The Use of DNA Markers in Stock Discrimination of Commercially Important Fish Species and their Application to Management of the Four-wing Flyingfish, *Hirundichthys Affinis*, in the Caribbean

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ABSTRACT

The ability to discriminate between genetically independent stocks of commercially important species is essential for the development of sound fisheries management and conservation strategies. Traditional techniques, such as the use of morphological, physiological and behavioural characteristics, which may be strongly affected by environmental factors, have now been largely superceded by molecular biology techniques which use genetic markers (i.e. protein enzyme markers and DNA markers) to examine the genome. This paper reviews the latest developments in the use of DNA markers for fish stock discrimination; reports on their application to testing the hypothesis of a single, shared stock of the four-wing flyingfish, *Hirundichthys affinis*, in the Caribbean; and discusses the management implications.

KEY WORDS: DNA, flyingfish, *Hirundichthys affinis*, stock assessment

INTRODUCTION AND REVIEW

Fish species are often divided into genetically discrete groups, unit stocks, that differ considerably intheir biological characteristics, including recruitment and mortality, and therefore react exploitation, thereby requiring independent management. Stock identification is therefore essential to fisheries management (Shaklee *et al.*, 1990; Carvalho and Hauser, 1995). Previously, differencesin behavioural, morphological and physiological characteristics within fish species have been used for stock discrimination. However, it is apparent that these features are highly sensitive to variations in environmental conditions such that they do not necessarily reflect genetic differences. One of the long-standing goals of fish molecular genetics research has been to find stock specific markers in an attempt to realise the goal of stock-based fisheries management.

Molecular studies of fish populations, in the sense of direct examination of macromolecules commenced some 35 years ago with the examination of protein variants (haemoglobin, transferrin and enzymes). In the past 15 years increasing

emphasis has been given to direct examination of deoxyribonucleic acids (DNA), firstly mitochondrial DNA (mtDNA) and then, as molecula rtechniques developed, nuclear DNA (nDNA). Although some DNA studies are more costly and technically demanding than protein studies, they offer a more accurate examination of intraspecific genetic variation of many fish species through examination of actual DNA sequences from the total genome rather than the products of limited regions of the genome (e.g. enzymes).

MtDNA, by virtue of its higher mutation rate compared with single copy nuclear DNA and its maternal mode of inheritance offers several advantages for population studies. Its relatively small size results in greater genetic differentiation than that which is apparent with nDNA owing to genetic drift and therefore it is likely to provide population specific markers. MtDNA markers are based on changes in DNA sequences generally as a result of point mutations involving base substitutions.

Restriction Fragment Length Polymorphism (RFLP) analysis was the first method used to examinemtDNA markers. RFLP analysis essentially involves comparisons of the products of restriction enzyme digests of DNA. Early studies of mtDNA variation required large tissue samples and timeconsuming protocols and many such studies were inadequate with respect to the sample size examined. The use of mtDNA probes and, more recently, amplification of selected regions by the Polymerase Chain Reaction (PCR) method has made examination of mtDNA variation considerably easier and faster. Universal vertebrate primers can be used to successfully amplify various mtDNA regions (e.g. D-loop, ND genes and cytochrome b gene) and now that complete mtDNA base pair sequences for several fish species are available in public-access databases, more specific fish primers can be designed. The combination of these techniques is now being widely applied to fish species (Cronin *et al.*, 1993; Martin *et al.*, 1992; Alvarado Bremer *et al.*, 1994; Chow, 1994: Shulman and Bermingham, 1995; Gomes *et al.*, in prep [a]) and has proven to be a very useful tool in stock discrimination studies (Carvalho and Hauser, 1995).

Recently attention has turned to another type of variation, that of differences in the number of repeated copies of a segment of nDNA. These sequences can be classified on the basis of decreasing size into satellites, minisatellites and microsatellites (Tautz, 1993). Satellites consist of units of up to several thousand base pairs (bp) repeated thousands or millions of times. Minisatellites consist of DNA sequences of about 9 to 100 bp in length which are repeated from 2 to several hundred times at a locus. Microsatellites have a unit length of 1 to 6 bp repeated up to about 100 times at eachl ocus. Individual variants at a given minisatellite or microsatellite locus (collectively known as variable number of tandem repeats [VNTRs]), differ in the number of tandem repeats of the unit sequence and as such can be differentiated by electrophoresis and

Southern hybridisation according to their lengths. Present constraints to using satellite markers in stock structure work are the fact that the initial development of a suite of satellite probes may be more costly and time-consuming than the development efforts needed with other markers, although it is reported that this effort is repaid with gains in efficiency at a later stage, as thousands of samples are readily assayed (Wright and Bentzen, 1995). However, locus-specific minisatellite and microsatellite primers have now been developed and used for population studies of salmonids, tilapias and sticklebacks (e.g. Taggart and Ferguson, 1990; Bentzen *et al.*, 1991).

Other techniques have been developed which make use of random parts of the nuclear genome. One approach involves PCR amplification of anonymous DNA fragments, commonly known as RandomAmplified Polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990). A single oligonucleotide primer is used to amplify intervening regions between two complementary, but inversely oriented sequences which are then separated by gel electrophoresis. Suitable primers include random G-C rich 10-mers and polymers complementary to microsatellite repeats (Ferguson *et al.*, 1995). However, homozygous and heterozygous states cannot be differentiated and the patterns are very sensitive to slight changes in amplification conditions giving problems of reproducibility. These problems have so far limited the application of RAPDs in fish population studies. However, Naish *et al.* (1995) and Gomes *et al.* (in prep[b]) have found the technique useful for detecting genetic diversity within strains of tilapia (*Oreochromis niloticus*) of African origin and flyingfish (*Hirundichthys affinis*) from thecentral western Atlantic respectively.

Flyingfishes are a significant component of the fish fauna of the Atlantic, Pacific and Indian Oceans (Parin, 1970). Of the thirteen species which inhabit the warm zones of the central western Atlantic ,the four-wing flyingfish, *Hirundichthys affinis*, is distributed throughout the epipelagic zone of thetropical Atlantic, with concentrations of abundance in the central western Atlantic occurring in the Caribbean Sea, Gulf of Mexico and off the northeast coast of Brazil (Brunn, 1935; Breder, 1938; Fischer, 1978; Oxenford *et al.*, 1995). It is a commercially valuable species throughout the eastern Caribbean, where it represents the first or second most important species landed by most of theislands (Mahon *et al.*, 1986; Oxenford *et al.*, 1993); in the southern Netherlands Antilles (Zaneveld, 1961; G. Van Buurt pers. comm.); and off the northeast coast of Brazil (Da Cruz, 1973; Monteiro *et al.*, 1996).

Expansion in both the number and sophistication of the fishing fleets in the eastern Caribbean (Oxenford, 1991; Lawrence, 1993; Samlalsingh *et al.*, 1993; Willoughby, 1993) as well as improved landing sites and market facilities have resulted in increased catches of flyingfish. Total annual catch by all eastern Caribbean Islands in 1995 was estimated to be in excess of 5,000 mt

(CARICOM Fisheries Unit, 1996a). Data are unavailable for the southern Netherlands Antilles. Despite its importance, very little is known about the stock structure of *H. affinis* and no form of fisheries resource management is currently practiced for this species by any of the countries harvesting flyingfish. This lack of knowledge is of particular concern to countries of the eastern Caribbean, and serious efforts are now being made to conduct resource assessments and to draw up rational fishery management plans (CARICOM Fisheries Unit, 1995 and 1996b). Although the fishery does not appear to be overexploited, perhaps because of the migratory nature of the fish (Mahon 1990;Oxenford 1991; Khokiattiwong *et al.*, 1993), the ability of the flyingfish stock in the eastern Caribbean to sustain increased exploitation, both in terms of heavier exploitation in the areas currently being fished as well as by means of expansion of the geographical range presently being fished by fleets, is a matter of concern for all islands participating in the fishery.

A tagging study in the eastern Caribbean (Oxenford, 1994) demonstrated that *H. affinis* do move freely between the islands, suggesting that eastern Caribbean nations share a common unit stock (Oxenford, 1994). However, tagging studies only address geographical movement of fish and cannot determine whether fish populations that appear to be mixing, are actually interbreeding (Milner *et al.*, 1985; Hynes *et al.*, 1989; Graves and McDowell, 1994). It remains possible then, that despite detected mixing of adult *H. affinis* in the eastern Caribbean, there may be a more complex stock structure in this region if adults are segregating to specific areas for spawning. Furthermore, two peaks in spawning activity of *H. affinis* have been detected off Barbados (Storey, 1983; Khokiattiwong, 1989; Lao, 1989), a minor one in December/January and a major one in April/May. This may indicate the presence of two genetically distinct, but sympatric, stocks of flyingfish, each with its own spawning period. If this is so, then management options will need to consider protection of both stocks(Oxenford *et al.*, 1993). Alternatively, since *H. affinis* are, at least in part, pelagic spawners with an extended spawning season (Oxenford, 1986; Hunte *et al.*, 1995) there is the potential for shared recruitment over a wide area of the Caribbean Sea that receives the northwesterly flowing Caribbean Current (Froelich *et al.*, 1978; Kjerfve, 1986; Gable, 1993) such that populations in the eastern Caribbean and the southern Netherlands Antilles may constitute a single stock, as has been found to be the case for several reeffish species (Shulman and Bermingham, 1995). It is, therefore, imperative that serious consideration be given to the matter of sustainability of the resource with stock discrimination being the first and most critical avenue to stock assessment and sustainability.

Given the constraints of comparisons based on behavioural, morphological and physiological characteristics for stock discrimination it is prudent to examine stock structure at the genetic level, using molecular biology techniques. This

paper examines the use of RFLP (mtDNA) and RAPD(nDNA) markers as suitable stock specific markers for the four-wing flyingfish, *H. affinis*, in the Caribbean and addresses the implications for management of the resource. Specifically, we test the hypothesis of a single Caribbean stock (i.e shared between the eastern Caribbean islands and the southern Netherlands Antilles).

MATERIALS AND METHODS

Sample Collection and Preservation

During the period January to July 1995, a total of 300 spawning *H. affinis* were sampled from two distinct geographical areas within the Caribbean (eastern Caribbean and southern Netherlands Antilles) during periods of peak spawning activity (Table 1). Samples were taken from different landing sites (Figure 1) and from different boats over a period of a few days wherever possible so as to increase the probability of sampling different schools of fish. Liver tissue was removed from all freshly landed fish and placed in preservative buffer.

DNA Extraction

Genomic DNA was isolated from liver tissue following an adaptation (involving the removal of mucous by washing with CTAB detergent) of the protocol described by Cheung *et al.* (1993). The extracted DNA pellet was then rinsed with 17 l ethanol, air-dried, resuspended in 150 µl TE buffer and stored at -20 °C.

Mitochondrial DNA Analysis

Amplification of Mitochondrial D-loop Region — Two primers:

5'-CTACCTCCAACTCCCAAAGC - 3'

and 5' - CCTGAAGTAGGACCAGATG - 3';

(Operon Technologies Incorporated) designed to target the tRNA genes which flank the D-loop region (Palumbi *et al.*, 1991) were used to amplify the D-loop region from genomic DNA. PCR was carried out in a final volume of 25 l as described by Kocher *et al.* (1989). Amplification parameters were as follows: preheating for four minutes at 94° C; 40 cycles of denaturation at 94° C for one minute, annealing at 55° C for one minute, primer extension at 72° C for two minutes; 72° C for 10 minutes.

Table 1. Sampling locations, dates and sample sizes for spawning flyingfish, (*H. affinis*) from the Caribbean.

Geographical area	Country	Landing site	Date	Number of fish
Eastern Caribbean	Barbados	Bridgetown	January 3 – 24	20
		Conset Bay		20
		Half Moon Fort		20
		Bridgetown	May1 – 22	20
		Conset Bay		20
		Half Moon Fort		20
	Dominica	Marigot	April 3 – 7	20
		Newtown		9
		St. Sauveur		22
	Tobago	Buccoo	April 14 – 17	60
Southern Netherlands Antilles	Curaçao	Boca Simon	July 25 – 28	1
		Caracasbaai		1
		Watamul		58
TOTAL				300

Restriction Endonuclease Digestion of Amplified mtDNA — Four restriction enzymes (HinP1I,MboI, MseI, RsaI) recognising tetranucleotide sequences which had successfully cut the D-loop amplicon in a sub-sample of 10 flyingfish (two from each sampled population) producing variable banding patterns, were selected for RFLP analysis of the 300 samples. Restriction enzyme digestion involved adding one unit of each enzyme and its buffer to 6 1 of the D-loop amplicon and incubating the reactants at a temperature appropriate for the particular enzyme. The digested mtDNA samples were separated through 2 % agarose gels in TAE buffer by electrophoresis. Gels were stained with ethidium bromide, fluoresced under ultraviolet light to visualise restriction fragment patterns (mitotypes), and photographed.

Genomic DNA Analysis

Amplification of Nuclear DNA — Twenty primers from the Operon "B" kit were screened for their suitability in randomly amplifying sections of genomic DNA. Of these three, and pairs of these three, produced many distinct bands

which could be easily scored and were therefore selected for RAPD analysis of all samples. PCR parameters were similar to those used in amplifying the D-loop region, except that the annealing temperature was reduced to 32 °C, and visualisation of the DNA bands followed the same method used for the D-loop region.

Data Analysis

Mitochondrial DNA — Following electrophoresis, the sizes of the digested fragments were estimatedand the presence or absence of restriction sites for all enzymes was inferred from comparisons offragment patterns (Palumbi *et al.*, 1991). Composite mitotypes were then determined for each individual Genetic heterogeneity across all sampled *H. affinis* in the Caribbean was investigated by examining composite mitotype sequence divergences. These were computed using the REAP package (McElroy *et al.*, 1992). Genetic relatedness of these fish, based on nucleotide sequence divergence estimates among them, was examined by cluster analysis of composite mitotypes using the Phylip software package (version 3.5c, Felsenstein, 1993) and the UPGMA algorithmn of Sneath and Sokal (1973). Composite mitotype frequencies among all sampled populations were compared through a MonteCarlo simulation (Roff and Bentzen, 1989), using the MONTE programme in REAP and 1000 bootstrapped replicates. Inter-population nucleotide sequence divergences were computed and clustered using the REAP and Phylip software packages, respectively, as explained above.

Genomic DNA — Following electrophoresis the size of each DNA band for all primers and over all samples was estimated and the bands were scored according to their presence or absence. Data forall primers were pooled. Similarity indices across all individual samples of flyingfish were estimated using the computer software RAPDPLOT (Black, 1994). Genetic relatedness of these fish was examined by cluster analysis of similarity indices using the Phylip software package (version 3.5c, Felsenstein, 1993) and the UPGMA algorithmn of Sneath and Sokal (1973).

RESULTS

Mitochondrial DNA

RFLP Analysis — Digestion of the D-loop amplicon with MseI produced a single mitotype (A) ,HinP1I and MboI each produced two mitotypes (A and B) and RsaI produced three mitotypes (A,B and C) giving a total of five composite mitotypes across the total sample size of 300 flyingfish.

Genetic heterogeneity of flyingfish over the Caribbean — Cluster analysis

resolved the five composite mitotypes into two major groups which aligned perfectly with the two major geographical areas studied in the Caribbean, i.e. the eastern Caribbean and the southern Netherlands Antilles (Figure 2).

Inter-population Genetic Heterogeneity — Interestingly, no composite mitotypes were shared between the two geographical areas (Figure 2). However, mitotypes were shared between sampled populations within the eastern Caribbean (Figure 2), although they occurred with significantly different frequencies in the different populations (Monte Carlo Chi squared 4 x 3 contingency test;max. $X^2r = 15.77 < X^2o = 384.85$, $p < 0.001$). Cluster analysis resolved the five sampled populations into two groups aligning perfectly with the two geographical areas (Figure 3).

Genomic DNA.

RAPD Analysis — Cluster analysis of the similarity indices revealed two main clusters, onecomprising the eastern Caribbean populations and the other the southern Netherlands Antilles population (Figure 4).

CONCLUSIONS

Flyingfish from the two geographical areas had distinct composite mitotypes indicating a lack of gene flow between them and the presence of at least two unit stocks in the Caribbean. This is supported by cluster analysis of the composite mitotypes (Figure 2) and inter-population cluster analyses (Figures 3 and 4) which indicate considerable genetic distance between flyingfish from the eastern Caribbean and from the southern Netherlands Antilles. Within the eastern Caribbean, mitotypes where shared (Figure 2), but inter-population mitotype frequencies were significantly different, implying restricted gene flow in this sub-region. However, the level of genetic variation among flyingfish from the eastern Caribbean island appears to be relatively small (Figures 3 and 4) and, as such, the island populations cannot be considered as separate stocks. This supports the inference of Oxenford (1994) that adult flyingfish from the eastern Caribbean are mixing but are not migrating the 890 km distance between the two regions, or if they are, they are not interbreeding. It also indicates that flyingfish eggs and larvae are not being entrained in, and transported by, the prevailing Caribbean Current, but may be trapped within the complex system of local gyres and tidal frontswithin the eastern Caribbean (Kingsford *et al.*, 1991), therefore allowing recruitment to be shared among eastern Caribbean islands but preventing their dispersal towards the southern Netherlands Antilles. Likewise, meanders and eddies around the southern Netherlands Antilles are likely to be retaining eggs and larvae within that sub-region (Kinder, 1983).

These results indicate that flyingfish from the Caribbean comprise at least

two distinct unit stocks, one in the eastern Caribbean and one in the southern Netherlands Antilles. This implies that the two sub-regions need not collaborate with management of flyingfish. However, flyingfish management will need to be a collaborative effort in the eastern Caribbean, since the separate island states appearto be sharing a common stock, whereas flyingfish management can be nation-specific in the Dutch-owned southern Netherlands Antilles.

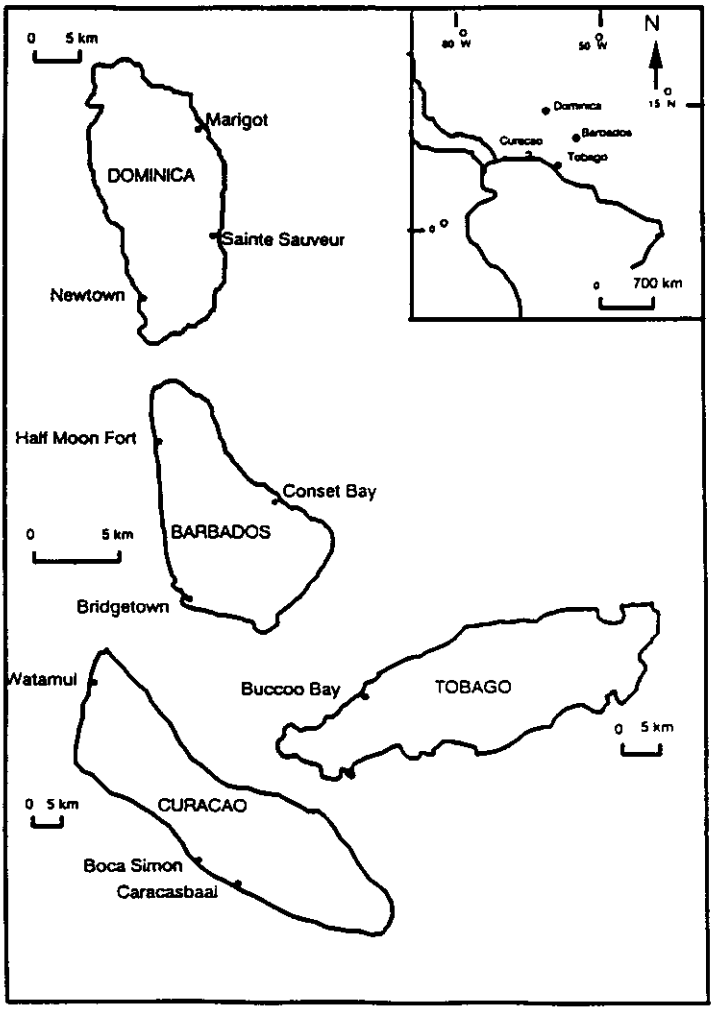

Figure 1. Geographical location of countries and position of sampling sites for 300 *H. affinis* within the Caribbean.

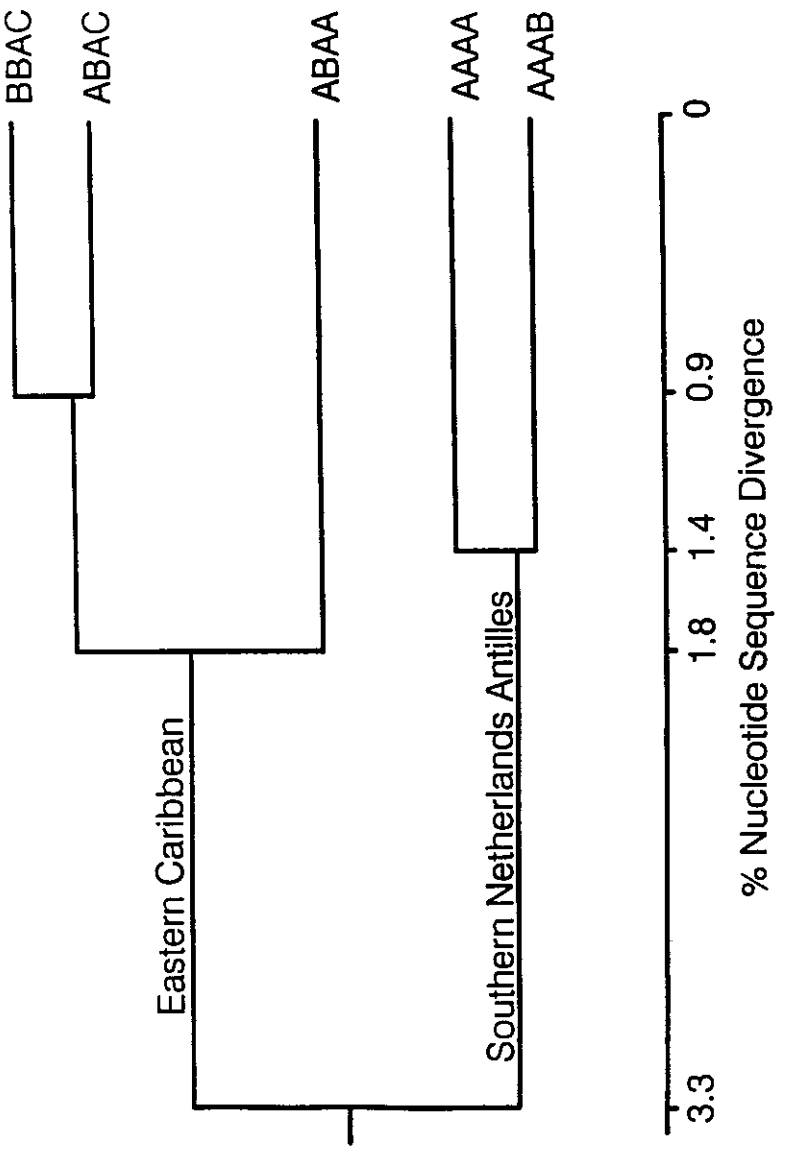

Figure 2. Dendrogram representing mitotype sequence divergence estimates of 300 *H. affinis* from the Caribbean

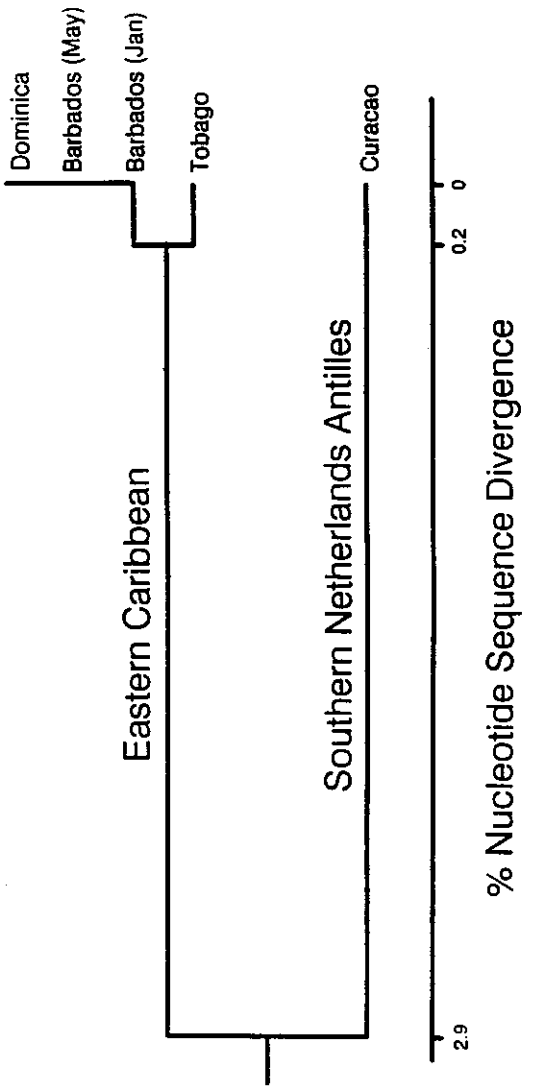

Figure 3. Dendrogram representing inter-population nucleotide sequence divergence estimates from 300 *H. affinis* from the Caribbean.

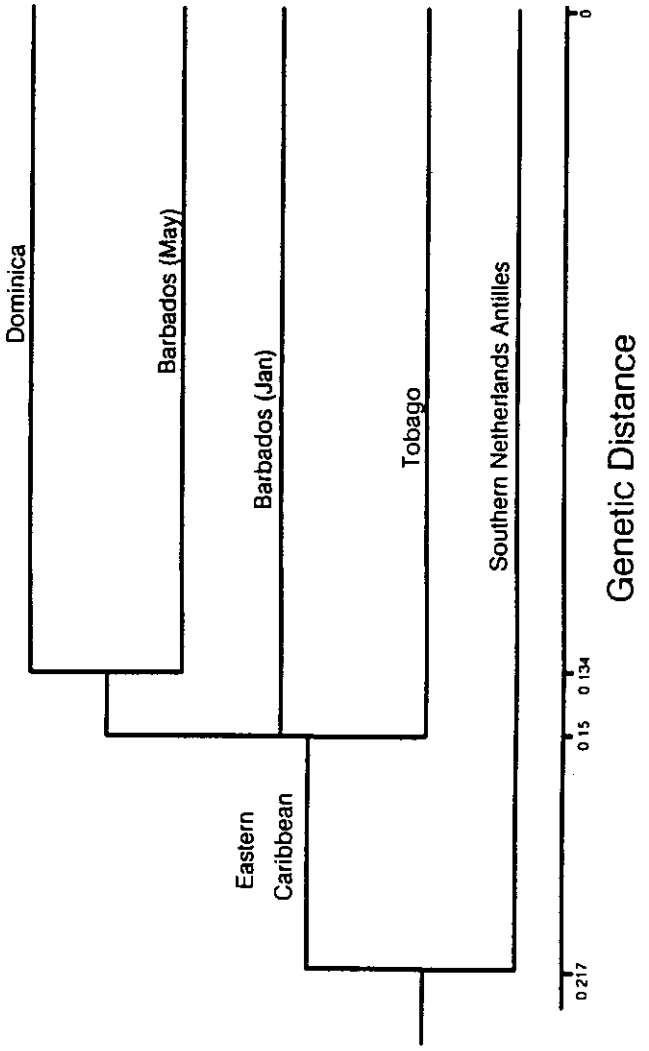

Figure 4. Dendrogram showing genetic relatedness of 300 *H. affinis* from five sampled poplations within the Caribbean.

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