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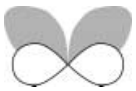

Phylogeography of the giant harlequin beetle (*Acrocinus longimanus*)

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Abstract

Aim The extent to which cryptic species contribute to neotropical diversity remains inadequately investigated. Based on its highly distinctive morphology, the giant harlequin beetle, *Acrocinus longimanus*, is currently described as a single species, ranging from southern Mexico to northern Argentina. However, the discovery of cryptic species in *Cordylochernes scorpioides*, a pseudoscorpion with obligate dependence on the harlequin beetle for dispersal, strongly suggests the existence of barriers to gene flow in *A. longimanus*. The aim of this study was therefore to determine whether levels of DNA divergence between geographical populations provided evidence of genetically distinct lineages in the harlequin beetle.

Location Trinidad and Panamá.

Methods Sequencing of 1245 bp of the mitochondrial cytochrome oxidase I (COI) gene of *A. longimanus* from seven locations in Trinidad and Panamá.

Results Mitochondrial haplotype diversity in the harlequin beetle shows limited evidence of geographical structuring, with a maximum sequence divergence between populations of only 1.29%. This is an order of magnitude less than the level of COI divergence between harlequin beetle riding pseudoscorpions from the same geographical locations.

Main conclusions The molecular data on populations from northern South America and Panamá are consistent with the current, morphologically based classification of *A. longimanus* as a single, pan-neotropical species. In addition, the relatively low level of population divergence detected in this study indicates that speciation in the hitchhiking pseudoscorpion has occurred in the absence of significant barriers to gene flow in its beetle host. It is proposed that, in the harlequin beetle, the phylogenetic signal of colonization and vicariance associated with the formation of the Isthmus of Panamá has been obscured, although not fully erased, by historical and contemporary gene flow.

Keywords
Acrocinus longimanus, *Cordylochernes scorpioides*, harlequin beetle, mitochondrial DNA, Neotropics, Panamá, phylogeography, Trinidad.

INTRODUCTION

As deforestation continues to fragment habitat at an accelerating pace, the need to map geographical boundaries of genetically distinct and reproductively cohesive populations is particularly pressing for species inhabiting neotropical rain forest environments. Traditional systematics based on morphological characters currently classifies many neotropical plants and animals as single species with extensive geographical ranges. 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 adequacy of such morphologically based species designations is being called into

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question as a result of recent molecular studies, demonstrating 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).

For neotropical, terrestrial invertebrates, much less is known of the geographical scale over which genetic divergence occurs. Exceptions are *Heliconius* butterflies (Brower, 1996) and *Cordylochernes scorpioides* pseudoscorpions (Wilcox *et al.*, 1997). In this pseudoscorpion, minimal morphological differentiation between populations is associated with up to 14% mitochondrial DNA (mtDNA) sequence divergence (Wilcox *et al.*, 1997) and either partial or complete breakdown in postzygotic reproductive compatibility (Zeh & Zeh, 1994a, J.A. Zeh, unpubl. data). The discovery of a *C. scorpioides* cryptic species complex, in what was previously described as a single, pan-neotropical species (Beier, 1948), suggests that the species boundaries of other apparently broadly distributed neotropical invertebrates may need to be reassessed. Such a reassessment seems particularly warranted in the case of the giant harlequin beetle, *Acrocinus longimanus*, the obligate dispersal agent of *C. scorpioides* (Beier, 1948; Zeh & Zeh, 1992, 1994a,b).

The harlequin beetle is currently described as a single species, ranging from southern Mexico to northern Argentina (see Duffy, 1960; Chemsak, 1983). Named for the swirling pattern of black, greenish-yellow and crimson or orange markings on its thorax and elytra, this large cerambycid beetle has a highly distinctive morphology, with males possessing greatly elongated forelegs (Zeh *et al.*, 1992b). Throughout its range, *A. longimanus* is a pioneer species in the diverse community of saproxylic invertebrates that utilizes decaying trees in the families Moraceae (e.g. *Ficus* spp., *Brosimum* spp. and *Bagassa guianensis*) and Apocynaceae (e.g. *Parahancornia fasciculata*). Newly dead and dying trees attract *A. longimanus* males and females for mating, oviposition and larval development (Zeh *et al.*, 1992b). The subsequent wood-boring activity of the harlequin beetle larvae is critical to creating the microhabitat of exfoliating bark and accumulated 'sawdust' that is used as a breeding site by the many invertebrate species that then colonize the dead tree. *Cordylochernes 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 beetles for mating and oviposition (Zeh *et al.*, 1992b). *Cordylochernes 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*.

As a consequence of the wood-boring activity of its larvae, the harlequin beetle serves as the keystone species for a diverse community of neotropical, organisms that utilize decaying trees (Zeh & Zeh, 1994b). Such saproxylic invertebrates play a critically important role in the process of nutrient recycling in forests, as has been recognized by the Council of Europe which established a special programme to develop conservation strategies for their protection (Speight, 1989). Given the goal of maximizing biodiversity under the constraint of limited resources, a major focus of conservation genetics is identifying genetically distinct populations/species as priorities for conservation (Avise, 1994, 2000; Moritz, 1994). With the discovery of cryptic species in the harlequin beetle riding pseudoscorpion, the aim of this study was to characterize levels of DNA divergence between geographical populations of *A. longimanus* in order to reevaluate the beetle's status as a single, pan-neotropical species.

METHODS

Collections

Beetles for this study were collected from six sites spanning 344 km within Panamá and from one location in north central Trinidad (Table 1). Individuals were collected either as adults attracted to newly dead or dying trees or as larvae from trees at a more advanced stage of decomposition. Specimens were either frozen on dry ice in the field or maintained alive before being stored at −80 °C.

DNA extraction, polymerase chain reaction, and sequencing

Total genomic DNA was extracted from each individual using the 2X CTAB protocol described elsewhere (Zeh *et al.*, 1992a). A *c.* 1263 bp fragment of the mitochondrial cytochrome oxidase subunit 1 (COI) gene, corresponding to the region between 1751 and 3014 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 TL2-N-3014 (alias Pat) (5′-TCCAATGCACTAATCTGCCATATTA-3′) (Simon *et al.*, 1994). The 25-μL polymerase chain reaction (PCR) mix contained *c.* 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 U Titanium *Taq* DNA polymerase (Clontech, Palo Alto, USA). PCR amplification conditions involved an initial 2 min melting step at 94 °C, followed by thirty-two iterations of the following cycle: 94 °C for 60 s, 56 °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, Carlsbad, USA) stained with ethidium bromide. Amplification products were excised from gels and purified using Promega Wizard minicolumns.

Attempts to obtain clean sequence using the primer C1-J-1751 were unsuccessful. Therefore, as an alternative to sequencing in both directions, the amplified COI fragment was first sequenced, using the TL2-N-3014 primer. From the resulting sequences, a second N-strand sequencing primer

Table 1 Specimen ID and locality of *A. longimanus* individuals sequenced for this study

Specimen ID	Collection site	Latitude/Longitude	Population
ODA1	Ojo de Agua, Chiriqui Province, Panamá	8°44′ N/82°44′ W	WPP
ODA2	Ojo de Agua, Chiriqui Province, Panamá	8°44′ N/82°44′ W	WPP
ODA3	Ojo de Agua, Chiriqui Province, Panamá	8°44′ N/82°44′ W	WPP
BDT1	Bocas del Toro Province, Panamá	9°00′ N/82°16′ W	WAP
BDT2	Bocas del Toro Province, Panamá	9°00′ N/82°16′ W	WAP
BDT3	Bocas del Toro Province, Panamá	9°00′ N/82°16′ W	WAP
SOLAR	Isla Solarte, Bocas del Toro, Panamá	9°21′ N/82°14′ W	WAP
ESTI	Rio Esti, Chiriqui Province, Panamá	8°26′ N/82°17′ W	WAP
FORT	La Fortuna, Chiriqui Province, Panamá	8°43′ N/82°14′ W	WAP
MAD1	Camino Madden, Panamá Province, Panamá	9°05′ N/79°37′ W	CP
MAD2	Camino Madden, Panamá Province, Panamá	9°05′ N/79°37′ W	CP
TRIN1	Blanchisseuse Road, Trinidad	10°45′ N/61°19′ W	TRIN
TRIN2	Blanchisseuse Road, Trinidad	10°45′ N/61°19′ W	TRIN
TRIN3	Blanchisseuse Road, Trinidad	10°45′ N/61°19′ W	TRIN

WPP, western Pacific Panamá; WAP, western Atlantic Panamá; CP, central Panamá; TRIN, Trinidad.

(5′-CATACAACAAATCCTAATAAACC-3′) was designed and used to obtain nearly complete sequence for the 1263-bp fragment. BigDye sequencing reactions (6 μL) containing *c.* 100 ng of purified PCR product and 3.2 pmol of primer were analysed using an ABI Prizm 3730 automated sequencer, according to the manufacturer's protocols (PE Applied Biosystems, Foster City, CA, USA).

Phylogenetic methods

Contig sequences were compiled in MacDNASIS (Hitachi software, v3.2, 1994, San Bruno, CA, USA) by joining the two N-strand sequences from each individual via a 34-bp overlapping region. As it is the J-strand that is transcribed for most insect mitochondrial genes (Simon *et al.*, 1994), phylogenetic analyses were performed on the complement of each sequence. Sequences were manually aligned using the MacDNASIS multiple sequence editor. An NCBI BLAST search of GenBank identified the milkweed beetle, *Phaea maryannae* (accession number AF267467; Farrell, 2001), as the most closely related cerambycid for which mtDNA COI sequence data are available. The *P. maryannae* sequence was therefore used as the outgroup in phylogenetic analyses in order to root the *A. longimanus* trees. Branch and bound parsimony and maximum likelihood (ML) searches were carried out on aligned sequences using PAUP v4.0b8 (Swofford, 2002) and all minimal trees were saved. To determine the most appropriate model for the likelihood analyses, hierarchical likelihood ratio tests were performed using MrModel Test v1.1b (Posada & Crandall, 1998). For both tree estimation procedures, support for each clade was estimated by analysing 1000 bootstrap replicate data sets (Felsenstein, 1985). Sequences were translated in McClade v4.05 (Maddison & Maddison, 2002) using the invertebrate mitochondrial code for analysis of amino acid substitutions.

To assess population genetic structure and to estimate gene flow, harlequin beetle sequences were assigned to one of four populations: (1) Trinidad (TRIN), (2) central Panamá (CP), (3) western Pacific Panamá (WPP), or (4) western Atlantic Panamá (WAP) (Table 1). The Panamá populations correspond to biogeographical regions predicted by a recently proposed model of Panamanian historical biogeography (Bermingham & Martin, 1998; see Discussion for explanation). An analysis of molecular variance (AMOVA), as implemented in Arlequin v2.000 (Schneider *et al.*, 2000), was used to calculate the proportion of total genetic variation occurring between populations (F_{st}). Gene flow between pairs of populations was estimated as the absolute number of migrants per generation (M), where $M = (1-F_{st})/2F_{st}$ (Schneider *et al.*, 2000, pp. 98–99).

RESULTS

The mean, uncorrected pairwise distance (p) between the *P. maryannae* sequence and the *A. longimanus* sequences was 13.86% (range: 13.67–13.99%; Table 2), with amino acid substitutions occurring at thirty-two of 415 positions. 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, suggesting that the sequences were obtained from genuine mitochondrial genes. A branch and bound search revealed two most parsimonious (MP) trees differing very slightly in topology (tree length = 210; CI = 0.938; RI = 0.772; RC = 0.724; HI = 0.062). Both MP trees identified the Trinidadian sequences as a monophyletic sister

Table 2 Estimated migration rates (above diagonal) and F_{st} values (below diagonal) for pairs of *A. longimanus* populations

Population	WPP	WAP	CP	TRIN
WPP		0.721	3.396	0.189
WAP	0.409		0.752	0.234
CP	0.128	0.399		0.290
TRIN	0.726	0.681	0.633	

WPP, western Pacific Panamá; WAP, western Atlantic Panamá; CP, central Panamá; TRIN, Trinidad.

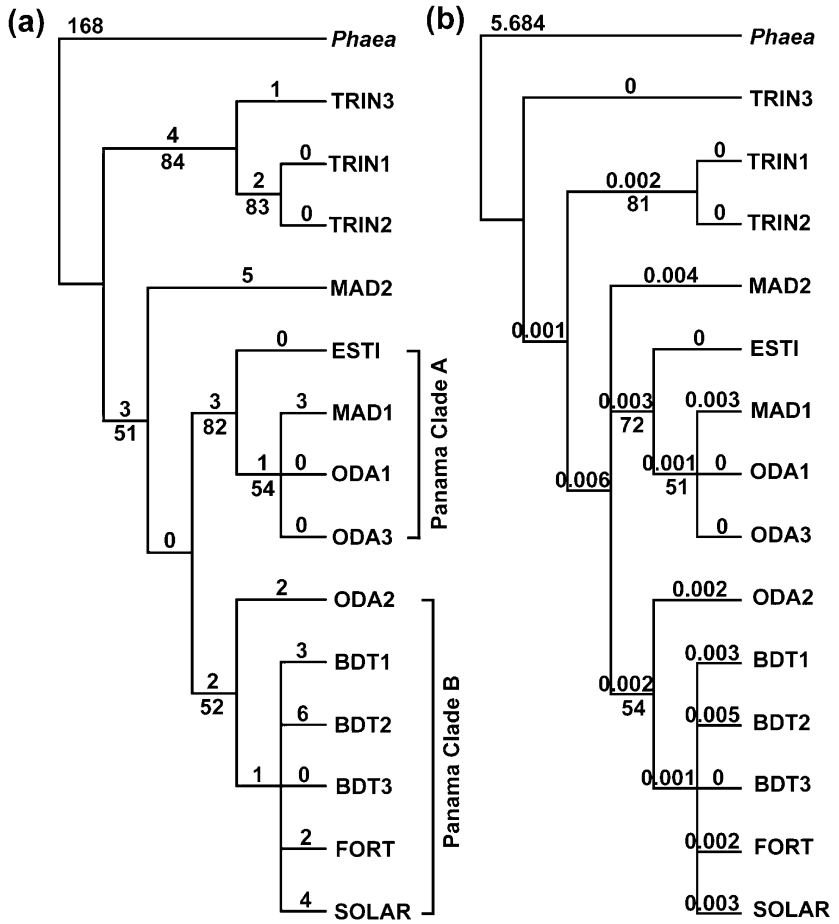

Figure 1 Phylogenetic relationships of *Acrocinus longimanus* populations from Trinidad and Panamá based on mtDNA COI sequences: (a) one of two MP trees, (b) one of two ML trees. In each case, the most highly resolved tree is shown. The alternative MP tree differs from the tree shown in that the MAD2 sequence, Panamá Clade A and Panamá Clade B comprise an unresolved trichotomy. In the alternative ML tree, TRIN3 is the sister taxon of the Panamá sequences. Numbers above branches indicate lengths (number of character state changes or likelihood distance) and values below branches represent bootstrap estimates (>50%). For specimen codes, see Table 1.

group of the Panamanian clade (Fig 1a). Likelihood analysis, based on the HKY+ I model of nucleotide substitution with a 21.66 transition/transversion ratio (Ti/Tv) and a proportion of invariant sites equal to 0.79, yielded two most probable trees ($-\ln L = 2527.63$). The likelihood trees differed from the parsimony trees in placing the Trinidadian sequences as a paraphyletic outgroup of the Panamanian clade (Fig. 1b).

Sequence variation in *A. longimanus*

Analysis of the COI sequence from fourteen *A. longimanus* Panamanian and Trinidadian individuals revealed twelve haplotypes (GenBank accession numbers AY248720–AY248731), with an average nucleotide composition of 31.8% A, 14.7% C, 14.9% G and 38.6% T. Pairwise sequence divergence ranged from 0% to 1.29%. Of the 1245 bp sequenced, 1208 sites were invariant and seventeen of the remaining thirty-seven polymorphic sites were parsimony informative. With the exception of one first-position transition, the informative substitutions all occurred at third positions, with a Ti/Tv of 16, and there were no amino acid substitutions. The mean pairwise divergence ($\bar{p}$) between the Panamanian and Trinidadian sequences was 1.06% but was reduced to 0.66% when corrected for within-site variation ($\bar{p}_{corr} = \bar{p} - 0.5[\bar{p}_{Trinidad} + \bar{p}_{Panama}]$); Avise, 1994, p. 96). Both MP and ML analyses grouped nine of the ten Panamanian haplotypes into two highly polymorphic clades (A and B), although bootstrap support was only moderate for Clade A and weak for Clade B (Fig. 1). Mean sequence divergence between these Panamanian A and B clades was 0.81% ($\bar{p}$) or 0.49% ($\bar{p}_{corr}$).

Population genetic structure and gene flow

Overall, between-population variation accounted for 54.3% of the total variation among haplotypes ($F_{st} = 0.543$). Between pairs of populations within Panamá, F_{st} values were between 0.128 and 0.409, with M values ranging from 0.721 to 3.396. Corresponding values between the Trinidadian population and each of the three Panamanian populations were: $F_{st} = 0.633$ to 0.726 and $M = 0.189$ to 0.290 (Table 2).

DISCUSSION

The results of this mtDNA COI sequencing study of populations from northern South America and Panamá are

consistent with the current, morphologically based classification of *A. longimanus* as a single, pan-neotropical species. Over a span of 2355 km, harlequin beetles from Panamá and Trinidad exhibited a maximum sequence divergence of 1.29%, a value markedly lower than that reported for many other neotropical species (e.g. Seutin *et al.*, 1993; da Silva & Patton, 1998; Perdices *et al.*, 2002). This relatively low level of variation between sites contrasted with high levels of polymorphism within locations. For example, three harlequin beetles, collected at the same time from a single fallen tree in Bocas del Toro, Panamá, possessed three distinct haplotypes, differing by as much as 0.72% in their sequence. Similarly, the haplotypes of two beetles collected <1 km apart along El Camino Madden in CP exhibited 0.80% sequence divergence. Within Panamá, haplotypes were clustered into two polymorphic clades, with interclade divergence nearly as great as that between Panamá and Trinidad.

The relatively low level of geographical structuring in *A. longimanus* detected here seems paradoxical, in light of the extreme geographical differentiation that exists in the pseudoscorpion, *C. scorpioides* (Wilcox *et al.*, 1997). The harlequin beetle riding pseudoscorpion co-occurs with *A. longimanus* throughout the Neotropics and depends exclusively on the beetle for dispersal between the ephemeral and patchily distributed habitats of decaying trees (Zeh & Zeh, 1992, 1994a,b). In *C. scorpioides*, the pattern of low between-site but high within-site mtDNA variation detected in *A. longimanus* is reversed. In a study of COI sequence divergence in *C. scorpioides* (Wilcox *et al.*, 1997), individuals from WPP, CP and TRIN clustered into three highly divergent geographical lineages, with little or no variation within clades. Mean, corrected sequence divergence in *C. scorpioides* was 13.10% between WPP and TRIN, 10.77% between CP and TRIN, and 7.86% between WPP and CP. This lack of concordance is unexpected since, barring intrinsic biological differences, the obligate association between *C. scorpioides* and *A. longimanus* (Beier, 1948; Zeh & Zeh, 1992) would be predicted to result in parallel cladogenesis in the two lineages as a consequence of their shared history of climatic and geological events (Hafner & Page, 1995; Page & Charleston, 1998).

Important geological events underlying phylogeographical patterns in *A. longimanus* and *C. scorpioides* are undoubtedly those associated with the emergence of the Isthmus of Panamá. This land bridge gave rise to the 'Great American Interchange' of species between the continents of North and South America (Marshall *et al.*, 1979; Stehli & Webb, 1985). Recent molecular phylogenies of neotropical freshwater fishes strongly suggest that formation of the Isthmus involved the emergence of a short-lived terrestrial corridor between 5.3 and 5.7 Ma, resulting in an early episode of colonization out of South America, across the corridor and into what is now Costa Rica (Bermingham & Martin, 1998). 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 taxa. According to the Bermingham/Martin (B/M) model, colonists surviving in Costa Rica's high-elevation Talamanca region became genetically isolated from source populations in north-western South America. Convergence of the South American and Caribbean plates, resulting in uplifting of eastern Panamá and north-western Colombia, brought about final completion of the Isthmus *c.* 3 Ma (Coates & Obando, 1996) and a second, geographically more limited wave of colonization extending as far as western Panamá. The B/M model for the historical biogeography of lower Central America is well supported in the marine record, with evidence of staggered genetic isolation between transisthmian sister taxa in snapping shrimp (Knowlton & Weigt, 1998), echinoderms (Lessios, 1998) and fishes (Bermingham *et al.*, 1997). Moreover, the hypothesized history of colonization, extinction, divergence and re-colonization of the Isthmus precisely predicts the pattern of deep divergence between clades exhibited by the *C. scorpioides* pseudoscorpion cryptic species complex (Wilcox *et al.*, 1997).

Because *C. scorpioides* capacity for colonization is strictly dictated by dispersal of the harlequin beetle, it was anticipated that our study of mtDNA sequence divergence in *A. longimanus* would yield clear phylogenetic signals of shared biogeographical history. This proved not to be the case. With a paraphyletic outgroup in Trinidad, and two main mtDNA clades in Panamá, the phylogeny of *A. longimanus* is 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 cladogenesis in the pseudoscorpion or the B/M model of dispersal and vicariance. Even with an 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. Assuming a COI divergence rate for Cerambycidae of *c.* 1.5% per million years (Farrell, 2001), the estimated divergence time in *A. longimanus* between the Panamanian and Trinidadian clades is 0.71 My, compared to a within-Panamá divergence between clade A and clade B of 0.54 My.

It is thus clear that a shared history defined by the geological development of the Isthmus of Panamá has led to very different genetic consequences in the two lineages. In *C. scorpioides*, phylogeny and contemporary patterns of reproductive isolation (Zeh & Zeh, 1994a; Wilcox *et al.*, 1997; J.A. Zeh, unpubl. data) suggest that, if the B/M model is correct, the allopatric divergence that followed inundation of the proto-Isthmus was sufficient to establish barriers to gene flow between the diverged mtDNA lineages when the Isthmus was completed and the second wave of colonization occurred. By contrast, the phylogeny of *A. longimanus* displays little evidence of barriers to gene flow. The F_{st} and M values reported here for the harlequin beetle fall within the range of those reported for other species exhibiting moderate levels of population structure (McKay & Latta, 2002). The high intra- but low inter-site haplotype diversity exhibited by the harlequin beetle is characteristic of a history of periodic separation and fusion of populations in which genetic

diversity accumulates but gene flow overwrites earlier history (Takahata, 1993; Bermingham & Martin, 1998).

How can taxa with such a tight dispersal relationship exhibit such differing phylogeographical patterns? One explanation for the lack of concordance might be that the pseudoscorpion's dispersal relationship with the harlequin beetle evolved only after the first postulated colonization of Central America from South America 5.3–5.7 Ma. However, this hypothesis is not parsimonious, as it would involve multiple, independent origins of the complex suite of behaviors associated with dispersal on harlequin beetles (Zeh & Zeh, 1992). Such an explanation therefore seems unlikely. As an alternative, we suggest that one potentially important contributing factor may be that *A. longimanus* and *C. scorpioides* differ in their mode of reproduction. The harlequin beetle is oviparous whereas the pseudoscorpion is viviparous (Zeh, 1997). 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 one (Newcomer *et al.*, 1999; 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 its egg-laying beetle host.

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