New Taxonomic Status of the Endangered Tiger Beetle *Cicindela limbata albissima* (Coleoptera: Cicindelidae): Evidence from mtDNA

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ABSTRACT The tiger beetle C*icindela limbata albissima* Rumpp is confined to the Coral Pink Sand Dunes formation in southern Utah where its habitat is under threat. To assess the conservation value of this population, the level of divergence in mtDNA throughout the range of *C. limbata* in the central and western parts of the United States and Canada was tested. A representative sample of 25 specimens from four subspecies was sequenced for 1,873 bp and three mtDNA regions. The data revealed the wide separation of *C. l. albissima* from the other subspecies. In a phylogenetic analysis that included all species in the *maritima* species group, *C. l. albissima* was placed as sister to seven species of a western North American clade. The remaining *C. limbata* populations formed a (weakly supported) monophyletic group within this western clade but were not closely related to *C. l. albissima.* These populations could not be further subdivided into discrete geographic entities defined by diagnostic characters. It was concluded that the distinct taxonomic status of *C. l. albissima* had not previously been recognized, possibly because of morphological similarity in elytral patterns and other characters that are convergent in sand dune-dwelling species. On the basis of the mtDNA data, *C. l. albissima* is eleveated to species status, *C. albissima* Rumpp [revised status]. The results highlight the importance of conservation efforts for *C. albissima* and its habitat.

KEY WORDS *Cicindela limbata albissima*, conservation genetics, evolutionarily significant unit, habitat loss, mtDNA, subspecies

MANY SPECIES OF tiger beetles occur in isolated patches of suitable habitat where populations of a few hundred or thousand individuals persist at large geographic distances from other populations. Habitat associations tend to be highly specific, and fragmentation of the species range is therefore caused mostly by the discontinous distribution of appropriate habitat (Shelford 1911, Knisley 1984, Schultz 1989). The geographic discontinuity is frequently reflected in morphological differences between local populations, in particular in the coloration of elytra and body size. This has caught the attention of taxonomists and resulted in prolific literature describing subspecific variation. Of the nearly 150 species of *Cicindela* in North America (Boyd 1982), the vast majority has been separated into several geographically confined subspecies, with up to 11 (e.g., *C. tranquebarica* Herbst) valid trinomials.

The traits upon which subspecific differences have been based, however, may be of little taxonomic value because they are under strong selection. In particular, elytral coloration differs in the extent of dark and light areas, which, in behavioral assays, has been shown to be a highly important aspect in thermoregulation (Schultz and Hadley 1987, Acorn 1992, Schultz 1998). Body coloration also plays an important role in predator evasion, reducing discovery and attack through soil matching (Pearson 1985, Schultz 1986, Schultz and Hadley 1987, Pearson et al. 1988, Hadley et al. 1992, Knisley and Schultz 1997, Vogler and Kelley 1998), mimicry (Acorn 1988, Kamoun 1991), and spectral properties in flight (Pearson et al. 1988, Schultz 1998). Populations therefore differ substantially in these traits, but their degree of morphological divergence may not reflect the degree of historical separation. However, historically widely separated populations may converge on similar phenotypes under similar habitat conditions, obscuring the existence of evolutionarily divergent groups.

The difficulty with recognizing patterns of phylogenetic relatedness in tiger beetles based on morphological characters precludes the analysis of convergence and diversification at the level of closely related species (e.g., Acorn 1992, Barraclough et al. 1999). It is also problematic for issues in conservation biology, because selection and morphological convergence complicate the recognition of evolutionarily distinct populations (Moritz 1994, Vogler and DeSalle 1994, Goldstein et al. 2000). This is significant because tiger beetles are highly suitable as indicators of environmental change because of their habitat specificity (Pearson 1992, Pearson and Cassola 1992) and have been shown to be a useful target for invertebrate conservation (e.g., Vogler et al. 1993a,b, Knisley and Schultz 1997) and an umbrella for the protection of sensitive habitats (Knisley and Hill 1992).

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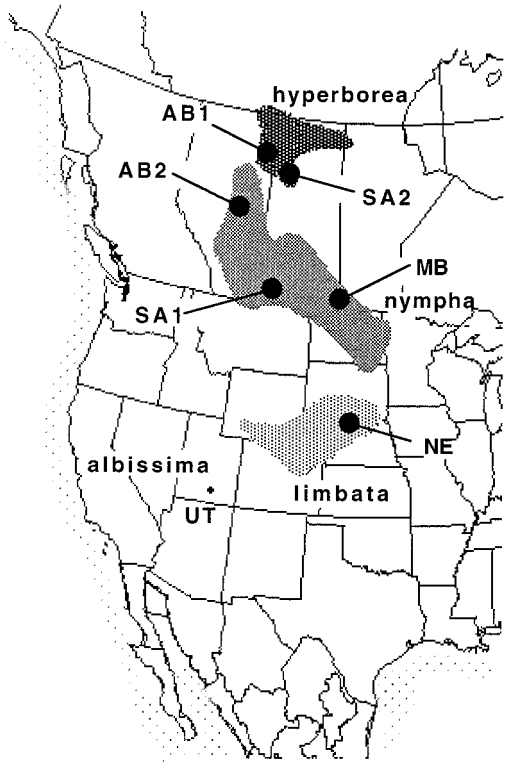

Fig. 1. Western half of North America with distributional ranges of four subspecies of *C. limbata.* The location of populations used in this study are indicated as follows: AB, Alberta; SA, Saskatchewan; MB, Manitoba; NE, Nebraska; UT, Utah.

A typical example of a morphologically and geographically subdivided species is *C. limbata* Say, which has a wide distribution in active dune fields of the central and northern parts of North America (Fig. 1; Table 1). Five subspecies are currently recognized based on elytral coloration (Rumpp 1961) and geographic range. Three subspecies occur in a largely continuous range from the western Great Plains (*C. l. nympha*), North Dakota, southern Canada (*C. l. limbata*), and northern Canada (*C. l. hyperborea*). The remaining two subspecies have widely disjunct ranges. *C. l. labradoriensis* is only known from Goose Bay, Labrador, and may have been introduced recently into the vicinity of an army base by military airplanes (Johnson 1989). *C. l. albissima* is limited to the Coral Pink Sand Dunes in Utah (Rumpp 1961), the only occurrence of *C. limbata* west of the Continental Divide. This is known from this single location only, despite careful surveys of other dune systems in Utah and adjacent states (C.B.K., unpublished data). The actual range of *C. l. albissima* is confined to an area of ≈500 acres that is regularly affected by recreational vehicle traffic, a well-established threat to tiger beetle populations (Schultz 1988, Knisley and Hill 1992). The Coral Pink Sand Dunes tiger beetle population is currently a candidate species for the Federally Endangered species list. It was proposed for listing before the listing moratorium in 1995.

Phylogenetically, *C. limbata* is part of the "western clade" within the Holarctic *maritima* species group (Rivalier 1950) that is composed of seven closely related species confined to the western half of the North American continent (Freitag 1965, Vogler et al. 1998). In addition to *C. limbata*, the western clade includes three other sand dune species, *C. theatina* Rotger and *C. arenicola* Rumpp, occurring in isolated dune formations of Utah and Idaho, respectively, and *C. bellissima* Leng, occurring in coastal dunes of Oregon and Washington. Two other species inhabit river edges, including the widespread *C. oregona* Leconte and *C. columbica* Hatch, a species confined to the Snake River of Idaho, whereas the remaining species, *C. depressula*, occurs in alpine grassland. Based on mtDNA sequences, hierarchical structure in the western clade is much more shallow than in the remainder of the *maritima* group. Pairwise sequence divergence between the species of the western clade is similar to the level observed between geographically disjunct populations of widespread species of North American *Cicindela*, perhaps consistent with the view that the western clade represents isolated, remnant populations of a formerly widespread and contiguously distributed taxon (Vogler et al. 1998). It is in this context that the different subspecies of *C. limbata* have to be placed; if *C. limbata* is indeed a monophyletic group, the separation of the subspecies must be even more recent and mtDNA differentiation between the various subspecies can be expected to be extremely low.

We present a survey of mtDNA variation in populations of *C. limbata*, investigating the level of divergence and the geographic structure within and between various subspecies. This analysis resulted in the unexpected finding that *C. l. albissima* is only distantly related to the other *C. limbata*, highlighting the im-

Table 1. Locality information for specimens used in this study

Population	No. specimens	Subspecies	Location
AB1	6	*hyperborea*[a]	Alberta, 32 km N Fort McMurray, Rt. 63 (Sancor)
AB2	2	*nympha*	Alberta, Hy 2, 6.3 mi NW Hwy. 2A. SE Slave Lake, 22-VIII-98
SA1	8	*nympha*	Saskatchewan, 5 km N Burstall Dunes, 13-IX-98
SA2	1	*hyperborea*	Saskatchewan, Rd 955, 16.6 mi NE La Loche, 28-VIII-98
MB	2	*nympha*	Manitoba, Spruce Woods Provencial Park, Hwy. 5, 16 mi S Carberry, 20-VIII-98
NE	2	*limbata*	Nebraska, 19 mi W Thetford, Rt. 2, 4-IX-98
UT	4	*albissima*	Utah, Kane Co., Coral Pink Sand Dunes, 2-IX-98

[a] Larval specimens could not be identified using morphological characters but were assigned to *C. l. hyperborea* based on the close proximity of the population containing the adult *C. l. hyperborea.*

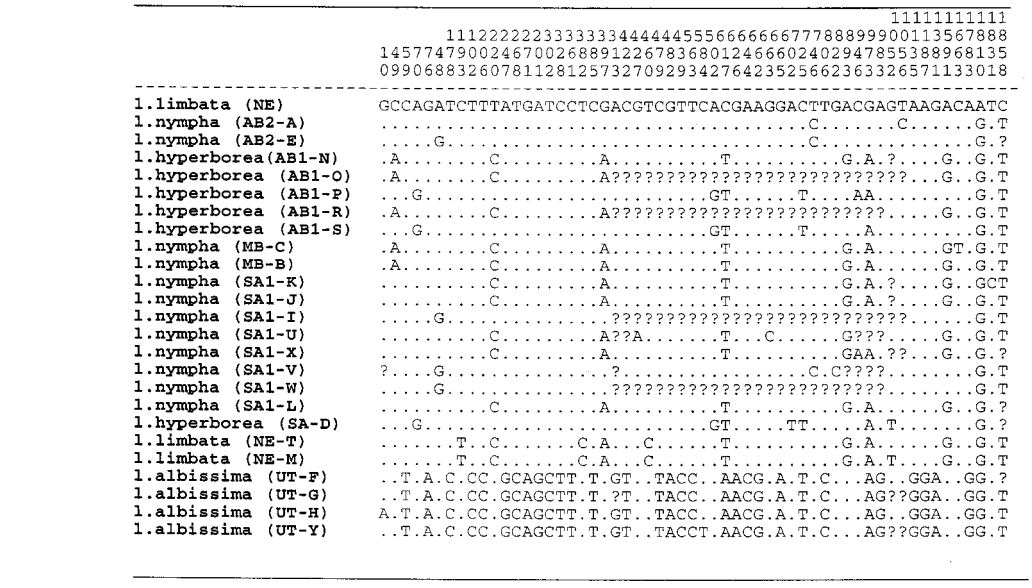

Fig.2. Polymorphic nucleotide positions. Position 1–411 corresponds to the gene encoding Cytb, 412-1049 to the COIII region, and 1050–1873 to 16S rRNA and adjacent $tRNA^{Gly}$ and ND1 genes. The letters after the subspecies name refer to the collecting locality and a one-letter designation for each haplotype.

portance of the Coral Pink Sand Dunes population for biodiversity conservation.

Materials and Methods

All experimental and analytical procedures follow those described in Vogler and Welsh (1997). DNA was extracted using a standard phenol/chloroform procedure, followed by polymerase chain reaction (PCR) amplification, and sequencing of three regions of the mitochondrial genome, coding for Cytochrome oxidase III (COIII), Cytochrome b (CytB) and 16S rRNA and adjacent genes. The three data sets include 634, 411, and 828 bp, respectively, and a total data matrix of 1873 nucleotide positions. Of these, 1078 positions are protein coding and the remainder codes for different RNAs. The full data matrix is available from http://www.bio.ic.ac.uk/research/tigerb/albissima.htm. Body parts of the beetles not used for the extraction were deposited in our frozen tissue collection.

Collection records are given in Table 1. The sample contains representatives of all subspecies of *C. limbata* except *C. l. labradoriensis.* Living specimens for the analysis were collected in the field and dried in vials containing silica gel. The amplification was not successful for the COIII region in five specimens (individuals I, N, O, R, and W). The mtDNA data were incorporated into an existing data set containing the equivalent sequences for all members of the North American *maritima* species group and several outgroups (Vogler et al. 1998), resulting in a matrix of 43 terminals. Data from all three regions of the mitochondrial genome were combined and analyzed simultaneously. Most parsimonious trees were determined using PAUP version 4.02b (Swofford 1999) with 10 random sequence additions and TBR branch swapping. Branch support was determined using bootstrap proportions and Bremer Support values (Bremer 1994). Constraint files for the estimation of shortest trees that do not contain the focal node were produced with TreeRot (Sorenson 1996). Nucleotide diversity was calculated according to Nei (1987) using equation 10.6.

Results

The data matrix included 25 newly sequenced haplotypes from various *C. limbata* populations, plus mtDNA for all North American species of the *maritima* species group and several outgroups from an earlier study (Vogler et al. 1998). The sequences did not exhibit any length variation, making alignment unambiguous. In total, the matrix contained 1,319 invariant, 185 variable uninformative and 314 potentially parsimony informative sites. Variation within *C. limbata* was confined to 57 polymorphic sites (Fig. 2). In a simultaneous analysis of all three mtDNA regions, 144 equally shortest cladograms of length 1,200 with CI = 0.45 and RI = 0.64 were found (Fig. 3). The tree is generally consistent with the relationships determined in our previous analysis (Vogler et al. 1998) that included only a single specimen of *C. limbata* (i.e., the species is closey related to *C. bellissima, C. oregona,* and *C. depressula,* but its position is either as sister to these three species or as the sister to the pair *C. depressula/C. oregona* only) (Fig. 2). Although the *C. limbata* haplotypes become paraphyletic under the first scenario, they form a monophyletic group under the latter.

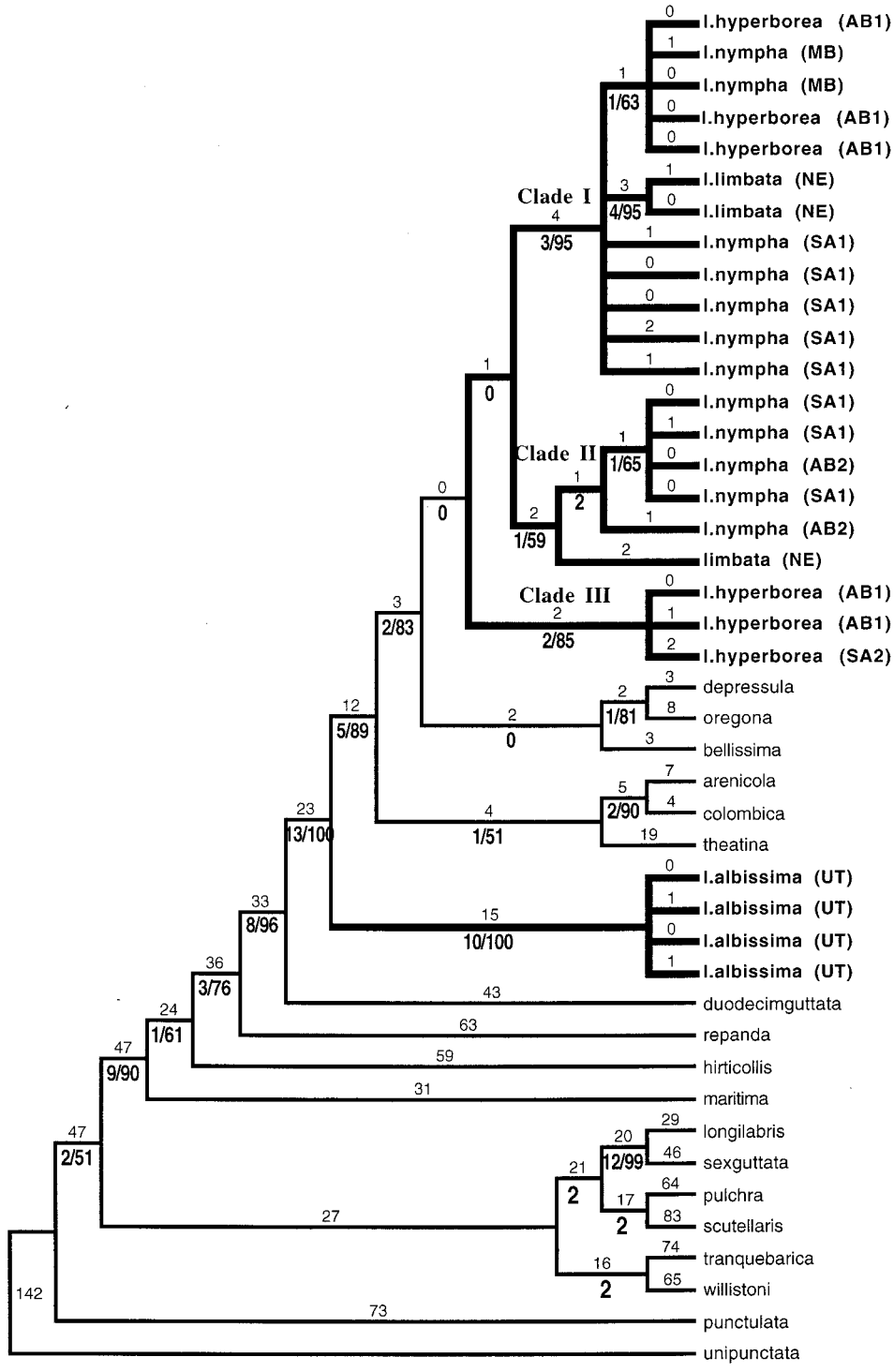

Fig. 3. Parsimony reconstruction of *C. limbata* haplotypes and close relatives in the *maritima* species group. Numbers above the branches refer to the number of inferred character changes, numbers below the branches are Bremer support values and bootstrap proportions (given only if >50). This is one of 144 shortest trees of 1,200 steps and CI = 0.45, as produced by PAUP4.0b2a. Note that the branch length below the node at the base of the *C. limbata* haplotypes is zero. In the tree selected here, this node is resolved arbitrarily to show that the data are consistent with monophyly of *C. limbata* (excluding *C. l. albissima)*, although there is no character support for this. Haplotype designations refer to the sampling localities as shown in Fig. 1.

Table 2. Comparison of intrapopulation diversity of haplotypes in two populations of *C. limbata* (Saskatchewan 1 and Alberta 1) with other North American species

Species	Location	No. individuals	No. haplotypes	Haplotype divergence	Nucleotide diversity
				(%)	
C. limbata	(SA-1)	8	6	0.053–0.48	0.00179
C. limbata	(AB-1)	5	3	0.053–0.48	0.00203
C. dorsalis	Martha's Vineyard (MA) 0.00051		12	3	0.15–0.31
C. dorsalis	Flag Pond (MD)	20	1	0	<0.00015
C. puritana	Connecticut River (CT)	13	3	0.22	0.0013
C. puritana	Chesapeake Bay (MD)	27	2	0.22	0.00032

Data for *C. (Habroscelimorpha) dorsalis* are from (Vogler and DeSalle 1993) and *C. (Ellipsoptera) puritana* are from (Vogler et al. 1993b).

The four haplotypes obtained from *C. l. albissima* are not part of this group. Instead they grouped together in a monophyletic and well-supported lineage at the base of the western clade, thus being the sister taxon to the other seven (including *C. limbata* proper) species of this group. The other haplotypes in *C. limbata* were grouped into three subclades (clades I, II, and III in Fig. 3) that were found consistently in all shortest trees, although the relationships with each other and with the nearest relatives were not fully resolved in the strict consensus tree (not shown). Clade I represented specimens from Sasketchewan, Manitoba (*C. l. nympha*), Alberta (*C. l. Hyperborea)*, and a divergent haplotype from Nebraska (*C. l. limbata*), clade II contained specimens from the same Sasketchewan site and a more southern Alberta site (all *C. l. nympha*), whereas clade III contained haplotypes from the first Alberta site and a second site in Sasketchewan (all *C. l. hyperborea*). These clades did not conform to any of the morphologically recognized subspecies, and individuals from a single population may exhibit haplotypes from more than one clade (e.g., at site SA1 clade I and II haplotypes, and at site AB1 clade I and III haplotypes were encountered). Whereas the clades did not conform strictly to the subspecies boundaries, a single haplotype was never encountered in more than one subspecies, with the exception of a haplotype in Clade I which occurred in *C. l. nympha* from Manitoba and *C. l. hyperborea* from the southern limit of that subspecies in Alberta, possibly indicating introgression of haplotypes in an area where morphological *C. l. nympha–C. l. hyperborea* intermediates occur (Johnson 1989).

Because of the small number of haplotypes analyzed to date, the analysis of their geographic structure has to be preliminary. The data were sufficient, however, to establish that local populations frequently contain haplotypes from two of the main clades. At the same time, haplotypes of any of the three main clades found in a single population were generally very closely related or even identical. The rather large amount of divergence between the two main groups of haplotypes, therefore, explains the relatively high haplotype diversity, expressed as Nei's *pi*, within populations. For the populations SA1 and AB1, where eight and five specimens, respectively, were analyzed, intrapopulation variation is rather high and not much lower than the level of haplotypes diversity across all *C. limbata* (excluding *C. l. albissima*) haplotypes combined (Table 2). Intrapopulational diversity also is high in comparison with two other species of North American tiger beetles, *C. dorsalis* Say and *C. puritana* G. Horn, for which similar data sets are available. *C. limbata* populations not only exhibited a higher number of different haplotypes in each population but also higher values of nucleotide diversity (Table 2).

Another remarkable observation is the high consistency of the character changes with the tree. Whereas the level of homoplasy in the western clade of the *maritima* group had been found to be very high (Vogler et al. 1998), this does not apply to the changes within *C. limbata.* Using only *C. limbata* haplotypes for phylogeny reconstruction results in a tree with CI = 0.97 and RI = 0.98 (in which the relationships suggested by the analysis of the full data set are almost exactly maintained). This finding is also unexpected given the high level of homoplasy encountered in populations of other North American species, such as *C. dorsalis* (Vogler and DeSalle 1993).

Discussion

MtDNA Diversification and Morphological Convergence. The most salient finding of this study is the phylogenetic position of *C. l. albissima*, distant from the other subspecies of *C. limbata* and sister to the entire "western clade" of the *maritima* species group. Although highly supported in the parsimony analysis, this proposition is based only on a single genetic locus and may be unique to the history of the mitochondrial genome rather than reflecting the species tree. Hybridization or ancestral polymorphisms could confound the mtDNA phylogeny (e.g., Doyle 1997), but this is unlikely because (1) no other species in the *maritima* group exhibit mtDNA haplotypes similar to those found in *C. l. albissima*, arguing against the possibility of recent gene flow; (2) all *C. l. albissima* haplotypes form a tight group of similar genotypes that are divergent from all others, indicating long periods of independent evolution; and (3) the position of *C. l. albissima* at the base of the western clade is plausible on biogeographic and ecological grounds. The occurrence of *C. l. albissima* is biogeographically nearest to another dune dwelling and locally restricted species of the Basin and Range Province, *C. theatina.* The latter is similarly divergent from other species in the *mari-*

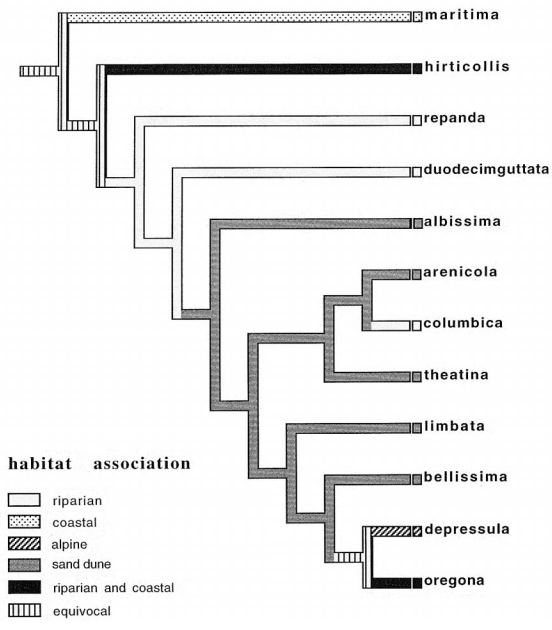

Fig. 4. Character optimization for habitat types in the *maritima* group.

tima group based on mtDNA sequences (Vogler et al. 1998), indicating a similarly long period of isolation in narrowly confined dune fields in this geomorphological region. In addition, mapping of habitat associations on the cladogram (Fig. 4) revealed that *C. l. albissima* occupies a basal position in a (paraphyletic) group that includes all dune-associated species, including the remaining *C. limbata* subspecies—*C. theatina*, *C. arenicola* and *C. bellissima* (the latter confined to coastal sand dunes). If habitat associations are phylogenetically informative, the inferred single evolutionary origin of sand dune association supports the mtDNA tree.

In the mtDNA tree, the Coral Pink Sand Dunes population is sister to a whole group of other species and is not more closely related to the other populations of *C. limbata* than to other species in the *maritima* group (whose species status is not under debate). The designation at the subspecific level is therefore misleading, and we propose to elevate the status of the Coral Pink Sand Dunes tiger beetle to that of a separate species, *C. albissima* Rumpp [new status].

The conclusions from mtDNA have to be evaluated against the morphological similarities that apparently exist between these unrelated groupings. The literature provides no specific justification for why *C. albissima* was grouped with other *C. limbata* populations beyond the elytral coloration patterns (Rumpp 1961). Populations and individuals of *C. limbata* differ in the extent of the dark markings, which in northern populations are more expanded presumably for improved infrared absorption under colder climatic conditions in this species (Acorn 1992) (although in most *Cicindela*, background matching is a more plausible explanation for geographic variation in the size of maculations, Hadley et al. 1992). The differences in the extent of markings are gradual, with specimens in the northern *C. l. hyperborea* generally the darkest, and, as the name suggests, *C. albissima* almost entirely white. Very light specimens also are found in Nebraska and considered *C. l. limbata*, but the degree to which this reflects historical relatedness has been unclear. The phylogenetic status of *C. albissima* as established in this study, however, clearly supports the hypothesis that the similarities in elytral patterns are convergent.

To corroborate this conclusion, it will be important to establish phylogenetic divergence also based on morphological characters that are presumably less constrained by habitat and climatic conditions, such as genitalia (Freitag 1972) and chaetotaxy. However, it can be expected that convergent patterns will be discernible in traits other than elytral coloration. For example, in unrelated sand dune species in the subgenus *Ellipsoptera* of the southeastern United States, parameters such as body size and mandible length (the latter closely correlated with prey size, Pearson 1985) seem to be very similar, more so than expected under a null model of random character change (Barraclough et al. 1999). Convergent evolution in these traits could be mediated by similar prey types, thermoregulatory requirements, or predators. The existence of independent lineages of *Cicindela* (*sensu lato*) in the same habitat types permits the identification of traits arising in response to the selective regime (*sensu* Baum and Larson 1991) exerted by the habitat. Sand dunes are particularly useful as a test system for the analysis of habitat-dependent convergence, because they represent a physically well-defined environment where specialized traits to cope with harsh conditions are needed.

Evolutionary Scenarios and Implication for Conservation. The phylogenetic position of *C. albissima* as the sister taxon to all other species in the western clade of the *maritima* group assigns equal age to this lineage and its sister clade comprising seven species. This reveals the relative antiquity of the lineage leading to *C. albissima* and raises the question whether a lineage as ancient as *C. albissima* could have persisted in a single dune formation. Estimates of the age of the Coral Pink Sand Dunes place their origin to the Quaternary only 10–15,000 yr BP (Gregory 1950), or even less, possibly dating to a warm period in the middle Holocene 6,000–2,500 yr BP when low lake levels and increased aridity may have produced conditions favorable for aeolian erosion and deposition (Shafer 1989). Although these dunes could not have sustained the lineage following the split of a common ancestor of *C. albissima* and its sister group, its deep separation and geographic isolation from other groups would suggest long-term persistence of the respective lineages within the intermountain basins (similar arguments apply to *C. theatina*). The changing environments of these areas (Thornbury 1965) would severely jeopardize lineage survival, a condition aggravated by the apparently low dispersal power of these species. The large number of dune formations in

the western United States occupied by only a single species of *Cicindela* or none at all may be indication of the high chances of extinction and low rates of colonization.

Careful surveys of other potential sites in the Great Basin have established that *C. albissima* is confined exclusively to the Coral Pink Sand Dunes formation (C.B.K., unpublished data). This makes it inherently vulnerable to extinction from human disturbance or natural factors. Our findings establish the value of this habitat for conservation. In the framework of the Endangered Species Act, arguments for the preservation of particular populations and their habitat are usually based on the degree of their evolutionary distinctiveness. Definitions for how to delimit these "evolutionarily significant units" (ESUs) are contentious but are principally based on either the level of genetic divergence (Dizon et al. 1992, Moritz 1994) or the presence of discrete differences between groups as an indication of the independence of their gene pools (criterion of diagnosability) (Vogler and DeSalle 1994). Under either criterion, *C. albissima* is clearly separable from the remaining populations of *C. limbata.* Even stronger arguments for the evolutionary divergence are based on tree topology, which clearly defines *C. albissima* as a separate species. Thus, the extinction of the Coral Pink Sand Dunes population would be a significant loss of biological diversity.

Whereas *C. albissima* is known to be severely at risk, the status of the other *limbata* subspecies is unclear. They are the only species in the "western clade" of the *maritima* group to occur east of the Continental Divide, presumably following dispersal from a western location. They have colonized a wide area that was covered in its entirety by an ice sheet during the most recent glaciation. This indicates a generally higher dispersal propensity compared with *C. albissima.* Populations depend on the subtle equilibrium of mild disturbance to keep a dune system active and the permanence of the habitat needed for larval development. Dune systems in the middle part of North America have seen periods of increasing and decreasing activity, mostly determined by changing climatic conditions, ungulate activity, fire regime, and, more recently, agricultural and land use practices. The current trend (since the earlier part of this century) appears to be toward increased dune stabilization and thus habitat loss, having led to local extinction of *C. limbata* populations in Canada (Acorn 1992).

The pattern of mtDNA variation is consistent with this dynamic process of colonization interspersed by periods of geographic isolation (Acorn 1992). There is substantial phylogenetic structure in these data (resulting in the well-supported clades I, II, and III in Fig. 3), but these divergent groups of haplotypes do not coincide with particular areas. Nevertheless, the distribution of haplotypes is also structured geographically, with very closely related or even identical haplotypes found side-by-side with a set of closely related types from a different lineage. The presence of widely different haplotypes in a single population is usually explained by secondary contact of allopatrically differentiated lineages (Avise 1994). Therefore, the observed patterns of mtDNA variation may be explained by secondary contact of divergent haplotypes during the postglacial colonization. Whereas different haplotypes colonized in different areas, leading to substantial differences between populations, in some cases the divergent haplotypes came into contact, forming a mixed assemblage of divergent haplotypes. Locally, these lineages apparently experienced further differentiation and gave rise to very similar haplotypes confined to a narrow area.

In conclusion, the fragmentation and dynamics of the habitat throughout the range of *C. limbata* led to substantial differences in molecular and morphological characters, but in contrast to the mtDNA markers, the elytral color pattern usually used to define the named subspecies did not reflect historical relationships. Changes of habitat associations are common in this fast-radiating group of tiger beetles in geologically and topographically complex western North America and appear to be associated with changes in morphology. Because of morphological convergences, the endangered *C. albissima* has not been recognized as a historically divergent entity. Further sampling across a range of populations and recognized subspecific taxa in the remainder of the *maritima* group would be desirable for a better understanding of the geographical and morphological differentiation in this group.

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