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Phylogeography of the harlequin beetle-riding pseudoscorpion and the rise of the Isthmus of Panamá

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Abstract

Molecular and geological evidence indicates that the emergence of the Isthmus of Panamá influenced the historical biogeography of the Neotropics in a complex, staggered manner dating back at least 9 Myr BP. To assess the influence of Isthmus formation on the biogeography of the harlequin beetle-riding pseudoscorpion, *Cordylochernes scorpioides*, we analysed mitochondrial COI sequence data from 71 individuals from 13 locations in Panamá and northern South America. Parsimony and likelihood-based phylogenies identified deep divergence between South American and Panamanian clades. In contrast to low haplotype diversity in South America, the Panamanian *Cordylochernes* clade is comprised of three highly divergent lineages: one clade consisting predominantly of individuals from central Panamá (PAN A), and two sister clades (PAN B1 and PAN B2) of western Panamanian pseudoscorpions. Breeding experiments demonstrated a strictly maternal mode of inheritance, indicating that our analyses were not confounded by nuclear-mitochondrial pseudogenes. Haplotype diversity is striking in western Atlantic Panamá, where all three Panamanian clades can occur in a single host tree. This sympatry points to the existence of a cryptic species hybrid zone in western Panamá, a conclusion supported by interclade crosses and coalescence-based migration rates. Molecular clock estimates yield a divergence time of ≈ 3 Myr between the central and western Panamanian clades. Taken together, these results are consistent with a recent model in which a transitory proto-Isthmus enabled an early wave of colonization out of South America at the close of the Miocene, followed by sea level rise, inundation of the terrestrial corridor and then a second wave of colonization that occurred when the Isthmus was completed ≈ 3 Myr bp.

Keywords: *COI* gene, *Cordylochernes scorpioides*, harlequin beetle, mitochondrial DNA, Neotropics, phylogeny

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Introduction

In joining North America to the island continent of South America, the rise of the Isthmus of Panamá was a geological event with profound consequences for the biogeography of the Neotropics (Simpson 1980). On land, the uplifting of the Isthmus enabled the 'Great American Interchange': a two-way migration and intermingling of terrestrial lineages that had been separated since the Cretaceous (Marshall *et al*. 1979; Stehli & Webb 1985; Pindell & Barrett 1990). In the ocean, the closure of the Central American Seaway severed gene flow and brought about evolutionary divergence between populations of marine species in the eastern Pacific and tropical western Atlantic Oceans. The resulting transisthmian sister-species pairs ('geminate species', Jordan 1908) have been used extensively to infer rates of molecular evolution in a wide range of marine taxa, including snapping shrimp (Knowlton *et al*. 1993; Knowlton & Weigt 1998), echinoderms (Lessios 1998) and fishes (Bermingham *et al*. 1997).

Studies of multiple transisthmian species pairs are providing growing evidence that cessation of gene flow did not occur simultaneously across pairs of sister species but was a staggered process spanning several million years (Bermingham & Lessios 1993; Knowlton *et al*. 1993; Knowlton & Weigt 1998; Marko 2002). These findings are consistent with palaeogeographical reconstructions that indicate a gradual formation of the Isthmus from its emergence as an

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island chain in the middle to late Miocene (15–6 Myr BP) to its completion as a terrestrial corridor ≈ 3 Myr BP (Coates & Obando 1996; Coates *et al*. 2003). The marine fossil record suggests equally gradual uplift-associated extinctions extending back more than 10 Myr (Jackson *et al*. 1993; Collins 1996; Vermeij 2001). Nonetheless, it is still commonly assumed that the final seaway closure date of 3 Myr BP provides a reliable basis for molecular clock calibrations (e.g. Baldwin *et al*. 1998; Metz *et al*. 1998; Stillman & Reeb 2001). Obviously, this assumption can yield greatly overestimated rates of nucleotide sequence divergence in marine geminate species whose isolation predated completion of the Isthmus (Knowlton *et al*. 1993; Knowlton & Weigt 1998; Marko 2002).

For terrestrial species, there is also ample evidence that the emergence of the Isthmus influenced the biogeography of the region in a complex, staggered manner dating back at least 9 Myr (Stehli & Webb 1985). The mammalian fossil record indicates a limited, but significant, exchange of taxa between North and South America during the early stages of Isthmus formation. Giant sloths of South American origin first appear in North America between 9.5 and 9.0 Myr BP, whereas procyonids (the racoon family) of North American origin are present in South America by 6.0 Myr BP (Marshall *et al*. 1979). This movement of terrestrial taxa between the two continents long before final completion of the land bridge has generally been attributed to 'island-hopping' dispersal along the emerging island chain (Simpson 1950). However, the island-hopping model for colonization that predated completion of the Isthmus provides a questionable explanation for the occurrence in extreme western Panamá of mitochondrial lineages of freshwater fishes estimated to have diverged from their putative South American source populations ≈ 5 Myr BP (Bermingham & Martin 1998). Marine conditions present a dispersal barrier to primary freshwater fishes (Myers 1938). As an alternative to the island-hopping model, Bermingham & Martin (1998) therefore proposed that formation of the Isthmus involved the emergence of a short-lived terrestrial corridor at the close of the Miocene (4–7 Myr BP).

Compared with what is known for marine species and terrestrial vertebrates, the impact of the rise of the Isthmus on the historical biogeography of terrestrial invertebrates is less well understood. Here, we present the results of a phylogeographical study of the harlequin beetle-riding pseudoscorpion, *Cordylochernes scorpioides*. This pseudoscorpion has proved to be an excellent model system for molecular, laboratory and field investigations of intrapopulation genetic incompatibility (Zeh 1997; Newcomer *et al*. 1999; Zeh & Zeh 2001), sexual selection (Zeh *et al*. 1997, 1998) and speciation (Zeh & Zeh 1994, 2000; Wilcox *et al*. 1997). This study significantly extends the findings of a previous investigation based on a limited geographical sampling of four populations (Wilcox *et al*. 1997). Phylogenetic analysis of mitochondrial DNA (mtDNA) sequence data from 71 individuals sampled from 13 locations in Panamá and northern South America reveals a pattern of deep divergence between *C. scorpioides* clades and a geographical distribution of mtDNA haplotypes consistent with the history of colonization, extinction, divergence and re-colonization hypothesized by the Bermingham/Martin (B/M) model of the formation of the Isthmus of Panamá.

Materials and methods

Collections

Pseudoscorpions for this study were collected from locations in north central Trinidad and eastern French Guiana and from 11 sites in Panamá (Table 1; Fig. 1). The Isthmus of Panamá is transected by a central cordillera that reaches an

Table 1 Locality data of 71 *Cordylochernes scorpioides* individuals sequenced for the phylogeographical component of the study. WPP, western Pacific Panamá; WAP, western Atlantic Panamá; CP, central Panamá

Collection site	Code	Latitude/longitude	Popn	No. of specimens
Ojo de Agua, Chiriquí Province, Panamá	ODA	08°44′N/82°44′W	WPP	10
Bocas del Toro Province, Panamá	BDT	09°00′N/82°16′W	WAP	14
La Fortuna, Chiriquí Province, Panamá	FORT	08°43′N/82°14′W	WAP	3
Tolé, Chiriquí Province, Panamá	TOLE	08°14′N/81°42′W	CP	1
Cerro Campana, Panamá Province, Panamá	CC	08°44′N/79°58′W	CP	4
Barro Colorado Island, Colon Province, Panamá	BCI	09°09′N/79°50′W	CP	3
Fort Sherman, Colon Province, Panamá	FS	09°17′N/79°56′W	CP	5
Bohio Peninsula, Colon Province, Panamá	BOHIO	09°12′N/79°50′W	CP	2
Camino Madden, Panamá Province, Panamá	MAD	09°05′N/79°37′W	CP	9
Río Platanares, Panamá Province, Panamá	PLAT	09°13′N/79°00′W	CP	1
El Llano Cartí, Panamá Province, Panamá	LC	09°18′N/78°58′W	CP	8
Blanchisseuse Road, Trinidad	TRIN	10°45′N/61°19′W	TRIN	6
Kaw Mountains, French Guiana	FG	04°32′N/52°04′W	FG	5

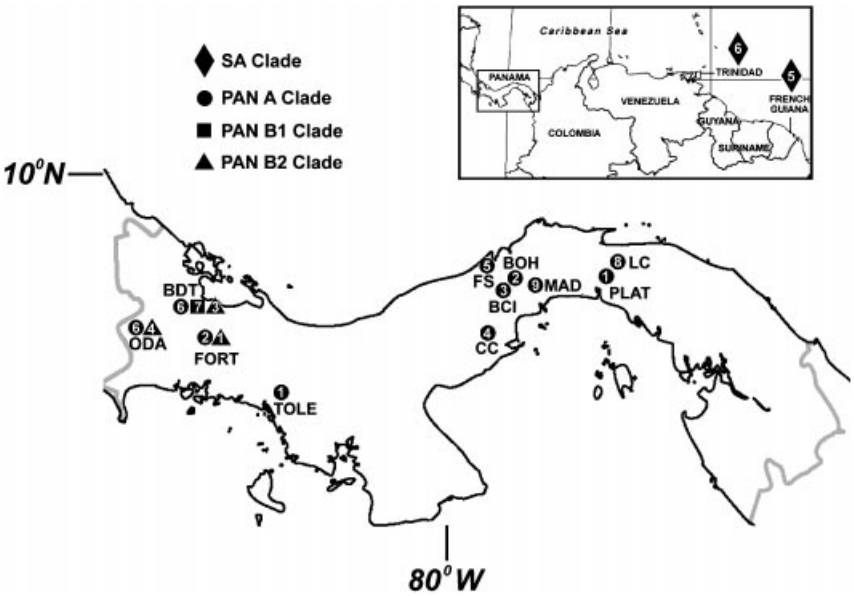

Fig. 1 Collection sites and geographical distribution of *Cordylochernes* mtDNA clades. Numbers within clade symbols indicate the number of individuals collected at the site belonging to that clade (see Fig. 2 for clade composition). Collection site codes, ODA: Ojo de Agua, BDT: Bocas del Toro, FORT: La Fortuna, TOLE: Tolé, CC: Cerro Campana, FS: Fort Sherman, BCI: Barro Colorado Island, BOH: Bohio Peninsula; MAD: Camino Madden, PLAT: Rio Platanares, LC: El Llano Cartí Road, TRIN: Trinidad, FG: French Guiana (see Table 1).

elevation of 3000 m in western Panamá and decreases to 200 m in central Panamá. Our collections were made over a region spanning 418 km from central to western Panamá where sites were located on either the Atlantic (northern) or the Pacific (southern) versant of the cordillera. Pseudoscorpions were collected from decaying trees (*Brosimum* spp. or *Ficus* spp. in Panamá and Trinidad; *Parahancornia fasciculata* in French Guiana) or were removed from harlequin beetles captured on newly dead or dying trees. Specimens were maintained alive before freezing at –80 °C.

DNA extraction, polymerase chain reaction and sequencing

Total genomic DNA was extracted from each individual using the 2× CTAB protocol described previously (Zeh *et al*. 1992a). An ≈ 450 bp fragment of the mitochondrial cytochrome oxidase subunit 1 (*COI*) gene, corresponding to the region between 1751 and 2191 bp in the *Drosophila yakuba* mtDNA sequence (Simon *et al*. 1994), was amplified using a degenerate version of the primer C1-J-1751 (alias Ron) (5′-GGAKCACCTGATATAGCATTYCC-3′) and the primer C1-N-2191 (alias Nancy) (5′-CCCGGTAARATT-AAAATATAAACTTC-3′) (Simon *et al*. 1994). The 25 μL polymerase chain reaction (PCR) mix contained ≈ 10 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 2 mM $MgCl_2$, 0.1 mM dNTPs and 0.5 units Titanium *Taq* DNA polymerase (Clontech). PCR amplification conditions involved an initial 2 min melting step at 94 °C, followed by 32 iterations of the following cycle: 94 °C for 60 s, 52 °C for 90 s and 72 °C for 105 s, with a final 7-min extension at 72 °C. PCR templates were prepared for sequencing by electrophoresing 12 μL of the reaction through a 1% agarose gel (Gibco-BRL) stained with ethidium bromide. Amplification products were excised from gels and purified using Promega Wizard minicolumns. BigDye sequencing reactions (6 μL) containing ≈ 100 ng of purified PCR product and 3.2 pmoles of the C1-J-1751 primer were analysed using an ABI Prizm 3730 automated sequencer, according to the manufacturer's protocols (PE Applied Biosystems).

Inheritance of mtDNA haplotype

To test for the possible presence of nuclear-mitochondrial pseudogenes (Numts) which can confound phylogenetic analysis (Bensasson *et al*. 2001), we examined the pattern of sequence inheritance (maternal or biparental) in several sets of offspring derived from matings between males and females of differing mtDNA haplotype. These offspring sequences were not included in the phylogenetic and gene flow analyses described below.

Phylogenetic reconstruction

Both morphological and molecular evidence indicates that *Lustrochernes* is the genus most closely related to *Cordylochernes* (Muchmore 1974; Wilcox *et al*. 1997), and *L. consocius* was therefore used as the outgroup in phylogenetic analyses in order to root the *C. scorpioides* trees. Maximum parsimony (MP) and maximum likelihood (ML) searches were carried out on aligned sequences, as implemented in PAUP Version 4.0b8 (Swofford 2002), and all minimal trees were saved. Hierarchical likelihood ratio tests were performed using MODELTEST Version 3.06 (Posada & Crandall 1998) in order to determine the best-fit ML substitution model available from the set of 56 models implemented

in the program. For both tree estimation procedures, support for each clade was estimated by analysing 500 bootstrap replicate datasets (Felsenstein 1985). Pairwise genetic distances between haplotypes were calculated using both Kimura's 2-parameter model (K2P) and maximum likelihood methods. Finally, sequences were translated in MCCLADE Version 4.05 (Maddison & Maddison 2002) using the invertebrate mitochondrial code for analysis of amino acid substitutions.

Migration rate estimation

We used a maximum likelihood approach based on coalescence theory (Beerli & Felsenstein 2001) to estimate patterns of gene flow between *C. scorpioides* populations. This method assumes neither symmetric migration rates nor equal subpopulation sizes and allows for the incorporation of both coalescence within subpopulations and migration events that switch lineages from one population to another (Beerli & Felsenstein 2001). Data were analysed using MIGRATE (Beerli 2002), a program that employs a Metropolis-Hastings Markov chain Monte Carlo algorithm (MH) to estimate ML effective population sizes ($\Theta = 2N_f\mu$) and migration rates ($2N_fm$), where N_f is the female effective population size, μ is the mutation rate per generation per site, and $2N_fm$ is the number of migrant females per generation. In our MIGRATE simulations, we used an empirically determined Ti/Tv (calculated from the ML tree using the 'state changes and stasis' function in MCCLADE), empirical base frequencies and starting parameters based on F_{ST} estimates calculated using the program (Beerli 2002). The search strategy for obtaining the ML estimates involved 10 short chains with 10 000 sampled trees and 3 long chains with 100 000 sampled trees. To obtain more precise estimates, a second analysis was performed with parameter estimates from the first run as starting values.

Results

Haplotypes

Examination of the mtDNA *COI* sequences of the 71 *Cordylochernes scorpioides* field-collected individuals identified 24 different haplotypes (GenBank Accession nos AY332244 to AY332267), with K2P pairwise distances ranging from 0.23 to 15.44%. Distances estimated using maximum likelihood were appreciably higher, particularly for more divergent sequences, and ranged from 0.23 to 32.61% (Table 2). The ML genetic distances were calculated, based on MODELTEST selection of the TrN + I substitution model (Akaike Information Criterion; Posada & Crandall 1998), with a rate matrix of $A \rightarrow C = 1.00$; $A \rightarrow G = 12.58$; $A \rightarrow T = 1.00$; $C \rightarrow G = 1.00$; $C \rightarrow T = 14.07$; $G \rightarrow T = 1.00$ and a proportion of invariable sites (I) equal to 0.73. Within French Guiana (FG), Trinidad (TRIN) and central Panamá, sequence variation was quite limited. A single haplotype was found in the six specimens from Trinidad, whereas the five individuals from French Guiana yielded two haplotypes that differed by a single nucleotide. Similarly, although 9 distinct haplotypes were identified among the 33 *C. scorpioides* collected from central Panamá, they differed by a maximum of only 3 nucleotides. By contrast, in western Panamá, mtDNA sequence variation was extremely high. Among the 17 individuals sampled from this region, we identified

Table 2 Pairwise genetic distances between major clades of *Cordylochernes scorpioides*. Distances were calculated from 439 nucleotide sites and corrected for multiple hits using either Kimura's 2-parameter model (K2P) or a maximum likelihood (TrN + I) substitution model (see Results). Approximate divergence times (Myr) are based on a divergence rate of 2.3% per Ma calculated for arthropod mtDNA (Brower 1994)

	Kimura's 2-parameter distances				Maximum likelihood distances (TrN + I)			
Comparison	Mean distance (%)	Range (%)	Mean divergence (Myr)	Range (Myr)	Mean distance (%)	Range (%)	Mean divergence (Myr)	Range (Myr)
South America vs. Panamá	13.26	11.72–15.44	5.77	5.10–6.71	25.03	22.22–32.61	10.88	9.66–14.18
Panamá A vs. Panamá B	7.15	6.22–7.76	3.11	2.70–3.38	9.82	8.21–10.76	4.27	3.57–4.68
Panamá B1 vs. Panamá B2	3.99	3.27–4.48	1.74	1.42–1.95	4.78	3.81–5.49	2.08	1.66–2.38
South America vs. Panamá A	12.62	11.72–13.45	5.49	5.10–5.85	23.57	22.22–25.46	10.25	9.66–11.07
South America vs. Panamá B	14.31	13.67–15.44	6.22	5.94–6.71	27.41	24.34–32.61	11.92	10.58–14.18
South America vs. Panamá B1	14.59	13.67–15.44	6.34	5.94–6.71	29.61	27.37–32.61	12.87	11.90–14.18
South America vs. Panamá B2	14.02	13.72–14.36	6.10	5.96–6.24	25.22	24.34–26.39	10.96	10.58–11.47
Panamá A vs. Panamá B	7.15	6.22–7.76	3.11	2.70–3.38	9.82	8.21–10.76	4.27	3.57–4.68
Panamá A vs. Panamá B1	6.99	6.22–7.49	3.04	2.70–3.26	9.67	8.21–10.76	4.20	3.57–4.68
Panamá A vs. Panamá B2	27.31	6.46–7.76	3.18	2.81–3.38	9.97	8.49–10.65	4.33	3.69–4.63
Panamá B1 vs. Panamá B2	3.99	3.27–4.48	1.74	1.42–1.95	4.78	3.81–5.49	2.08	1.66–2.38

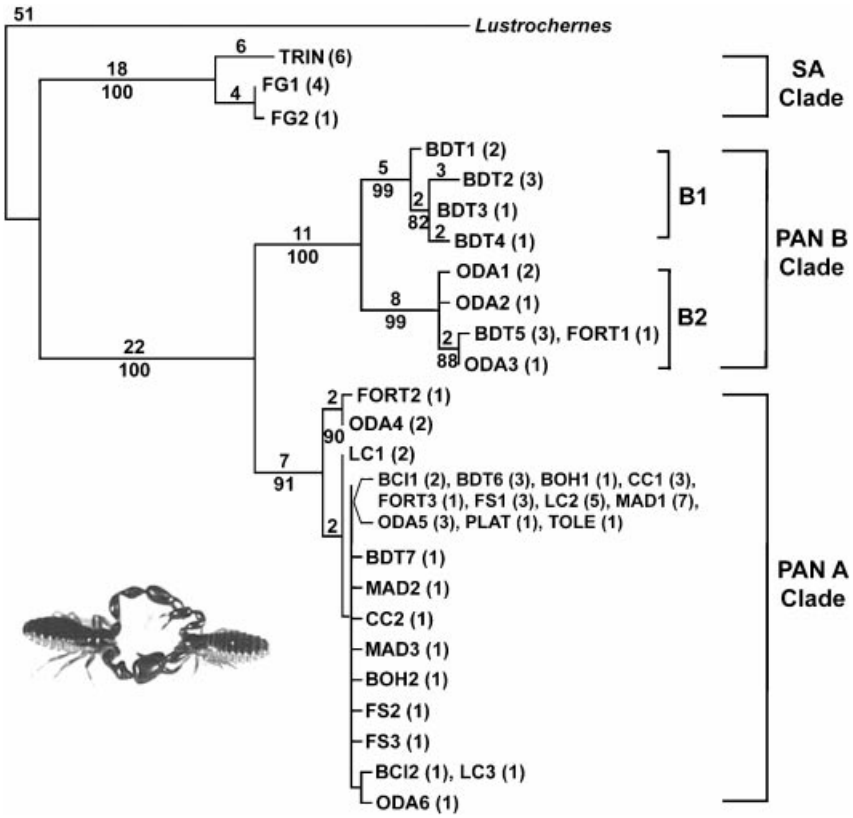

Fig. 2 *Cordylochernes* phylogenetic relationships based on 24 unique mtDNA *COI* haplotypes of pseudoscorpions sampled from French Guiana, Trinidad and Panamá. The phylogram is one of 28 most parsimonious trees and is topologically equivalent to the maximum likelihood tree (TrN + I substitution model) of the *Cordylochernes* clade (see Results). Numbers above branches indicate number of nucleotide substitutions (but note that terminal branches of length 1 are unlabelled). Values below branches represent bootstrap estimates (> 80%). Haplotypes are identified by collection site, with numbers in parentheses indicating number of individuals from a particular site that exhibit the haplotype. For collection site codes, see Fig. 1. The *Cordylochernes* tree was rooted using sequence from *Lustrochernes consocius* (Wilcox *et al*. 1997).

16 different haplotypes distinguishable by up to 33 nucleotide substitutions (Fig. 2). Of these, 12 haplotypes were unique to western Panamá and 4 were almost identical to haplotypes found in individuals sampled from central Panamá.

Phylogenetic analysis

We used unambiguously aligned sequence from *Lustrochernes consocius* (Wilcox *et al*. 1997) to root phylogenetic trees of the 24 different *C. scorpioides* haplotypes. Unweighted MP analysis identified a South American clade (SA = FG + TRIN; Fig. 2) as the strongly supported monophyletic sister taxon of a more diverged and polymorphic Panamanian clade (bootstrap = 100%; tree length = 162; CI = 0.815). The South American (SA) and Panamanian (PAN) pseudoscorpions are separated by mean K2P and ML distances of 13.26 and 25.03%, respectively (Table 2). Within the Panamanian clade, *C. scorpioides* haplotypes are further divided into two main groups, a clade consisting largely of individuals from central Panamá (PAN A) and a clade composed entirely of individuals from western Panamá (PAN B). Mean K2P and ML distances between the PAN A and PAN B clades are 7.15 and 9.82%. The PAN B clade is further subdivided into two well-differentiated, monophyletic lineages, PAN B1 and PAN B2, separated by mean K2P and ML distances of 4.00 and 4.78%. These two clades correspond loosely to western Atlantic Panamá (WAP) and western Pacific Panamá (WPP), respectively (Fig. 2). Support for the three clades within Panamá was strong, with bootstrap values ranging from 91 to 100%.

Rooting of trees on *L. consocius* proved problematic for ML analyses based on the two substitution models chosen by MODELTEST. The first model, selected by the Akaike Information Criterion, was TrN + I with a rate matrix of A → C = 1.00; A → G = 12.69; A → T = 1.00; C → G = 1.00; C → T = 11.16; G → T = 1.00 and a proportion of invariable sites equal to 0.67 (–LnL = 1352.67). The second model, selected by the hierarchical likelihood ratio test, was K81uf + G with a substitution rate matrix of A → C = 1.00; A → G = 13.36; A → T = 1.20; C → G = 1.20; C → T = 13.36; G → T = 1.00 and a gamma parameter equal to 0.13 (–LnL = 1353.84). The two models recovered the same poorly resolved and weakly supported tree (data not shown) in which the South American *C. scorpioides* clade is placed between the PAN B1 and PAN A clades. The ML tree was appreciably longer (17 steps) and had a higher level of homoplasy than the MP trees (tree length = 179; CI = 0.737). Because the *L. consocius* and the *C. scorpioides* haplotypes exhibit high levels of sequence divergence at third positions (mean uncorrected pairwise distance equal to 45.4%), we investigated the possibility that the ML tree

was misleading as a consequence of substitutional saturation at third-positions. Restriction of phylogenetic analysis to first and second positions recovered a single MP (tree length = 21; CI = 0.952) and a single ML tree (–LnL = 512.67; HKY substitution model with Ti/Tv = 4.43) that were identical in topology and that placed the TRIN and FG haplotypes as outgroup taxa to the PAN A and PAN B clades.

When *L. consocius* was excluded from analysis and trees were rooted on the SA clade, MP and ML analyses carried out on all three codon positions produced concordant results. Of 439 total characters, 81 were polymorphic and 75 were parsimony informative, with an average nucleotide composition of 24.01% A, 17.65% C, 16.39% G and 41.94% T. A branch and bound parsimony analysis produced 28 MP trees that differed only slightly in topology (tree length = 111; CI = 0.820). Likelihood analysis, based on the TrN + I substitution model described above (see 'Haplotypes'), produced a most probable tree (–lnL = 1153.52; Fig. 2) that was almost identical to the *C. scorpioides* topology obtained from MP analysis of the *Lustrochernes*-rooted tree shown in Fig. 2. To test for rate constancy of nucleotide substitutions in *C. scorpioides*, we carried out a log-likelihood ratio test using PAUP. This analysis indicated that evolutionary substitutions in *C. scorpioides* mtDNA occur in an approximately clock-like fashion (–ln = 1162.50 clock enforced tree vs. 1153.52 nonclock tree, $\chi^2 = 17.96$, d.f. = 22, $P > 0.10$).

Inheritance of mtDNA haplotype

The translated sequences exhibited high amino acid sequence homology with mtDNA COI proteins from other arthropods (data not shown) and no missense or stop codons were found. The pattern of inheritance of mtDNA haplotype was examined in 12 crosses between males and females of differing mtDNA haplotype, as follows: (i) PAN A maternal haplotype × PAN B1 paternal haplotype ($N = 8$); (ii) PAN B1 maternal haplotype × PAN A paternal haplotype ($N = 3$); and (iii) PAN A maternal haplotype × PAN B2 paternal haplotype ($N = 1$). In all the 24 offspring assayed (1 male and 1 female offspring per cross), DNA sequencing demonstrated maternal inheritance of the mtDNA *COI* sequence. Taken together, these data provide clear evidence that our sequence analyses were not confounded by nuclear-mitochondrial pseudogenes.

Gene flow estimation

To estimate gene flow, *C. scorpioides* sequences were initially assigned to one of three populations representing major geographical regions: (i) South America (SA, comprising Trinidad and French Guiana), (ii) central Panamá (CP), and (iii) western Panamá (WP). This analysis was carried out using a three-island, full migration model, in which there were no a priori restrictions on gene flow. Pairwise estimates indicate moderate, unidirectional gene flow from central to western Panamá (MLE, $2N_fm = 1.507$; see Table 3). Migration between the two Panamanian populations and South America was estimated to be either nonexistent or extremely low. Based on calculation of the 95% profile confidence intervals, as implemented in MIGRATE (Table 3), only the South America to central Panamá migration rate was estimated to be significantly greater than zero (MLE, $2N_fm = 0.0467$). Given these initial results, CP was specified as the source population in a second MIGRATE simulation was run to estimate gene flow between the Panamanian populations. For this analysis, WP was subdivided into the two geographical areas separated by the central cordillera: western Atlantic Panamá (WAP) and western Pacific Panamá (WPP). Gene flow from central to western Panamá was highest on the Pacific slope of the cordillera (CP → WPP, $2N_fm = 1.2613$; CP → WAP, $2N_fm = 0.6090$). Within western Panamá, gene flow across the cordillera was unidirectional

Table 3 Maximum likelihood estimates of gene flow between *Cordylochernes scorpioides* populations from South America (SA), central Panamá (CP) and western Panamá (WP). The analysis was carried out using an unrestricted migration matrix model with variable subpopulation size. The ML estimate (bold) and the 95% profile confidence intervals (in parentheses, below) are shown for population sizes ($\Theta = 2N_f\mu$) and number of immigrant females per generation ($2N_fm$), where N_f is the female effective population size and μ is the mutation rate per generation per site

Population *i*	Θ	South America → *i*	Central Panamá → *i*	Western Panamá → *i*
South America	**0.0059** (0.0035–0.0115)	—	**0.0000** (0.0000–0.1430)	**0.0000** (0.0000–0.2718)
Central Panamá	**0.0037** (0.0026–0.0094)	**0.0467** (0.0351–0.2048)	—	**0.0000** (0.0000–0.0895)
Western Panamá	**0.0220** (0.0144–0.0433)	**0.0000** (0.0000–0.5626)	**1.5070** (0.5487–3.1605)	—

Table 4 Maximum likelihood estimates of gene flow between *Cordylochernes scorpioides* populations from central Panamá (CP), western Atlantic Panamá (WAP) and western Pacific Panamá (WPP). Based on the results shown in Table 3, the analysis was performed with central Panamá specified as the source population. Abbreviations as in Table 3

Population *i*	Θ	Central Panamá → *i*	Western Atlantic Panamá → *i*	Western Pacific Panamá → *i*
Central Panamá	**0.0076** (0.0053–0.0164)	—	—	—
Western Atlantic Panamá	**0.0069** (0.0033–0.0169)	**0.6090** (0.4318–2.3606)	—	**0.9770** (0.1821–3.5262)
Western Pacific Panamá	**0.0149** (0.0059–0.0558)	**1.2613** (0.3626–5.3773)	**0.0000** (0.0000–0.0006)	—

from the Pacific to the Atlantic side (WPP → WAP, $2N_fm$ = 0.9770; WAP → WPP, $2N_fm$ = 0.0000 (Table 4).

Discussion

Classical taxonomy based on morphological characters classifies many plants and animals as single species with pan-neotropical distributions. For example, the fig tree, *Ficus insipida* (Croat 1978), the boas, *Boa constrictor*, *Corallus enydris* and *Epicrates cenchria* (Henderson & Hedges 1995), the macaws, *Ara macao* (Ridgely 1981) and *Ara militaris* (Wege & Long 1995), and the paca, *Agouti paca* (Eisenberg 1989) are each classified as a single species, ranging throughout the Neotropics from Central America to southern Brazil or northern Argentina. However, the validity of such morphologically based species designations is being called into question, as a growing number of molecular phylogeographical studies demonstrates pronounced genetic differentiation over relatively short distances in several putative species of neotropical birds (Seutin *et al*. 1993), freshwater fishes (Bermingham & Martin 1998; Perdices *et al*. 2002), frogs (Ryan *et al*. 1996) mammals (da Silva & Patton 1998) and snakes (Henderson & Hedges 1995).

Similarly, this phylogeographical study of *Cordylochernes scorpioides* suggests that the species status of many terrestrial arthropods with apparently pan-neotropical distributions may also need to be reassessed. The harlequin beetle-riding pseudoscorpion was described by Beier (1948) as a single species, ranging from Costa Rica to southern Brazil, on the basis of his morphological examination of hundreds of specimens from several countries in South and Central America. However, the results of our mtDNA *COI* sequencing study demonstrate extensive genetic differentiation between *C. scorpioides* populations from Panamá and northern South America, with a maximum ML nucleotide divergence of almost 33%. This extreme level of divergence is associated with complete postzygotic incompatibility between *C. scorpioides* individuals from central Panamá and both French Guiana (Zeh & Zeh 1994) and Trinidad (JA Zeh, unpublished data). Not surprisingly, our MIGRATE simulations suggest essentially no gene flow between the South American and Isthmian populations.

Populations of *C. scorpioides* from South and Central America also differ dramatically in levels of haplotype diversity. Whereas sequence variation both within and between populations in Trinidad and French Guiana is quite limited, the Panamanian isthmus supports three polymorphic and highly divergent clades (PAN A, PAN B1 and PAN B2), with mtDNA haplotypes that differ by up to 11% in ML genetic distance. In the Bocas del Toro province of western Atlantic Panamá, the level of within-population sequence polymorphism is particularly striking, with representatives of all three Panamanian clades collected from a single decaying *Ficus* tree. Likewise, haplotypes from both the PAN B1 and PAN B2 clades were found in a group of three WAP pseudoscorpions dispersing together on a harlequin beetle. Only in the central region of Panamá was sequence diversity limited, with all individuals clustering into the relatively uniform PAN A clade. The sympatry of the three highly divergent mtDNA haplotype clades in western Panamá points to the existence of a cryptic species boundary in this region. Consistent with this interpretation are preliminary results from ongoing laboratory crosses that indicate partial breakdown in postzygotic reproductive compatibility between individuals from the PAN A, PAN B1 and PAN B2 clades (JA Zeh, unpublished data). In addition, the pattern of gene flow that emerges from our coalescent analysis of migration rates indicates moderate levels of gene flow from central to western Panamá along both sides of the central cordillera. Within western Panamá, gene flow appears to be unidirectional from the Pacific to the Atlantic versant of the cordillera. Presumably, western Panamá represents the southeastern limit of the PAN B clade, and sampling of *C. scorpoides* populations from Costa Rica is now required to determine the geographical distribution of this mtDNA lineage.

Our analysis of mtDNA evolutionary rate constancy failed to reject a molecular clock for *Cordylochernes*, indicating that genetic distances can be used to estimate approximate divergence times between *C. scorpioides* clades. We therefore used a molecular clock approach to provide a temporal framework for assessing the diversification of *C. scorpioides* mitochondrial lineages within the context of the major geological events that shaped the formation of the Isthmus of Panamá. The absence of appropriate data for direct calibration of a *Cordylochernes* mtDNA clock necessitated reliance on external rate calibration. We adopted the calibration of 2.3% pairwise divergence per million years, estimated by Brower (1994) on the basis of uncorrected or K2P mtDNA nucleotide distances in a variety of recently diverged arthropods. When applied to the K2P distances for *C. scorpioides*, this rate resulted in an estimate of divergence time between populations from South America and Panamá that corresponds approximately to the Miocene/Pliocene boundary (mean clade divergence of 5.3 Myr; Table 2). Within the Panamanian clades, estimated divergence times were 2.9 Myr between central and western Panamá (PANA and PANB), and 1.6 Myr between western Atlantic Panamá and western Pacific Panamá (PAN B1 and PAN B2). The 2.3% calibration yielded higher divergence times when applied to the ML nucleotide distances, particularly for the more diverged clades: 10.0 Myr between the clades from South America and Panamá; 3.9 Myr between central and western Panamanian clades, and 1.9 Myr between the two western Panamanian clades. These ML-based estimates, although still providing western and central Panamá divergence times consistent with the B/M model, may also shed light on the deep divergence between *C. scorpioides* from Panamá and South America east of the Andes mountains. The 10 Myr, ML-based age of the split between South American and Panamanian *C. scorpioides* lineages corresponds approximately to the timing of final uplifting of the Andes, calculated from geological data to have occurred 8.5–8 Ma (Lundberg *et al*. 1998). However, it is important to note that the divergence times based on ML genetic distances are highly tentative, given the absence of an appropriate maximum likelihood calibrated clock for terrestrial arthropods.

The biogeographical distribution of *C. scorpioides* mtDNA lineages detected in this study yields a phylogenetic signal of the geological events associated with the emergence of the Isthmus of Panamá. Our analysis reveals a deep split between western and central Panamanian clades that provides compelling evidence for an early colonization event followed by isolation and re-colonization of the Isthmus. The estimated divergence time between the two main Panamanian clades is largely consistent with the emergence of a short-lived terrestrial corridor at the close of the Miocene (5–7 Myr BP), as proposed by Bermingham & Martin (1998). This would have enabled a first wave of colonization out of South America, across the corridor and into what is now Costa Rica. Subsequent inundation of this nascent Isthmus during the Pliocene sea level rise (Haq *et al*. 1987) would have resulted in mass extinction of isthmian terrestrial and freshwater taxa. According to the B/M model, colonists surviving in Costa Rica's high-elevation Talamanca region became genetically isolated from source populations in northwestern South America. Interestingly, the most highly diverged of the *C. scorpioides* mtDNA haplotypes in this study (the PAN B clade) were collected from western Panama, near the eastern edge of the Talamanca range. The B/M model then posits a second, geographically more limited wave of colonization that reached western Panamá. This coincided with final completion of the Isthmus ≈3 Myr BP (Coates & Obando 1996), after convergence of the South American and Caribbean plates resulted in uplifting of eastern Panamá and northwestern Colombia. We propose that a second wave of colonization out of northwestern South America is responsible for the current distribution of the *C. scorpioides* PAN A clade that extends across much of the Isthmus as far as western Panamá. The absence of phylogeographical structure within central Panamá suggests that the late-Pliocene colonization of the Isthmus by *C. scorpioides* occurred with a rate of spread even more rapid than that documented for freshwater fishes (Bermingham & Martin 1998), presumably as a consequence of the dispersal ability of the pseudoscorpion's harlequin-beetle host (see Zeh *et al*. 2003).

Because the Bocas del Toro region of western Panama harbours the greatest diversity of mtDNA haplotypes, it might be argued that this region represents a 'centre of dispersal' and that the posited second wave of colonization of the Isthmus took place in a west-to-east direction, that is, with an origin in Costa Rica rather than in South America. However, this hypothesis is not consistent with the results obtained from our MIGRATE analysis. Unlike traditional F_{ST}-based methods for estimating migration rates, the coalescent-based, maximum-likelihood approach in MIGRATE is capable of evaluating not only the magnitude, but also the direction of gene flow. Results from the MIGRATE simulations identified central Panamá as the source of the PAN A (central Panamá) clade individuals in western Panamá and not vice versa. Moreover, the western origin hypothesis requires additional, ad hoc explanations to account for the absence of the PAN B1 and B2 clades in central Panamá. It therefore seems to provide a less parsimonious explanation for the geographical distribution of *C. scorpioides* haplotypes in Panamá than the South American origin hypothesis. Nonetheless, determining the mtDNA haplotypes of individuals from Costa Rica and northwestern Colombia is clearly required to resolve this issue.

Intriguingly, a shared history defined by the geological development of the Isthmus has led to very different genetic consequences in the case of the pseudoscorpion's

obligate dispersal agent, the giant harlequin beetle, *Acrocinus longimanus* (Beier 1948; Zeh & Zeh 1992). Throughout its range, the pan-neotropical *A. longimanus* co-occurs with *C. scorpioides* and is a pioneer species in the diverse community of saproxylic invertebrates that utilizes decaying trees in the families Moraceae and Apocynaceae. *C. scorpioides* gains access to these rich but patchily distributed and ephemeral habitats by hitchhiking under the elytra of the beetle (Zeh & Zeh 1992). Because of this obligate association, pseudoscorpion colonization is restricted to the brief period when newly fallen or dying trees attract *A. longimanus* males and females for mating and oviposition (Zeh *et al*. 1992b). The *C. scorpioides* populations in dead trees then remain marooned for two or three generations until the harlequin beetle larvae complete development and the pseudoscorpions can climb on board newly eclosed adult beetles to disperse *en masse*.

Because *C. scorpioides*' capacity for colonization is strictly dictated by dispersal of the harlequin beetle, it was anticipated that the pattern and depth of cladogenesis in the pseudoscorpion and the beetle would yield clear phylogenetic signals of shared biogeographical history. This proved not to be the case. A recent mtDNA *COI* phylogenetic study of *A. longimanus* (Zeh *et al*. 2003) produced a phylogeny of the harlequin beetle topologically similar to that of its hitchhiking pseudoscorpion. However, there is no apparent congruence between timing of branching events in the harlequin beetle phylogeny and either the temporal pattern of phylogenesis in the pseudoscorpion or the B/M model of dispersal and vicariance. Even with adjustment for an approximate twofold difference in generation time, the pseudoscorpions still exhibit estimated divergence times that are an order of magnitude greater than those in the beetles (Zeh *et al*. 2003).

Such a lack of concordance could be explained by a pseudoscorpion/harlequin beetle relationship that evolved only after the first postulated colonization of Central America from South America 5–7 Ma. However, this hypothesis seems unlikely and is not parsimonious, involving, as it would, multiple, independent origins of the complex suite of behaviours associated with dispersal on harlequin beetles (Zeh & Zeh 1992). An alternative hypothesis invokes difference between *C. scorpioides* and *A. longimanus* in their mode of reproduction (Zeh *et al*. 2003). Whereas the pseudoscorpion is viviparous (Zeh 1997), the harlequin beetle is oviparous. It has been argued elsewhere that, because viviparity involves an intimate relationship between the developing embryo and its mother, postzygotic isolation should evolve more rapidly in live-bearing species than in oviparous ones (Zeh & Zeh 2000, 2001). Consequently, the threshold above which genetic divergence translates into genetic incompatibility and reproductive isolation may be significantly lower in the viviparous pseudoscorpion than in the egg-laying beetle. In the context of the reproductive mode hypothesis, the ≈ 3 Myr hiatus between the postulated early wave of colonization across the Panamanian terrestrial corridor and the second wave appears to have resulted in genetic divergence sufficient to create barriers to gene flow in *C. scorpioides* but not in *A. longimanus*. We hypothesize that, in the harlequin beetle, the phylogenetic signal of colonization and vicariance associated with the formation of the Isthmus of Panamá has been obscured, though not fully erased, by historical and contemporary gene flow. Intrinsic biological characteristics may thus have played a significant role in determining the extent to which the extrinsic geological events associated with the rise of the Isthmus of Panamá have shaped phylogeographical patterns in the pseudoscorpion and its harlequin beetle host.

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