



# Biogeography of *Trimerotropis pallidipennis* (Acrididae: Oedipodinae): deep divergence across the Americas

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## ABSTRACT

**Aim** *Trimerotropis pallidipennis* represents a species complex of band-winged grasshopper distributed over North and South America. Previous studies indicated a North American origin of the species and suggested that colonization of South America occurred during the Pleistocene after the closure of the Isthmus of Panama. Here we use a phylogeographical approach in order to test different biogeographical scenarios and determine how many distinct units exist within the species complex.

**Location** North and South America with specific emphasis on the Andes mountains of South America.

**Methods** We sequenced two mitochondrial and two nuclear genes for multiple specimens belonging to each taxonomic unit. Using the concatenated dataset and a coalescent-based approach we estimated the phylogeny of the complex. In order to distinguish the different biogeographical and species delimitation hypotheses we constrained our dataset to different taxon sets and ran Bayesian analyses in \*BEAST. Posterior probabilities and DensiTree plots allowed us to determine the best hypotheses. We used a molecular clock approach to correlate geological events with observed phylogenetic splits.

**Results** All analyses indicate the existence of at least three distinct genetic lineages: *Trimerotropis pallidipennis* from North America, *Trimerotropis ochraceipennis* from Chile and an undescribed *Trimerotropis* species from Argentina. The split between North and South American forms took place about 1.3 Ma, long after the Isthmus of Panama had been completed. Biogeographical analyses suggest a first dispersal event from North to South America. Subsequent dispersion and vicariance probably led to the differentiation of the endemics now found in Chile and Argentina.

**Main conclusions** We demonstrate the existence of three distinct genetic lineages in the *Trimerotropis pallidipennis* species complex. These lineages are also chromosomally differentiated as previous studies have indicated. Dispersion of *T. pallidipennis* from North to South America probably occurred during the early Pleistocene, when climatic conditions were more suitable. Subsequent diversification in South America was the result of range expansion and vicariance, possibly in response to later Pleistocene glaciations of the Andes.

## Keywords

Andes, band-winged grasshoppers, Bayesian species delimitation, Panama Isthmus, phylogeography, Pleistocene glaciations.

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## INTRODUCTION

The biogeography of Pan-American taxa is strongly influenced by the geological history of the two continents. At the

beginning of the Tertiary, North and South America were separated by more than 1300 km of ocean; this distance gradually diminished, and the number of intervening islands and land patches increased (Carbonell, 1977). The consensus in

the literature is that after a 12 Myr process, the Isthmus of Panama was completed about 2.8 Ma in the late Pliocene (Coates & Obando, 1996; Coates *et al.*, 2005). The subsequent migration of terrestrial animals led to huge range expansions, and multiple diversification events, but also to extinctions in both North and South America (also known as the 'Great American Interchange'; Marshall *et al.*, 1982; MacPhee & Iturralde-Vinent, 1995). In addition, a second major geological event, the uplift of the Andes mountains, occurred in two stages, 10–5 Ma and 5–2 Ma (Hoorn *et al.*, 1995; Gregory-Wodzicki, 2000), resulting in a new migration barrier to many taxa. Both of these large-scale geological events have strongly influenced the diversification of the American biota (Hines, 2008; Cigliano & Amédégato, 2010).

While tectonic movements characterized the late Tertiary, dramatic fluctuations in the climatic conditions marked the beginning of the Quaternary, triggering the evolution of new taxa during the last 2.5 Myr (Sanmartín *et al.*, 2001; Sanmartín & Ronquist, 2004). Indeed, there is an ongoing debate about the relative importance of Tertiary palaeogeographical reorganizations versus Quaternary glacial–interglacial cycles in shaping the present-day Neotropical biota (Hewitt, 2000; Rull, 2008).

The Acrididae are a globally distributed family of grasshoppers (Orthoptera) composed of 25 subfamilies (Eades *et al.*, 2011), 13 of which inhabit the Neotropical region (Cigliano *et al.*, 2012). Eight of these 13 subfamilies are endemic to the Neotropics, while the remaining five (Melanoplinae, Acridinae, Gomphocerinae, Cyrtacanthacridinae and Oedipodinae) have representatives elsewhere in the world as well. According to Carbonell (1977), these latter subfamilies probably invaded the Neotropical region during the Cenozoic and South America in late Cenozoic times.

Most Oedipodinae, or band-winged grasshoppers, are inhabitants of the drier, temperate to tropical regions throughout the world (Carbonell, 1977). The subfamily currently consists of 135 valid genera and 812 known species (Eades *et al.*, 2011) and occurs throughout all major continents, thus being the most cosmopolitan subfamily of acridids (Otte, 1995). The distribution of band-winged grasshoppers spreads over all six zoogeographical zones but their centres of diversity are the Afrotropical, Palaearctic and Nearctic regions, where the majority of species can be found (Otte, 1984). They are fairly poorly represented in the Neotropics. Here, the most common Oedipodinae species belong to the genus *Trimerotropis* Stål, 1873. This genus is found from Canada to Chile and Argentina, but has distributional gaps in central and northern South America, where habitats are unsuitable (Otte, 1984). *Trimerotropis* is one of the dominant Oedipodinae genera in the western United States and northern Mexico, where it is represented by more than 40 species (Rehn, 1939; Otte, 1984; Eades *et al.*, 2011). The genus *Trimerotropis* is also interesting from a cytological viewpoint because it has a high incidence of pericentric inversions (White, 1949; Guzman & Confalonieri, 2010). Within the genus, White (1949, 1973) distinguished two

subgroups based on their chromosome morphology. Members of group A have only acrocentric chromosomes, while in group B some chromosomes are metacentric (White, 1949, 1973). Within group B, *Trimerotropis pallidipennis* (Burmeister, 1838) has been extensively studied (Confalonieri, 1988, 1994; Confalonieri & Colombo, 1989; Colombo & Confalonieri, 1996; Confalonieri *et al.*, 1998; Guzman & Confalonieri, 2010).

While their cytology is well described, the taxonomic and systematic relationships within the group of species consisting of *T. pallidipennis* and several closely related species are poorly understood. Otte (1984) suggested that the species *T. pallidipennis*, *Trimerotropis saxatilis*, *Trimerotropis huroniana* and *Trimerotropis schaefferi* together form a systematic unit, that he informally called the 'Pallidipennis group'. Furthermore, he differentiated *T. pallidipennis* from the morphologically very similar *Trimerotropis salina* and *Trimerotropis diversellus*, but did not include these species in the 'Pallidipennis group' (Otte, 1984). Instead he described these as a complex of three distinct species. Thus, no clear definition of the North American 'Pallidipennis group' has been established. Interestingly, both North and South America host populations of *T. pallidipennis sensu stricto*.

The taxonomic status of the South American representatives of the *T. pallidipennis* species group resembles the relationships described for North American species in its high degree of uncertainty. Originally, six species had been described for South America: *T. pallidipennis*, with the subspecies *T. pallidipennis pallidipennis* (Burmeister, 1838) and *T. pallidipennis andeana* Rehn, 1939; *Trimerotropis ochraceipennis* (Blanchard, 1851), *Trimerotropis atacamensis* (Philippi, 1860), *Trimerotropis chloris* (Philippi, 1863), *Trimerotropis flavipennis* (Philippi, 1863) and *Trimerotropis irrorata* (Philippi, 1863). Yet, the latter four names are now considered synonyms of *T. ochraceipennis* (Amédégato & Carbonell, 2001), while *T. pallidipennis andeana* has been raised to the status of species (Otte, 1995). Therefore, currently three species are recognized for *Trimerotropis* in South America: *T. pallidipennis*, *T. andeana* and *T. ochraceipennis*. However, diagnostic characters among these taxa are vague and intra-specific variability is high, making taxonomic decisions based on morphological traits difficult (M.M.C. & M.H., unpublished data).

Contrasting with this apparent confusion about species delimitation within the *Trimerotropis pallidipennis* species complex in South America, there is general agreement about its Nearctic origin (Rehn, 1939; White, 1973; Carbonell, 1977; Confalonieri *et al.*, 1998). In particular, Rehn (1939) proposed that *Trimerotropis* probably invaded South America at least twice: he suggested that 'the isolation of possibly the first invasion in the present Chile by the last great elevation of the Andes resulted in the development there of the endemic *T. ochraceipennis*' (p. 414). According to Rehn (1939) a second invasion brought in a 'form' of *T. pallidipennis* that invaded most areas with suitable habitats in South America. Furthermore, he suggested that during the Pleistocene, these

new southern lineages of *T. pallidipennis* invaded the Peruvian Puna, giving rise to *T. andeana*. Alternatively, Carbonell (1977) proposed that the Oedipodinae invaded South America during the Pleistocene glaciations after the land bridge connection was already established. Finally, Confalonieri *et al.* (1998) provided further support for this idea based on age estimates from haplotype divergence among South American populations of *T. pallidipennis*. This analysis yielded an average age estimate of 3 Ma, which corresponds to the emergence of the land bridge connecting both continents.

In this study we performed a molecular phylogeographical analysis of members of the '*Trimerotropis pallidipennis* species complex' from South America, including North American specimens of the species, aiming to shed light on the diversification and speciation processes that could have led to current diversity patterns. We test several biogeographical hypotheses in order to explain its present genetic structure.

## MATERIALS AND METHODS

### Study species

*Trimerotropis pallidipennis* ranges from south-western Canada to southern Argentina. It is widely distributed across the western United States, extending into the arid and semi-arid regions of Mexico. It generally inhabits dry grasslands, such as the western prairies, desert and semi-desert grasslands and scrublands. In North America it is generally found at lower elevations, being replaced at higher elevations by other species of the genus. From central Mexico southwards it is absent until sub-Andean conditions are reached in southern Ecuador and on the Pacific coast of Peru. From there its range extends to the Santa Cruz province in Argentina where it inhabits a wide elevational range, from low salt flats to mountain grasslands at 3900 m a.s.l. (Rehn, 1939; Otte, 1984; Carbonell *et al.*, 2006). Rehn (1939) suggested that on the high Puna grasslands of Peru *T. pallidipennis* is represented by the endemic subspecies *T. p. andeana* (currently *T. andeana*), while in Chile the genus is exclusively represented by *T. ochraceipennis*. All these species will be referred to hereafter as the '*Trimerotropis pallidipennis* species complex'.

### Sample collection and storage

Adult specimens of the *T. pallidipennis* species complex ( $n = 77$ ) were collected during the summers of 2007–2010 across 27 locations in the USA, Mexico, Peru, Chile and Argentina (Fig. 1 and Appendix S1 in Supporting Information) spanning most of its total distribution range. We included the following species as outgroups: *Sphingonotus corsicus* ( $n = 1$ ), *Conozoa texana* ( $n = 2$ ), *Trimerotropis maritima* ( $n = 2$ ), *Circotettix rabula* ( $n = 2$ ) and *T. saxatilis* ( $n = 3$ ). Hind legs were dried or preserved in 100% ethanol until DNA extraction.

### DNA extraction and sequencing

Genomic DNA was extracted from dried or ethanol-preserved hindleg tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol for tissue samples. We amplified two mitochondrial [NADH dehydrogenase subunit 5 (*ND5*) and cytochrome *c* oxidase subunit I (*COI*)] and two nuclear gene fragments [internal transcribed spacer 2 (*ITS2*) and histone 3 (*H3*)] using a standard polymerase chain reaction (PCR) protocol. Previous studies demonstrated that these markers are informative for Oedipodinae (Hochkirch & Husemann, 2008; Guzman & Confalonieri, 2010; Husemann *et al.*, 2012), covering a range of evolutionary rates useful for comparisons within and between species and genera. Detailed information about primers is provided in Appendix S2.

PCRs were performed using the following setup: 36.6  $\mu$ L of deionized H<sub>2</sub>O, 6  $\mu$ L of 10  $\times$  PCR buffer (reaction concentration 1 $\times$ ), 4.8  $\mu$ L of dNTP mixture (0.2  $\mu$ M each), 0.6  $\mu$ L of DyNAzyme DNA Polymerase (1.2 U, Finnzymes, Vantaa, Finland), 3  $\mu$ L of each primer (0.5  $\mu$ M, Integrated DNA Technologies, Coralville, IA, USA) and 6  $\mu$ L of DNA template adding up to a total volume of 60  $\mu$ L. Amplification conditions were as follows: 94  $^{\circ}$ C for 3 min, followed by 30 cycles of 94  $^{\circ}$ C for 1 min, 48–60  $^{\circ}$ C for 1 min (*ND5*, 48  $^{\circ}$ C; *COI*, 53–43  $^{\circ}$ C; *H3*, 57  $^{\circ}$ C; *ITS2*, 60–55  $^{\circ}$ C) and 72  $^{\circ}$ C for 2 min, with a final step at 72  $^{\circ}$ C for 10 min.

The PCR products were visualized on a 1% agarose gel stained with Gel Red (0.1 $\times$ , Biotium, Hayward, CA, USA) and purified using solid-phase reversible immobilization (SPRI; DeAngelis *et al.*, 1995) with carboxylated magnetic beads (Bangs Laboratories, Fishers, IN, USA) and a 96-ring SPRIplate (Agencourt, Beverly, MA, USA). The purified PCR products were sequenced at the Yale Sequencing Facility (New Haven, CT, USA) and at the 'Unidad de Secuenciación y Genotipificado' (FCEyN, UBA, Buenos Aires, Argentina). Sequences were deposited at GenBank (Appendix S1).

Sequences were inspected, trimmed and aligned using GENEIOUS 5.0.3 (Drummond *et al.*, 2006) and SEQUENCHER v.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

### Phylogenetic analysis

#### Gene tree reconstruction

Phylogenetic analyses were performed employing Bayesian inference (BI) and maximum parsimony (MP) using a concatenated dataset of all four gene fragments for a subsample of 33 individuals ( $n = 33$ ), representing all species, subspecies and locations. jMODELTEST (Posada, 2008) was used to infer the most appropriate model of molecular evolution for each dataset based on the Akaike information criterion (AIC) (Akaike, 1973). Bayesian phylogenetic analysis was performed using the 'Metropolis-coupled Markov chain Monte Carlo' (MCMCMC) algorithm implemented in MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001). A partitioned algorithm



**Figure 1** Map showing the sampling locations of species in the ‘*Trimerotropis pallidipennis* complex’ in North and South America. Location IDs correspond to those in Appendix S1.

was used to account for heterogeneity between the four datasets. Program defaults were used for estimation of priors. Two independent analyses were run: each over 1,500,000 generations with sampling every 100 generations. The tree space was explored using four chains (one cold chain and three incrementally heated). Stationarity was checked with TRACER 1.5 (Rambaut & Drummond, 2007). We discarded a burn-in of 10%. The remaining trees were used to construct a 50% majority-rule consensus tree with mean branch length estimates. Posterior probabilities are given as branch support (Huelsenbeck & Ronquist, 2001).

The MP analysis was performed with the program TNT 1.1 (Goloboff *et al.*, 2003). The heuristic search procedure consisted of ‘tree bisection–reconnection (TBR) branch swapping’ applied to a series of 100 random addition sequences, retaining 10 trees per replicate. No further search strategies were adopted when optimal trees were found for all the replicates. Characters were treated as unweighted, and gaps were

read as an additional state. To estimate the support of each node, 1000 bootstrap permutations were performed.

#### *Species tree reconstruction*

In order to estimate the coalescent-based species tree of the ‘*Trimerotropis pallidipennis* species group’, all four genetic regions were simultaneously analysed using \*BEAST (Heled & Drummond, 2009). The program \*BEAST implements a coalescent-based Bayesian method that finds the most likely species tree when multiple individuals per taxon are sequenced. Here, we were able to include all 84 individuals assayed (Appendix S1), because it is not necessary to have the same individuals analysed for each marker, if partitions are considered as unlinked. We constrained the individuals into different units in order to test several alternative phylogenetic hypotheses: (1) the whole species complex represents a single species (one species); (2) individuals of *T. pallidipennis* from

North America represent one species, while all individuals from South America (including *T. pallidipennis*, *T. andeana* and *T. ochraceipennis*) belong to a second distinct species (two species); (3) *T. pallidipennis*, *T. andeana* and *T. ochraceipennis* represent three distinct species (three species); (4) *T. pallidipennis* from North America, *T. pallidipennis* from South America, *T. andeana* and *T. ochraceipennis* represent four different species (four species); (5) *T. pallidipennis* from North America, *T. pallidipennis* from South America (including *T. andeana*) and *T. ochraceipennis* represent three different species (three species); and, finally, (6) similar to hypothesis 5, but instead of belonging to *T. pallidipennis* from South America, the Peruvian *T. andeana* belongs to *T. ochraceipennis* (three species).

An input file for \*BEAST was generated using the program BEAUTI v.1.6.1 (Drummond & Rambaut, 2007). All four genetic regions were simultaneously analysed; each gene was treated as unlinked, except from both mitochondrial partitions, which were considered as linked. The ploidy level of *ND5* and *COI* was analysed as 'mitochondrial' and the remaining genes were designated as the default 'nuclear autosomal'. The Yule process was chosen as the tree prior and the population size prior was set to constant. The analysis was run five times for 200,000,000 generations to ensure convergence; trees were sampled every 20,000 generations. Convergence was inferred through the effective sample sizes (ESS) of parameters of interest (gene trees, species tree, root age), which were all above 200. The first 1000 saved trees (10%) from each run were discarded as burn-in after ensuring the likelihood scores reached a plateau which was determined with the program TRACER 1.5 (Drummond & Rambaut, 2007). Trees were visualized in FIGTREE 1.2 (Rambaut, 2011) displaying posterior probabilities as branch support.

The best 'species delimitation' hypothesis was chosen based on the posterior probabilities of individual branches and on a qualitative analysis of all 'species trees' through the DENSITREE 2.01 program (Bouckaert, 2010). Posteriors above 95% were considered as evidence for substantial support at a node. DENSITREE draws all trees gained from a \*BEAST run using transparent lines. In areas where many trees agree on a specific topology and branch length, a densely coloured area will be observed.

Node ages of 'species trees' were estimated through \*BEAST, using a generalized *COI* substitution rate of 2.3% pairwise divergence per million years, estimated from several arthropod datasets (Brower, 1994), giving a value of 0.0115 substitutions/site/lineage/Myr. Rate constancy across these lineages was tested with Felsenstein's (1988, 1993) global test of rate heterogeneity as described in the manual of the PHYLIP software package version 3.5c. Accordingly, we used a 'strict' clock model and estimated clock rates relative to *COI*.

### Historical biogeography

Dispersal–vicariance analyses (DIVA; Ronquist, 1997) were performed to evaluate different biogeographical scenarios

possibly explaining the distribution of the *T. pallidipennis* complex in North and South America. Four geographical regions were defined: North America (A), Andean Peru (B), Chile (C) and Argentina (D). We conducted a statistical DIVA using the program s-DIVA 1.9 (Yu *et al.*, 2010). This method calculates the optimized areas over a set of trees, thus taking topological uncertainty into account. We used the 15,000 trees retained from Bayesian analysis of the concatenated dataset and discarded a burn-in of 1500 trees. The number of maximum areas was set to four to match the number of defined distribution areas.

A DIVA analysis is prone to errors when the biogeographical history includes numerous dispersal events, and it often finds very widespread ancestors towards the base of the tree (Ronquist, 1997; Kodandaramaiah, 2009). Therefore we also carried out an analysis of ancestral ranges using LAGRANGE (Ree & Smith, 2008). This program performs ancestral area reconstruction by inheriting areas at each node based on a dispersal–extinction–cladogenesis (DEC) model and allows model comparison. We used the tree generated by MRBAYES, and set equal coefficients (=1.0) in the adjacency and dispersal matrices. This allows species to be co-distributed freely among regions, and to disperse with equal probabilities among them.

## RESULTS

### Genetic data analyses

We analysed four gene fragments [two of mitochondrial DNA (mtDNA), two of nuclear DNA (nDNA)] for 84 specimens from two continents and five countries (Argentina, Chile, Mexico, Peru, USA). The resulting alignments for *COI*, *ND5*, *H3* and ITS2 sequences consisted of 661, 1011, 359 and 315 bp, respectively. Appendix S1 displays the number of sequenced individuals for each taxon and gene fragment and the corresponding GenBank accession numbers. None of the three coding genes (*COI*, *ND5* and *H3*) had either gaps or stop-codons that would alter the reading frame, minimizing the possibility of pseudo-gene amplifications. Conversely, ITS2 showed a variable number of insertions/deletions. Genetic diversity greatly varied among all four data partitions: mitochondrial genes had far higher numbers of informative sites compared with nuclear loci (Table 1). Substitution rates/lineage/Myr inferred by \*BEAST for *ND5*, *H3* and ITS2 (in relation to a fixed rate of 0.0115 for *COI*; Brower, 1994) were also higher for mitochondrial genes than for nuclear ones (Table 1).

### Gene tree reconstruction

The topology of the tree obtained from partitioned Bayesian analysis is shown in Fig. 2. *Sphingonotus corsicus* was defined as an outgroup. The first taxa branching off the root are *Conozoa texana* and both individuals of *T. maritima*, which together form a monophyletic clade. Both species belong to

‘group A’, according to White’s (1949) chromosomal classification. Subsequently, *T. saxatilis*, *Circotettix rabula* and a cluster containing all individuals belonging to the ‘*T. pallidipennis* species complex’ successively split off; the relationships are supported by high Bayesian posterior probabilities (BPP). All species from this monophyletic clade belong to White’s (1949) ‘group B’. The *T. pallidipennis* species group itself is separated into two well-resolved sister groups, each one supported by maximum BPP: (1) a group containing all *T. pallidipennis* individuals from North and Central America (clade I, Fig. 2), and (2) a group containing all individuals from South America (clade II, Fig. 2). Within this South American clade, individuals are grouped according to their geographical location and taxonomic classification, except for *T. andeana*. In detail, two monophyletic groups supported

by high BPP can be identified, one including all specimens from Argentina (clade IIa, Fig. 2) and the other one including all individuals from Chile (i.e. *T. ochraceipennis*) (clade IIb, Fig. 2). The specimens morphologically identified as *T. andeana*, on the other hand, are found on two branches in the tree. Two individuals, P827 and P1030, from the more northern locality of Ancash in Peru group basally to clade ‘II’, while the individual P25 from Cusco groups at the basis of the Chilean clade (clade IIb, BPP = 0.93). No geographical pattern was recovered within the North and Central American clade (clade I, Fig. 2).

Nine most parsimonious trees of 647 steps in length were obtained through cladistic analysis (MP) based on the same alignment of concatenated genes. The topology of the strict consensus tree is similar to that obtained through Bayesian inference (Fig. 2). However, the Peruvian samples are now grouped together and form a monophyletic clade; yet this group only receives moderate bootstrap support (68%). Besides, this Peruvian group along with clade IIa and clade IIb form a trichotomy within the South American clade II.

**Table 1** Information about datasets for each gene fragment analysed in the study of the ‘*Trimerotropis pallidipennis* species complex’.

Gene	<i>n</i>	<i>L</i>	PI	Model	<i>S</i>
<i>COI</i>	67	661	43	GTR + G	0.0115*
<i>ND5</i>	68	1011	92	HKW + I	0.0113†
<i>H3</i>	65	359	6	HKW	0.0022†
<i>ITS2</i>	54	315	15	HKW + G	0.0015†

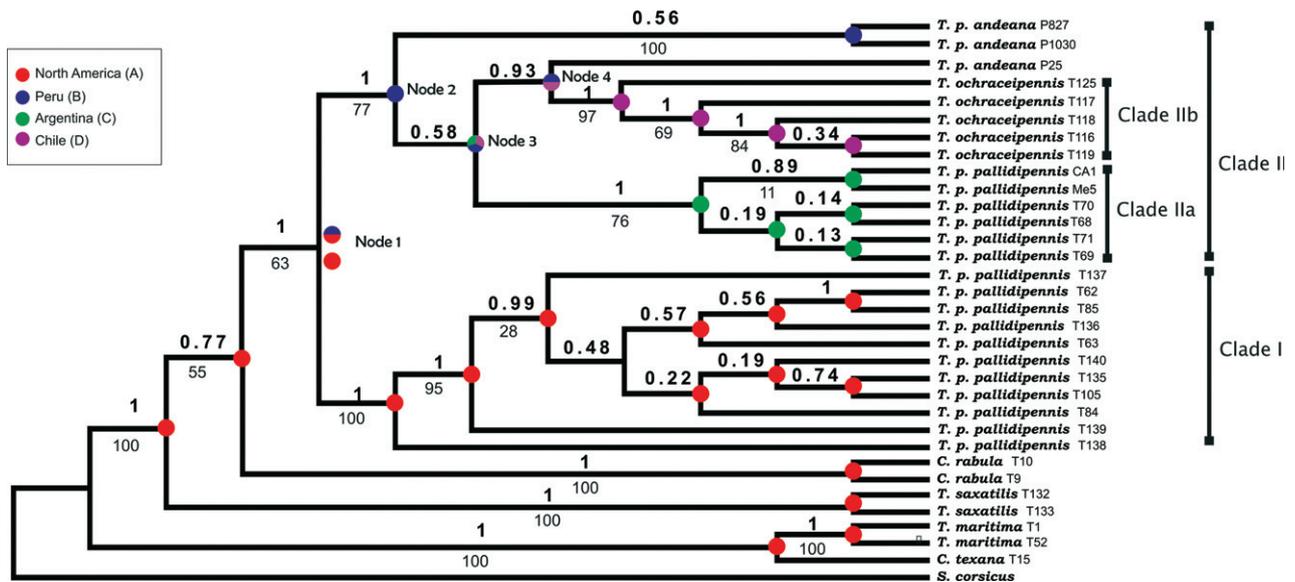
\*Inferred by Brower (1994).

†inferred by \*BEAST in this study.

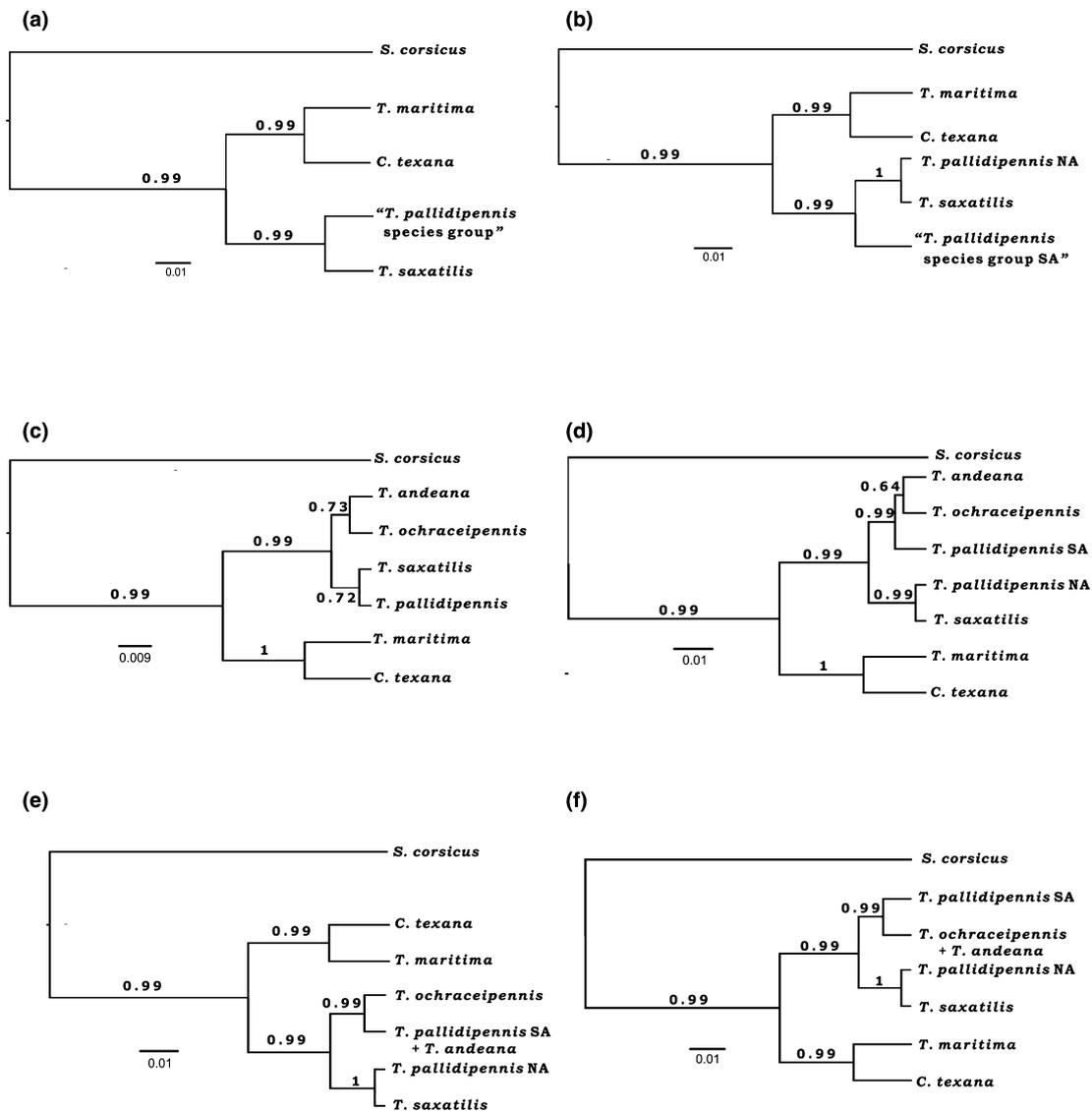
*n*, number of individuals analysed for each gene fragment; *L*, length of aligned sequences (in bp); PI, number of parsimony informative sites; Model, model of molecular evolution; *S*, substitution rate per site/Myr/lineage.

**Species delimitation**

We tested different hypotheses of speciation by comparing BPP retrieved by individual branches (Fig. 3a–f), and by visualizing the whole set of trees using the DensiTREE program (Fig. 4a–f). The clade consisting of *Conozoa texana*–*T. maritima* (chromosomal group A) was always resolved as the sister group of the remaining ‘taxa’ (chromosomal group B), independently of the hypotheses being tested. However, the position of the other outgroup species, i.e. *T. saxatilis*,



**Figure 2** Tree topology obtained from the partitioned Bayesian analysis of the concatenated *COI*, *ND5*, *ITS2* and *H3* dataset for the ‘*Trimerotropis pallidipennis* complex’ from North and South America and related species; *T.* = *Trimerotropis*; *T. p.* = *Trimerotropis pallidipennis*; *S. corsicus* = *Sphingonotus corsicus*; *C. texana* = *Conozoa texana*; *C. rabula* = *Circotettix rabula*. Ancestral area optimization obtained from the programs *s-DIVA* and *LAGRANGE* is indicated. Numbers above branches indicate posterior probabilities. Numbers below branches indicate bootstrap support retrieved from maximum parsimony (MP) analysis. Individual IDs correspond to those in Appendix S1.

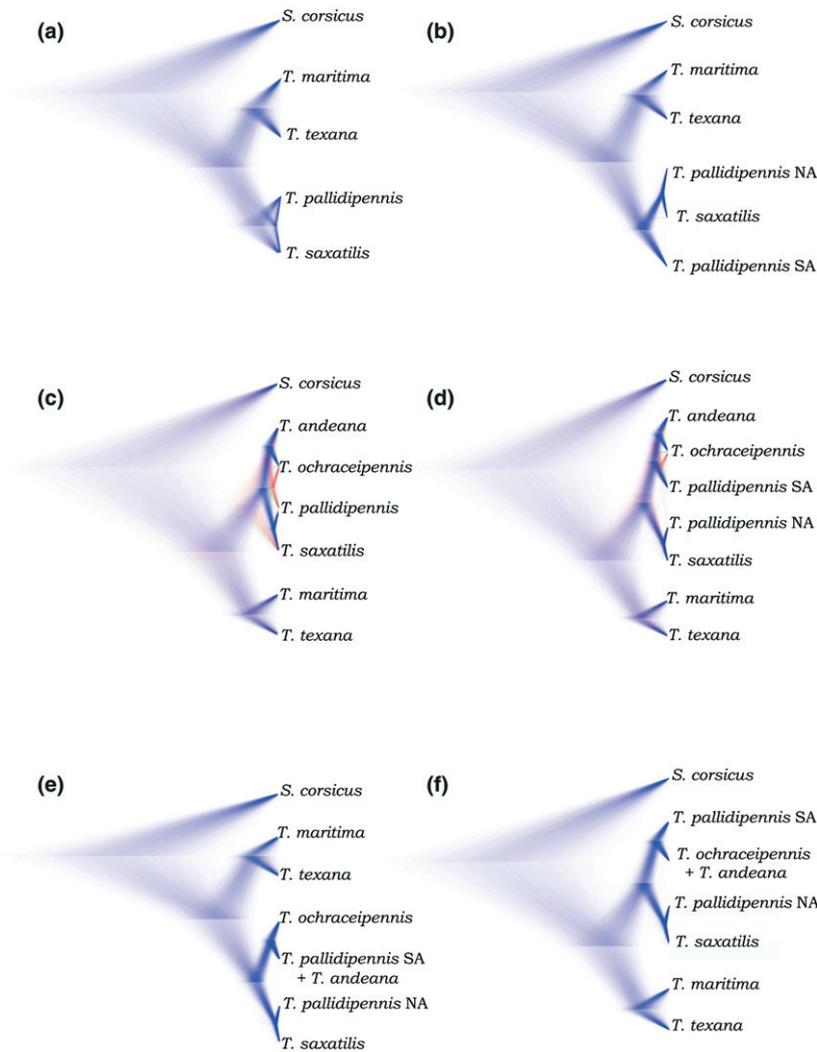


**Figure 3** Species trees obtained from \*BEAST analyses of all hypotheses being tested for the *Trimerotropis pallidipennis* complex from North America (NA) and South America (SA), and related species: (a) hypothesis 1, (b) hypothesis 2, (c) hypothesis 3, (d) hypothesis 4, (e) hypothesis 5, and (f) hypothesis 6 (for a description of the hypotheses see Materials and Methods). *S.* = *Sphingonotus*; *C.* = *Conozoa*; *T.* = *Trimerotropis*. Numbers above branches indicate posterior probabilities. Scale bars represent substitutions per site.

varied depending on the hypothesis tested (Fig. 3). When all specimens of the '*T. pallidipennis* species complex' were considered as the same taxon (hypothesis 1), *T. saxatilis* was the sister taxon to this group. Within this hypothetical framework all nodes received high support (Fig. 3a). When all South American individuals were considered as a separate taxon with respect to North American *T. pallidipennis* specimens (hypothesis 2; Fig. 3b), the latter formed a sister group with *T. saxatilis*, with maximum BPP support. The lowest values of BPP for most individual branches were obtained when species were delimited according to the current taxonomic nomenclature (hypothesis 3; Fig. 3c), suggesting that *T. pallidipennis* from North America and South America are indeed separate units. This result is further supported by the position of *T. saxatilis* as sister group of the North American

*T. pallidipennis* lineage in all species tree hypotheses that recognize the latter as a separate unit (hypotheses 2, 4, 5 and 6; Fig. 3b–f).

Hypotheses 4, 5 and 6 were specifically designed to test for species delimitation within the South American clade (Figs. 3d–f). In hypothesis 4 three separate species occurring in South America are defined, while hypotheses 5 and 6 only define two distinct species. *Trimerotropis andeana* was not supported as a separate species by any of these analyses. In fact, the putative speciation event between this Peruvian lineage and *T. ochraceipennis* only received low support (BPP = 0.64; hypothesis 4; Fig. 3d). Yet, the BPP of individual branches belonging to the South American clade remains high either when *T. andeana* specimens are included within *T. pallidipennis* from Argentina (hypothesis 5; Fig. 3e) or



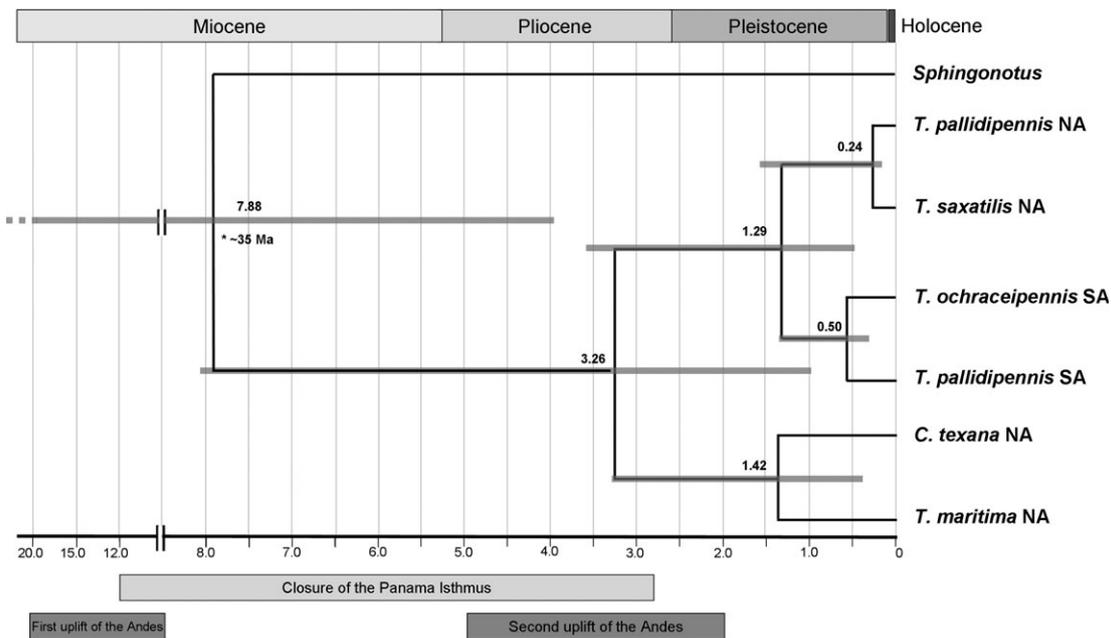
**Figure 4** Density plots of all trees obtained from \*BEAST analyses of all hypotheses being tested for the *Trimerotropis pallidipennis* complex from North America (NA) and South America (SA), and related species. Blue signals branches in concert with the consensus topology while red shows branches in conflict with it: (a) hypothesis 1, (b) hypothesis 2, (c) hypothesis 3, (d) hypothesis 4, (e) hypothesis 5, and (f) hypothesis 6 (for a description of the hypotheses see Materials and Methods). *S.* = *Sphingonotus*; *C.* = *Conozoa*; *T.* = *Trimerotropis*.

when they are included within *T. ochraceipennis* (hypothesis 6; Fig. 3f).

DENSITREE analysis gave additional support for the previously described relationships (Fig. 4a–f). In detail, the whole set of ‘species trees’ retrieved from hypothesis 1 (Fig. 4a) showed that the *T. pallidipennis*–*T. saxatilis* clade is resolved in all of them, but with two markedly different ‘node ages’. The younger node age corresponds to gene trees including *T. pallidipennis* individuals from North America, which are closely related to *T. saxatilis*. The older node age corresponds to all other individuals of *T. pallidipennis* from South America, which are more distantly related to *T. saxatilis*. DENSITREE analysis of species trees based on hypotheses 3 and 4 (Fig. 4c,d) showed large uncertainties among topologies within the *T. pallidipennis*–*T. saxatilis*–*T. ochraceipennis*–

*T. andeana* clade (Fig. 4c) and within the *T. pallidipennis* from South America–*T. ochraceipennis*–*T. andeana* clade (Fig. 4d). Conversely, the same analysis for hypothesis 2 showed strong congruence within the whole set of ‘species trees’ (Fig. 4b), confirming the natural separation of *T. pallidipennis* from North and South America. Finally, this same analysis demonstrated that hypotheses 5 and 6 were indistinguishably good, because most trees in both sets overlap without large conflicts (Fig. 4e, f).

Our molecular clock analysis suggested that the North America–South America split occurred around 1.3 Ma [3.5–0.5 Ma 95% high confidence interval (HCI); Fig. 5]. The time of diversification between *T. ochraceipennis* and *T. pallidipennis* from South America was estimated to be between 0.7 and 0.5 Ma (1.25–0.25 Ma 95% HCI) (Fig. 5).



**Figure 5** Chronogram showing node ages corresponding to the hypothesis 5 proposed for the relationships among species of the *Trimerotropis pallidipennis* complex from North (NA) and South America (SA) (for a description of hypothesis 5 see Materials and Methods); node bars indicate 95% high confidence interval for node ages; major geological epochs are given along with two major geological events influencing the biogeography of the species complex. NA = North America, SA = South America. C. = *Conozoa*; T. = *Trimerotropis*; \*age estimate obtained for this split with a more comprehensive taxon sampling (Husemann *et al.*, 2012).

## Biogeography

The results of s-DIVA and LAGRANGE analyses are consistent (Table 2, Fig 2), with the exception of node 1 which corre-

**Table 2** Results of biogeographical analyses of the *Trimerotropis pallidipennis* species complex performed with the programs s-DIVA and LAGRANGE. Node IDs correspond to those in Fig. 2.

Node	s-DIVA		LAGRANGE	
	Ancestral area	Frequency (%)	Ancestral area	Relative probability
1	AB	100	A	0.64
			AB	0.11
2	B	100	B	0.28
			CB	0.18
			DCB	0.17
			DB	0.12
			C	0.11
			D	0.07
			DC	0.07
3	BCD	50.40	BCD	0.49
			BC	0.24
4	BD	100	B	0.14
			CB	0.11
			BD	0.78
			B	0.20

Geographical areas: A, North America; B, Peru; C, Argentina; D, Chile. s-DIVA: for each node the percentage of trees where a particular ancestral area optimization was obtained is indicated.

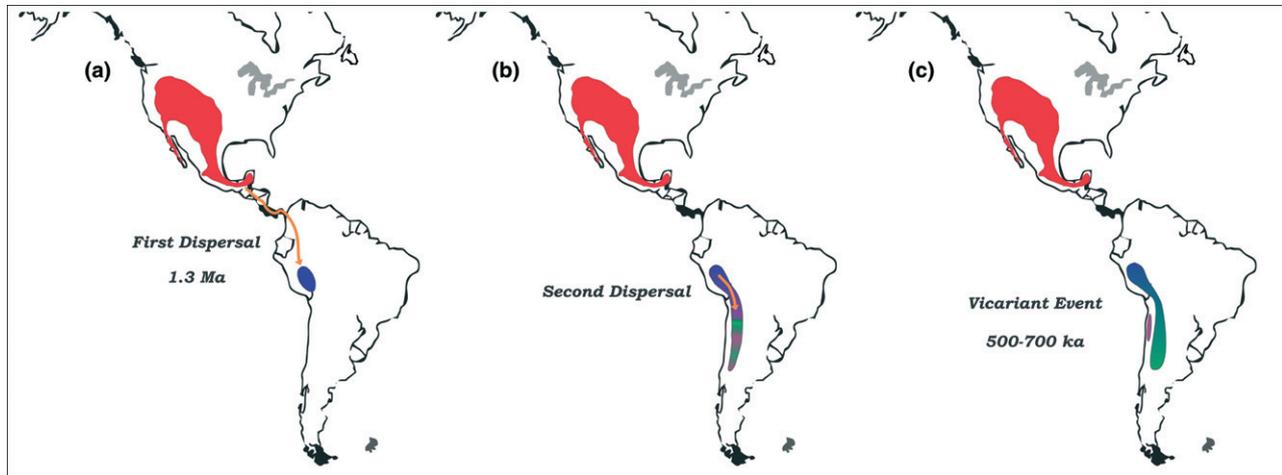
LAGRANGE: for each node the alternative ancestral area optimization within 2 log-likelihood units is given along with the relative probabilities.

sponds to the ancestor of the *T. pallidipennis* species complex. According to s-DIVA analysis, the ancestor of the group was distributed in North America and Peru (Fig. 2, Table 2). LAGRANGE analysis, however, suggested a strictly North American distribution ( $P = 0.64$ ) as opposed to a North American and Peruvian one ( $P = 0.11$ ). Yet, the latter hypothesis is within 2 log-likelihood units of the former, i.e.  $\ln L = -21.6$  and  $\ln L = -23.35$ . Therefore, the dispersal from North America to Peru would have taken place either before (s-DIVA) or after (LAGRANGE) the cladogenetic event represented by node 1 (Fig. 2). For node 2, both analyses indicate a higher probability for Peru as the ancestral distribution range of the South American clade. Subsequent dispersal is suggested to have occurred to Argentina and Chile, giving rise to a wide ancestral distribution at node 3. Alternative scenarios for the distribution areas of these later nodes were suggested by the LAGRANGE analysis with lower probabilities (Table 2). Finally, a vicariance event probably gave rise to *T. ochraceipennis* (Fig. 2, Table 2).

## DISCUSSION

### How many biological units?

We found strong evidence for the existence of at least three distinct species within the *T. pallidipennis* species complex: *T. pallidipennis* from North America, *T. pallidipennis* from South America and *T. ochraceipennis* endemic to Chile. All analyses independently showed that specimens from North and South America represent two clearly distinct units. In



**Figure 6** Biogeographical scenario displaying the stepwise diversification of the ‘*Trimerotropis pallidipennis* species complex’ in North and South America.

addition, two units can be distinguished within South America: *T. ochraceipennis* from Chile and specimens from Argentina. On the other hand, all approaches agreed that the status and position of Peruvian specimens is ambiguous. Indeed, MP analysis suggested, though with moderate bootstrap, that Peruvian specimens might form a separate clade.

Interestingly, these recovered genetic lineages can also be distinguished on cytological grounds: in the undescribed species *Trimerotropis* sp. from Argentina the four medium chromosomes are polymorphic for inversions (Confalonieri & Colombo, 1989), a karyotypic feature that differentiates this species from North American *T. pallidipennis*, in which the same chromosomes are always monomorphic (White, 1951). In contrast, in *T. ochraceipennis* two of the medium-sized chromosomes are fixed for a submetacentric state (Lafuente *et al.*, 1968). However, the Bayesian analysis indicated that *T. andeana* is paraphyletic; it splits into two clearly distinct groups: one basal to the whole South American clade (clade II, Fig. 2) with low BPP value, and a second group related to the Chilean clade (clade IIb, Fig. 2), receiving high BPP support. Although paraphyly can be the consequence of incomplete lineage sorting in incipient species, this scenario was not supported by the coalescent-based approach because Peruvian specimens were joined either to *T. pallidipennis* from Argentina or to *T. ochraceipennis*.

Rehn (1939) described two distinct forms coexisting in Peru, the most typical ‘*T. p. pallidipennis* form’ and ‘*T. p. andeana*’, a geographical race endemic to the high Puna area. Although some morphological features distinguished ‘*T. p. andeana*’ from the other subspecies (i.e. it is proportionately more robust, the head is broader and less compressed, the genae are more inflated, eyes are less prominent both dorsally and laterally, etc.), Rehn (1939) recognized that ‘these differences become less clearly marked as one goes southward’ (p. 408). Moreover, although the Peruvian samples were collected in high Puna habitats, specimens from Ancash come from the Cordillera Blanca, the highest

snow-capped tropical range in the world, with 27 peaks above 6000 m a.s.l. This region is characterized by high levels of endemism. A recent survey of grasshoppers in the region yielded large numbers of undescribed species. All specimens found during these surveys, except for *T. andeana*, belonged to species new to science (Cigliano *et al.*, 2011). Thus, it is not surprising that specimens of *T. andeana* from Ancash, although not yet morphologically differentiated, may even constitute a distinct unit from those of Cusco. Therefore, the Peruvian specimens analysed here are most probably composed by an admixture of two distinct biological units, which could correspond to both *T. pallidipennis* from Argentina and *T. ochraceipennis*, or even to a third distinct biological unit that currently is undergoing a differentiation process. A more comprehensive sampling of Peruvian specimens is needed in order to clarify these relationships.

### Species differentiation and historical biogeography

Our results indicate that at least two dispersal events and one vicariance event have occurred during the diversification process of the *T. pallidipennis* species complex (Fig. 6). The s-DIVA analysis suggests that the ancestor of the species complex inhabited both continents. This distribution was the result of dispersion from North to South America at some time in the past. Conversely, LAGRANGE analysis suggests that this ancestor was most probably restricted to North and Central America. This in turn would mean that dispersal to South America occurred at a later time (Fig. 6a).

The estimated age of the phylogenetic split between North and South America is around 1.3 Ma (3.5–0.5 Ma 95% HCI). During this time, the Isthmus of Panama had already been completed for almost a million years, allowing for an intensive faunal interchange between both continents. However, although the emergence of this land bridge was undoubtedly necessary for terrestrial animals to migrate, the shift from humid tropical to drier conditions in Central

America as a consequence of Quaternary glaciations was equally important. The drier climate allowed for the migration of temperate and xerophilic organisms, such as oedipodines. Therefore, dispersion of *T. pallidipennis* from North to South America in this time frame is a more plausible scenario for the North America–South America split, than the alternative vicariance scenario. The latter appears unlikely because it would imply an earlier widespread distribution of *T. pallidipennis* across North and South America at a time when the land bridge connection had not been established. Even if the Isthmus of Panama had been closed, the climatic conditions of Central America would have been an important barrier for temperate species.

The second dispersal event detected in both analyses led to a range expansion from Ecuador and Peru to southern latitudes, reaching Chile and Argentina (Figs 2 & 6b). The species belonging to the *T. pallidipennis* complex are adapted to a wide elevational range and occur at high elevations, especially in South America. Indeed, some of the analysed populations were collected at elevations of more than 3500 m and included specimens caught at Mirador Quitapampa in Ancash, Peru, at 3852 m a.s.l. and in Jujuy, Argentina at 3969 m a.s.l. (Appendix S1). This modern distribution indicates that high elevations do not necessarily represent important physical barriers. Hence, dispersal could have occurred along the Andes mountains unless ice caps were present. The early Pleistocene (2.6–0.8 Ma) was characterized by climatic fluctuations dominated by the 41-kyr obliquity and 26-kyr precession Milankovitch cycles, during which substantial ice sheets developed (Ehlers & Gibbard, 2007). However, it was not until the transition to the 100-kyr eccentricity cycles (beginning about 1.2 Ma and fully established by about 800 ka), that the cold periods (glacials) were regularly cold and long enough to allow for significant development of ice sheets on a continental scale, outside the polar regions (Ehlers & Gibbard, 2007). As in Europe and North America, in South America glaciations increased in intensity throughout the Andean mountain chain from 800 ka until the Last Glacial Maximum about 20 ka (Rabassa *et al.*, 2011). The vicariance event that probably led to the split between the Argentinean form of *T. pallidipennis* and the Chilean *T. ochraceipennis* was detected by both analytical approaches and was dated at around 0.7–0.5 Ma (0.25–1.25 Ma 95% HCl) (Figs 5 & 6c). This corresponds to a time when the Andes were substantially covered with ice sheets, undoubtedly constituting a physical barrier leading to allopatric diversification resulting in these southernmost *Trimerotropis* species.

## ACKNOWLEDGEMENTS

We are grateful to all colleagues who provided samples: Ted Cohn, Dave Ferguson, Dario Salas, Jaime Pizarro, Axel Hochkirch and Ralph Scott. M.M.C. thanks Ricardo Solano Morales, SENASA, Peru, for his collaboration with the collecting permits. We thank Suk Namkung for help with sequencing, Joseph Heled for his advice on the use of \*BEAST

and DensiTREE programs, and members of the Danley lab and two anonymous referees for valuable comments on previous versions of this manuscript. Financial support to V.A.C. was provided by the University of Buenos Aires, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica (ANCyPT) (Argentina). An Undergraduate Research and Scholarly Achievement grant from Baylor University and the Orthopterists' Society provided funding to P.D.D. and M.H. A grant from National Geographic Society provided funding to M.M.C. V.A.C. and M.M.C. are members of the CONICET, Argentina.

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## SUPPORTING INFORMATION

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

**Appendix S1** Sampling locations of species in the ‘*Trimerotropis pallidipennis* complex’ and GenBank accession numbers for each individual and gene.

**Appendix S2** Primer sequences used for amplification of gene fragments.

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## BIOSKETCH

**Martin Husemann** is a PhD candidate in the Biology Department at Baylor University under the supervision of Patrick D. Danley. He is using population genetic and genomic tools and morphometric methods to study the evolution of East African cichlid fish. In addition he is interested in the phylogeny and diversification of Oedipodinae mainly belonging to the tribes Sphingonotini, Trimerotropini and Bryodemini.

Author contributions: M.H., V.A.C. and N.V.G. conceived the ideas; M.H., N.V.G., V.A.C. and M.M.C. collected specimens; M.H. and N.V.G. carried out the molecular work; V.A.C., M.H. and N.V.G. analysed the data; V.A.C., M.H. and N.V.G. led the writing. All authors contributed to the final analyses and writing of the manuscript.

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Editor: Malte Ebach