). There were six missing or partially missing sequences for the nuclear data set, comprising 1.2% of the total data set. The mitochondrial data set contained 20 missing and 28 partially missing sequences, representing 3.9% and 3.5% of the total data set, respectively.

Sequences were edited in GENEIOUS v5.4.2 (Biomatters, Aukland, New Zealand) and aligned using the GENEIOUS alignment algorithm. Due to ambiguity in alignment, the intron from *LR* was excluded from all subsequent analyses. Nuclear copies of mitochondrial DNA (numts) were recovered for some mitochondrial gene segments (*Cytb* and *COI*). To address this issue, numt sequences were first identified by inspection for indels and stop codons and removed. We discovered additional numts in preliminary phylogenetic analyses as sequences that formed distinct clades but represented morphologically disparate species. These sequences had unusual patterns of amino acid substitutions and were also excluded from all subsequent analyses. We re-extracted DNA from as many taxa as possible, designed alternative sets of primers for each gene region with numts and modified PCR protocols to reduce numt amplification. We removed any sequence suspected as a numt from analysis. Alignments were deposited in TreeBASE (Submission Number 14990), and sequences were accessioned in GenBank (Accession Numbers KC205480–205554, 208511–208592, 335575–335655, 335656–335726, 335727–335808, 335809–335891, 335892–335974; Table S4).

We incorporated a published 131 character morphological data set that includes all described extant and fossil turtle ant species [115 and 16 species, respectively (de Andrade & Baroni-Urbani, 1999)]. Characters are based on spines and integumental expansions, coloration patterns and microsculpturing of the workers, soldiers, gynes and males. These data were used to examine the effects of combined data on tree topology and nodal support, as well as to place fossils in a phylogenetic context for dating purposes (see below). We coded characters for *Procryptocerus* as ambiguous because the same species were not used as outgroups in the molecular and morphological matrices. Undescribed turtle ant species were also coded as ambiguous for morphological characters.

Phylogenetic inference

We used Bayesian inference to infer the turtle ant phylogeny in the program MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) accessed on the TeraGrid through the CIPRES Science Gateway (Miller *et al.*, 2010). We first analysed the molecular data set alone. Molecular data were partitioned by nuclear gene segment and each mitochondrial codon position (mitochondrial data were considered as one segment). We applied a GTR + G model to each partition to allow for rate heterogeneity without an invariant sites model (Huelsenbeck & Rannala, 2004; Lemmon & Moriarty, 2004; Revell *et al.*, 2005). We then performed a combined analysis of the molecular and morphological data sets for extant species only. Molecular data were partitioned and modelled as above. For the morphological data set, we applied the Markov model (Lewis, 2001) with gamma-distributed rates. Analyses were run for 20 million generations, sampling every 1000 generations, with a heating parameter of 0.1 to increase mixing between chains. Convergence diagnostics were assessed in TRACER v1.5 (Rambaut & Drummond, 2007) and ARE WE THERE YET? (Wilgenbusch *et al.*, 2004). After ensuring that both runs reached stationarity and effective sample sizes for all parameters were sufficient (> 200), we discarded the first 25% of sampled trees as burn-in and computed a majority rule consensus tree from the remaining sample.

Due to potential discordance between gene trees (Maddison, 1997), we also used Bayesian concordance

analysis (BCA) on the molecular data set implemented in the program BUCKY v1.4.0 (Ané *et al.*, 2007; Larget *et al.*, 2010). We first analysed each gene separately with MrBayes. Each analysis was run for 15 million generations with the same parameter settings used in the species tree analyses. In BUCKY, the alpha parameter (the value for the Dirichlet process prior) was set to both 5 and 10, placing a high prior distribution on between three and four distinct trees, respectively. Both values resulted in the same output values and tree topology. We ran BUCKY for 1 million generations with four chains (one cold, three heated). Burn-in was left at the default value of 10%.

Divergence dating

To determine fossil calibration points for divergence time estimation, we ran an analysis in MrBayes of the combined data set, including extant species sampled for this study and all fossil species. As some of the morphological characters are based on soldier, gyne and male traits, which were not available for any fossil species, we trimmed those characters from the matrix, resulting in 77 morphological characters. The analysis was run for 10 million generations using the same partitioning scheme as for extant taxa only. Given the large amount of missing data for fossil species, most nodes in this analysis were not well supported (Fig. S2). However, we utilized fossils as calibration points if they fell within groups that were well supported [posterior probability (PP) ≥ 0.95] in molecular-only analyses (Fig. 1). Although other fossil species fell within well-supported clades, we limited calibration points to the shallowest nodes of the phylogeny to reflect the minimum age of the divergence events represented by the fossils. Based on these criteria, we were able to define three fossil calibration points: (i) *C. bloosi* (Dominican amber; de Andrade & Baroni-Urbani, 1999) was used to calibrate the node uniting the *coffeae* and *crenaticeps* groups; (2) *C. caribicus* (Dominican amber; de Andrade & Baroni-Urbani, 1999) was used to calibrate the node uniting the *texanus* group and (*C. kukulcan*, *C. scutulatus*); and (iii) *C. maya* and *C. olmecus* (Mexican amber; de Andrade & Baroni-Urbani, 1999) were used to calibrate the node uniting the *grandinosus* group and *C. maculatus*.

All turtle ant fossils are from Dominican or Mexican amber. Dominican amber has been challenging to age (Poinar & Mastalerz, 2000; Poinar, 2010), and different ages have been proposed using inferential dating techniques. They range from 15 to 45 mya (Baroni-Urbani & Saunders, 1980; Lambert *et al.*, 1985; Schlee, 1990; Grimaldi, 1996; Iturralde-Vinent & MacPhee, 1996), but currently, the most robust age for Dominican amber is 15–20 mya based on multiple lines of evidence (Iturralde-Vinent & MacPhee, 1996; Grimaldi & Engel, 2005). There is general agreement that Mexican fossil dates are similar to those of Dominican amber

(Kraemer, 2007). Nodes with fossil calibration points were assigned a lognormal prior distribution with a zero offset value of 15 million years, corresponding to the youngest suggested age of the fossils. We assigned a median value of 25 million years (mean of lognormal = 2.303) to account for the fact that fossils represent minimum ages and that the true node age is likely to be older than the fossil. The 95% upper bound was 65 million years (standard deviation of lognormal = 0.9782) based on a previous estimate for the *Cephalotes-Procryptocerus* split (Moreau *et al.*, 2006). Because the root prior is a secondary calibration point, we assigned a broad normal prior distribution to the root node to reflect divergence age uncertainty (Ho & Phillips, 2009). The mean was 65 million years (Moreau *et al.*, 2006) with a standard deviation of 12.753 million years corresponding to 2.5% and 97.5% quantiles of 90 and 40 million years, respectively.

Divergence dating analysis was performed using BEAST v1.6.1 (Drummond & Rambaut, 2007). We constrained the analyses to search topologies that included well-supported nodes (PP values ≥ 0.95) from the tree generated with morphological and molecular data in MrBayes. Data were partitioned using the same scheme as for the MrBayes analyses, and the GTR + G model was applied to all partitions. We used an uncorrelated lognormal relaxed clock model with a birth–death process as the tree prior. Two MCMC analyses were run from independent starting points for 100 million generations, sampling every 10 000 generations. The results from both sets of analyses were evaluated in TRACER to ensure convergence. We discarded the first 20% of each run as burn-in, combined the remaining output using LOGCOMBINER v1.6.1 (Drummond & Rambaut, 2007) and produced a maximum clade credibility (MCC) tree using TREEANNOTATOR v.1.6.1 (Drummond & Rambaut, 2007). Finally, we ran BEAST without data to only sample the effects of our priors on the resulting trees.

Estimating diversification rates

To explore diversification dynamics within the turtle ants, we applied a series of tests that do not make the assumption of a completely sampled phylogeny. Each address different aspects of the pattern of diversification within a lineage. To test for deviations from a constant rates pure birth process, we calculated the gamma statistic using both the MCC tree and the post-burn-in distribution of trees from the divergence dating analysis (Pybus & Harvey, 2000). This was conducted using the LASER v2.3 package (Rabosky, 2006) in R 2.13.0 (R Development Core Team). The Monte Carlo constant rates (MCCR) test was used to compute a critical value for gamma while accounting for incomplete taxon sampling (Pybus & Harvey, 2000). As input for the MCCR test, the total number of turtle ant species used was

118 [all extant described species (de Andrade & Baroni-Urbani, 1999) and three new species discovered by SP].

Changes in rates of diversification through time were examined using TREEPAR v1.4 (Stadler, 2011) in R. This algorithm assumes a birth–death process, but allows for multiple shifts in rates of speciation and extinction across the entire phylogeny at specified time intervals. We limited our search to a maximum of 10 shift points with shifts assessed at 1 million-year intervals from root to tip. Likelihood ratio tests were used to determine whether models with multiple temporal shifts were favoured over a single birth–death process. Incomplete sampling was accounted for following Stadler (2011). We assigned the proportion of sampled species (0.52) as the probability of a species surviving to the present day (Stadler, 2011). Survival probability assumes random sampling, nevertheless, simulations show that it is not sensitive to biased sampling (Stadler, 2011).

Shifts in rates of diversification at different nodes of the tree were investigated using MEDUSA (Alfaro *et al.*, 2009) in R. MEDUSA initially fits a simple birth–death model to the entire tree and then fits models allowing for additional birth and death rates at different nodes. The AIC score is calculated for each model, and the algorithm stops when the difference in AIC scores for each model reaches a defined threshold. A strength of this method is that the MEDUSA algorithm uses branch lengths and species richness data without requiring complete taxon sampling. To account for missing taxa, we collapsed terminal branches and assigned species richness data to those branches based on the morphological species-group designations of de Andrade & Baroni-Urbani (1999; Fig. 1). In most cases, our molecular phylogeny recovered these previously proposed morphological species groups as monophyletic (Fig. 1), so assigning richness data was straightforward. Three previously proposed morphological groups fell within another clade (Fig. 1). MEDUSA assumes the monophyly of incompletely sampled clades, so those branches were collapsed and the species richness of both groups were assigned to the branch. In addition, the *angustus* morphological group was recovered as paraphyletic, with the monophyletic *fiebrigi* group nested within it (Fig. 1). The considerable diversity of the *angustus* and *fiebrigi* groups means that much information is lost when collapsing the clade comprising these groups and estimating diversification rates from the stem age. Consequently, we contrasted this highly conservative assignment against three more realistic richness assignments. We first retained the two *angustus* branches with

deepest dated splits and assigned the unsampled taxa equally to each terminal branch. Second, we assigned species unequally to branches by placing one species in the earliest branching *angustus* clade and the remaining species in the later branching clade. Finally, we assigned one species to the later branching clade and the remaining species to the earliest branching clade.

Only one morphological group was recovered as polyphyletic in the molecular phylogeny (*pinelii*); one clade contains primarily species distributed in Central America whereas the other clade contains South American species. The missing species are only found in South America, so they were assigned to the South American clade. Five monotypic or bitypic morphological groups that were not sampled, totalling six species, were not represented in the phylogeny, and thus were not able to be assigned to clades in the analysis.

Testing the influence of biogeographic regions

We used a community phylogenetics approach to explore the possibility of a phylogenetic signal in the geographic distribution of the turtle ants. We divided the geographic range into eight biogeographic regions based on Morrone's (2006) classification of areas of Neotropical insect endemism: Nearctic, Mexican transition zone, Mesoamerican, Antillean, Northwestern South American, Amazonian, Chacoan and Paraná (Fig. 2). Turtle ant species were coded as present or absent in each region based on distribution data in de Andrade & Baroni-Urbani (1999). Species were allowed to occur in multiple regions, and undescribed species were not included in the analysis. Although community phylogenetics metrics assume no missing taxa, they are used here as a valuable exploratory tool. Our findings are interrogated with subsequent analyses that explicitly account for an incomplete phylogeny. For each community (biogeographic region), we calculated mean pairwise distance (MPD) and mean nearest taxon distance (MNTD; Webb *et al.*, 2002) using the PICANTE v1.3 library (Kembel *et al.*, 2010) in R. MPD is a measure of the average branch length distance among all species pairs in a community (representing phylogenetic distance across the phylogeny), whereas MNTD is the branch length distance between each species and its closest relative in a community (representing phylogenetic distance at the tips; Webb *et al.*, 2002). Standardized effect size was calculated for both indices by randomizing tip values 999 times to generate a null distribution for each metric and comparing the observed value to the null distribution.

Fig. 3 Chronogram showing that crown group *Cephalotes* originated around 46 mya (indicated by white circle and dotted line) in the Middle Eocene (Oligo., Oligocene; Plio., Pliocene; P., Pleistocene). The tree was calibrated with three fossil calibration points and constrained with well-supported nodes (posterior probability values ≥ 0.95) from the combined molecular and morphological analysis. Node bars represent 95% highest posterior density intervals. The arrow on the log lineage through time plot, shown with 95% confidence intervals from the post-burn-in distribution of trees, indicates a significant shift in the rate of diversification in the Late Miocene (around 9 mya).
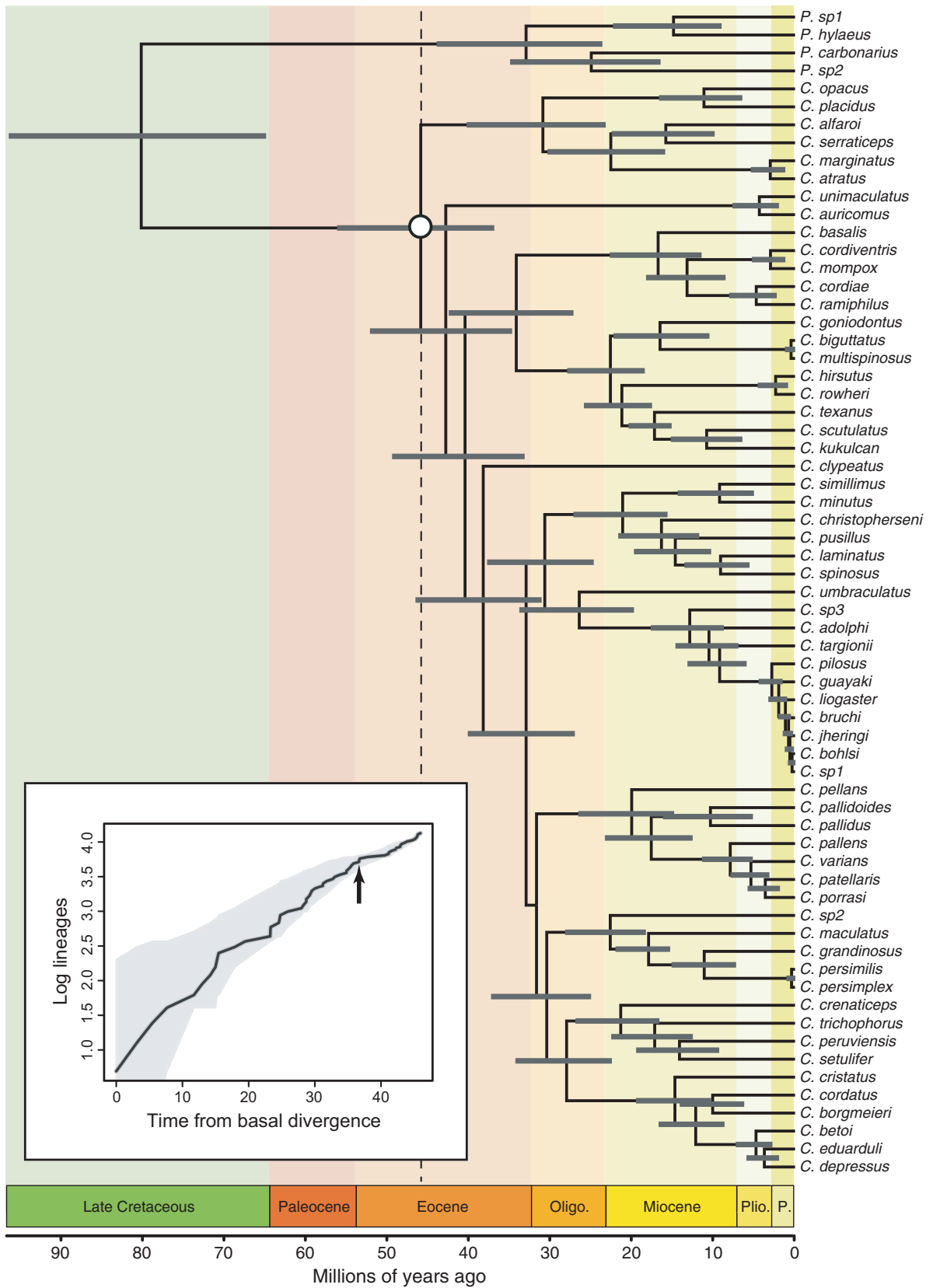
Guided by the results from the diversification and community phylogenetics analyses, we tested whether the Chacoan region is associated with higher levels of turtle ant diversification than the other regions, using the geographic state speciation and extinction model (GeoSSE; Goldberg *et al.*, 2011) implemented in DIVERSI-TREE v0.7 (FitzJohn, 2012) in R. GeoSSE examines patterns of geographic range evolution by testing for differences in rates of speciation and extinction associated with distinct regions. Range expansion occurs through dispersal, and range contraction occurs through local extinction. Taxa may only inhabit two regions, so we classified species as inhabiting the Chacoan region (C), other regions (O) or both the Chacoan and other regions (CO). The full GeoSSE model includes seven parameters: speciation (s_C, s_O) and extinction (x_C, x_O) rates for lineages within each region, dispersal rates between regions (d_C, d_O) and a parameter that allows for speciation in taxa inhabiting both regions through vicariance (s_{CO}). We first fit models with and without the last parameter using maximum likelihood and compared model fits using Akaike weights. The model without the vicariance parameter was preferred (Table 1). For this model, we subsequently used 10 000 generations of MCMC to sample speciation and extinction rates within each region and dispersal rates between regions from their posterior distributions. To test whether rates of speciation, extinction and dispersal were greater in the Chacoan region relative to other regions, we computed the PP as the proportion of Chacoan values for each of the three rates that were greater than the non-Chacoan values, following Goldberg *et al.* (2011). We accounted for missing species in GeoSSE using a method that assumes the phylogeny represents a sample of species distributed evenly across the phylogeny whose geographic states are known (FitzJohn *et al.*, 2009), which is the case for our data. This method includes a parameter for the probability of a particular state being sampled, which is modelled as the proportion of tips in each geographic state that are sampled vs. missing in the phylogeny (C = 0.467, O = 0.786, CO = 0.406). We did not include undescribed species or four described species whose character states were not known due to the lack of specific locality data (de Andrade & Baroni-Urbani, 1999). This method is a conservative way to account for incomplete sampling, because it does not incorporate known information on the phylogenetic placement of missing turtle ant taxa.

Results

Phylogenetic inference and divergence dating

Analyses using both the molecular data set and the combined molecular and morphological data set recovered the same tree topology, with most nodes strongly supported (Fig. 1). The combined analysis had mixed effects on node support, with higher PP values at two nodes and lower values at three nodes, suggesting a level of conflict between molecular and morphological data. The tree topology from BCA was the same as the one from the concatenated analysis, but nodal support was relatively low (Fig. S3).

The topology of our phylogeny differed in several important ways from a previously published most parsimonious tree based on morphological data (Fig. 1; de Andrade & Baroni-Urbani, 1999). Although de Andrade & Baroni-Urbani (1999) proposed the endemic Antillean *hamulus* group to be sister to all other *Cephalotes*, we recovered the *atratus* group in this position, with the *hamulus* group diverging at the next node (Figs 1 and 2). In our analyses, the Central and North American groups (e.g. *texanus*, *wheeleri*) formed a monophyletic lineage that resulted from early, rather than late branching events in our topology (Figs 1 and 2). A reanalysis of the morphological data in a model-based framework (see Methods in Supporting information) revealed that morphological data alone cannot resolve deeper nodes in the phylogeny, but results do provide support for previously proposed morphological groups (Fig. S4). Most of the proposed morphological groups (de Andrade & Baroni-Urbani, 1999) were recovered as monophyletic (Fig. 1). In almost all cases where proposed morphological groups were not recovered as such, this was because they were rendered paraphyletic by the placement of another group within them. Only the *pinelii* group was found to be polyphyletic. One *pinelii* clade has a primarily Central American distribution and forms a monophyletic lineage with the other Central American clades, whereas the other *pinelii* clade occurs largely in South America (Figs 1 and 2).

The chronogram from BEAST dates the split of *Cephalotes* from *Procryptocerus* at around 80 mya [95% highest posterior density (HDP): 65–96 mya]. Crown group *Cephalotes* originated around 46 mya (95% HDP: 37–56 mya) in the Middle Eocene (Fig. 3). A visual inspection of the summary of the prior distribution in the absence of data compared with the summary of the output of the posterior distribution containing molecular data indicated our timetree results were driven by the molecular data and not the priors.

Diversification rates

We recovered a negative gamma statistic for the MCC tree and the posterior distribution of trees ($\gamma_{MCC} = -1.55$; $\gamma_{distribution} = -2.55$ to -0.53); however, the MCCR test to account for incomplete sampling was not significant ($\gamma_{critical\ value} = -3.03$; $p_{MCC} = 0.44$). Analysis with TREEPAR, which uses a model-based approach and incorporates incomplete taxon sampling, identified a tree-wide shift to lower rates of diversification in the Late Miocene (9 mya; Fig. 3 insert; background net

Table 1 AIC scores and weights for geographic state speciation and extinction models. Unequal rates models allow speciation, extinction and dispersal rates to vary between regions. Equal rates models constrain rates to be equal across regions. The s_{CO} parameter represents speciation due to vicariance, and models were run with and without this parameter.

Model	AIC	ΔAIC	Weights
Unequal rates, no s_{CO}	499.71	0.00	0.73
Unequal rates, s_{CO}	501.71	2.00	0.27
Equal rates, no s_{CO}	521.17	21.46	<0.01
Equal rates, s_{CO}	523.17	23.46	<0.01

Table 2 Results from TREEPAR examining shifts in rates of diversification at 10 different time periods. Shift points, the log likelihood (ln(L)) score for each inferred rate shift and P-values are given.

Shifts	Time (my)	ln(L)	P-value
0	–	−223.22	–
1	9	−219.31	0.05
2	23	−217.58	0.33
3	30	−215.55	0.26
4	18	−213.56	0.26
5	20	−211.37	0.22
6	26	−210.06	0.45
7	13	−209.05	0.57
8	31	−207.97	0.54
9	21	−207.31	0.72

diversification rate = 0.083, background rate of turnover = 0.91, diversification rate at 9 mya = −0.17, turnover at 9 mya = 1.98; Table 2).

In contrast with the lineage-wide pattern of a shift to low net diversification rates towards the present, MEDUSA detected a recent increase in diversification rate at the *fiebrigi* species group plus portions of the *angustus* group depending on the species assignment scheme (Table 3). Because the *fiebrigi* group is nested within the paraphyletic *angustus* group, the location of the increase in diversification rate changed from the node uniting the *fiebrigi* group (minus *C. pilosus*), to the *fiebrigi* group plus a part of the *angustus* group, to the *fiebrigi* plus *angustus* groups (Table 3). The most conservative and information-poor species assignment scheme did not recover evidence for a rate shift. However, this scheme ignores the relatively young crown age of the diverse *fiebrigi* group, which is included in the other assignment schemes.

Testing the influence of biogeographic regions

Analyses using both MPD and MNTD recovered a significant pattern of phylogenetic clustering (a.k.a. underdispersion) for turtle ants within the Chacoan region (Fig. 2; Table 4). We also found significant phy-

logenetic clustering for turtle ants within the Antillean region (Fig. 2), but our sample size (*n* = 3) is too small to draw meaningful conclusions from this result.

Using GeoSSE to further interrogate biogeographic community patterns, we found strong support for higher rates of speciation within the Chacoan community (Fig. 4a; s_C: mode = 0.072, 95% credible interval = 0.045, 0.106) than outside of it (s_O: mode = 0.037, 95% credible interval = 0.021, 0.063; PP = 0.96). Extinction rates were similar (Fig. 4b; x_C: mode = 0.004, 95% credible interval = 0, 0.080; x_O: mode = 0.003, 95% credible interval = 0, 0.039; PP = 0.56). We also found strong support for asymmetric dispersal rates between regions (Fig. 4c; PP = 0.97), with lower rates of range expansion into the Chacoan region (d_O: mode = 0.003, 95% credible interval = 0, 0.113) than out of it (d_C: mode = 0.194, 95% credible interval = 0.076, 0.405). Binary state speciation and extinction models, such as GeoSSE, may lack the power to detect trait-dependent rates of diversification (Type II error) when taxon sampling is incomplete or when a phylogeny does not have large numbers of tips (Davis *et al.*, 2013). However, the significant differences in rates of speciation and dispersal that we recovered indicate that our analyses do not suffer this issue.

Discussion

Overall patterns and timing of diversification in turtle ants

Our results indicate that crown group turtle ants originated in the Middle Eocene (approximately 46 mya). The origin of turtle ants is coincident with the diversification of disparate groups of taxa (e.g. Pellmyr & Leebens-Mack, 1999; Bininda-Emonds *et al.*, 2007; Jaramillo *et al.*, 2010), including the fungus-gardening ants (Schultz & Brady, 2008), which also have a New World and predominantly Neotropical distribution. The Early Eocene saw an increase in global temperatures (Zachos *et al.*, 2008) and concurrently a rapid increase in plant diversity and the expansion of tropical rainforests (Burnham & Graham, 1999; Burnham & Johnson, 2004; Wing *et al.*, 2009; Graham, 2011). The expansion of a diverse rainforest habitat at this time likely allowed for the origination and initial diversification of arboreal arthropod lineages like the turtle ants.

We detected a significant lineage-wide shift to lower rates of diversification in the Late Miocene (9 mya; Fig. 3 insert; Table 2). The decline in the net diversification rate has several potential explanations, such as environmental change (Cornell, 2013), geographic patterns where parent and daughter range sizes are reduced during speciation (Pigot *et al.*, 2010) or a niche-filling model of cladogenesis (Simpson, 1944; Freckleton & Harvey, 2006; Rabosky, 2009, 2010; Rabosky & Glor, 2010). Our understanding of the

Table 3 Results from MEDUSA showing the ways unsampled taxa were assigned to the paraphyletic *angustus* species group, in which the *fiebrigi* group is nested. Complete tree, assumption that all taxa are sampled; collapsed, *angustus* and *fiebrigi* groups collapsed to one node; equal, taxa assigned evenly to the two earliest branching *angustus* clades; unequal 1, one species assigned to the *C. adolphi* terminal (the earliest branching *angustus* clade) and the remaining species to the *C.* sp3 terminal; unequal 2, one species assigned to the *C. adolphi* terminal and the remaining species to the *C.* sp3 terminal. Shown are whether a one or two rate model was preferred, the Akaike weights, background diversification rates (net rate, turnover rate), the rate of diversification if the shift was significant (net rate, turnover rate) and the node the shift occurred in.

Analysis	Model	AIC weights	Background rates	Shift rates	Shift node
Complete tree	2 rate	0.99	$0.056, 4.6 \times 10^{-8}$	$0.636, 4.0 \times 10^{-7}$	*fiebrigi* (-*C. pilosus*)
Collapsed	1 rate	1.00	$0.078, 1.1 \times 10^{-6}$	–	–
Equal	2 rate	0.75	$0.074, 2.5 \times 10^{-6}$	$0.206, 6.4 \times 10^{-7}$	*fiebrigi* + *C. targionii* + *C. adolphi* terminal
Unequal 1	2 rate	0.95	$0.071, 1.6 \times 10^{-8}$	$0.222, 9.0 \times 10^{-8}$	*fiebrigi* + *C. targionii* + *C. adolphi* terminal
Unequal 2	2 rate	0.65	$0.072, 3.3 \times 10^{-7}$	$0.107, 0.604$	*fiebrigi* + *angustus*

Table 4 Community phylogenetics results for the eight biogeographic regions. *N* is the number of sampled species in each region. Observed values, randomized values, *z*-values for the observed value based on the randomization tests and *P*-values were calculated for MPDs and MNTDs.

		MPD				MNTD			
	N	obs.	rand.	*z*-value	*P*-value	obs.	rand.	*z*-value	*P*-value
Antillean	3	40.06	48.60	−1.18	0.12	34.43	65.64	−2.09	0.05
Nearctic	3	45.46	48.98	−0.47	0.30	55.35	66.16	−0.73	0.19
Mexican Trans. Zone	1	NA	NA	NA	NA	NA	NA	NA	NA
Mesoamerica	19	70.10	69.20	0.34	0.59	33.73	33.85	−0.02	0.48
NW South America	18	68.85	68.93	−0.03	0.45	36.91	34.93	0.41	0.66
Amazonian	33	70.72	70.86	−0.09	0.42	25.76	25.60	0.06	0.51
Chacoan	29	60.41	70.64	−5.60	<0.01	22.32	27.27	−1.62	0.05
Paraná	15	65.32	68.23	−0.94	0.18	37.78	37.85	−0.01	0.48

MNTD, mean nearest taxon distance; MPD, mean pairwise distance.

ecology of this lineage provides an explanation for a niche-filling scenario. Specifically, we know that turtle ants need particular sizes of beetle-produced arboreal cavities for nesting (Creighton, 1963; Powell, 2008, 2009). We propose that this dependency on a critical resource base that is produced by other organisms may limit diversification rates as new species accumulate and the bounds of the niche space are approached. Although this scenario is speculative, it provides a suite of predictions that can be tested by future studies, such as those of trait function and organismal performance. Improved taxon sampling will be central to future comparative analyses, including further detailing of the lineage-wide decline in diversification rates detected in this study (Nee *et al.*, 1994; Nee, 2001; Cusimano & Renner, 2010).

Novel ecological opportunity and diversification in the Chacoan region

A lineage-wide slowdown in turtle ant diversification is strongly contrasted against the renewed burst of diversification that we detected in the *fiebrigi* group and members of the *angustus* group. Classic adaptive radia-tion theory predicts that following the invasion of a new ecological region, or 'adaptive zone', a lineage should exploit the new ecological opportunity and diversify rapidly (Simpson, 1944; Yoder *et al.*, 2010). This ecological process fits closely with our observed elevated diversification rates of the *fiebrigi* group and its tight association with the Chacoan biogeographic region (Figs 2 and 4). The Amazon has the highest total *Cephalotes* species richness of any region, but this pattern can be explained most parsimoniously as a function of time. This area has likely been occupied for longer and should therefore have more species. In contrast, time does not account for levels of turtle ant diversity within the Chacoan region. Here, species richness is better explained by a higher rate of diversification than is seen in other regions. In particular, the young *fiebrigi* group has diversified faster than other turtle ant lineages (Fig. 4; Table 3), and most species in the group are exclusively Chacoan in distribution.

In addition to the remarkable species richness of the young *fiebrigi* group being coupled with the Chacoan biogeographic region, the timing of this burst of diversification is coincident with the probable recent diversification of the unique Chacoan vegetation. The Chacoan
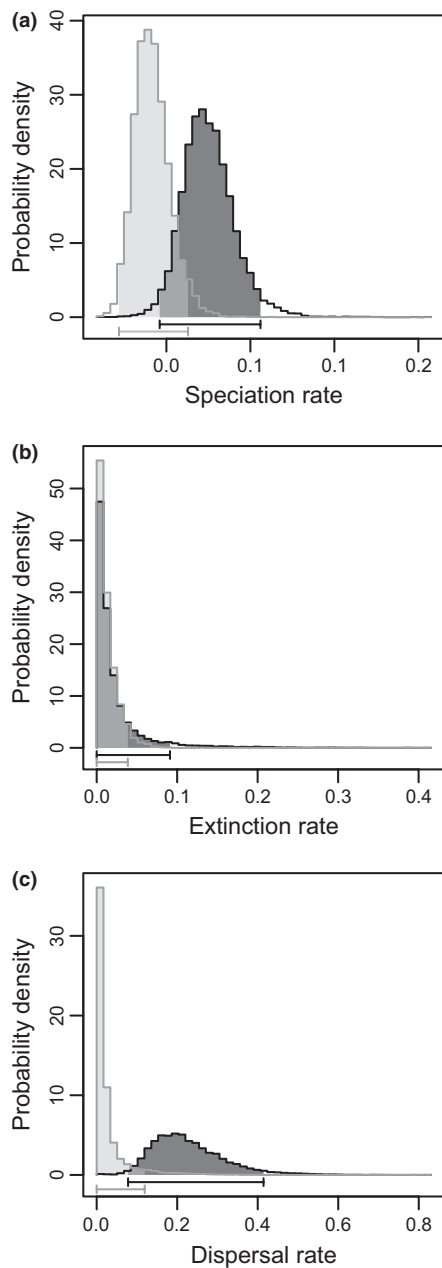
Fig. 4 Speciation, extinction and dispersal rates for turtle ants in the Chacoan vs. other biogeographic regions. The *Cephalotes* community in the Chacoan region is associated with higher levels of diversification than other regions, as well as higher rates of dispersal out of the Chacoan region than into it. Posterior probability distributions for estimates of speciation and extinction rates in the Chacoan (dark grey) and non-Chacoan (light grey) regions as well as of dispersal rates from the Chacoan region into other regions (dark grey) and from other regions into the Chacoan region (light grey) are shown.

biogeographic region is comprised of three major biomes – Caatinga (thorn forest), Cerrado (Neotropical savanna) and Chaco (dry forest). These biomes have

been referred to historically as the 'dry diagonal' (Vanzolini, 1963) of South America. Although not uniform in environmental conditions or vegetation, they are all characterized by long dry seasons, relatively open and heterogeneously distributed vegetation, and distinct floras and faunas with high endemism and diversity that are adapted to drought stress (Pennington *et al.*, 2000; Pennington *et al.*, 2006; Werneck, 2011). The age of the Chacoan region biomes and their distinctive vegetation types has been the subject of recent research, and there is growing agreement that the modern Chacoan flora is relatively young. Studies based on time-calibrated phylogenies show that many Cerrado lineages diversified at the end of the Miocene or later (Simon *et al.*, 2009; Simon & Pennington, 2012; Hughes *et al.*, 2013; Roncal *et al.*, 2013; Trovó *et al.*, 2013). In particular, several dominant Cerrado lineages diversified 4 mya or less (Simon *et al.*, 2009). The ages of the Chaco and Caatinga biomes are less well understood, but the Chaco flora is thought to be no older than the Early Pliocene (5.3 mya; Iriondo, 1993; Spichiger *et al.*, 2004), and evidence suggests that Caatinga vegetation is < 10 my old (Pennington *et al.*, 2009). These dates coincide closely with the recent, explosive diversification of the Chacoan fiebrigi group (Fig. 3) and strongly suggest that turtle ants colonized and diversified in the Chacoan region relatively recently.

In further support of this interpretation, we found that the rates of dispersal out of the Chacoan region were an order of magnitude higher than rates of dispersal into the region (Fig. 4). This suggests that the Chacoan region has been difficult for turtle ants to colonize, but once established, species readily dispersed out of the region. The open and seasonally dry habitats shared by the Chacoan biomes undoubtedly exert strong desiccation pressure, especially on exposed arboreal taxa. Indeed, many taxa in the region show pronounced behavioural and physiological adaptations to the seasonal scarcity of water (Eiten, 1978; Navas *et al.*, 2002; Olalla-Tárraga *et al.*, 2009). Moreover, turtle ants are thought to have a suite of traits to mitigate the desiccation pressures from arboreal life (Wilson, 1976), and these may have been elaborated upon by species inhabiting the Chacoan region. Recovering similar patterns in rates of ecomorphological trait evolution would lend further support to the hypothesis that the Chacoan region is a driver of diversification in turtle ants (Mahler *et al.*, 2010; Slater *et al.*, 2010).

Other biogeographic patterns of diversification

Our new phylogeny contains major topological changes over the previously published morphological phylogeny (de Andrade & Baroni-Urbani, 1999), and these highlight other important biogeographic signatures in turtle ant diversification. First, species groups with a primarily Central and North American distribution form

a monophyletic lineage that branches early in the turtle ant phylogeny (Fig. 2), suggesting a single colonization of Central America. All of the remaining groups have a primarily South American geographic distribution. The Central American groups are nested within the South American groups, indicating that turtle ants originated in South America. Second, results from one of the exploratory community phylogenetics analyses (MNTD; Table 4) show that the Antillean region is significantly phylogenetically clustered. The low sample size for that region (three species) precludes making strong inferences, but the Antilles have a unique *Cephalotes* biota and geologic history (Iturralde-Vinent & MacPhee, 1999; Hedges, 2006; Ricklefs & Bermingham, 2008; Ali, 2012). Most notably, the island of Hispaniola is the only known locality of all six extant members of the previously described *hamulus* species group (represented by *C. unimaculatus* and *C. auricomus* in Figs 1–3), which are unusual in that they all lack soldiers. The Antilles region as a whole has relatively few extant turtle ant species, but most fossil species are from amber deposits in the Dominican Republic, on the Eastern half of the island of Hispaniola. These fossils are distributed throughout the phylogeny, suggesting that the Antilles harboured higher species richness in the past than currently. Further research will help to uncover whether ecological opportunity played a role in limiting the number of Antillean species currently present.

Conclusion

It is increasingly apparent that the high levels of diversity in the Neotropics cannot be attributed to any one temporal period or geologic event (Bush, 1994; Zink *et al.*, 2004; Bush & Oliveira, 2006; Rull, 2008, 2011; Hoorn *et al.*, 2010). Moreover, it is unclear how generally the process of adaptive diversification, largely studied in vertebrate diversification on islands, plays out on continents (but see Hughes & Eastwood, 2006; Burbrink & Pyron, 2009; Derryberry *et al.*, 2011; Claramunt *et al.*, 2012; Drummond *et al.*, 2012; Day *et al.*, 2013). Informative study systems that represent successful continental radiations in understudied taxa are likely to be particularly important in tackling these issues. Here, we have produced the first dated molecular phylogeny for the turtle ants, a highly successful ant lineage that has diversified throughout the Neotropics. We have identified the origin of modern turtle ants in the Eocene, with a marked slowdown in diversification rates in the Miocene. This lineage-wide pattern is contrasted against a recent burst of diversification in a species group inhabiting the seasonally dry, environmentally harsh and relatively young Chacoan region. The importance of the Chacoan in turtle ant diversification is corroborated by a significant signature of phylogenetic clustering in extant species from the region, and evidence that diversification rates are higher in the Chacoan than elsewhere. Taken together, these findings strongly suggest that recent ecological opportunity from successful colonization of the Chacoan has facilitated renewed diversification. We have further identified distinct Central and South American species groups, and a preliminary but intriguing signature of the Antilles being important in turtle ant diversification. Broadly, our findings suggest a central role of ecological opportunity at different phylogenetic and temporal scales within the continental diversification of turtle ants. The functional ecology of turtle ants also suggests a possible adaptive scenario to explain these patterns, as well as some possible tests for future work. It will be particularly valuable to ask whether lineage diversification patterns identified here are mirrored by patterns of morphological diversification, as predicted by adaptive diversification theory (e.g. Mahler *et al.*, 2010; Slater *et al.*, 2010). The biogeographic signatures that we have identified in turtle ant lineage diversification will provide a number of valuable contrasts for such analyses.

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Supporting information

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

Appendix S1 Supplemental molecular and phylogenetic methods.

Appendix S2 Supplemental results about species monophyly.

Table S1 Species sequenced, voucher numbers, detailed locality information and deposit location for specimens used in phylogenetic analyses.

Table S2 Table of number of species and percentage of groups sampled from previously defined clades based on morphological characters.

Table S3 Primers used and annealing temperatures for PCR amplification and sequencing of long wavelength rhodopsin, wingless, elongation factor 1α F2, cytochrome oxidase I, cytochrome oxidase II and cytochrome *b*.

Table S4 Species sequenced, voucher numbers and GenBank accession numbers for specimens used in phylogenetic analyses.

Figure S1 Bayesian consensus phylogram of *Cephalotes* using the molecular data set, which includes multiple individuals for 12 species.

Figure S2 Bayesiann consensus phylogram of extant and extinct *Cephalotes* species, used to determine fossil calibration points for divergence dating analysis.

Figure S3 Primary concordance tree obtained from the Bayesian concordance analysis of the molecular data set.

Figure S4 Bayesian consensus phylogram of the 131 character morphological data sets for *Cephalotes*.