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SHORT COMMUNICATION

Calibrating phylogenetic species formation in a threatened insect using DNA from historical specimens

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Abstract

Museum specimens from the late 19th and early 20th centuries were surveyed for the single nucleotide polymorphism identified previously and used to diagnose populations of the federally threatened Northeastern Beach Tiger Beetle *Cicindela d. dorsalis* (Coleoptera: Carabidae). Widespread polymorphism was revealed throughout the historical range of this species, suggesting a relatively recent anthropogenic character fixation event associated with the extinction and fragmentation of populations. Implications for the phylogenetic species criterion and for the reintroduction of individuals to formerly occupied sites are discussed.

Keywords: ancient DNA, *Cicindela dorsalis*, conservation genetics, mtDNA, phylogenetic species, population aggregation analysis

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Introduction

The northeastern beach tiger beetle *Cicindela d. dorsalis* has been the focus of several recent molecular studies focusing on the distribution of sequence polymorphism among extant populations and the diagnosis of biological units for conservation. This species has undergone a precipitous decline during the last century: formerly distributed along sandy beaches between Massachusetts and the Chesapeake Bay, it was believed extirpated from Massachusetts, Connecticut and Long Island (Knisley *et al*. 1987) and was listed as threatened under the federal Endangered Species Act in 1991 following the rediscovery of a single Massachusetts population on the island of Martha's Vineyard. Suitable beach habitat has been decimated on much of the Atlantic coast by a combination of beach stabilization initiatives (jetties, revetments, etc.; see Dean 1999) and recreational off-road vehicle traffic, which decimates the larval burrows of tiger beetles. Restoration of populations to formerly occupied sites, mandated by the federal recovery plan for *C. d. dorsalis* (U.S. Fish & Wildlife Service 1994) and currently under way, has required at least a rudimentary understanding of region-wide genetic differences, if only to suggest protocols for identifying source populations. Chief among concerns for the reintroduction effort have been the impact on source populations of extracting individuals for reintroduction and the potential for corrupting local gene pools by introducing less closely related individuals than might be desirable. Molecular data of Vogler & DeSalle (1993a,b 1994) and Vogler *et al*. (1993a,b) did not yield fixed diagnostic characters for any of the four named subspecies of *C. dorsalis*, nor did any obtain as reciprocally monophyletic based on the mtDNA data. However, among the extant populations, the one remaining on Martha's Vineyard was diagnosable from all others, but that by virtue of only a single base pair (position 5016 on the cytochrome oxidase III gene). A specimen from a second, smaller remnant population of *C. d. dorsalis* rediscovered on mainland Massachusetts in 1994 was found to bear the same character state as individuals from the Martha's Vineyard population (A. Vogler, pers. comm.) This population appears to have since been extinguished. As such, under the strictest possible definition, these populations comprise a phylogenetic species (Nixon & Wheeler 1991). In this paper we explore the implications of such criteria for species recognition and conservation management and the role of extinction in precipitating the diagnosis of phylogenetic species.

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Under the phylogenetic species concept (Cracraft 1983; Nixon & Wheeler 1991; Davis & Nixon 1992), only when alternative character states are fixed in independent populations may those groups of individuals be defined as separate species. The phylogenetic species 'concept' has since been characterized — perhaps more accurately — as a criterion (De Queiroz 1998; Goldstein & DeSalle 2000). Recently, the utility of the phylogenetic species concept (PSC) has been discussed and reviewed with respect to the furtherance of both taxonomy and conservation biology (Goldstein & DeSalle 2000; Goldstein *et al*. 2000; Goldstein & Brower 2002). While many authors endorse or apply criteria of fixed character states to their delimitation of species, others have argued variously that PSC does not necessarily correspond to biologically 'real' entities on epistemological grounds (e.g. Frost & Kluge 1994), that strict application of the PSC is uniquely vulnerable to sampling data on statistical grounds (Walsh 2000) and that adoption of the PSC in formal taxonomy will lead to an unacceptable proliferation of names or entities in need of formal conservation management (Avise 1989; Moritz 1994). Perhaps more pointedly, many authors find fault with the PSC by virtue of its involving 'speciation by remote control' (e.g. Templeton 2001). To the extent that biologists wish their species to be evolutionarily equivalent or represent reproductively stable and cohesive lineages, all these criticisms are valid, but may not necessarily serve the endeavors of describing or protecting nature. For the purpose of this study, we wish to put aside some of the more arcane philosophical questions associated with species concepts. As has been described elsewhere (e.g. Goldstein & DeSalle 2000; Goldstein *et al*. 2000; Wheeler & Meier 2000; Goldstein & Brower 2002), many rely on philosophical schemata that by definition cannot be addressed empirically. Here, instead, we concentrate on a *reductio ad absurdum*, an example of a phylogenetic species characterized by a single base pair substitution, and its logical and practical ramifications for the PSC.

Using DNA extracted from museum specimens from the 19th century and early 20th century, we explore the historical distribution of the single nucleotide polymorphism used to diagnose extant metapopulations of *C. d. dorsalis*.

Materials and methods

Samples

Single hind legs were removed from 92 museum specimens of *Cicindela d. dorsalis* from the Museum of Comparative Zoology (Harvard) collected between 1885 and 1971 from 21 towns in Massachusetts, Rhode Island, New Jersey and New York. With the exception of older specimens from the two extant Massachusetts sites, none of these locations currently supports beetle populations. Thus, none were sampled by Vogler & DeSalle (1993a) in their original study, which included only specimens from extant populations.

DNA isolation and manipulation

DNA from recently collected specimens was isolated using the protocol described for small scale preparations in DeSalle *et al*. (1993). DNA from legs of historic beetle specimens was isolated using a DTAB/CTAB preparation as follows: the leg was first dispersed into several pieces by crushing in a glassine envelope. The crushed semi-powdered particles were then transfered to an Eppendorf tube and treated as in Phillips & Simon (1995). The DNA from these isolations was kept at –20 °C until used in polymerase chain reactions (PCR).

Amplification and sequencing

PCR primers were designed to amplify a 73 base pair fragment (including primer lengths) so that degraded DNA could be used as target template for PCR. The primers used in this study were ALF101 (5′ GGAATAATTTTATTTATTACATCAG-3′) and ALF104 (5′ TCCTAGTTCAACTGCAGGAG-3′), which amplify a fragment between positions 4953–5026 of the mitochondrial COIII gene that includes the polymorphic site (at position 5016) discovered by Vogler & DeSalle (1993a). Reaction conditions were 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s for 28 cycles in an ABI 496 in 30-μL reaction volumes. All experimental PCR runs were accomplished alongside negative controls. Any PCR runs that showed a band in the negative controls were discarded in their entirety. PCR products were ethanol-precipitated and resuspended in 5 μL of water. 1 μl of the resuspended PCR product was used in cycle sequencing reactions with ABI BigDye ready reaction kits (PE Biosystems). Sequences were run on 5% polyacrylamide gels and analysed on an ABI 377 DNA sequencer (PE Biosystems). Sequences were compiled and aligned using SEQUENCHER software.

Results and discussion

Of 92 specimens from which DNA was extracted, we amplified and sequenced successfully the COIII fragment (described above) from 42 individuals from the range of *C. dorsalis* between Massachusetts and New Jersey (individuals from 20 sites altogether; Table 1). Typical amplifications were faint but easily identifiable single bands. Base calling of the position in question was unambiguous in all sequences. Nucleotide data for position 5016 in the mtDNA COIII gene are presented in Table 1.

Table 1 Locality data and year of collection for *Cicindela dorsalis* beetles sequenced successfully. Character states at the polymorphic position 5016 on the COIII gene are given in the right-hand column

Locality	Year	Char. state	Locality	Year	Char. state
Martha's Vineyard, MA	*c.* 1870	A	Long Beach, NY	1900	G
Martha's Vineyard, MA	*c.* 1870	G	Rockaway, NY	*c.* 1900	G
Martha's Vineyard, MA	1924	G	Rockaway, NY	1903	G
Martha's Vineyard, MA	1930	G	Rockaway, NY	1903	A
Martha's Vineyard, MA	1935	G	Edgmere, NY	1895	A
Nantucket, MA	1900	G	Coney Island, NY	*c.* 1900	G
Chatham, MA	1919	G	Coney Island, NY	*c.* 1900	A
Westport, MA	1932	G	Coney Island, NY	*c.* 1900	T
Westport, MA	1971	G	Point Pleasant, NJ	1905	G
Narragansett, RI	1914	A	Mantoloking, NJ	1907	G
Narragansett, RI	1914	G	Mantoloking, NJ	1907	G
Narragansett, RI	1914	G	Atlantic City, NJ	*c.* 1900	A
Kingston, RI	1900	G	Ventnor, NJ	1905	G
Montauk, NY	*c.* 1900	G	Ventnor, NJ	1905	G
Fire Island, NY	1895	G	Ventnor, NJ	1906	G
Fire Island, NY	1895	G	Ocean City, NJ	1905	G
Fire Island, NY	1900	G	Ocean City, NJ	1905	G
Oak Island, NY	1915	A	Cape May, NJ	1908	G
Oak Island, NY	1915	G	Cape May, NJ	1908	G
Oak Island, NY	1915	G	Cape May, NJ	1910	G
Jones Beach, NY	1930	G			

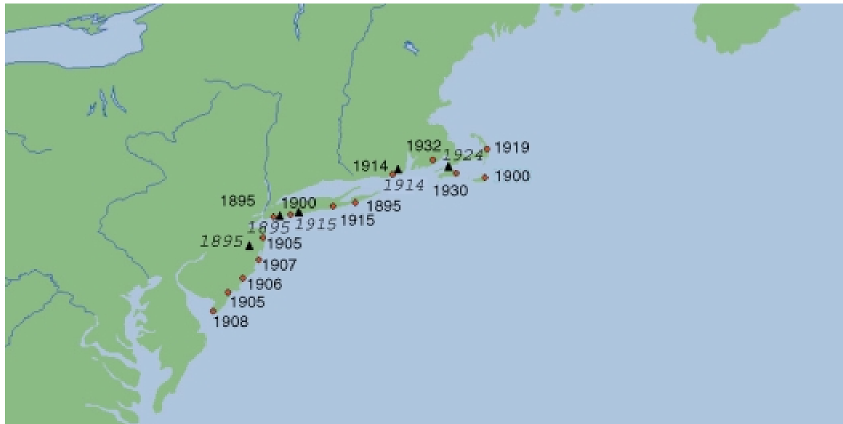

Fig. 1 Locations with collecting years of specimens identified as sharing currently diagnostic haplotype (guanine at COIII position 5016, designated by red circles) of extant New England population. Locations with collecting years of specimens identified as sharing alternative haplotype (adenosine at COIII position 5016) currently characterizing Chesapeake Bay populations are designated with black triangles.

According to our data, the nucleotide at position 5016 in COIII was polymorphic at a number of sites between New England and New York. The guanine at this position that diagnosed the Martha's Vineyard population in Vogler & DeSalle 1993a) study was indeed predominant within historical populations on Cape Cod, in Rhode Island and in numerous sites on Long Island (NY) and coastal New Jersey, as far south as Cape May (Fig. 1). However, individuals from a number of locations — including one 1924 specimen from the Martha's Vineyard population itself as well as individuals from Rhode Island and five sites on Long Island, New York — exhibited adenosine at this site, confirming polymorphism throughout the northernmost portion of the species' historical range (Fig. 1). Polymorphism was discovered at Martha's Vineyard, Massachusetts, Narragansett, Rhode Island (1914) and at Oak Island (1915), Rockaway (*c.* 1903) and Coney Island, NY (the latter three of which are Long Island sites; Fig. 1). Although collecting data were limited for some of these specimens, notably the Coney Island specimens, one of the Martha's Vineyard specimens and two of the Rockaway specimens, labels from specimens with similar histories suggest they were collected in the late 1800s or early 1900s. The 'southern' nucleotide character state (adenosine) also appeared at

Edgmere, Long Island, NY, which abuts Coney Island. A unique polymorphism was discovered in the sample of Coney Island specimens, such that adenosine, thymine and guanine were all represented in their sequences.

The fixation of the single nucleotide in the northernmost remnant populations of *Cicindela d. dorsalis* — and their concomitant diagnosability — appears to have followed the anthropogenic extinction of intermediate populations that maintained a clinal polymorphism. This is an example of how fragmentation of habitat can result in an increase in recognizable species and, when those are threatened critically, of units of independent management. A misguided conclusion of the empirical phenomenon in this context would be that fragmentation enhances biodiversity by virtue of creating more phylogenetic species, and should therefore be disregarded (if not encouraged). A more reasonable conclusion is that such fragmentation merely increases the number of diagnosable populations regardless of their instability or conservation value. In the case of threatened species such as *C. dorsalis* already under active management, that management will be encumbered to the extent that molecular diagnosibility is the exclusive arbiter to decisions of whether to manage such populations independently. This conclusion certainly supports Avise's (1989) observation about the proliferation of conservation units under the PSC if no behavioural or ecological data are considered. In this case, ignoring one of those units would result in the extirpation of a species from a majority of its historical range. We suggest that this example, however, simplified, illustrates the context in which genetic data must be taken in making conservation decisions as well as the important role of fixation (= the extinction of polymorphism) in our ability to understand the divergence process generally.

So far as protection and restoration of this species in the northeast are concerned, these data present an interesting question. Previous studies suggest a southern origin of *C. d. dorsalis*, yet the current configuration of genetic diversity among remaining populations raises the question of whether reintroduction stock for New England should be derived from the existing Massachusetts populations in order to maintain their uniqueness, or from the more southerly populations in order to restore past genetic diversity. Arguments could be made for either option. In this case, ecological and behavioural considerations, consistent with the molecular data, argue for the maintenance of the current pattern given that the Massachusetts populations are ocean-fronted: those in the Chesapeake Bay are not exposed to the same storm regimes, and the northern populations appear to have evolved behavioural adaptations such as larval retreat into backdune areas during the winter months (Nothnagle & Simmons 1990). In this case, the molecular genetic data and ecological/behavioural data corroborate one another in the current strategy to restore *C. d. dorsalis* to as much of its historical New England range as possible using the northernmost extant populations.

On the surface, this study represents little more than the documentation of an apparently recent and eminently reversible character fixation event. Few (if any) systematists would advocate assigning formal epithets to clusters of organisms identifiable by such paltry evidence as a single mitochondrial nucleotide substitution (Ballard *et al*. 2002). None the less, these data highlight some important features of the phylogenetic species criterion that warrant further discussion.

Many discussions of species concepts emphasize the incorporation of various well-established tenets of microevolutionary theory. This emphasis is based on a desire to understand the evolution of reproductive isolation and achieve some level of evolutionary comparability to the entities we call species. It has been argued that to the extent such approaches are practised, taxonomy and phylogenetic exploration will be beholden to population genetics (Nixon & Wheeler 1991; Luckow 1995), and the description of life on Earth encumbered. Character-based approaches accept that species are not comparable except by virtue of observable character state distributions that serve to determine whether or not an aggregate of individuals may serve legitimately as a terminal in phylogenetic analysis suitable for the exploration of historical affinities. As such, they have emphasized criteria that enable the effective exploration of evolutionary history first and foremost, regardless of whether phylogenetic species are isolated reproductively or stable over time. In his arrangement of species concepts according to divergence time, Harrison (1998) emphasized that different species concepts refer legitimately to different kinds of entities, some barely diagnosable, some the products of long histories of reproductive isolation and adaptation. Clearly, the empirical utility of the PSC is in identifying recently diverged or otherwise cryptic species. Identification of management units below formal taxonomic levels appears to represent an arena in which systematics and population genetics complement one another, and we suggest the character fixation approach to delineating management units provides a framework from within which to enable this complementarity.

With its focus on character fixation, i.e. the extinction of polymorphism, the phylogenetic species emphasizes discontinuity among character state distributions as the arbiter of recognition rather than reproductive continuity (Goldstein & Brower 2002). Extinction of species has long been recognized as an important aspect of macroevolution (Lundberg & Chernoff 1992), and the extinction of characters, or more precisely character states or haplotypes, has been articulated with respect to both the recognition of species generally (Nixon & Wheeler 1990) and, with

considerable prescience, to the *C. dorsalis* complex in particular (Vogler 1994). Although it probably has little to do with the evolution of reproductive isolation, the example of *C. dorsalis* illustrates the formation of recognizably phylogenetic species over time by virtue of the polymorphism's disappearance.

A common concern about the PSC is its instability and its sensitivity to sampling from natural populations (Walsh 2000). In the case we outline, further sampling of live organisms from threatened populations may add little either to our understanding of their evolution or to our ability to protect them unless novel sources of genetic data (e.g. more genes, microsatellites) are examined, and indeed when diagnostic features of a prospective management unit are so few in number as to be potentially refuted by an individual bearing a single nucleotide change, it must be recognized that a case for independent management is weakened. The trade-off between availability of data and impact on threatened populations is general to conservation endeavours and not resolved easily in this or any other context. We can offer only that major conservation or management decisions should not be made exclusively on the basis of single nucleotide polymorphisms, and that to the extent such data do enjoy a role they should be gathered with as much rigour as possible without further endangering the population in question.

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