Interspecific phylogeography of the *Stator limbatus* species complex: The geographic context of speciation and specialization

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Received 3 March 2004; revised 28 March 2005

Abstract

Diversification in phytophagous insects is often attributed to a propensity toward specialization and to a tendency for speciation to be associated with host-shifts. Phylogenetic analysis revealed a sister relationship between the generalist *Stator limbatus* and the specialist host-shifted *Stator beali*, providing a system to examine the genealogical and geographic origins of the main processes involved in this diversification: host-shifts, specialization, and reproductive isolation. We examine the interspecific phylogeographic relationships between these species using mitochondrial DNA sequence data. *S. beali* is derived within *S. limbatus*, rendering the latter paraphyletic and suggesting a budding process of speciation. The inherent polarity in this genealogical pattern indicates that the specialist habit, clumping oviposition behavior, and distinct genitalia of *S. beali* are all derived from the ancestral *S. limbatus*. The phylogeography of *S. limbatus* also shows strong geographic structure with divergences corresponding to known biogeographic boundaries, indicating that this evolutionary signal has not been erased by the vagaries of history. However, the derivation of *S. beali* and the evolution of reproductive isolation between the two species does not correspond to these known biogeographic boundaries, as *S. beali* and its sister clade of *S. limbatus* are restricted to the same geographic province. The geographic proximity of diversification combined with a divergence time estimated at the beginning of the Pleistocene indicates that speciation likely occurred very rapidly, although further genetic and ecological work is necessary to examine the mode of speciation. This study provides the historical context for ongoing evolutionary, ecological, and quantitative genetic research on the divergence in diet breadth between these species.

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Keywords: Phylogeography; Specialization; Speciation; Stator; Bruchinae; Molecular systematics; Paraphyly; Cytochrome Oxidase I; Mitochondrial DNA

1. Introduction

According to the ecological theory of adaptive radiation, evolutionary diversification (that is, genetic or species diversification) occurs as lineages enter previously unoccupied ecological adaptive zones. These lineages diverge from the parental species, specialize onto discrete resources, and species diversity increases with the onset of reproductive isolation (Schluter, 2000; Simpson, 1944). There appears to be considerable evidence in support of this model, including evidence from phytophagous insects. The observation that the majority of plant species serve as hosts to insect species that are specialized to one degree or another (Bernays and Chapman, 1994; Futuyma and Moreno, 1988; Jaenike, 1990; Thompson, 1994), and that the majority of speciation events in phytophagous insects are accompanied by
shifts in host plants (Ehrlich and Raven, 1964; Farrell, 1998; Funk et al., 1995; Futuyma and Mitter, 1996; Mitter et al., 1988; Thompson, 1994) has been advanced as supporting the idea that the divergent evolution of plant defenses has led to the subsequent divergence of insect species (Ehrlich and Raven, 1964). This model also suggests that selection favors specialization through competitive interactions and trade-offs in performance, and causes resource generalists to give rise to specialized daughter species more often than the reverse occurs (Schluter, 2000; Thompson, 1994). Phylogenetic results “provide little support for the generalists-to-specialists hypothesis” (Schluter, 2000, p. 48), and the results from phytophagous insects are certainly mixed (Dobler et al., 1996; Kelley and Farrell, 1998; Moran, 1988; Termonia et al., 2001; see review in Nosil, 2002). However, the recent review of species polyphyly by Funk and Omland (2003) indicates that a process of budding speciation may be common, and species-level phylogenies would not adequately identify the generalist-to-specialist polarity inherent in such a process. In budding speciation, widespread, eurytopic species give rise to geographically and ecologically restricted stenotopic species by a number of speciation mechanisms. Explicit phylogeographic investigations of species complexes showing variation in diet breadth have provided some insights into this process in phytophagous insects (Brown et al., 1994; Funk, 1998, 1999; Knowles et al., 1999). In this study, we extend this by examining the phylogeography of the S. limbatus species complex of seed beetles. Species of Stator are obligate endophagous seed parasitoids of legumes (Fabaceae) and show considerable variation in host plant specialization (Johnson, 1981a; Johnson et al., 1989; Morse and Farrell, in press). The two nominal species in this complex, S. limbatus (Horn) and S. beali Johnson, have been the subject of considerable research into their divergent generalist and specialist habits, and this paper provides the historical and geographic context for the interpretation of their evolutionary and ecological divergence.

Stator limbatus has been a focus of ecological and quantitative genetic research ever since Janzen (1980) noted its propensity towards being a generalist within both local populations and across its geographic range (see Fig. 1). Along with its congener S. pruininus, S. limbatus has by far the most generalist habit of any species of bruchine seed beetles, feeding on over 70 species of legumes from all three subfamilies. Janzen noted that:

![Fig. 1. Map of sites and phylogeographic clades of monophyletic lineages of S. limbatus and S. beali as determined by the mtDNA phylogeny. Site numbers correspond to the sites listed in Supplementary materials. The inset shows the degree of sampling for the area of sympatry. S. beali is found entirely within the Tamaulipan region with the Mexican xerophytic province.](image-url)
“Such a varied diet requires the ability to detoxify or avoid a large variety of secondary compounds. Viewed over their entire range (southern USA to northern South America), these two beetles have really remarkable predatory abilities (1980, p. 939).” Because this generalism runs contrary to the propensity of phytophagous insects toward specialization in general and bruchine seed beetles in particular (Janzen, 1980; Johnson, 1981b, 1990), S. limbatus has attracted attention from researchers interested in the mechanisms underlying variation in host specialization. This research supports the contention that S. limbatus individuals are generalists, and this generalist designation is not the result of a conglomerate of specialized local populations (Fox and Morrow, 1981). Johnson (1981a) surveyed numerous species in the genus Stator for their ability to use alternative hosts and consistently found S. limbatus, S. pruinusus, and S. sordidus to show abilities to complete development inside hosts from which they had not yet been reared in the wild. All other species of Stator appeared to be limited to their natal hosts. More recently, Fox (Fox and Savalli, 2000; Fox et al., 1994, 1995, 1996a,b, 1997) has shown that generalism in S. limbatus, at least in study populations from Arizona, is a property of individuals (although modified significantly by both maternal and paternal effects), rather than a property distributed between different host races. While this quantitative genetic and ecological research has not been undertaken in other areas in the geographic range of S. limbatus, the long host lists from within these other areas (Johnson, 1963, 1967, 1981a, 1984, 1995, 1998; Johnson and Kingsolver, 1976; Johnson et al., 1989, 1991) suggest that this is likely to be a species-wide phenomenon.

However, there is some evidence for geographically related divergence in morphological characters within S. limbatus, suggesting the potential for geographically based genetic structure. The historical separation of North and South American populations into S. limbatus and Stator ceearusus (Pic) was based on geographically varying coloration, but the two were synonymized due to the uniform shape and armature of the genitalia (Johnson, 1995), a feature that shows high consistency and low variability across geographic ranges of species of seed beetles (Johnson and Kingsolver, 1976; Kingsolver, 1970). In addition, body size in S. limbatus appears to vary with respect to temperature and/or latitude (C. Stillwell, G.E. Morse, C. Fox, unpublished data). If this morphological difference is in part due to phylogeographic structure in S. limbatus that reflects historical boundaries to dispersal, it would suggest that the signal of divergence within this species has not necessarily been erased by the vagaries of history, such as climatic cycling (Avise, 2000).

When Johnson (1963) described S. beali, he noted its close resemblance to S. limbatus and indicated that genital characters presented the most reliable means of differentiating the two. Subsequent research has shown that S. beali is ecologically different from S. limbatus, particularly in regard to host use. First, it is a strict specialist on the Texas Ebony tree, Ebenopsis ebano (Berland.) Barneby & Grimes, and its range is completely restricted to that of its host: below about 500m in the coastal flood plain of the Gulf of Mexico (Fig. 1). This is in contrast to the generalist habit and extensive range (from Venezuela to the southwestern USA, Fig. 1) of S. limbatus (Fox and Mousseau, 1995b; Johnson and Kingsolver, 1976; Johnson and Siemens, 1995). Second, females of S. beali lay their eggs in clumps (Fig. 2A) that can exceed 20 eggs and manipulate this number depending on the size of the host seed, whereas S. limbatus females oviposit eggs singly (Fig. 2A), generally depositing one egg per seed unless seeds are limiting, in which case they disperse their eggs across a seed (Fox and Mousseau, 1995b; Fox et al., 1996b). Interestingly, while S. limbatus can complete development in E. ebano in laboratory experiments or in areas where it is introduced as an ornamental planting, populations of S. limbatus maintained on this host plant alone eventually go extinct because of very low levels of survivorship and fecundity (Fox and Savalli, 2000). This implies that the successful shift to and subsequent specialization onto E. ebano by a proto S. beali population (if it is derived from within S. limbatus) required adaptations (clumping of eggs and adaptive maternal effects, for example, see Fox and Mousseau, 1995b; Fox et al., 1996b) that may have facilitated isolation from and/or permitted co-existence with S. limbatus, thereby accelerating speciation and preventing extinction. While these topics are beyond the scope of the current paper, the geographic context of the relationship between S. beali and haplotypes of S. limbatus may provide some insights into the rapidity of the evolution of reproductive isolation (Knowles et al., 1999).

In addition to differences in host use, the two species have diverged in the armature of the male genitalia (Johnson and Kingsolver, 1976), a character highly relevant to sexual conflict in bruchine beetles (Crudgington and Siva Jothy, 2000): S. beali lacks the recurved serrations extending from the apical sclerite of the median lobe that consistently characterize the male genitalia in S. limbatus throughout its range (Johnson, 1995; Johnson and Kingsolver, 1976; Johnson et al., 1989, 1991). Finally, there is evidence of fairly recent speciation with incomplete prezygotic reproductive isolation and complete postzygotic reproductive isolation between S. limbatus and S. beali: heterospecific pairs will readily copulate, but only male S. beali × female S. limbatus crosses produce viable offspring, and these are sterile (Fox and Mousseau, 1995a; Nilsson and Johnson, 1993).

The host specialization in S. beali combined with its behavioral differences, genitalic differences, and restricted range in relation to S. limbatus are suggestive
of a budding type of divergence. If this is true, then we may expect that the phylogeographic patterns underlying the divergence of \textit{S. beali} from \textit{S. limbatus} are different than the phylogeographic patterns underlying the divergence of lineages within \textit{S. limbatus}, which shows very little variation in ecologically (generalist, dispersed oviposition throughout) or evolutionarily (genitalic morphology) relevant characters throughout its range. However, the close relationship between \textit{S. beali} and \textit{S. limbatus} is based on taxonomy and a willingness to copulate, which say nothing about genealogical relationship. While molecular phylogenetic analyses of the genus \textit{Stator} based on the mitochondrial gene Cytochrome oxidase I (COI) and the nuclear gene Elongation Factor 1-\alpha (EF1\alpha) analyzed separately or together show strong support for the nesting of \textit{S. beali} within \textit{S. limbatus} (Morse, 2003; Morse and Farrell, in press), the results of these analyses were limited in their ability to discern the geographic context of the divergence of \textit{S. beali} from \textit{S. limbatus}, as sampling was not sufficient to adequately address the combined intra- and interspecific phylogeography necessary for this purpose.

The goal of this paper was to examine the phylogeographic structure of mitochondrial haplotypes of the \textit{S. limbatus} complex with respect to four questions: (1) Does intense sampling support the nesting of \textit{S. beali} within \textit{S. limbatus}? (2) Does the phylogeography of the \textit{S. limbatus} complex reflect historical boundaries to dispersal in both space and time? (3) Does the phylogeographic divergence of the ecologically and morphologically distinct lineage of \textit{S. beali} from \textit{S. limbatus} resemble phylogeographic diversions of lineages within \textit{S. limbatus}? And (4) What is the geographic context of the relationships between haplotypes of \textit{S. beali} and \textit{S. limbatus} and how can this guide future research into the mechanisms of divergence between these two species?

2. Methods

2.1. Specimens examined

Host seeds were collected from numerous locales throughout the range of both species during collecting trips between 1998 and 2001 (see Supplementary material). Adults were collected as they emerged and were immediately frozen and stored in a $-80^\circ$C freezer. In total, 108 specimens of \textit{S. beali} from 18 different localities were included in this study (Fig. 1); and 162 specimens of
S. limbatus were included in this analysis: 13 specimens from South America and 149 specimens from 28 different localities in North America. South American specimens of S. limbatus were sampled less extensively because the phylogeny of the genus showed such strong support for a separate clade of South American specimens.

Initially, two specimens each of Stator testudinarius, Stator generalis, and Stator furcatus were included as outgroups. All analyses including these outgroups individually or combined supported a sister relationship between the South American and North American specimens (100% bootstrap for both nodes in all cases). Because rooting in a phylogeographic analysis is quite difficult due to the low ratio of divergence within the ingroup to any outgroup species (Templeton, 1998), this information was used to root the phylogenetic analyses with the South American clade of specimens.

2.2. Molecular methods

Total genomic DNA was isolated using the ‘salting out’ protocol of Sunnucks and Hales (1996). A ~700 bp fragment of 3’ end of the mtDNA gene cytochrome oxidase I (COI) was amplified using the primers CI-J-1859 (Simon et al., 1994) and CI-N-2590 (GCTCTATTGAT ARWACATARTGRAAATG), resulting in sequences of 665 consistently readable base pairs. PCR amplifications were performed in 50 μl reactions to produce double-stranded DNA product under the following conditions: 0.2 μM each primer, 0.15 mM each dNTP, 2.5 μM MgCl2, 1× buffer, and 1 U of Taq polymerase (Qiagen, Valencia, CA) and 1.5 μl of genomic DNA template. Temperature profiles for amplification of fragments consisted of 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1.5 min at 72 °C, followed by a 5 min extension at 72 °C. PCR products were purified directly or after gel extraction using QIAquick columns (Qiagen). Cycle sequencing reactions were performed using one of the primers from the PCR amplifications and Dye Deoxy FS Terminator or BigDye premix (PE Biosystems, Foster City, CA). The majority of sequencing was done using an ABI 373 automated DNA sequencer, with some sequencing done using an ABI 3100 automated sequencer (PE Biosystems). Both directions of the PCR product were sequenced and contigs were assembled, edited, and aligned with Sequencher ver. 4.1 (GeneCodes, Ann Arbor, MI). Because the fragment consists of protein-coding sequences with no insertions or deletions, alignment was unambiguous. Sequences have been deposited in GenBank under Accession Nos. AY963901–AY964023.

2.3. Data analysis

Identical haplotypes were condensed into representative haplotypes and phylogenetic analyses were performed on these using maximum parsimony, maximum-likelihood, and Bayesian inference. We used Fitch parsimony, with heuristic searches implemented using the parsimony ratchet algorithm (Nixon, 1999) implemented in PAUP* (Swofford, 2000) as a batch file (PAUPPrat) written by Sikes and Lewis (2001). Three separate searches of 1000 iterations were employed, with reweighting of 10, 15, and 20% of the characters in the separate searches. The resulting 3000 trees were then compiled into a single file, duplicates were discarded, and trees were filtered to include only the most parsimonious trees. Because of the low levels of divergence between haplotypes, a modified approach similar to that taken by Althoff et al. (2001) was used to estimate bootstraps. Clades that were present in the strict consensus tree of the parsimony analysis, that had greater than 95% posterior probabilities in the Bayesian analysis, and were present in the maximum-likelihood analysis were each represented by five randomly chosen specimens and bootstrap support for these clades were then estimated based on 100 bootstrap replicates, each using starting trees based on 100 random addition sequences. The analyses agreed between five different clades, as well as one individual that did not associate with any of the clades in the Bayesian analysis. Because all of the outgroup specimens were included, each of the bootstrap replicates required 42 taxa. This procedure was repeated 50 times, and the bootstrap values were added together and divided by 50 (total number of replicates).

The program ModelTest 3.0 (Posada and Crandall, 1998) was used to determine the appropriate model of molecular evolution for the maximum likelihood analysis. The model selected was a variant of the HKY85 model (Hasegawa et al., 1985) with rates estimated based on a γ distribution (x-parameter estimated from the data) with an estimated proportion of sites assumed to be invariable (HK85 + I + G). The parameters estimated from the ModelTest algorithm were incorporated into the searches to decrease the search to a reasonable time limit. The maximum-likelihood analysis was implemented using PAUP*, and consisted of 50 heuristic searches from the random addition of taxa, with each search allowed to examine 1,000,000 trees. The Bayesian search was implemented using MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). The search was run with four simultaneous chains for 1,000,000 generations, sampling every 100 generations, and applying temperatures of 0.2 and 0.5. Trees with likelihood scores lower than those at stationarity (the burn-in) were discarded from the analysis. Posterior probabilities of nodes were estimated based on the majority rule consensus of the trees, and branch lengths for the phylogram are based on the mean branch lengths from these trees. The analysis used a model of evolution estimating different γ distributions for the site partitions based on codon position (SS + G).

Divergence times were estimated based on a rate of 2.3% mitochondrial sequence divergence per million
years, as estimated for numerous groups of recently derived arthropod taxa by Brower (1994). Sequence divergence was based on the Hasegawa et al. (1985) model of evolution to correct for multiple substitution events.

3. Results

One hundred and twenty-four unique haplotypes were detected among the 270 specimens included in the analysis. For S. beali, 39 distinct haplotypes were detected among the 108 specimens included, 12 of which were found in single specimens (Table 1). For North American S. limbatus, 74 distinct haplotypes were detected among the 150 specimens included, 54 of which were found in single specimens (Table 2). For South American S. limbatus, 11 distinct haplotypes were detected among the 13 specimens included, 10 of which were found in single specimens (numbered 47–59 on Fig. 3). The only shared haplotype is indicated in Fig. 3 as haplotype AL (found at localities 51, 55, and 56).

All analyses support three major clades of haplotypes that correspond to divisions between major biogeographic regions of the Americas (Figs. 1 and 3, Brown et al., 1998; Rivas-Martínez et al., 1999): Clade 1 is restricted to the Colombian–Venezuelan region of South America, with a distinct clade occurring to the west of the Andes in the Equatorial coastal province; Clade 2 is restricted to the Mesoamerican region, with extension into the western lowlands of the Madrean province; and Clade 3 is limited to the Mexican xerophytic region. This last clade consists of four distinct lineages, labeled 3a–3d in Figs. 1 and 3. The first three lineages are of the generalist S. limbatus and only clade 3c is limited to a single province (the Tamaulipan province) within this region. Clades 3a and 3b are found in the Tamaulipan, the Chihuahuan, and the Sonoran provinces. The fourth, 3d, consists of only S. beali haplotypes and is found exclusively within the Tamaulipan province. There is no resolution between the four lineages found in the Mexican xerophytic region (clade 3) in the strict consensus of the parsimony tree, although the three clades of S. limbatus form a monophyletic clade sister to S. beali in both the Bayesian and maximum-likelihood analyses. This resolution is also present in 85% of the 1265 most parsimonious trees (MPTs).

The deepest divergence is between the North and South American clades, with the corrected pairwise divergence between North and South American taxa averaging 10.2% (SD 1.40%). The pairwise divergence separating the Mesoamerican and Mexican xerophytic clades averages 5.5% (SD 0.80%). Divergence between S. beali and its sister clade in S. limbatus ranged from 1.7 to 5.3% (mean = 3.3%, SD 0.58%). Using the calibration of Brower (1994), these divergence times indicate a split of 4.4 mya (SD 0.61 mya) between the North American and South American specimens; a split of 2.4 mya (SD 0.34 mya) between the two North American phylogroups; and a split of 1.4 mya (SD 0.25) between S. beali and its sister clade of S. limbatus. Sequence variation is greatest within the Mexican xerophytic phylogroup of S. limbatus, with sequence divergence ranging between 0.2 and 5.4% (mean = 1.9%). The South American and

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This species is only found in the Tamaulipan province of the Mexican xerophytic biogeographic region. Site numbers correspond to the sites listed in Supplementary materials. Shared haplotypes are given letter codes, as indicated across the top, with numbers in the table referring to the number of individuals with that haplotype from each respective locality. Unique haplotypes have number-letter codes (e.g., 3a) and are shown in the right-hand column.
Mesoamerican phylogroups show more moderate levels of sequence divergence, with divergences between 0.2 and 3.1% (mean = 1.6%) and 0.2 and 2.5% (mean = 1.4%), respectively. This lower divergence could reflect the lower levels of sampling in these two areas versus the Mexican xerophytic region. *S. beali* showed the least divergence of any of the phylogroups, with sequence divergence between 0.2 and 1.7% (mean = 0.7%). The phylogram of the maximum-likelihood analysis gives a graphical representation of the partitioning of haplotypic divergences within and between regions and clades (Fig. 3). Clade 3 includes a single haplotype found within the range of the Mesoamerican clade in the Madrean region (Fig. 1). This represents the only area of overlap between the three major phylogroups, although this could be due to the relatively low levels of sampling along the western coast of both the Mexican xerophytic and Mesoamerican regions.

### 4. Discussion

The purpose of this study was to examine phylogeographic divergence within the *S. limbatus* complex of seed beetles, and in particular to compare the circumstances underlying the divergence of the specialist *S. beali* from the generalist *S. limbatus* to lineage divergence within the generalist species itself. These data provide insight into the geographic circumstances...
Fig. 3. Phylogram from the maximum-likelihood analysis based on HKY85+G+I model. The results agree with the six numbered clades present in the ML analysis, Bayesian analysis, and parsimony analysis, although the two nodes indicated with * were not completely resolved in the parsimony analysis (present in 85 and 92% of the MPTs, read left to right). Bayesian posteriors, and bootstraps and branch lengths from the parsimony analysis are shown on these clades. Letter codes refer to haplotypes that are represented by multiple individuals, while unique haplotypes are represented by their number codes (see Tables 1 and 2); see Supplementary materials for exact localities.
involved in what appears to be a case of a generalist species budding off a specialist species, provide the appropriate historical context within which to understand recent ecological and quantitative genetic work into this species complex, and establish a basis for future research into the evolution of reproductive isolation between these species.

The results strongly support the conclusion from the phylogenetic analysis of Morse and Farrell (in press) that *S. beali* is a daughter species of *S. limbatus*, indicating that the specialist habit, clumping oviposition behavior, and loss of genitalic armature are all derived from the *S. limbatus* ancestral state. This pattern of a geographically restricted specialist species genealogically nested within a geographically widespread generalist species or species complex supports a budding type of speciation process, whereby widespread generalists give rise to localized specialists. The reliance on mitochondrial DNA sequence data alone here has the potential to be misleading, particularly when viewed in the context of recent evidence documenting the effects of mitochondrial introgression (Machado and Hey, 2003; Shaw, 2002). However, all haplotypes agree with the a priori morphological species designations and form cohesive clades, indicating that recent mitochondrial introgression is unlikely (e.g., Shaw, 2002). The agreement with the tree obtained from limited sampling of the nuclear gene EF1α indicates that the paraphyletic mitochondrial genealogy is not likely to be due to ancient mitochondrial introgression, although further sampling of both specimens and loci is needed to verify this.

The phylogeography of *S. limbatus* corresponds to well-established biogeographic boundaries that represent known barriers to dispersal. *S. limbatus* likely colonized North America from the south, as all closely related species of *Stator* are South American (Morse, 2003; Morse and Farrell, in press), and the deepest divergence within the species separates South American from North American populations. This split is estimated to have occurred approximately 4.4 mya, an estimate considerably older than the appearance of the Panamanian land bridge connecting the two continents around 3 mya (Keigwin, 1982). While this could be due to population divergence prior to crossing the land bridge (Edwards and Beerli, 2000), stochastic variation in this single non-recombining locus (Hudson and Turrelli, 2003), different rates of evolution in the genus *Stator*, or open-water dispersal by *S. limbatus* (it is found on Caribbean islands), this divergence does follow a biologically likely scenario of a barrier to gene flow. The phylogeography of *S. limbatus* in general indicates that geography plays an important role in structuring lineages in this species (type IV concordance, Avise, 2000): The remainder of the lineages of *S. limbatus* also correspond to major biogeographic boundaries, with ranges of clades delimited by the Andes mountains, the Isthmus of Panama, the Cordillera Transvolcanica, the Sierra Madre Oriental, and an increasingly temperate climate in the north.

The divergences within *S. limbatus* may indicate that it consists of multiple allopatric species. The deep genetic divergence between North and South American lineages (mean of 10.4%) provides support for the resurrection of *S. cearamus* as the South American species, and would require reversing the synonymy by Johnson (1995). Analyses regarding mating compatibilities between the different areas will be required to explore this further. However, the uniformity of male genitalia throughout the species supports Johnson’s (1995) contention that these are one species. Male genitalia are known to evolve rapidly in insects, particularly in taxa with a known tendency toward antagonistic sexual selection (Crudgington and Siva Jothy, 2000; Eberhard, 1985; Eberhard, 2004). The most distinctive morphological difference between *S. limbatus* and *S. beali* involves the absence of recurved spines on the median apical sclerite of the male genitalia. If these spines function in damaging female genitalia in the same way that genitalic spines in *Callosobruchus maculatus* do (Crudgington and Siva Jothy, 2000), then this could represent the loss of a character relevant to sexual conflict in the *S. limbatus* complex. Regardless, these deep genetic divergences within *S. limbatus* do not correspond to divergent ecologically important (host use) or evolutionarily important (genitalic morphology) features that characterize the divergence of *S. beali*, and therefore are not pertinent to our understanding of the link between ecological and evolutionary diversification in the ecological theory of adaptive radiation.

The phylogeography of *S. limbatus* reflects well-known boundaries to dispersal, indicating that historical events have not erased the evolutionary signals of divergence within this generalist species. Interestingly, the divergence of the morphologically, behaviorally, and ecologically distinct, and reproductively isolated lineage represented by *S. beali* does not correspond to such biogeographic boundaries. It is found in sympathy with haplotypes from all lineages of its sister clade of *S. limbatus* (clades 3a–3c, Fig. 3), and it is the divergence of this entire clade (*S. beali* + *S. limbatus* clades 3a–3c) that corresponds to the biogeographic break of the Cordillera Transvolcanica in Central Mexico. The divergence of *S. beali* within *S. limbatus* appears to have occurred within a biogeographic region with no known or hypothesized barriers to dispersal: the coastal floodplain of the Gulf of Mexico. This observation, combined with the observation that the phylogeographic pattern of *S. limbatus*, shows a signature of long-term geographic stability, indicates that the diversification of this species from *S. limbatus* has followed a different trajectory than the diversification of lineages within *S. limbatus*: long-term barriers to dispersal were not necessary for the
divergence of *S. beali* from *S. limbatus*, as they were for lineage divergences within *S. limbatus*. Instead, ecological divergence appears to have permitted lineage divergence without either short-term boundaries to dispersal or even none at all.

Given that biogeographic boundaries are concordant with phylogenetic relationships within *S. limbatus*, the divergence of *S. beali* from *S. limbatus* occurred within the Mexican xerophytic region. This region contains not only *S. beali*, but also its sister lineage of *S. limbatus*, only one of which (clade 3c) shows any range limitation, being restricted to the same Tamaulipan province to which *S. beali* is restricted. The remaining two clades both have haplotypes that are distributed through the Tamaulipan, Sonoran, and Chihuahuan provinces (all subsets of the Mexican xerophytic region). This lack of geographic structure in the Mexican xerophytic lineages indicates that divergences within this area are not significantly affected by major boundaries to dispersal. Regardless, within the Mexican xerophytic region, the best predictor of haplotype differentiation is no longer geography, but specializing (or not) on *E. ebano*. This result is in contrast to recent phylogeographic study of the bogus yucca moth *Prodoxus quinquepunctellus* in which it was found that biogeographic distributions were better predictors of differentiation than host use patterns (Althoff et al., 2001); but it agrees with the result found by Brown et al. (1996) for the goldenrod ball gall-maker, *Europoda solidaginis*, in which host use is a predictor of haplotype differentiation for a derived host association.

The results strongly support a generalist-to-specialist polarity in the derivation of *S. beali* from *S. limbatus*, supporting the hypothesis that selection for specialization coupled with reproductive isolation can result in increases in species diversity in adaptive radiation. The results also show that the divergence of *S. beali* from *S. limbatus* followed a different geographic trajectory than divergences between lineages of the generalist. And the results indicate that the genealogy of the widespread generalist *S. limbatus* maintains the signal of the geographic origins of lineage diversification, whereas the evolution of reproductive isolation and a specialist habitat in *S. beali* does not reflect the signal of such a geographic origin.

The insights that these results provide into the nature of the divergence between *S. limbatus* and *S. beali* are primarily inferential. The divergence of *S. beali* from *S. limbatus* is estimated to have occurred during the early Pleistocene (1.4 mya, SD 0.25 mya), a period marked by the first of the Pleistocene glaciations (Graham, 1999). The subsequent divergences within both species would have occurred during a time of dramatic and rapid climatic isolations, and could readily explain the low level of phylogeographic structure within the Mexican xerophytic region. These rapid climatic isolations would have resulted in numerous opportunities for the type of gene flow that is expected to saturate the local adaptations of the type necessary to colonize and successfully specialize on *E. ebano* (Fox and Mousseau, 1995b; Fox and Savalli, 2000; Fox et al., 1996b), unless these adaptations are maintained by reproductive isolation (Futuyma, 1987). Furthermore, the ancestral generalist habit of *S. limbatus* makes extended periods of isolation of a specialist proto *S. beali* unlikely. Paleobotanical studies of plant communities and current distributions suggest that it is unlikely that *E. ebano* would have been completely allopatric from one of the numerous hosts of *S. limbatus* for any extended period of time, as these hosts (e.g., *Acacia*) include some of the persistent and regular taxa from paleoecological communities in Mexico and the Neotropics (Graham, 1976, 1987, 1988; Magallón and Cevallos F., 1994) and are currently ubiquitous throughout the tropical and subtropical regions of North and South America (*S. limbatus* is known to use 15 different species of legumes as hosts in the current area of sympatry alone). In the absence of data concerning the phylogeographic history of the host plant of *S. beali*, one could make the argument that the current geographic proximity between *S. beali* and *S. limbatus* is due to large-scale range changes in the host *E. ebano*, which may have moved from biogeographic areas where it was in long-term isolation from any *S. limbatus* hosts. While information from *E. ebano* would be highly informative, this scenario is less parsimonious than one that takes the strong historical signal in the phylogeography of the beetles into consideration.

Given that the ancestors of *S. beali* would not likely have been isolated from *S. limbatus* for an extended period of time (if at all), reproductive isolation and specialization would have evolved rapidly—reproductive isolation would have prevented the saturation of adaptations to use *E. ebano*, and these adaptations would have given the ancestors of *S. beali* the means to outcompete the ancestral generalist and thereby persist to the present. There are reasons to hypothesize that specialization and reproductive isolation could both have evolved rapidly in *S. beali*. In the absence of alternative host plants or in a community dominated by *E. ebano*, natural selection to increase survivorship and fecundity would have been intense, likely selecting for the adaptive maternal effects and egg-clumping behavior that we see today (Fox and Mousseau, 1995b; Fox and Savalli, 2000; Fox et al., 1996b). In addition, if the loss of genitalic armature in *S. beali* indicates a decrease in intersexual conflict, then it would imply that the host shift may have created a decrease in levels of sperm competition. This could drive the rapid changes in genitalic structures in *S. beali* and result in subsequent reproductive isolation between *S. beali* and *S. limbatus* when brought into sympatry (Eberhard, 2004; Gavrilets, 2000), perhaps aided by reinforcement (Noor, 1995, 1999). If host use were
indeed coupled with levels of sperm competition, then host adaptation and sexual selection would be linked, further accelerating the evolution of reproductive isolation.

Similar arguments concerning the rapid evolution of reproductive isolation amidst climatic oscillations have been put forward to explain diversification patterns in Ophiuraella leaf beetles (Knowles et al., 1999) and Melanoplus grasshoppers (Knowles, 2000, 2001a,b). It is worth noting that the current study involves the northernmost clade of S. limbatus, the clade that would have been most affected by glacial cycling due to the penetration of grasslands and boreal forest further south, supporting the possibility that isolation in refugia during glaciations, when coupled with natural and/or sexual selection may have enhanced diversification instead of impeding it (Hewitt, 1996).

Note that this study cannot directly address the mode of speciation involved in this diversification event, it only has implications for the rapidity and coarse-grained geographic context of speciation. Genealogical information into the mode of speciation (e.g., sympatric, peripatric, and allopatric) must come from evidence of gene flow and demographic circumstances during the initial speciation stages, information that can only be gleaned by examining multiple neutral loci, by examining loci responsible for reproductive isolation or ecological diversification, and by examining taxa that have diverged recently enough that this information is still informative (Kliman et al., 2000; Wakeley and Hey, 1998). Additionally, the conclusions in this study are based on a single non-recombining locus and thus represent a single record of the phylogeographic history of the maternal genome. Sex-biased patterns of dispersal, selective sweeps on a mitochondrial locus, or mitochondrial introgression could all have serious implications for interpreting these results. Sampling of multiple loci would certainly increase our confidence in these results, but the general agreement of the genealogy with known phylogeographic boundaries and the concordance with the smaller EF1α dataset suggest that these results are not misleading.

This study was intended to provide the geographic and historical context for understanding divergences within the S. limbatus complex. It sets the appropriate historical framework for research into the adaptations involved in the specialization of S. beali onto E. ebano and into the genetic and morphological architecture underlying its evolution of reproductive isolation from S. limbatus. It also provides the phylogeographic and phylogeographic background for specific hypothesis-based analyses into the historical demography accompanying the divergence between these two species. This analysis provides clear evidence that S. beali is derived within S. limbatus and that its unique features are departures from the ancestral S. limbatus features. By establishing that lineage diversification within S. limbatus maintains the signature of history by generally reflecting geographic boundaries, we are also able to establish that the divergence of S. beali did not correspond to any known geographic boundaries. This phylogeographic investigation into the history of lineage differentiation when it is and is not associated with ecological differentiation provides insights into the role of specialization and speciation in the generation of phytophagous insect diversity.

Acknowledgments

We thank J. Romero, D. Windsor, and J.A. Clavijo for logistic assistance in Mexico, Panama, and Venezuela, respectively. Two anonymous reviewers greatly improved the quality of the manuscript and their comments and criticisms were a valuable contribution. We also thank D. Futuyma, D. Funk, and the laboratories of J. Thompson and C. Fox for discussion of the results and their implications. Funding for this research was provided by a Dissertation Improvement Grant from the National Science Foundation (DEB-0073330), the Smithsonian Tropical Research Institute, the Putnam Expeditionary Fund at the Museum of Comparative Zoology, and the Department of Organismic and Evolutionary Biology at Harvard University. We dedicate this paper to C. Dan Johnson, whose advice, encouragement, and research in and knowledge of bruchine systematics made much of this research possible.

Appendix A. Supplementary data


References


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