Use of Random Amplified Polymorphic DNA (RAPD) Markers to Assess Relationships Among Beach Clams of the Genus *Donax*

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ABSTRACT

The polymerase chain reaction was used to amplify genomic DNA from nine populations of donacid clams representing six taxa occurring in three sympatric pairs. The randomly amplified polymorphic DNA (RAPD) markers produced by this technique successfully distinguished among all taxa. Each taxon possessed a unique subset of markers and one member of each sympatric pair differed from the other by several markers. The taxa also separated clearly into two groups, one North American and the other Caribbean. Use of RAPD markers as characters in a cladistic analysis produced well resolved phylogenetic trees of high consistency.

Key words: RAPD, PCR, phylogeny, biogeography, *Donax*.

INTRODUCTION

Comparisons of closely related species are often informative with regard to the functional biology of their shared characters, while comparisons of sympatric congeners can provide insights to the selective forces and adaptive complexes that are important in speciation (Larson, 1989). Western Atlantic species of beach clams in the genus *Donax* are particularly appropriate candidates for such comparative studies. Six species or subspecies have been described from the coastal waters of the eastern United States, and nearly as many have been reported from the Caribbean (Morrison, 1971). All of these species are highly polymorphic for shell colors and patterns, and one (*Donax variabilis* Say, 1822) serves as a classic example of a hyper-variable species (Moment, 1962). Both in the western Atlantic and world-wide, *Donax* often occur as sympatric species pairs or triplets (Abbott, 1974; Ansell, 1983; Morrison, 1971) that partition their shared habitat. The selective forces that promote the rise and maintenance of hyper-variable polymorphisms are poorly understood (Allen, 1988; Owen & Whitely, 1988) and may be clarified by comparative studies. Furthermore, the interaction of habitat partitioning and hyper-variability has been studied only in the snail genus *Cepaea* (Clarke, 1960). Our long-term goal is to investigate these questions in *Donax*. However, the sympatric co-occurrences of two or more similar and often highly polymorphic species have placed the systematic status of some of these taxa in dispute.

Morrison (1971) recognized six taxa along the Atlantic and Gulf coasts of the United States: 1) *Donax fossor* Say, 1822, which ranges from New York to North Carolina; 2) *Donax variabilis variabilis* Say, 1822 (as *Donax roemeri protracta* Conrad, 1849[1]), which occurs from Virginia southward along both coasts of Florida and westward along the Gulf coast to Mississippi; 3) *Donax parvulus* Philippi, 1849, with a range that extends from North Carolina to the eastern coast of Florida; 4) *Donax dorotheae* Morrison, 1971, which occurs along the Gulf coast from Florida to Louisiana; 5) *Donax variabilis roemeri* Philippi, 1849 (as *Donax roemeri roemeri* Philippi, 1849), which ranges from the Mississippi delta westward along the coasts of Texas and Mexico; and 6) *Donax texasianus* Philippi, 1847 with the same range as *D. variabilis roemeri*. Only in the northern-most part of its American range is *Donax* represented by a single species, *D. fossor*. Elsewhere, species of *Donax* generally occur as sympatric pairs. In the Caribbean and along the coast of northern South America, the genus is represented by *Donax denticulatus denticulatus* Linné, 1758, *D. denticulatus stephaniae* Petuch, 1992, *D. striatus* Linné, 1767, and *D. vellicatus* Reeve, 1855, with two or three species occurring together. *Donax denticulatus* is the type species of the subgenus *Chion* Scopoli, 1777 (Gray, 1847).

Where species of *Donax* co-occur, they subdivide the habitat in much the same way. As described by Morrison (1971), *Donax parvulus*, *D. dorotheae*, and *D. texasianus* all occupy the same habitat and are allopatric, replacing one another along the coast, each co-occurring with *Donax variabilis*. In every case, *D. variabilis* is larger, occurs

[1] See Boss (1970) and Melville (1976) for details on the nomenclature of this taxon.

higher in the inter-tidal zone, and migrates more actively with the tides. The other three species are much smaller, occur at the bottom of the inter-tidal zone and spend much of the year sub-tidally. During at least some parts of the year, these species occur with *D. variabilis* and both can be collected in the same handful of sand. This co-occurrence, combined with morphological similarity, has placed the status and rank of several taxa in dispute. Abbott (1974) considered *D. parvulus* to be an offshore ecological form of *D. variabilis*. Loesch (1957) reviewed the names of donacid taxa reported from the Texas coast and stated that morphological intergrades had been reported between *D. texasianus* and what may have been *D. dorotheae* near Louisiana and between *D. texasianus* and *D. variabilis roemeri* along the Texas coast. Chanley (1969) suggested that *D. fossor* represents a [temporary, seasonal] summer extension of the range of *D. variabilis*.

In the Caribbean, *D. vellicatus*, like several of the American taxa, remains primarily sub-tidal. *Donax denticulatus* and *D. striatus* partition their habitat somewhat differently, but the division is still based on the tendency to migrate with the tide, as well as a preferred position in the inter-tidal zone. Wade (1967, 1968) has observed that, although *D. denticulatus* and *D. striatus* did sometimes occur together, *D. denticulatus* migrated actively throughout the tidal cycle while *D. striatus* maintained a constant position higher in the inter-tidal zone.

Historically, separations of donacid species and subspecies have been based exclusively on shell morphology (primarily on size, inflation [obesity], and degree of striation), which is subject to environmentally induced variation. Molecular data are often suitable for resolving taxonomic questions of this nature where the cause of morphological differences cannot be ascribed to either genetic or environmental differences. The restriction of a molecular character, either an allele in an allozyme system or a DNA marker, to one member of a sympatric species pair is taken as evidence of the absence of interbreeding between the two taxa. While molecular evidence cannot confirm genetic isolation when two populations are allopatric, it can at a minimum demonstrate genetic divergence. A previous attempt to differentiate between sympatric populations of *D. parvulus* and *D. variabilis* using allozyme data yielded ambiguous results (Nelson *et al.*, 1993). While allele frequencies differed between the two groups, no alleles unique to either taxon were found. Because the allozyme data could not distinguish the two taxa, additional molecular markers were sought.

The use of randomly amplified polymorphic DNA (RAPD) markers to differentiate between closely related individuals, populations and species was introduced in 1990 by Williams *et al.* and by Welsh and McClelland. In essence, segments of genomic DNA are amplified by the polymerase chain reaction (PCR) using a single very short primer (9 to 11 nucleotides) whose sequence might occur multiple times within the genome. The RAPD amplification of genomic DNA produces a set of fragments of various molecular weights, their number and size depending upon the number of times the primer sequence occurs in the genome, as well as the distances between pairs of primer sites. The RAPD technique has already been shown to produce genetic markers for Mendelian segregational analysis (Klein-Lankhorst *et al.*, 1991), genetic markers to distinguish among individuals within one population (Smith *et al.*, 1992), genetic markers that identify cultivars within a species (Hu & Quiros, 1991), and genetic markers that discriminate among species within a genus (Kambhampati *et al.*, 1992).

As a necessary prelude to the long-term goal of investigating morphological polymorphisms and ecological niche-partitioning in *Donax*, the present study seeks to resolve the systematic status of several western Atlantic donacid taxa and to discern their phylogenetic relationships. Failure of the allozyme data to resolve these taxa definitively has led us to investigate the utility of randomly amplified polymorphic DNA (RAPD) markers to distinguish between populations, subspecies and species in the genus *Donax*, as well as to determine the relationships of these populations and taxa using cladistic methodology.

MATERIALS AND METHODS

1. *COLLECTION OF SPECIMENS*

Donax were collected at the six locations shown on the map in Figure 7. These collections included samples from nine populations, representing six taxa, which are listed in Table 1 and are further identified as follows. 1) *Donax variabilis variabilis* (DVF) and 2) *Donax parvula* (DPF) were collected in the spring of 1990 at Indiatlantic Beach, on the Atlantic coast of Florida. 3) *Donax variabilis roemeri* (DVR) and 4) *Donax texasianus* (DTT) were collected at Corpus Christi, on the Gulf coast of Texas, in spring of 1990. 5) *Donax variabilis variabilis* (DVG) were collected in 1992 at Alligator Point on the Gulf coast of Florida. Collections were made in Jamaica in both May and November of 1991 for 6) *Donax denticulatus* (DDM) from Port Maria, a town on the northern coast of the island, along the town's seawall 7) *Donax denticulatus* (DDN) from Negril, a town on the western side of the island, at the Cosmos Beach Club 8) *Donax denticulatus* (DDB) and 9) *Donax striatus* (DSB) from Black River, a town on the southern coast of the island, at the Bridge House Inn.

Donax were collected by sieving sand from the intertidal zone of a beach. The animals were placed in plastic bags and kept cool until they could be identified to species, then frozen at −20° C and shipped to George Mason University. Thereafter, clams were maintained at −60° C until DNA was extracted. Attempts to collect *Donax fossor* and *D. dorotheae* at their respective type localities were unsuccessful, and these taxa are not included in the present study. Shells of the samples used in this study are deposited in the collections of the National Museum of Natural History, Smithsonian Institution. Catalogue numbers for voucher lots are listed in table 1.

Table 1. Sources of the nine populations of *Donax* used in this study. Collecting sites are those shown on the map in figure 7.

Sample designation	Taxon	Collecting site	USNM catalogue number
DVF	*Donax variabilis variabilis*	Atlantic coast of Florida	869538
DVG	*Donax variabilis variabilis*	Gulf coast of Florida	869539
DVR	*Donax variabilis roemeri*	Gulf coast of Texas	869540
DPF	*Donax parvulus*	Atlantic coast of Florida	869541
DTT	*Donax texasianus*	Gulf coast of Texas	869542
DDM	*Donax denticulatus*	Port Maria, Jamaica	869543
DDN	*Donax denticulatus*	Negril, Jamaica	869544
DDB	*Donax denticulatus*	Black River, Jamaica	869545
DSB	*Donax striatus*	Black River, Jamaica	869546

2. *EXTRACTION OF DNA*

To avoid contamination from food organisms, only muscle dissected from the foot of each clam was used to extract DNA. Approximately 30 mg of tissue was treated according to a protocol derived from that of Reeb and Avise (1990). Tissue was macerated in a 1.7 ml microcentrifuge tube containing 400 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) for about 30 seconds with a pestle driven by an electric drill. After adding 25 μl of 10% sodium dodecyl sulfate (SDS), the extract was incubated at 65° C for 30 to 60 minutes. Next, 70 μl of 8M potassium acetate was added, the mixture shaken, and chilled on ice for 60 minutes. After the extract was centrifuged at 14,000 × g for 10 minutes, the supernatant was transferred to a clean tube and the pellet discarded. The supernatant was chilled at −20° C for 2 minutes, spun again for 10 minutes, and again transferred to a clean tube. Next, 400 μl of chloroform and 400 μl of tris-saturated phenol were added, the tube shaken, and centrifuged for 5 minutes at 14,000 × g. The upper, aqueous layer was transferred to a clean tube, 400 μl of chloroform added, the tube shaken, and centrifuged for 5 minutes. The upper, aqueous layer was again decanted to a clean tube, treated with chloroform, and centrifuged. After the upper layer was transferred to yet another clean tube, 1ml of cold 95% ethanol was added and mixed by gently inverting the tube. The sample was kept at −20°C for two minutes and centrifuged again for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml of 80% ethanol. After another centrifugation for 5 minutes at 14,000 × g, the ethanol was discarded and the pellet was dried in an incubator at 38° C for about 30 minutes. The cleaned DNA pellet was dissolved in 300 μl of TE buffer and kept at −20° C until needed.

This procedure yielded DNA at concentrations ranging from 5 to 35 μg/ml. If initial PCR amplification failed, the DNA was further purified with "GeneClean II" (BIO 101 Inc., P.O. Box 2284, La Jolla, CA 92038) after which it amplified satisfactorily.

3. *PCR AMPLIFICATION OF DNA*

The amplification protocol of Bowditch *et al.* (1993) was used in this study. "Amplitaq" DNA polymerase, supplied by Perkin-Elmer/Cetus at an activity of 8 units per μl, was used at a concentration of 0.5 units per sample (0.06 μl). The four nucleotide triphosphates were supplied by Pharmacia as 100mM stocks and mixed to make a single stock 0.25 mM for each dNTP. A special RAPD buffer was prepared according to the recipe: 100mM Tris, 500mM KCl, 19mM $MgCl_2$, and 10 mg/ml bovine serum albumin (not acetylated). Primers came from two sources: 20 (designated OP-E) were from Operon Technologies Kit E and 40 (designated LMS-P) were provided by the Laboratory for Molecular Systematics, National Museum of Natural History, Smithsonian Institution, where they had been synthesized.

Between 5 and 15ng of DNA from an individual clam and 50ng of a single, short primer (10mer in all cases) were combined in 25 μl of a reaction mixture comprised of: 18 μl sterile distilled water, 2.5 μl RAPD buffer, 2.5 μl deoxynucleotide mix, 1 μl primer, and 1 μl target DNA. The reaction mix was topped with mineral oil and placed in a Perkin-Elmer 4800 Thermocycler for 45 cycles of a RAPD amplification profile as follows: dissociation of DNA for 1 minute at 94°C, annealing of primer for 1 minute at 36°C, polymerization of DNA for 2 minutes at 72°C. The amplification products were loaded onto a 1.4% agarose gel, electrophoresed in TBE buffer (89mM Tris, 89mM Boric Acid, 2mM EDTA) and visualized with ethidium bromide. Each primer produced a characteristic set of amplification products with sizes ranging from 0.3 to 3.0 kilobases, which appeared as bright bands on the agarose gels (Figure 1). Rather than measuring distances from the origin, approximate sizes were determined by comparison to fragments of known size in a mixture of lambda DNA cut with HindIII and ϕX174 cut with HaeIII. To confirm that two bands were identical, samples were run in adjacent lanes of a gel. Throughout this paper, these products are referred to interchangeably as "amplification products," "DNA fragments," or "RAPD markers."

4. *SELECTION OF PRIMERS AND MARKERS*

Sixty primers were screened on a panel of 24 individuals comprised of three clams from each of the sample populations except *D. striatus* (DBS). Each primer was used

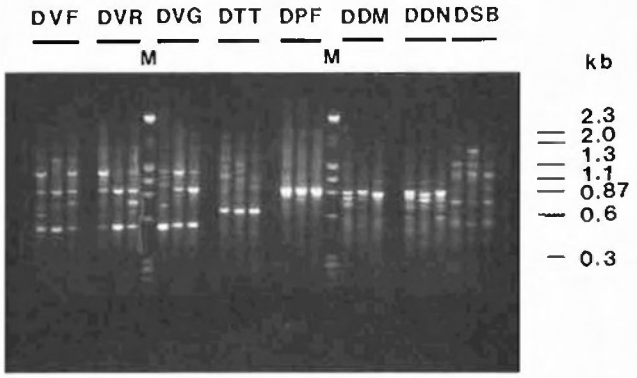


Figure 1. RAPD amplifications generated by primer OP-E18. The horizontal bars over the gel join the three individuals from each population that were run on each gel. Sample designations above the bars refer to taxa and populations listed in table 1. The letter M identifies lanes containing molecular weight standards (lambda DNA cut with HindIII + ϕX174 cut with HaeIII). Standard bands and sizes in kilobases are depicted to the right of the gel.

at least twice to amplify each screening DNA. A primer was judged to be suitable for use in this study if it met the criteria of: 1) amplification, that is, the production of clearly resolved DNA fragments, 2) reproducibility, with at least one DNA fragment appearing consistently and reproducibly in repeated assays of the same individuals, and 3) commonality, or the presence of at least one DNA fragment in two or more populations (but not necessarily two or more taxa). Primers that met these requirements were not common. Approximately one fourth of the primers tested failed to meet criterion 1, with most of the remainder failing criterion 3. Criterion 2, reproducibility, was not a serious problem. For all of the markers chosen, amplification of DNA from the same individual produced the same results whether the amplification was repeated in separate PCR experiments or replicated within the same PCR experiment. Identical results were produced when amplifications were repeated using a Coy thermocycler, in which temperature changes much more slowly than in a Perkin-Elmer machine.

The initial screening procedure identified five primers that produced a total of 17 RAPD markers that were informative for the purposes of this study. These primers and markers are described in Table 2 and representative results are shown in Figure 1. Of the two primers from the Laboratory for Molecular Systematics, primer LMS-P01 is the same as primer AP8g of Williams et al. (1990) while primer LMS-P56 was designed and synthesized at LMS. A total of nine individuals from each population were assayed at least twice with each of the five primers. Samples with similar markers were run side-by-side in the replicate assay in order to facilitate direct comparisons.

5. *PHYLOGENETIC ANALYSIS*

Data were analyzed and trees produced using Hennig86 version 1.5 software (Farris, 1988). The implicit enumeration (ie;) algorithm was used in each series of analyses to insure that all shortest, equally parsimonious trees were found.

Each RAPD marker was treated as a separate character regardless of which primer was used to generate it or which other bands from the same or other primers co-occurred with it. In an initial analysis (Analysis 1), the 17 markers listed in Table 4 were scored as either absent (0) or present (1) for each population, and a hypothetical outgroup, scored as lacking all 17 RAPD markers (all characters = 0), was used (for data matrix, see Appendix 1). In subsequent analyses, the markers were scored as absent (0), polymorphic (1), *i.e.* present in some but not all members of the population, or fixed (2), *i.e.* present in all members of the population. In Analysis 2, this data set was run unordered, using the same hypothetical outgroup as in Analysis 1 (data matrix in Appendix 2). A third series of analyses used the same data matrix but, instead of the hypothetical outgroup, each of the basal taxa from Analysis 2 (DSB, DDN) was used in turn as the outgroup. The arrangement of character states on the resulting trees were examined using the Dos Equis (*xx*) and *xsteps* tree diagnostic commands.

Table 2. DNA primers used in this study. For each primer the table shows: the sequence; the average number of DNA amplification products detected in an individual, with the range of averages among the nine groups following in parentheses; and the sizes of those DNA amplification products used as markers. Primers designated OP-E are from Operon Technologies Kit E and primers designated LMS-P were provided by the Laboratory for Molecular Systematics. Those fragments followed by an asterisk (*) are characteristic of the Caribbean taxa, while those followed by an ampersand (&) are characteristic of the Carolinian taxa. Note that, for any given primer, the smaller fragments are always characteristic of the Carolinian taxa.

Primer	Sequence	Average number of RAPD DNA fragments per clam (range)	Size (kb) of useful RAPD markers
OP-E07	5′-AGATGCAGCC	3.8 (3.1–4.3)	0.6 1.5
OP-E16	5′-GGTGACTGTG	3.4 (2.6–4.3)	0.3& 0.5& 0.6* 0.9 1.1
OP-E18	5′-GGACTGCAGA	3.0 (1.6–4.2)	0.5& 0.6* 0.9
LMS-P01	5′-TGGTCAGTGA	2.8 (2.0–3.2)	0.5& 1.0* 1.2*
LMS-P56	5′-AGATCTGCAG	3.0 (2.2–3.6)	0.3& 0.6& 1.1* 1.2*

Table 3. Distribution of RAPD DNA markers in May and November samples of *Donax denticulatus* from Port Maria, Jamaica. As in tables 2 and 4, the RAPD marker identification designates the primer that produced the marker, the approximate size of each marker in kilobases, and our identifying marker number. Each entry in the matrix shows the number of individuals in which the RAPD marker was detected over the number of individuals tested (*e.g.*, 3/9). Because Primer OP-E07 was not used with the May sample, data from this sample were not included in table 4, which serves as the data matrix for phylogenetic analysis.

Primer:	OP E16	OP E16	OP E18	LMS P01	LMS P56	LMS P56	OP E16	OP E18	LMS P01
Marker size (kb):	0.9	1.1	0.9	1.2	1.1	1.2	0.6	0.6	1.0
Marker number:	7	9	10	12	13	14	15	16	17
May	9/9	9/9	9/9	0/9	4/8	8/8	9/9	9/9	9/9
November	9/9	9/9	9/9	1/9	6/6	5/8	8/9	9/9	8/9

RESULTS

1. *STABILITY OF RAPD MARKERS OVER TIME*

To assess the stability of the RAPD markers used in this study, two different samples of *Donax denticulatus* from Port Maria, Jamaica, were examined, one taken in May and the other in November of 1991. These two samples produced identical RAPD markers with only slight differences in frequencies between the two collections (Table 3). The only marker not present in both samples, marker 12 (LMS-P01/1.2kb), was the rarest, appearing in only 3 of a total of 21 individuals assayed.

2. *DISTRIBUTION OF RAPD MARKERS AMONG POPULATIONS*

Table 4 summarizes the distribution of the 17 RAPD markers among the nine assayed populations, but does not contain data from the May sample of *Donax denticulatus* from Port Maria. Because the presence of two species in the sample from Black River was not discovered until after the laboratory work was completed, these two populations have reduced sample sizes (3 *D. denticulatus*, 6 *D. striatus*).

Of the 17 RAPD markers assayed, two (markers 8 and 9), each produced by a different primer, were present at varying frequencies in all nine populations. All three samples of *D. variabilis* (DVF, DVG, DVR), including the subspecies *D. variabilis roemeri*, had the same 11 markers appearing in at least one member of each population. Of these 11 markers, two appeared in no other taxon. Similarly, all three populations of *D. denticulatus* (DDM, DDN, DDB) shared a set of 11 markers, three of which appeared in no other taxon. Twelve of the 17 RAPD markers were not shared between *D. variabilis* (DVF, DVG,DVR) and *D. denticulatus* (DDM, DDN, DDB). The three remaining taxa, each represented by a single population, showed clear affinities with either *D. variabilis* or *D. denticulatus*. The absence of unique markers in these taxa was an artifact of the criteria for primer selection (i.e.- that bands occur in at least two sample populations). Nevertheless, *Donax parvulus* (DPF) was distinguished from its sympatric congener *D. variabilis variabilis* (DVF) by the absence of RAPD markers 1 and 2, while *D. texasianus* (DTT) differed from its sympatric congener *D. variabilis roemeri* (DVR) in lacking markers 1, 2, 3, and 7. Marker 2, which was fixed in all populations of *Donax variabilis* and present in no other taxon, appears to be a diagnostic marker for this species. The Caribbean species *D. striatus* (DSB) differed from *D. denticulatus* (DDB) in lacking 5 RAPD markers (10, 11, 15, 16, 17), of which two (10, 16) were fixed in *D. denticulatus*. Marker 16, which occurred in all individuals of *D. denticulatus* tested, was unique to this species and may be used as a diagnostic marker for this species.

Although diagnostic markers were not identified for some taxa, the absence of multiple RAPD markers in one member of each sympatric pair (including markers fixed in the other member of the sympatric pair) is taken as evidence that these pairs do not exchange genetic material. An empirical observation is that when primers produced RAPD markers characteristic of both Carolinian and Caribbean taxa, markers that distinguished Carolinian taxa were invariably shorter than markers that were diagnostic of Caribbean taxa (Table 2). The significance of this observation is not yet clear.

3. *PHYLOGENETIC ANALYSES*

An initial cladistic analysis (Analysis 1), scoring each of the 17 RAPD markers as absent or present (regardless of frequency) in each sample population and using a hypothetical outgroup in which all characters were scored as absent, produced a single most parsimonious tree (length = 20, ci = 85, ri = 91) that resolved all species level taxa but left populations and/or subspecies unresolved (Figure 2). Fourteen of the 17 character transformations plotted unambiguously onto this tree (Figure 2). Each of the remaining three characters (markers 7, 10, 11) could either have been present in the common ancestor of all the taxa in this study and subsequently lost in a single taxon (Figure 2), or have arisen twice independently (Figure 3). An analysis of character polarity using the out-group comparison method (Watrous & Wheeler, 1981) indicated that the presence of markers 7, 10, and 11 is plesiomorphic, as they occur in both the in-group and the out-group, while their loss, in each case

Table 4. Distribution of the RAPD DNA markers among sample populations. For each RAPD marker, the band designation identifies the primer, the approximate size of the marker in kilobases and a sequential number to identify the marker. Each entry in the matrix shows the number of individuals in which the RAPD marker was detected over the number of individuals tested (*e.g.*, 3/9). In a few cases, the number of individuals tested was less than nine, either because fewer DNA samples were available (DDB, DSB) or because we were unable to score an individual for a particular marker (markers 1, 13, 14). When no individuals produced a marker, the entry is marked "—" rather than 0/n. Data are grouped to emphasize affinities among sample populations, rather than by primer.

	OP	OP	LMS	OP	LMS	LMS	OP	OP	OP	OP	LMS	LMS	LMS	OP	OP	LMS
Primer:	E16	E18	P56	E16	P01	P56	E16	E07	E16	E18	E07	P01	P56	E16	E18	P01
Size (kb):	0.3	0.5	0.3	0.5	0.5	0.6	0.9	1.5	1.1	0.9	0.6	1.2	1.1	0.6	0.6	1.0
Number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
DVF	2/9	9/9	8/9	9/9	9/9	9/9	5/9	9/9	3/9	9/9	9/9	—	—	—	—	—	—
DVG	1/9	9/9	9/9	9/9	9/9	9/9	5/9	9/9	4/9	9/9	9/9	—	—	—	—	—	—
DVR	4/8	9/9	6/9	9/9	9/9	9/9	5/9	9/9	6/9	9/9	7/9	—	—	—	—	—	—
DPF	—	—	5/9	7/9	9/9	9/9	7/9	6/9	2/9	9/9	2/9	—	—	—	—	—	—
DTT	—	—	—	9/9	3/9	9/9	—	9/9	9/9	2/9	2/9	—	—	—	—	—	—
DDM	—	—	—	—	—	—	9/9	2/9	9/9	9/9	5/9	1/9	6/6	5/8	8/9	9/9	8/9
DDN	—	—	—	—	—	—	9/9	7/9	9/9	9/9	7/9	1/9	5/9	6/9	8/9	9/9	9/9
DDB[1]	—	—	—	—	—	—	3/3	2/3	3/3	3/3	2/3	1/3	3/3	1/3	2/3	3/3	3/3
DSB[2]	—	—	—	—	—	—	6/6	5/6	6/6	—	—	6/6	4/6	6/6	—	—	—

[1] Only 3 individuals available for testing.
[2] Only 6 individuals available for testing.

restricted to a functional in-group, is apomorphic (Fig 2).

A second analysis, scoring the RAPD markers as absent (0), polymorphic (1) or fixed (2), and employing the same hypothetical outgroup, produced four equally parsimonious trees (length = 32, ci = 90, ri = 92) when the data were run unordered. One tree, supported by all markers except 8, 10, and 11, matched the topology of the tree in Figure 3, except that all sample populations were resolved. In the other three trees, which differed only in the resolution of *D. denticulatus* populations and which were supported by all markers except 7, 8, and 13, *Donax striatus* emerged as the sister group to all remaining taxa. The nelsen consensus tree (length = 34, ci = 85, ri = 87) of these four trees is shown in Figure 4. Twelve of the 17 character transformations plotted uniquely onto the consensus tree, while markers (7, 8, 10, 11, 13) could be interpreted as evolving in several equally parsimonious scenarios. Analyses of character polarity using the outgroup comparison method (Watrous & Wheeler, 1981) suggest that marker 7 was fixed in the *Donax* ancestor, became polymorphic in the North American clade, and eventually lost in *Donax texasianus*, while marker 11, which was polymorphic in the *Donax* ancestor, was lost in *Donax striatus*, but became fixed in Florida populations of *Donax variabilis*. The remaining markers could not be mapped onto the consensus tree (Figure 4) without reversals (markers 8, 13) or convergences (marker 10). Only marker 8 was incompatible with all of the initial trees.

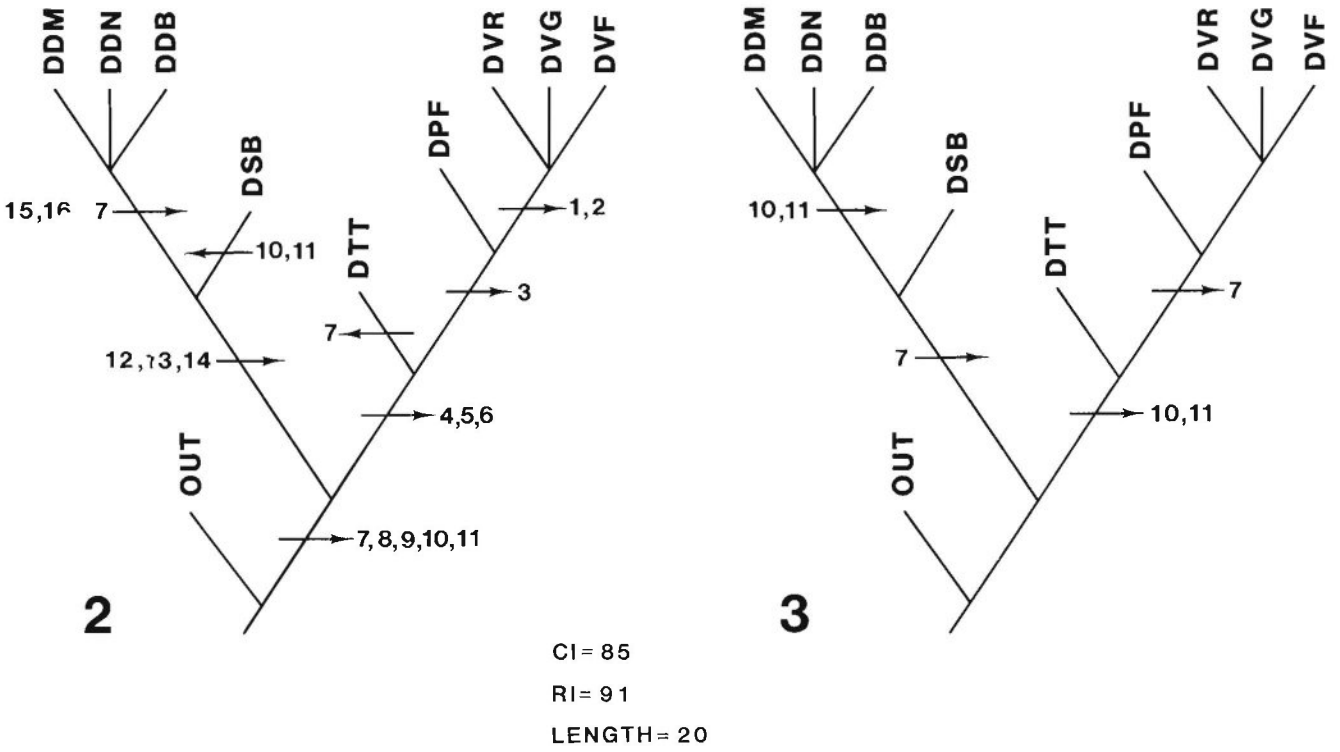
When the data were reanalyzed using *Donax striatus* as the outgroup, one most parsimonious tree (length = 28, ci =96, ri = 97), resulted (Figure 5). Likewise, when the Black River population of *Donax denticulatus*, served as the outgroup, a single, equally parsimonious tree (length = 28, ci =96, ri = 97), was produced (Figure 6).

All analyses that employed an intermediate character state produced identical tree topologies for the Carolinian samples but differed in the resolution and/or relationships of the Caribbean taxa and populations.

DISCUSSION

RAPD markers observed in the nine samples, representing six species or subspecies of *Donax*, showed a high degree of polymorphism both within and among taxa. Nevertheless, the polymorphisms did not obscure relationships among the samples and the presence of these markers was stable over time. The distribution of these RAPD markers supports previously disputed distinctions between members of the following three sympatric pairs of species: *Donax parvulus* and *D. variabilis variabilis*, *D. texasianus* and *D. variabilis roemeri*, and *D. denticulatus* and *D. striatus*, as well as between the similar but allopatric pair *D. parvulus* and *D. texasianus*.

Among the earlier applications of RAPD methodology, the technique was used to distinguish between individuals, strains, cultivars, populations and species (e.g. Hu & Quiros, 1991, Kambhampati et al. 1992). The char-



Figures 2–3. Phylogenetic tree resulting from analysis 1, in which all RAPD markers listed in table 4 were scored as absent or present in each sample population, regardless of frequency (data matrix in appendix 1). **2.** Character transformations plotted onto tree. Markers 7, 10 and 11 are plotted as having arisen once and been subsequently lost in a single taxon. **3.** Markers 7, 10 and 11 are plotted as having arisen twice independently.

acterization of diagnostic markers that would identify *Donax* species was beyond the scope of this study, and our criteria for primer selection precluded the recognition of markers unique to taxa represented by single samples. Even with this screening bias, fixed, species specific RAPD markers were discovered for *Donax variabilis* s.l. and for *D. denticulatus*, the two species in this study that were represented by multiple samples.

Kambhampati *et al.* (1992) successfully applied RAPD methodology to identify mosquito species and were able to cluster individuals of the same species correctly by applying phenetic algorithms (UPGMA) to markers generated by two primers. Although the resulting phenogram did not reflect the ancestral relationships of the mosquito species, these authors did not rule out the utility of RAPD data for phylogeny reconstruction but suggested that a greater number of primers (>20) should be tested to find lineage-specific markers. Our results confirm their conjecture. A survey of 60 primers was necessary to select the 5 primers and 17 markers that we

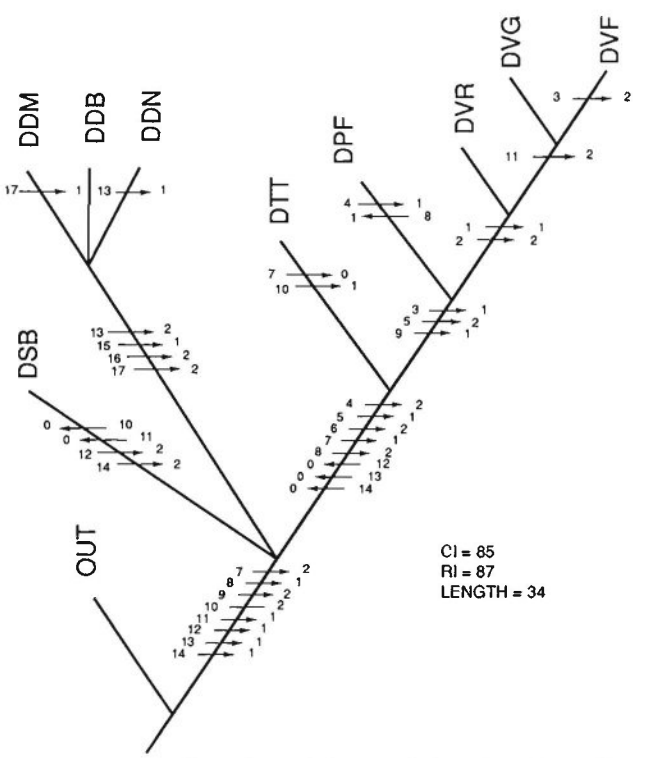


Figure 4. Nelson Consensus Tree of four equally parsimonious trees produced when all RAPD markers listed in table 4 were scored as absent, polymorphic, or fixed (data matrix in appendix 2) and all characters were run unordered. Character transformations are plotted onto the tree. Where alternative, equally parsimonious character transformations were possible, preference was given to the ordered, but not polarized, transformation series (0 <-> 1 <-> 2) and to reversals (loss of marker) over convergent origins of a marker.

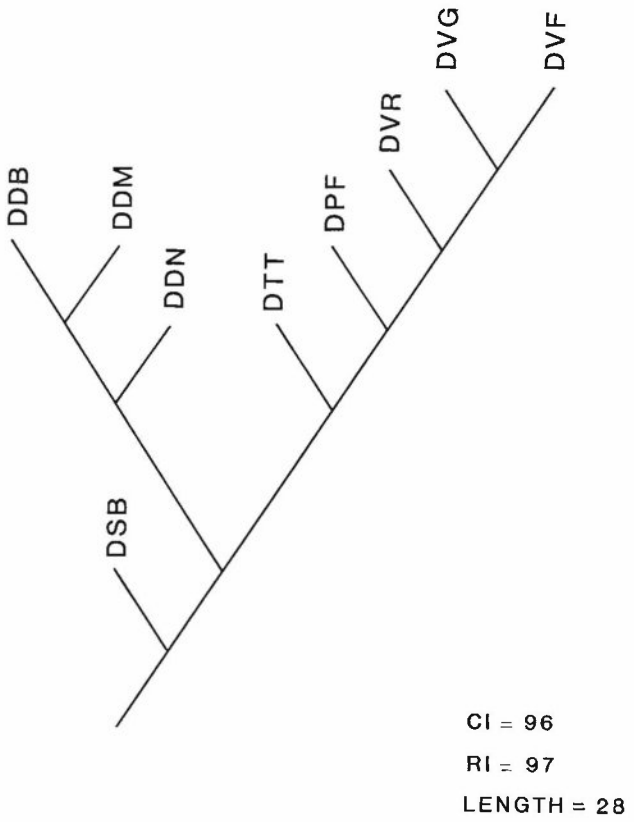


Figure 5. Most parsimonious tree produced when all RAPD markers listed in table 4 were scored as absent, polymorphic, or fixed, and all characters were run unordered. Based upon results of the previous analysis (Figure 4), *Donax striatus* was selected as the outgroup.

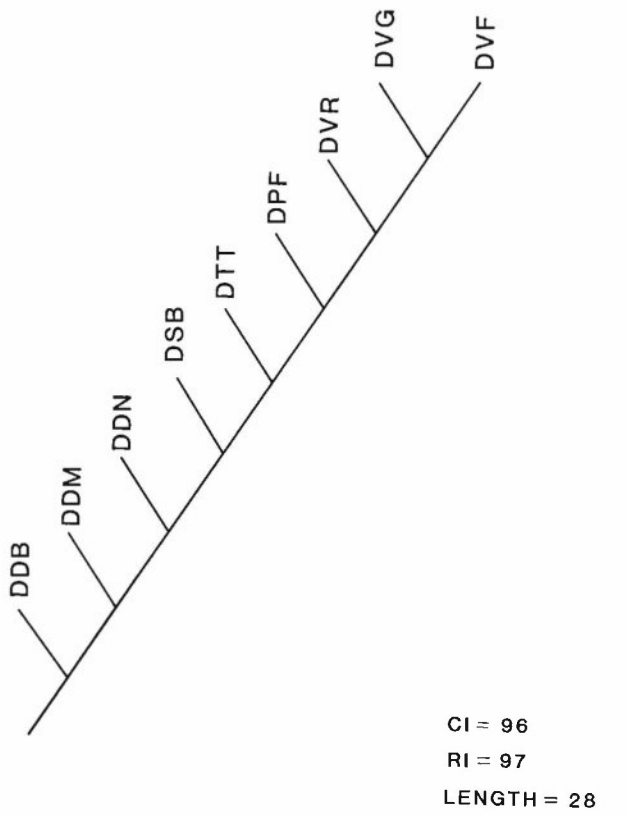


Figure 6. Most parsimonious tree produced when all RAPD markers listed in table 4 were scored as absent, polymorphic, or fixed, and all characters were run unordered. Based upon results of a previous analysis (Figure 4), the Black River population *Donax denticulatus*, was selected as the outgroup.

used to construct phylogenies. As RAPD markers have previously been shown to segregate in a Mendelian manner, behaving as dominant alleles (Williams *et al.*, 1990), we treated the individual markers as homologous characters suitable for cladistic analyses. Although the analyses were run unordered, when character transformations could be plotted onto the resulting tree in several equally parsimonious ways, preference was given to the ordered, but not polarized, transformation series (0 <-> 1 <-> 2) because this series reflects the manner in which alleles enter, are distributed within, and leave populations. Preference was also given to reversal (loss of a marker) over convergent evolution of a marker. Despite differences in outgroup selection and data scoring, all cladistic analyses produced the same, single, highly consistent tree for the five Carolinian samples. The inability to stably resolve the Caribbean samples is attributed in large part to the low number of samples.

A plot of the consensus tree (Figure 4) on a map of geographic distributions of the taxa (Figure 7) illustrates that the Carolinian *Donax* species form a monophyletic clade, while the Caribbean species may represent either a clade (Figure 2) or grade (Figs. 5,6) depending on how the limited data are analyzed. Although the genus *Donax*

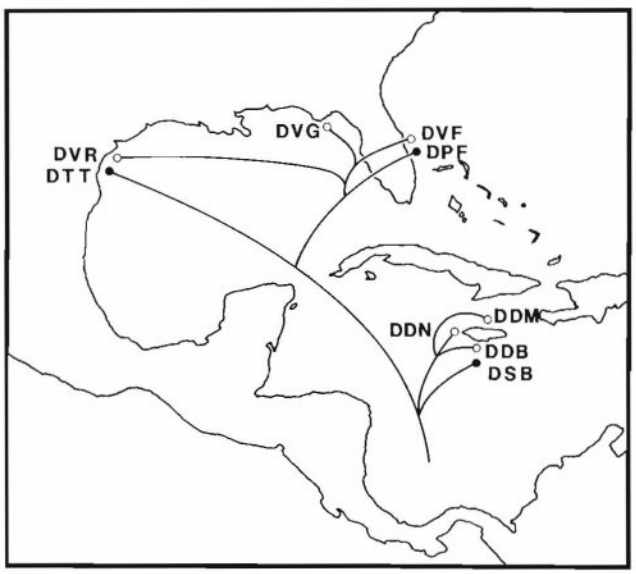


Figure 7. A plot of the consensus tree (Figure 4) on a map of the locations of the collection sites for the nine sample populations of *Donax* used in this study. Open circles indicate species that migrate with the tide. Solid circles indicate subtidal species. Sample designations as in Table 1.

has been represented in the fossil record of the western Atlantic since the Oligocene (Gardner, 1943:105), provincial boundaries have existed between molluscan faunas of the Gulf of Mexico and the Caribbean Sea since the late Oligocene or early Miocene (Petuch, 1988:48). The Recent Carolinian and Caribbean Provinces comprise, respectively, the Caloosahatchian Province and a portion of the larger Gatunian Province, both ranging from the late Oligocene to the early Pleistocene (Petuch, 1988:fig.1). Thus, the considerable divergence between Carolinian and Caribbean *Donax* faunas, which share at most five of 17 markers (29% similarity), may have accumulated over a period of approximately 25 million years.

The topology of the phylogenetic tree indicates that, within the Carolinian province, the non-migratory, lower intertidal to subtidal habitat is the more primitive among *Donax*. Species occupying this habitat (*D. texasianus*, *D. parvulus* and *D. dorotheae*) likely diverged as a result of barriers to gene flow posed by the Mississippi River and the emergence of peninsular Florida, respectively. As *D. parvulus* appears to be the sister species of *D. variabilis*, it is likely that *D. variabilis* originated in the eastern Carolinian Province, although comparable RAPD data on *D. dorotheae* may make possible a more precise localization of the area of origin of *D. variabilis*.

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Appendix 1. Data matrix for initial cladistic analysis of *Donax* phylogeny. RAPD markers scored as absent (0) or present (1), regardless of frequency. For sample designations see Table 1.

	RAPD markers																
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Outgroup	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DVF	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
DVG	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
DVR	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
DPF	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
DTT	0	0	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0
DDM	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
DDN	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
DDB	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
DSB	0	0	0	0	0	0	1	1	1	0	0	1	1	1	0	0	0

Appendix 2. Data matrix for subsequent cladistic analyses of *Donax* phylogeny. RAPD markers scored as absent (0), polymorphic (1), or fixed (2). For sample designations see Table 1.

	RAPD markers																
Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Outgroup	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DVF	1	2	1	2	2	2	1	2	1	2	2	0	0	0	0	0	0
DVG	1	2	2	2	2	2	1	2	1	2	2	0	0	0	0	0	0
DVR	1	2	1	2	2	2	1	2	1	2	1	0	0	0	0	0	0
DPF	0	0	1	1	2	2	1	1	1	2	1	0	0	0	0	0	0
DTT	0	0	0	2	1	2	0	2	2	1	1	0	0	0	0	0	0
DDM	0	0	0	0	0	0	2	1	2	2	1	1	2	1	1	2	1
DDN	0	0	0	0	0	0	2	1	2	2	1	1	1	1	1	2	2
DDB	0	0	0	0	0	0	2	1	2	2	1	1	2	1	1	2	2
DSB	0	0	0	0	0	0	2	1	2	0	0	2	1	2	0	0	0