Evolution and Phylogenetic Information Content of the ITS-1 Region in the Tiger Beetle *Cicindela dorsalis*

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Sequence divergence in the internal transcribed spacer region 1 (ITS-1) of the ribosomal DNA locus was assessed in subspecies of the coastal North American tiger beetle, *Cicindela dorsalis*. The spacer region was amplified using the polymerase chain reaction and cloned for sequencing. Of a total of 50 clones obtained from 12 specimens, 42 clones were different in at least one nucleotide position. In a parsimony analysis of these sequences, the main phylogenetic distinction was found to separate sequences from the Gulf of Mexico and the Atlantic Ocean. Within these two assemblages phylogenetic resolution was low, and the variation within individuals was almost as high as the variation within the entire lineage. The pattern of sequence variation suggests the existence of two forms of the ITS-1 that are maintained on different chromosomes. Polymorphisms of limited geographical distribution could be detected, and 41 additional clones were partly sequenced, to assess the geographic distribution of these polymorphisms in more detail. In a population aggregation analysis, the geographic pattern of ITS-1 distribution was basically congruent with that obtained in earlier studies from mitochondrial DNA in the same *C. dorsalis* populations.

Introduction

Ribosomal RNA (rRNA) genes have long been recognized as an attractive marker for phylogenetic studies (Hillis and Davis 1986; Mindell and Honeycutt 1990). The rRNA genes are organized in clusters of tandemly repeated units, each of which consist of coding sequences and several transcribed and nontranscribed spacer regions. Coding regions and spacers differ widely in their rate of evolution, and hence the rDNA gene clusters have the potential to reveal phylogenetic relationships ranging from the level of major phyla of living organisms to the population level (Hillis and Dixon 1991). The level of divergence observed in the spacer regions is appropriate for detecting differences between conspecific individuals, which provides a potentially useful marker with which to study the relationships of populations and closely related species.

In the past, DNA variation in these spacer regions was analyzed using restriction fragment length polymorphisms (RFLP) (for a list of taxa analyzed, see Hillis and Dixon 1991). Recently, these regions and, in particular, the internal transcribed spacers (ITS) have been analyzed more frequently using polymerase chain reaction (PCR) technology. Conserved stretches of DNA in the coding rDNA adjacent to the ITS can be conveniently used to design "universal" primers for the amplification of highly variable spacer regions. Several studies have applied this methodology to the analysis of phylogenetic relationships among closely related taxa and populations (Pleyte et al. 1992; Wesson et al. 1993; Schlötterer et al. 1994).

A difficulty with the use of the rDNA locus in phylogenetic analysis stems from the existence of polymorphisms among repeat units, which may cause extensive differentiation even within a single individual. However, it is generally assumed that concerted evolution results in the homogenization of individual repeats and produces a mostly uniform sequence in all repeats of a given species. Different mechanisms have been proposed for the process of concerted evolution including unequal crossing over and gene conversion. Unequal crossing over assumes recombination among tandem repeats either within or between (homologous or heterologous) chromosomes, resulting in the stochastic elimination of variation in individuals and populations. In contrast, nonstochastic processes, such as directed gene conversion, assume selection or "drive" for the homogenization of tandem repeats (Dover 1982). Empirical evidence for the latter scenario comes from studies on parthenogenetic lizards (Hillis et al. 1991).

The use of the rDNA locus in phylogenetic analysis of closely related organisms has been a matter of con-

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tention (Hillis and Davis 1988; Williams et al. 1988). In particular, the contradictory findings of no intraspecific variation from RFLP studies in frogs of the genus *Rana* (Hillis and Davis 1986) and substantial variation within certain *Drosophila* species (Williams et al. 1987) fueled this debate. A more recent DNA sequencing study on internal transcribed spacer region 1 (ITS-1) of Salmonid fishes does not address the problem of intraindividual and intraspecific variation (Pleyte et al. 1992), while another study revealed a substantial degree of such variation in mosquitoes (Wesson et al. 1993).

We investigated the utility of the ITS-1 region for the phylogenetic analysis of closely related taxa and for the study of phylogeographic variation by using the tiger beetle *Cicindela dorsalis* as a model system. On the basis of morphological studies (Boyd and Rust 1982), the species represents a complex of four subspecies and a closely related species, *C. curvata,* that occur along the coastline of North America from Massachusetts to Veracruz, Mexico (fig. 1). Our extensive analysis of mitochondrial DNA (mtDNA) revealed that haplotypes within this species complex exhibit various degrees of phylogenetic hierarchy. The geographic distribution of haplotype variation was highly structured, and individual populations differed in haplotype composition or frequency. The most notable feature of the mtDNA phylogeny is the deep phyletic separation of haplotypes from the Atlantic Ocean and the Gulf of Mexico (Vogler and DeSalle 1993, and in press). A similar pattern of phylogeographic differentiation of Atlantic and Gulf of Mexico assemblages has been observed in a variety of unrelated species (Avise 1992). Within the Atlantic assemblage, a high degree of geographic structuring of haplotypes was observed, but the phylogenetic relationships of haplotypes were only poorly resolved (fig. 1). The *C. dorsalis* complex provides a system in which to study the dynamics and molecular evolution of rDNA in natural populations at different hierarchic levels.

In particular, our study addresses the following problems. First, we analyze the extent of rDNA differentiation within and between single individuals, by sequencing the ITS-1 region of selected individuals from throughout the geographic range of the species complex. Second, the observed pattern of sequence variation is used to assess the congruence of rDNA data with evidence from mtDNA and morphology. Third, we address the problem of reconstructing phylogenies from multiple-copy loci by using a methodology based on the assumptions of the phylogenetic species concept. Last, we relate our findings to the tenets of concerted evolution theory; the differentiation of genotypes in populations may follow either stochastic or deterministic processes that potentially can be discerned from the patterns of genotype distribution.

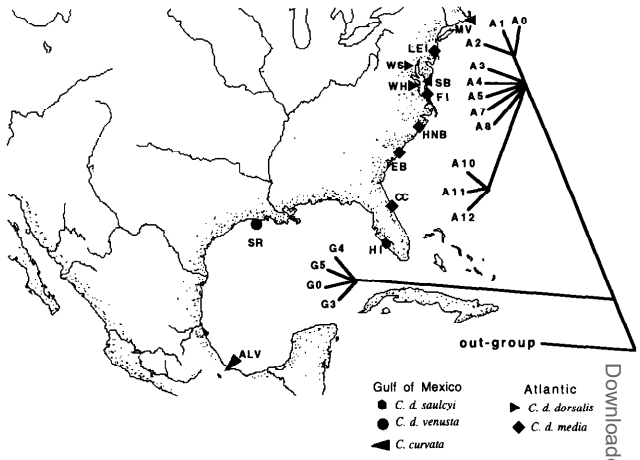


FIG. 1.—Location of *Cicindela dorsalis* populations and phylogeny of mtDNA haplotypes. Different symbols mark the locations of the four described *C. dorsalis* subspecies and the closely related *C. curvata.* A cladogram reconstructed from mtDNA haplotype sequences is superimposed on the map, giving a schematic representation of the location of haplotypes. For the exact sites associated with each haplotype, see table 1. The branch length in the cladogram is approximately proportional to the number of steps defining the respective nodes. Haplotypes A0 through A12 are from the Atlantic ocean populations, and haplotypes G0 through G5 are from the Gulf of Mexico populations. For details on the phylogenetic analysis of mtDNA haplotypes and on the geographic distribution of haplotypes, see Vogler and DeSalle (1993, and in press).

Material and Methods

DNA Amplification and Sequencing

Total genomic DNA was isolated from individual beetles as described elsewhere (Vogler et al. 1993). The ITS-1 was amplified using the primers 5′GCGTTCGAARTGCGATGATCAA and 5′GTAGGTGAACCTGCAGAAGG, which were designed to match the 5.8SrRNA (Hillis and Dixon 1991) and the 3′ end of the 18SrRNA coding regions using the reverse complement of the 3′ primer of Medlin et al. (1988). Amplification was carried out in a 100-μl volume using a *Thermus aquaticus* (*Taq*) polymerase kit (Perkin Elmer Cetus) following the manufacturer's instructions. Two nanograms of genomic DNA and 0.2 unit of polymerase were used in each reaction. Each cycle of the PCR consisted of denaturation for 1 min at 94°C, hybridization for 1.5 min at 50°C, and extension for 1.5 min at 72°C. The cycle was repeated 30 times. An aliquot of the reaction was subjected to electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. In most cases a single PCR product of ~400 bp was visible.

Approximately 0.1 μg of the PCR product was ligated into the vector pCR1000 provided with the TA Cloning Kit (Invitrogen, La Jolla, Calif.), according to the instructions of the manufacturer, and subsequently

transformed into competent cells that are provided with the kit. The transformation mixture was incubated in Luria medium (LM) at 37°C under shaking and subsequently plated on LM ampicillin plates (100 μg/ml) and X-Gal (100 μg/ml). Care was taken so that the incubation time did not exceed 45 min, to avoid cell division of transformants before plating. Thus, the majority of clones should result from independent ligation and transformation events).

White colonies were grown in LM supplemented with kanamycin (25 μg/ml), and plasmid DNA was isolated according to a miniprep protocol (Sambrook et al. 1989). Plasmids were sequenced as described by Toneguzzo et al. (1988), using the same primers as in the amplification reaction to determine the DNA sequence in both directions. Both strands could usually be read over the full length of the ITS-1 region.

Data Analysis

Individual sequences were aligned using MALIGN, version 1.87 (Wheeler and Gladstein 1993), which optimizes the alignment according to parsimony criteria. A gap-to-change cost of 20:15 was chosen (leading and trailing cost = 19). The cost of "extragaps," i.e., the gap cost after the initial gap, was set to 8. These parameters were empirically chosen among other gap-to-change cost ratios, because they resulted in a meaningful alignment between the two more distantly related clades while also aligning sequences within these two clades. A heuristic search procedure ("quick" option) was used for the alignment. With these specifications, the alignment of 42 different sequences took ~24 h on a 486DX/66 personal computer.

The resulting data matrix was subsequently used to reconstruct the most-parsimonious cladograms with the heuristic search option in PAUP version 3.0s (Swofford 1990). To obtain as accurate a representation of parsimony trees as possible, 10 replicates of a heuristic search using the random stepwise addition was used. Searches were also performed with the mhennig* option in HENNIG86 (Farris 1988). The display of phylogenetic trees with mapped character changes was done with CLADOS version 1.1 (Nixon 1991). Homoplastic characters were determined with the "hom 0" option that indicates only parallel or convergent forward changes as homoplastic. Statistical analysis of character changes was done using MacClade version 3.01 (Maddison and Maddison 1992).

Results

ITS-1 in *Cicindela dorsalis*

The extent of the intergenic region between the 18S and 5.8S coding regions referred to as ITS-1 was determined by similarity with the published sequences of *Drosophila melanogaster* (Tautz et al. 1988) and of the mosquito *Aedes aegypti* (Wesson et al. 1993). The length of the ITS-1 region in different clones of *C. dorsalis* was between 255 and 268 bp. In insects, the sequence of the ITS-1 segment is known in various species of the genus *Drosophila* (Schlötterer et al. 1994) and has a range of 529 to 851 bp in length. The ITS-1 region in *A. aegypti* comprises 419 bp (Wesson et al. 1993). In vertebrates, considerable length variation in the ITS-1 has been observed, ranging from 343 bp in the loach to 1,095 bp in humans (Gonzalez et al. 1990). The ITS-1 in *C. dorsalis* is the shortest recorded in animals. The GC content of 21.4% is lower than that in most reported sequences (Torres et al. 1990).

The analysis of the ITS-1 in *C. dorsalis* included a total of 12 different populations covering the entire geographic range of the species complex (fig. 1 and table 1). One individual from each of these populations was randomly selected for the PCR amplification, and between three and five independent clones covering the ITS-1 were sequenced from each of these individuals. Clones recovered from a single individual were labeled with the same letter code according to the location in figure 1. We analyzed 15 clones from three Gulf of Mexico populations and 35 clones from nine Atlantic Ocean populations. Of these 50 clones examined, 42 clones were different. One sequence was encountered five times (clones WS3, SB25, WH2, and FI5 were identical to WS1), and several haplotypes were represented twice (MV2 like MV1; LEI5 like LEI1; CC4 like CC1; and ALV5 like ALV4). Four clones (LEI1, FI2, HNB1, and EB2) have been inferred to be the result of recombination events and will be discussed in more detail in a separate study (Vogler and DeSalle, submitted). These putative recombinants were omitted from the cladistic analysis. This treatment is appropriate if the recombinants represent only a minor portion of the sample size (Templeton et al. 1987; Crandall and Templeton 1993). The aligned sequences for the remaining 38 different clones are given in figure 2, and all subsequent phylogenetic analyses are based on this data set. In addition to the sequences presented, 6 bp from the 3′ end of the 18SrRNA gene and 75 bp from the 5′ end of the 5.8SrRNA end were amplified and sequenced. No variation was found at these nucleotide positions. We also amplified and sequenced a single clone obtained from the closely related *C. chlorocepahala* for use as a potential outgroup but found the sequence to be too divergent for a reliable alignment to the *C. dorsalis* sequences.

The Type of Sequence Variation

Of the 301 positions in the aligned data matrix, 112 positions were variable, and 67 positions were phylo-

Table 1
Individuals of *Cincindela dorsalis* and *C. curvata* Used in This Study, and Their Location

(Individual) and Location	(Sub)species	mtDNA Haplotypes
(MV) Martha's Vineyard, Mass.	*dorsalis*	A0, A1, A2
(LEI) Little Egg Island, N.J.	*dorsalis/media*[a]	Not determined
(WS) Western Shore, Chesapeake Bay, Md.	*dorsalis*	A3
(SB) Silver Beach, Chesapeake Bay, Va.	*dorsalis*	A3, A5
(WH) Winter Harbor, Chesapeake Bay, Va.	*dorsalis*	A3, A4, A5
(FI) Fisherman Island, Atlantic Ocean, Va.	*media*	A5, A6
(HNB) Holden Beach, N.C.	*media*	A7, A10
(EB) Edisto Beach, S.C.	*media*	A10, A11, A12
(CC) Cape Canaveral, Atlantic Ocean, Fla.	*media*	A12
(HI) Honeymoon Island, Gulf of Mexico, Fla.	*saulcyi*	G4
(SR) Sea Rim State Park, Tex.	*venusta*	G0, G5
(ALV) Alvarado, Veracruz, Mexico	*curvata*	G3

SOURCE.—mtDNA data are from Vogler and DeSalle (1993, and in press).
[a] Subspecies designation was not entirely clear on the basis of morphological characters.

genetically informative. If only the phylogenetically informative positions are considered, five clones (LEI2, WH1, WH3, SR2, and ALV3) are identical to other clones in the data set. The variation can be divided into three classes. Characters of the first class are insertions/deletions in the aligned sequences that are longer than 2 bp. Sequence variation of this type distinguishes the two main groups of clones that represent the individuals from the coast of the Gulf of Mexico and the Atlantic ocean (see below). A total of 26 positions define such indels in the alignment. The remainder of the nucleotide variation can be divided into two additional classes that mostly reflect the variation within each of these two major assemblages. The majority of variable sites results from single-base mutations that are transformed into another base or to a gap. A total of 59 variable sites (27 phylogenetically informative) belong to this class. Another type of variation results from simple length variation in 1- or 2-bp repeats. A total of 27 variable sites, 16 of which are phylogenetically informative, group in this class. Most notable is the length variation in a string of adenosine residues starting at nucleotide position 19 and the number of AT repeats around positions 140, 222, and 272 (fig. 2). This type of sequence variation has been inferred to be the product of slippage events in other ITS sequences in vivo (Tautz et al. 1988; Wesson et al. 1993) and in vitro (Schlötterer and Tautz 1991). This class of nucleotide polymorphisms exhibits a high degree of homoplasy, which limits its importance in the phylogenetic reconstruction.

Phylogenetic Analysis

Critical for phylogenetic reconstruction is the treatment of gaps in the data matrix. We assume that the gaps in the alignment are the result of point mutations or single mutational events and thus can be used for the phylogenetic reconstruction in the same way that other types of base exchanges can. We coded the gaps in the data matrix of figure 2 in three different ways for the phylogenetic reconstruction.

First, each gap was treated as a single character, regardless of the length of the gap. If the length of a given gap was not identical in all sequences, each length variant was coded as a single character. This treatment assumes that length differences in a given gap result from more than one mutational event. Under this weighting scheme a total of six cladograms of 125 steps in length and a consistency index (CI) = 0.776 (excluding uninformative characters) and retention index (RI) = 0.931 were obtained using PAUP. The mhennig* option in Hennig86 (Farris 1988) also arrived at parsimony trees of 125 steps. One of these most-parsimonious solutions was arbitrarily chosen for representation in figure 3.

Second, each single position of a gap was coded as an independent character, thus increasing the relative weight of gaps in the phylogenetic analysis according to their length. This treatment resulted in three most-parsimonious cladograms of 167 steps (CI = 0.698; RI = 0.947) that were very similar in topology to the cladogram presented in figure 3 and did not contradict any of the relationships of clones established there.

Third, we completely excluded the gaps from the phylogenetic analysis (treated as missing data). Using PAUP, several thousand shortest cladograms of 69 steps, with a CI = 0.743 and RI = 0.944, were detected under this treatment of gaps. Phylogenetic relationships in the most-parsimonious trees and, in particular, in the strict-consensus tree derived from these trees were poorly re-

solved but did not contradict the relationships in trees that include the information from gaps (analysis not shown). Thus, the inclusion of gaps as characters substantially increases the amount of phylogenetic information. The similar homoplasy measures in cladograms that do and do not use the information from gaps indicates that evolutionary dynamics of gap positions is similar to that of other positions in the data matrix.

In the cladogram presented in figure 3, the haplotypes encountered in individuals from the Gulf of Mexico form a well-defined clade (clade III) that is distinguished from the haplotypes of the Atlantic Ocean by a minimum of 18 characters. Within the Atlantic lineage two clades can be distinguished. Clade I is diagnosed by three character changes and comprises 11 clones (MV4, LEI2 and 3, FI1 and 4, WS1 and 2, WH1 and 3, and SB1 and 3). The second clade (clade II), comprising all other clones of the Atlantic lineage, is defined by four synapomorphies. These clades can further be subdivided on the basis of geographically restricted polymorphisms, as discussed below.

The frequency of unambiguous character state changes was calculated for one of the most-parsimonious trees (fig. 4). The character state "gap" accounts for the majority of changes (53 changes) in this tree. Transitions (34 changes) and transversions (26 changes) are almost equal in number. Thus, the ITS-1 region does not exhibit the pronounced bias toward transitions observed in mtDNA of the same populations (Vogler and DeSalle, in press). This result is in accordance with data from sequence variation in the 18S rRNA coding region (Vawter and Brown 1993) and the ITS-2 (Schlötterer et al. 1994), which were also found to exhibit a transition/transversion ratio close to the rate of 1:2, which is expected when equal probability of change for all nucleotides is assumed.

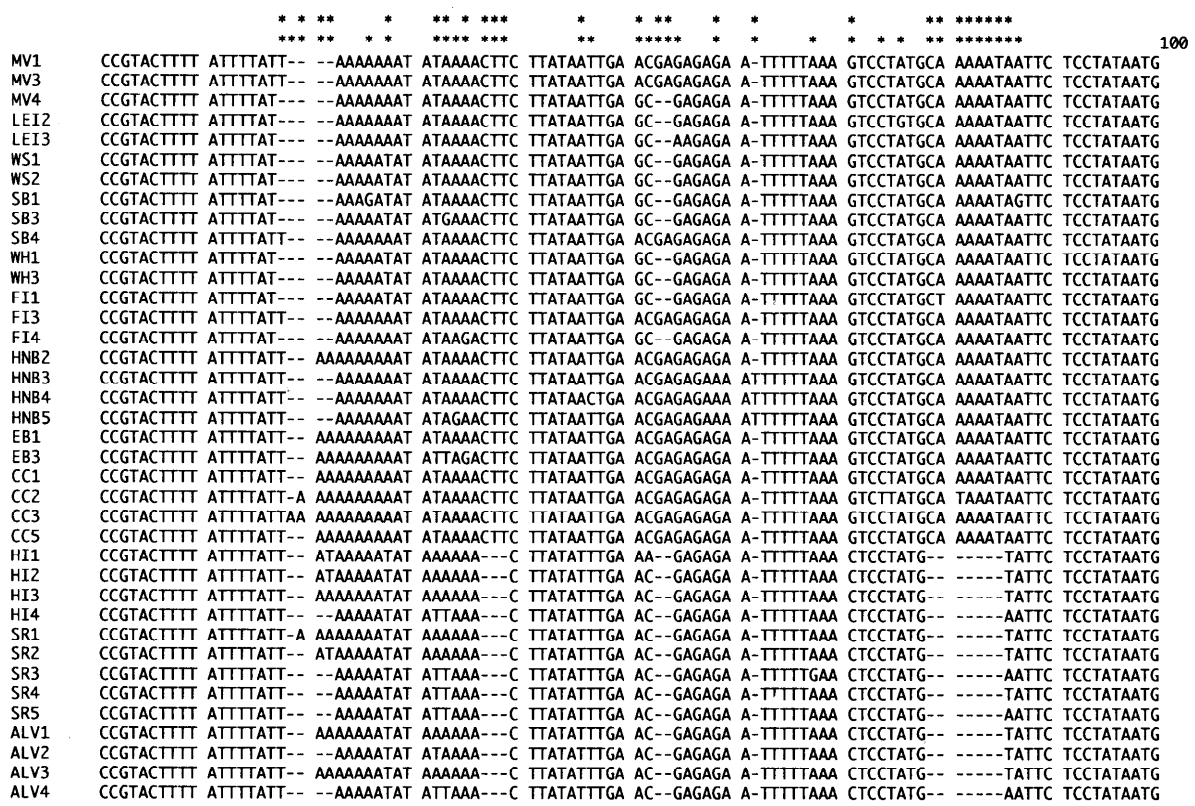
The Distribution of Variation

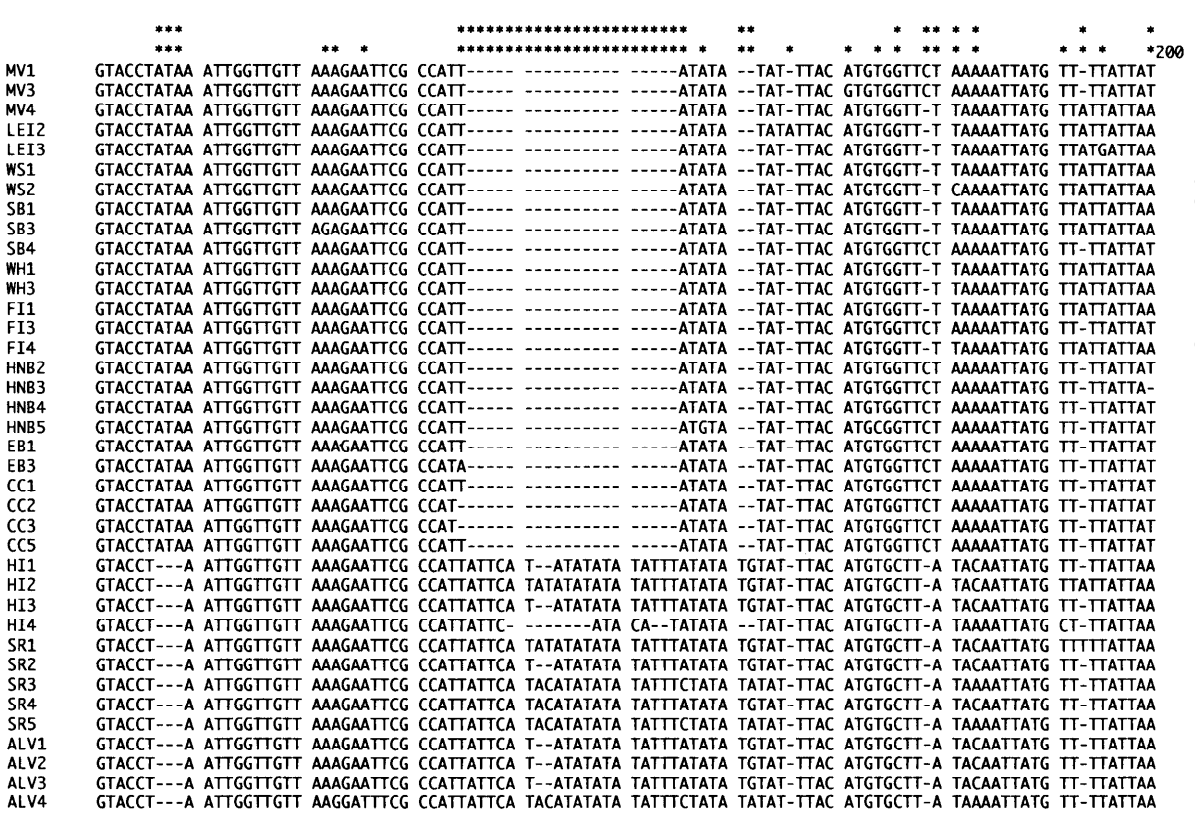
The main phylogenetic separation of ITS-1 sequences in the *C. dorsalis* complex coincides with the geographic separation of the populations from the Atlantic Ocean (clades I and II) and the Gulf of Mexico (clade III). This is consistent with the observation of a deep phylogenetic separation of Gulf and Atlantic populations, as inferred from mtDNA (Vogler and DeSalle, in press).

Within both of these major assemblages the geographic structuring of ITS-1 distribution was less pronounced, mostly due to the comparatively large intra-individual variation. Sequences recovered from a single individual are not always closely related to other sequences from the same individual. A tree constrained to group all clones obtained from a single individual together resulted in shortest trees of 174 steps and CI = 0.430 (compared with 125 steps and CI = 0.766 for the most-parsimonious solution). Most notable is the observation that within the Atlantic assemblage the distribution of variation does not coincide with the phylogenetic associations of the haplotypes. In particular, several individuals maintain haplotypes of both of the well-defined clades I and II. Similarly, in the Gulf Coast assemblage the haplotypes found within an individual are no more closely related to each other than to haplotypes of other individuals.

To analyze the distribution of clade I and II genotypes in single individuals in more detail, an extended data set was generated that included a total of 41 additional clones from the Atlantic Coast individuals, for which only those nucleotide positions that define clades I and II were scored. We also tried to assess the variation within a single population by studying a second individual from the Martha's Vineyard (MV) population (individual MV28). When the clones described above are included, the extended data set comprises a total of 76 clones from 10 individuals and nine populations of the Atlantic lineage (table 2). Seven of these clones belong to a category of possible chimerical sequences that are the subject of a separate communication (Vogler and DeSalle, submitted). There is a tendency for clade I clones to be found more frequently in individuals from the Chesapeake Bay and the Atlantic Coast from Virginia north, whereas clade II clones were more prominent in the populations from the southern part of the range. Several of the individuals contained both clades I and II sequences. The two individuals taken from the same population (MV and MV28) differed in the composition of their ITS-1 repeats in that MV has an equal number of clade I and clade II repeats, whereas MV28 exclusively exhibits clade II sequences. Thus, the presence of clades I and II repeats is not indicative of any particular population or geographic region.

Although the distribution of clades I and II haplotypes is not congruent with geographic patterns, we note that some nucleotide polymorphisms detected either in clade I or clade II haplotypes are confined to certain geographic regions. These polymorphisms include the character state at position 28 which is T in populations in the Chesapeake Bay and the surrounding areas and A in other regions. Another geographically confined polymorphism is found at position 21/22 at which haplotypes from the southern Atlantic states exhibit an AA insertion not observed in other regions. It is interesting that these locally confined polymorphisms are only detected in either clade I (position 28) or clade II (positions 21/22) haplotypes even if haplotypes of the other clade are present in the same individual. We interpret these polymorphisms as phyletic events that





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MV1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTATATATAT ATATACTAAA AG-TTTGATAA
MV3      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTATATATAT ATATACTAAA AG-TTTGATAA
MV4      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
LEI2     TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
LEI3     TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTTTATATAT ATATACTAAA AGTTTTGAT-A
WS1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
WS2      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
SB1      TAAATAGACT TTGATAGTCA G--TATATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
SB3      TAAATAGACT TTGATAGTCA G--TATATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
SB4      TAAATAGACT TTGATAGTCA G----TATAT ATATATGTAT --ACATTCAG GTATGTATGT GTTTCTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
WH1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTGTGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
WH3      TAAATAGACT TTGATAGTCA GTATATATAT ATATATATAT ACACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAGA AGTTTTGAT-A
FI1      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
FI3      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTGTAT ATATATATAT ATATACTAAA AGTTTTGAT-A
FI4      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
HNB2     TAAATAGACT TTGATAGTCA G----TATAT ATATATATGT --ACATTCAG GTATGTATGT GTTTTTGTGT ATATATATAT ATATACTAAA AG-TTTGATAA
HNB3     TAGATAGACT TTGTTAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATACACTAAA AGTTTTGAC-A
HNB4     TAAATAGGCT TTGATAGTCA G--TATATAT ATATGTATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
HNB5     TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
EB1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTA-GT GTATATATAT ATATACTAAA AGTTTTGAT-A
EB3      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTC-GT GTATATATAT ATATACTAAA AGTTTTGAT-A
CC1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACTAAA AGTTTTGAT-A
CC2      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT G--TATATAT ATATACTAAA AGTTTTGAT-A
CC3      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT G--TATATAT ATATACTAAA AGTTTTGAT-A
CC5      TAAATAGACT TTGATAGTCA G--TATATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTTCTGT GTATATATAT ATATACT-AA AGTTTTGAT-A
HI1      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATACAAAA AG-TTTGAT-A
HI2      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATACAAAA AG-TTTGAT-A
HI3      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTATATATAT ATACA--AAA AG-TTTTAT-A
HI4      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATAC-AAA AG-TTTGAT-A
SR1      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATAC-AAA AG-TTTGAT-A
SR2      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATACAAAA AG-TTTGAT-A
SR3      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATAC-AAA AG-TTTGAT-A
SR4      TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATACA--AAA AG-TTTGAT-A
SR5      TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATAC-AAA AG-TTTGAT-A
ALV1     TAAATAGACT TTGATAGTCA G------TAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATACA--AAA AG-TTTGAT-A
ALV2     TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATACAAAA AG-TTTGAT-A
ALV3     TAAATAGACC TTGATAGTCA G--------T ATATATATAT --ACATTCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATACA--AAA AG-TTTGAT-A
ALV4     TAAATAGACT TTGATAGTCA G----TATAT ATATATATAT --ACAATCAG GTATGTATGT GTTTTT--GT GTGTATATAT ATATACAAAA AG-TTTGAT-A
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FIG. 2.—Aligned sequences for 38 *Cicindela dorsalis* ITS-1 clones. The alignment was generated by MALIGN (Wheeler and Gladstein 1993) using the parameters described in Material and Methods. Variable nucleotide positions are indicated by an asterisk, and phylogenetically informative positions are indicated by double asterisks.

occurred subsequent to the split of clades I and II. These polymorphisms are apparently able to spread through individuals and subsequently through geographic regions. These polymorphisms do not spread, however, through all repeat units within an individual, indicating some barrier to the homogenization between clades I and II haplotypes.

Population Aggregation Analysis

The phylogeny and the geographic distribution of ITS-1 sequences can be analyzed in the context of a comprehensive survey of mtDNA available for *C. dorsalis.* One possible way of comparing the degree of resolution provided by both data sets is population aggregation analysis, which was developed for the delimitation of phylogenetic species by Davis and Nixon (1992). The usefulness of the ITS-1 as a phylogenetic marker at low taxonomic levels can be addressed by comparing the basic phylogenetic units recognized from the analysis of different genetic loci. We therefore investigated whether in the analysis of both the mtDNA and the ITS-1 data sets the same groups of populations are recognized to be phylogenetic species.

In a population aggregation analysis, characters are scored for individuals from local populations. If no character consistently distinguishes the individuals from two populations, these populations belong to the same phylogenetic species. Only diagnostic characters that distinguish all individuals in a group of populations from other such groups are considered sufficient evidence to infer hierarchic descent relationships among these populations. Hence, only those groups that exhibit such characters are the units amenable to phylogenetic analysis, i.e., are phylogenetic species (Davis and Nixon 1992).

For the purpose of this analysis the ITS-1 clones encountered in single individuals of *C. dorsalis* were considered to represent a sample of the total diversity in a given population. In the population aggregation analysis we, therefore, treated each clone as an individual of a population, and the specimens of *C. dorsalis* from which these clones were obtained are considered the populations in the analysis. The character states at polymorphic sites were scored for each clone, and a profile of character states (character state 0, 1, or inconstant ±) for each population was recorded (fig. 5, *upper panel*). The distribution of characters grouped the Atlantic populations and the Gulf of Mexico populations into two different groups that can be consistently separated by a total of 15 characters. Within both of these groups, no

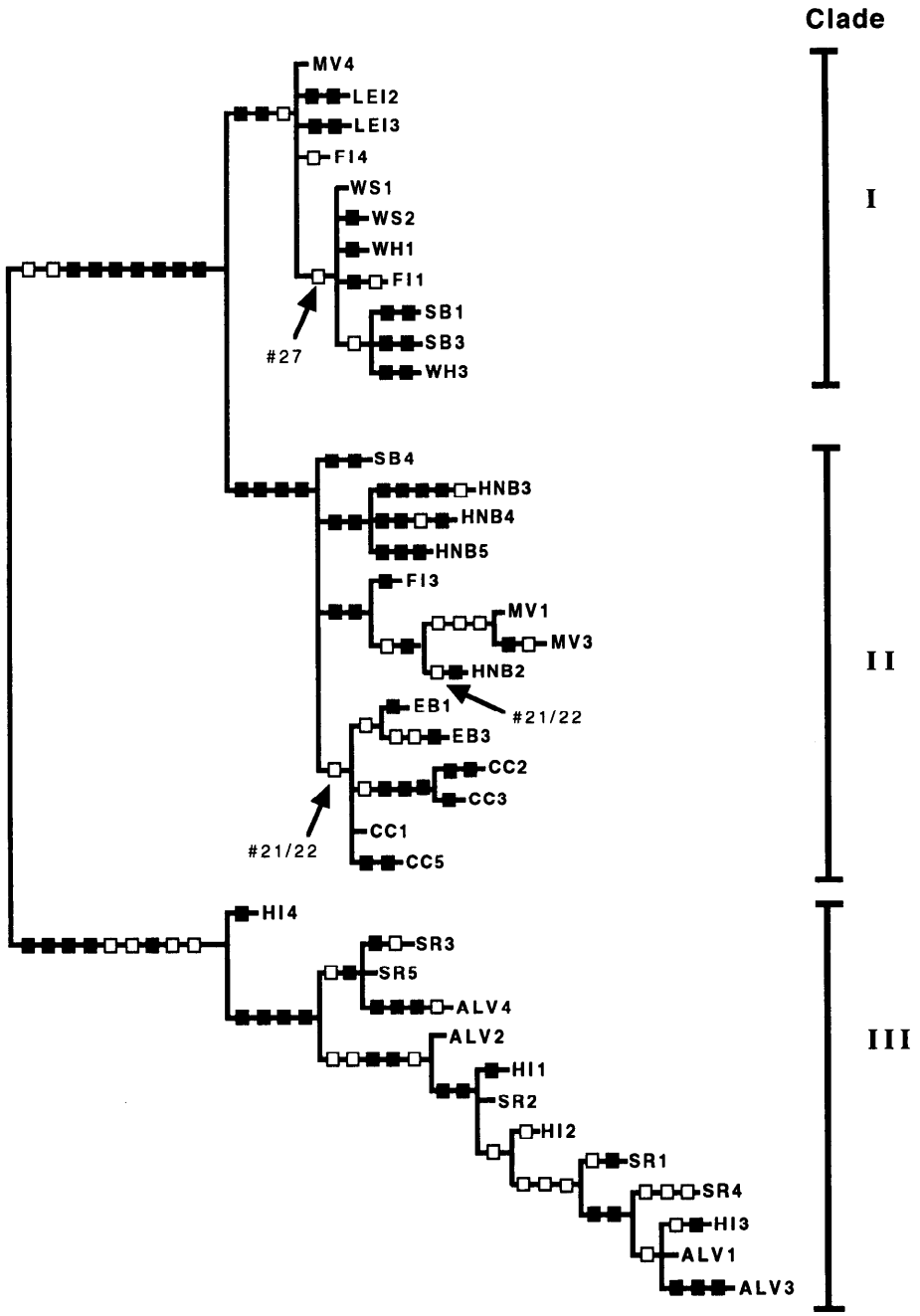

FIG. 3.—One of six most-parsimonious trees generated from 38 ITS-1 clones shown in fig. 2. Gaps in the alignment were treated as a single character only, regardless of their length. The topology of the most-parsimonious trees only differed in the relative position of clones in clade I in which relationships are unresolved. Characters were mapped on the tree after midpoint rooting by using CLADOS (Nixon 1991). Unblackened boxes indicate homoplastic changes. The clones were subdivided into three clades (I–III) on the basis of phylogenetically informative characters. Polymorphisms in nucleotide positions 21/22 and 28, which had geographically limited distribution in the Chesapeake Bay and in the southern Atlantic states, respectively, are marked with arrows.

further subsets of populations exhibit any character that distinguishes it to the exclusion of other populations. Consequently, the Atlantic and the Gulf of Mexico assemblage cannot be further subdivided into additional phylogenetic species. Some of the polymorphisms that distinguish haplotypes within the Atlantic region seem to follow a pattern in which haplotypes from the northern parts of the range are different from the haplotypes in the south. However, in most of these cases the variation is gradual. As a result, the Atlantic lineage, com-

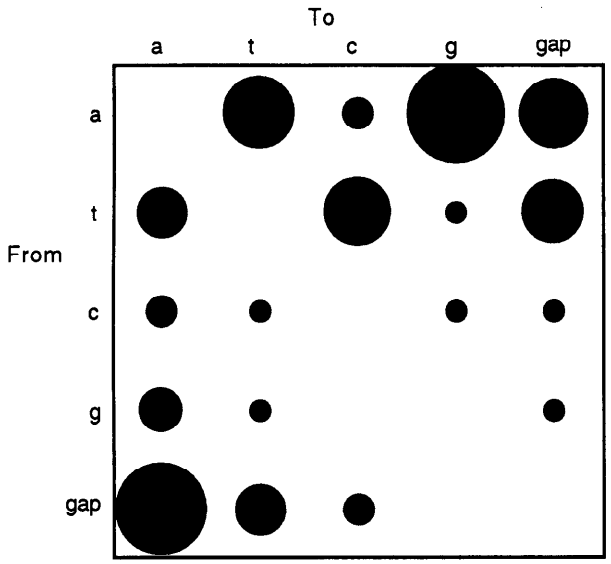

FIG. 4.—Frequency of character changes in one of the three most-parsimonious trees. The frequency of change was calculated using the State Change & Stasis menu in MacClade (Maddison and Maddison 1992). The size of the circles is proportional to the number of character transformation on the tree in fig. 3.

prising two morphologically distinguishable subspecies, cannot be subdivided into more than one phylogenetic species on the basis of ITS-1 sequences.

The distribution of ITS-1 clones is compared with the occurrence of mtDNA haplotypes that have been analyzed for the same set of populations in figure 5 (*lower panel*). The grouping of populations using the mtDNA information is basically the same: Atlantic and Gulf of Mexico populations are separated by a total of 39 distinct characters, clearly subdividing them into two phylogenetic species. Within the Atlantic lineage there is only a single character that diagnoses a population (Martha's Vineyard) to the exclusion of other populations. Therefore we concluded elsewhere (Vogler and DeSalle 1993) that there are no populations that are diagnosed as phylogenetic species on the basis of the mtDNA data, except for the population at Martha's Vineyard whose geographic and genetic separation presumably is the result of recent habitat destruction and extinction of adjacent populations (Vogler et al. 1993).

In summary, the distribution of genetic variation in mtDNA and ITS-1 is very similar. Both data sets provide strong evidence for two phylogenetic species corresponding to Gulf of Mexico and Atlantic populations. The data also agree with the observation that the Atlantic lineage of *C. dorsalis* is a cluster of closely related populations that are weakly separated genetically according to their geographic distance.

Discussion

ITS-1 Sequences in Phylogenetic Analysis

The *Cicindela dorsalis* complex presents a unique system in which to investigate the differentiation of the ITS-1 region and its use for phylogenetic analysis because of the existence of isolated populations of several morphologically recognized subspecies covering different biogeographic regions, thus providing different levels of phylogenetic differentiation. We find a substantial amount of variation in *C. dorsalis* that is partly coincident with major phylogeographic regions but to a large extent has to be attributed to variation within individuals. This finding counters concerted-evolution theories and complicates the use of the ITS-1 region as a marker in phylogenetic analysis.

Our sequencing approach to detect ITS-1 variation differs from that of earlier studies that use RFLP to address intraspecific variation in the rDNA locus. One concern of our technical approach is the reliability of the amplification reaction, which is the first step in the generation of the clones. It is possible that some of the

Table 2
Number of Clones That Can Be Assigned to Either Clade I or Clade II or to Presumed Hybrid Sequences

Individual	Sex	Clade I	Clade II	Hybrid Sequences	*n*	Subspecies	Location
MV	F	6	6	0	12	*dorsalis*	Massachusetts
MV28	M	0	10	0	10	*dorsalis*	Massachusetts
LEI	F	6	0	4	10	*dorsalis/media*	New Jersey
WS	M	6	1	0	7	*dorsalis*	Chesapeake Bay
SB	F	3	1	0	4	*dorsalis*	Chesapeake Bay
WH	F	6	2	0	8	*dorsalis*	Chesapeake Bay
FI	F	6	2	1	9	*media*	Virginia
HNB	M	0	4	1	5	*media*	North Carolina
EB	?	0	2	1	3	*media*	South Carolina
CC	F	0	9	0	9	*media*	Florida

NOTE.—The numbers refer to the extended data set that comprises the clones presented in fig. 2, as well as 41 additional clones for which only the character states defining clade I and clade II sequences were scored.

ITS-1

Pop	Clones	Attribute # 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Characters
MV	4	±	1	0	1	1	±	±	1	1	1	±	±	±	±	1	±	±	±	1	1	1	±	±	0
LEI	3	0	1	±	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	±	0	1	0
WS	3	0	1	1	1	1	0	1	1	1	1	1	±	0	1	1	0	0	0	1	1	1	0	1	0
SB	4	±	1	±	±	1	±	±	1	1	1	±	±	±	±	±	0	0	0	1	1	1	0	1	0
WH	3	0	1	1	1	1	0	1	1	1	1	1	1	0	1	±	0	0	0	1	1	1	0	1	0
FI	4	±	1	±	1	1	±	±	1	1	1	±	±	±	±	1	±	±	0	±	±	1	0	1	0
HNB	4	1	1	0	1	1	1	0	±	±	1	0	0	1	±	±	0	±	0	1	±	1	±	±	0
EB	2	1	1	0	±	±	1	0	1	1	±	0	0	1	1	1	0	±	±	1	1	1	0	1	0
CC	5	1	±	0	1	1	1	0	1	1	±	0	0	1	1	±	0	0	±	±	1	±	0	1	0
Gulf of Mexico		(15 clones)																							15

mtDNA

Pop	Indiv	Attribute # 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Characters
MV	22	±	0	1	1	1	±	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
WS	4	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0	0
WH	24	1	1	1	1	1	1	1	1	1	1	±	±	1	±	1	1	±	1	1	1	±	1	1	1	±	0
SB	21	1	1	1	1	1	1	1	1	1	1	1	±	1	±	1	1	1	1	1	1	±	1	1	1	±	0
FI	29	1	1	1	1	1	1	1	1	1	1	1	0	1	1	±	1	1	1	1	1	0	1	1	1	1	0
HNB	16	1	1	1	±	±	1	±	±	±	±	±	1	1	1	1	±	1	±	±	1	±	1	±	1	1	0
EB	4	1	1	1	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1	0
CC	1	1	1	1	0	0	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1	0
Gulf of Mexico		(45 indivduals)																									39

FIG. 5.—Population aggregation analysis for the *Cicindela dorsalis* complex, based on ITS-1 (*upper panel*) and mtDNA (*lower panel*). The ITS-1 data are based on the aligned sequences of fig. 2, with each gap scored as a single character. The mtDNA data are based on 650 bp of sequence information (attributes 1–12) and 13 RFLP polymorphisms that distinguish Atlantic individuals. These sequences were the basis for the haplotype designations given in fig. 1 and table 1. No mtDNA data for the population at LEI were available. The number of attributes that consistently distinguished all Atlantic haplotypes from the Gulf of Mexico haplotypes is given in the last line of both panels. There were several additional attributes that distinguished haplotypes within the Gulf of Mexico assemblage, none of which defined any subgroups in this assemblage (not shown).

observed variation is the result of mistakes introduced by the *Taq* polymerase during amplification. In particular, simple repetitious sequences have been shown to increase in length when amplified by various DNA polymerases in vitro (Schlötterer and Tautz 1991). This type of error could account for the observed length variability in AT repeats. The amplification by the *Taq* polymerase seems to be accurate for the nonrepetitive sequences, as is evident from the 75 bp of 5.8S rRNA coding region that did not differ in any of the 50 clones analyzed. Thus, the variation in single base pairs is not likely to be an artifact of the amplification and cloning strategy. Any variation introduced in vitro would predominantly generate phylogenetically uninformative (autapomorphic or homoplastic) character changes that do not substantially affect the results of our phylogenetic analysis of ITS-1 sequences.

The rRNA array in animal species typically consists of several hundred tandemly repeated copies (Hillis and Dixon 1991). In *C. dorsalis,* the large diversity in the sequence of these rRNA clusters is reflected in the number of different clones that were isolated from each individual. From a total of 50 clones analyzed, 42 clones were different in their DNA sequence, and only one of these sequences was observed more than twice (clone WS1 was identical to four other clones isolated from four different individuals collected in the Chesapeake Bay and adjacent areas). It is, therefore, unlikely that the total amount of genetic variation within a population or even within a single individual can be comprehensively described with the cloning and sequencing technique used here.

However, we conclude that the sequence information obtained in this study is sufficient to give an

adequate representation of the main characteristics of the genetic variation of the ITS-1 in *C. dorsalis.* The characters that define the two major phylogenetic subdivisions, the split of Atlantic and Gulf of Mexico lineages, were consistently observed in the extended data set of 15 Gulf of Mexico and 76 Atlantic clones. We expect that these characters will also be found if the variation within and between more individuals and populations is analyzed. Similarly, all Atlantic clones could be assigned unequivocally to either clade I or clade II (or to a class of hybrid sequences of presumed clade I/II recombinants). Therefore, while it cannot be ruled out that haplotypes will be found that do not fall into any of the described clades, it is unlikely that a larger sample size would change the conclusions on the genealogy of major ITS-1 types and on the distribution of variation in the *C. dorsalis* complex. Variation within these main groups can be expected to be substantial and will consist mostly of autapomorphic and homoplastic changes that do not interfere with the cladistic analysis of haplotype sequences.

The use of these clades in a phylogenetic analysis, however, needs to take into account the distribution of the haplotypes in individuals and populations. Our approach to extract information from the ITS-1 sequences is based on the assumptions of the phylogenetic-species concept. Under this concept, all individuals of a phylogenetic species can be diagnosed by characters, to the exclusion of other such species. DNA markers that occur in a single individual (or in a single population), even if cladistically well resolved, cannot be used in a phylogenetic analysis of either populations or closely related species. Only those characters that are diagnostic for all individuals of an entire population can be used in the phylogenetic reconstruction of populations. When the ITS-1 or other multiple-copy markers are used in phylogenetic reconstruction at low taxonomic levels, it is necessary to establish that the nucleotide polymorphisms used indeed fulfill these requirements. In *C. dorsalis* only the nucleotide variation that distinguishes Gulf and Atlantic haplotypes is phylogenetically informative, according to these criteria.

Below the level of the phylogenetically relevant information we detected a small class of characters within the Atlantic lineage that exhibited local endemism. Through the investigation of these characters it could be shown that, in fact, substantial geographic coherence exists among some of the populations. While these characters cannot strictly be used as evidence for hierarchic descent, they possibly provide suitable markers for a more comprehensive study, under the assumptions of classical population genetics. They also provide some information on the processes underlying the sequence evolution of the ITS-1 region and the rDNA locus in natural populations, as discussed below.

Concerted Evolution

In an early discussion of the importance of concerted evolution, Williams et al. (1988) cautioned against the idea that rDNA homogenization within a species requires only short periods of evolutionary time. In *C. dorsalis,* the large number of different rDNA repeats that have been isolated from single individuals and the presence of both clade I and clade II repeats in single individuals argue for the lack of an efficient homogenization system. Polymorphisms in rDNAs repeats are apparently maintained for periods of time that are sufficient for the differentiation of morphologically distinct subspecies.

Despite the lack of homogenization of rDNA repeats in general, several local polymorphisms endemic to certain geographic regions were encountered. These polymorphisms are subtypes of either clade I or clade II repeats that apparently went to fixation in the presence of the other main type in the same individual (or population). Thus, the homogenization of these local polymorphisms is under different dynamics than the homogenization of clade I and clade II variants.

This observation could be explained if clade I and clade II clusters were located on different chromosomes. It is conceivable that the rDNA homogenization proceeds more efficiently within a single chromosome than among different chromosomes. This hypothesis is consistent with the observation of different rates and types of sequence variation for rDNA repeats located on the X and Y chromosomes in *Drosophila melanogaster* (Tartof and Dawid 1976; Williams et al. 1987). Consequently, polymorphisms can be fixed on one chromosome (e.g., in all clade I repeats, as in position 28 of the Chesapeake Bay individuals), while the other chromosome (harboring clade II sequences) in the same population is also present and homogenized for a particular clade II type. This interpretation of the phylogenetic analysis of the ITS-1 sequences would provide an explanation for the seemingly random presence or absence of clade I or clade II types in some individuals. In one example, individuals in the population at Martha's Vineyard were found to exhibit either both clade I and clade II (MV) or only clade II repeats (MV28), indicating "heterozygous" or "homozygous" conditions, respectively. One of the two types could be eliminated from certain populations by stochastic processes, an explanation that could account for the absence of clade I repeats in the populations from the southern Atlantic states. (Alternatively, these observations could be the result of limited sample numbers.)

Under the concept of molecular drive, the directed homogenization of rDNA repeats would always lead to the fixation of the same ITS-1 type. In *C. dorsalis* two ITS-1 types are maintained for sufficiently long periods of time to render different subspecies paraphyletic with respect to their ITS-1 sequences. This is clearly different from the situation in parthenogenetic lizards, where homogenization for a particular rDNA type occurs after very few generations (Hillis et al. 1991). As the authors of that study noted, the results obtained in a parthenogenetic system may be atypical and not applicable to sexual species. Alternatively, selection for one of the two types can play a role in the observed efficient homogenization. The occurrence of more fit rDNA types could also account for the elimination of large numbers of diverse rDNA repeats ("selective sweeps") resulting in the temporary loss of all variation. This would also explain why certain species apparently are homogeneous for their ITS repeats, while others, presumably not affected by such recent selection processes, exhibit high levels of rDNA variation.

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