20

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The application of RAPD markers in stock discrimination of the four-wing flyingfish, *Hirundichthys affinis* in the central western Atlantic

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Abstract

The polymerase chain reaction–random amplified polymorphic DNA (PCR–RAPD) technique was used to examine genetic variability and population structuring in the four-wing flyingfish, *Hirundichthys affinis* within the central western Atlantic. Three random decamer primers and pairs of these primers were used to amplify nuclear DNA from 360 fish sampled from six populations (at five locations) across the region. A total of 58 polymorphic RAPD markers were identified, 20 of which were population-specific and six of which were subregional or stock-specific markers. Cluster analysis of similarity indices indicated the presence of three genetically distinct subregional stocks located in the eastern Caribbean, southern Netherlands Antilles and Brazil, respectively. Estimates of gene diversity (ϕ) and gene flow (*Nm*) are consistent with this three-stock hypothesis. Furthermore, partially restricted gene flow was apparent among spatially and temporally separate sampled populations within the eastern Caribbean subregional stock, indicating the possible presence of different spawning groups. These results are entirely consistent with those obtained from PCR–RFLP analysis of the mtDNA D-loop in the same fish, indicating the presence of barriers to dispersal and interbreeding in both sexes. We conclude that the PCR–RAPD technique is suitable for determining population stock structure in this species and that a three-stock approach to managing *H. affinis* within the central western Atlantic would be appropriate.

Keywords: flyingfish stock structure, nuclear DNA, RAPD markers

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Introduction

The four-wing flyingfish, *Hirundichthys affinis*, is found throughout the tropical Atlantic (Gibbs 1978, 1981). In the central western Atlantic it is a commercially important species in the eastern Caribbean (Mahon *et al*. 1986; Oxenford *et al*. 1993), the southern Netherlands Antilles (Zaneveld 1961) and Brazil (Da Cruz 1965; Monteiro 1996). Total annual catch of flyingfish by all eastern Caribbean islands is currently estimated to be in excess of 5000 metric tonnes (CFRAMP 1996a) and in northeast Brazil around 403 metric tonnes (Monteiro *et al*. 1996), whilst catch data for the southern Netherlands Antilles are not routinely collected (G. Van Buurt, personal communication). However, recent and continuing expansion in both the number and sophistication of the fleets (Oxenford 1991; Lawrence 1993; Samlalsingh *et al*. 1993; Willoughby 1993) as well as improved landing sites and market facilities have resulted in the potential for greatly increased catches of flyingfish.

Recognition of the importance of flyingfish as a fishery resource and increasing concern for its sustainability have prompted research efforts over the last decade to examine the basic biology of *H. affinis* (e.g. Storey 1983; Mahon *et al*. 1986; Khokiattiwong 1988; Lao 1989; Oxenford *et al*. 1993; Oxenford *et al*. 1994), in an attempt to provide more informed management advice. Many of the eastern Caribbean islands are now attempting to develop species-specific fishery management plans (CFRAMP 1996b, McConney & Mahon 1998) and recent research has focused on resolving the stock structure of

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the flyingfish resource in the central western Atlantic in order to determine the appropriate geographical scale for any management initiatives for this species (Oxenford 1994; Gomes *et al*. 1996, 1998).

An examination of reproductive characteristics of flyingfish in Barbados indicates that whilst individuals are multiple batch spawners, two periods of peak spawning activity occur there (December/January and April/May; Storey 1983; Khokiattiwong 1988; Lao 1989). This results in higher catches per unit effort (CPUEs) in the Barbados flyingfish fishery during these two spawning peaks. A similar pattern of bimodal CPUEs is also seen in the other eastern Caribbean fisheries (Oxenford *et al*. 1993). This raises the possibility of two separate spawning populations occurring in the eastern Caribbean (Oxenford *et al*. 1993).

Tag and recapture results from the Caribbean have indicated free mixing of both male and female adult flyingfish among the eastern Caribbean islands, but have not detected movement between the eastern Caribbean and the southern Netherlands Antilles subregions (Oxenford 1994). Tag and recapture results from an earlier study in Brazil did not detect movement, although this may have been the result of a small sample size and very short mean time span between release and recapture (Barroso 1967). However, tagging studies cannot detect whether fish, which appear to be mixing, are actually interbreeding (Milner *et al*. 1985, Hynes *et al*. 1989; Graves & McDowell 1994). This is better determined by examination of the genome (Carvalho & Pitcher 1995).

Analysis of mtDNA markers (restriction fragment length polymorphisms (RFLPs) of the D-loop region) in flyingfish sampled from five sites across the central western Atlantic has indicated the presence of three genetically distinct matrilineages: one located in the eastern Caribbean, one in the southern Netherlands Antilles and one in northeast Brazil (Gomes *et al*. 1996, 1998). It has also detected at least partially restricted maternal gene flow among spatially and temporally separated populations in the eastern Caribbean, although this subregional population structuring remains unresolved (Gomes *et al*. 1998). However, a potential constraint of using mtDNA markers in population structure studies to design an appropriate resource management strategy is their maternal mode of inheritance, which means that they are not necessarily representative of variation in the nuclear genome (Billington & Hebert 1991; Wirgin *et al*. 1991). For example, almost nothing is known about spawning migration and/or spawning behaviour in central western Atlantic flyingfish. Therefore it remains possible in this case that although female flyingfish must be displaying natal area philopatry as evidenced by the distinct matrilines observed in the three major geographical subregions (Gomes *et al*. 1996, 1998), males could be travelling and interbreeding between these areas. Although tagging studies did not detect any difference between the sexes in movement (Oxenford 1994) the comparison was necessarily limited to the eastern Caribbean, and was not designed to test for differences in homing behaviour. Movement and interbreeding by males could result in sufficient gene flow to homogenize interpopulation variation in the nuclear genome (Takahata & Palumbi 1985; Billington & Hebert 1991).

Determination of whether the observed variation in the mitochondrial genome is also reflected in the nuclear genome (i.e. barriers to dispersal and interbreeding are effective for both sexes), and further resolution of the eastern Caribbean subregional population structuring has important management implications for this resource.

The development of the polymerase chain reaction (PCR) technique (Saiki *et al*. 1988), and its subsequent application in the development of the PCR–RAPD (randomly amplified polymorphic DNA) technique (Welsh & McClelland 1990; Williams *et al*. 1990) has provided a relatively simple and inexpensive method for examining variation in the total genome (Hadrys *et al.* 1992; Ward & Grewe 1995). This technique therefore has the potential for greatly enhancing population structure studies, as it is less laborious than the currently popular mtDNA RFLP technique, and the detected polymorphisms (multiple RAPD markers) reflect variation in nuclear DNA and can presumably therefore provide a more comprehensive picture of the population genetic structure, particularly in association with information on mtDNA variation.

In this study we utilize the PCR–RAPD technique for identifying nuclear DNA markers to examine variation in the nuclear genome of the four-wing flyingfish in the central western Atlantic. We compare this with previously detected genetic variability in the mitochondrial genome to determine whether barriers to dispersal and interbreeding are present for both sexes, and thus to test the unit stock structure of flyingfish in this region reported by Gomes *et al*. (1998). We also attempt to further resolve the local population structure of flyingfish within the eastern Caribbean subregion by considering nuclear DNA variation among spatially separated populations, and between temporally separated populations in Barbados.

Materials and methods

Collection of flyingfish samples

Details of sample collection are given in Gomes *et al*. (1998). In brief, 60 ripe female flyingfish were sampled during periods of peak spawning activity between January and August 1995 from populations in each of Dominica (April), Barbados (January), Barbados (May) and Tobago (April) in the eastern Caribbean; from Curaçao in the southern Netherlands Antilles (July); and from Caiçara, Brazil

Fig. 1 Geographical position of sampling sites for *Hirundichthys affinis* from the central western Atlantic. SNA, southern Netherlands Antilles; EC, eastern Caribbean.

(August; Fig. 1). Flyingfish liver tissue was selected for analysis because it proved to be a richer source of DNA than gonads or heart, and homogenization was easier. The liver tissue was removed from freshly caught flyingfish which were kept on ice, and immediately placed in preservative buffer (20% (w/v) DMSO and 0.25 M Na_2EDTA saturated with 1 M NaCl).

Extraction and amplification of genomic DNA

Genomic DNA was isolated from 5 mg samples of liver tissue according to the protocol of Cheung *et al*. (1993) with slight modification as described in detail by Gomes *et al*. (1998). Concentrations of all DNA isolates were determined by UV spectrophotometric analysis, and working solutions were prepared at a concentration of 5 ng/μL in TE + RNase and stored at – 20 °C. Seventeen decamer primers from the Operon B kit (60% G + C content; Operon Technologies Inc.) were screened on a subsample of two fish from each region, to test their suitability for amplifying DNA sequences which could be accurately scored. Of the 17 primers, three, and pairs of these three were selected for amplification of genomic DNA (Table 1). Pairs of primers were used in an attempt to screen additional sections of the genome not amplified by the single primers (i.e. sections primed by a combination of the two primers).

PCR–RAPD amplifications were performed on each DNA sample in a reaction mix comprising 2.5 μL of 2 mM

Table 1 Maximum number of distinct genomic DNA bands and polymorphic bands amplified by the PCR–RAPD technique, using individual primers and pairs of primers for 360 *Hirundichthys affinis* from six sampled populations within the central western Atlantic

Primer	Total bands	Polymorphic bands
OPB 6	8	7
OPB 9	14	11
OPB 20	12	10
OPB 6 & OPB 9	9	7
OPB 6 & OPB 20	12	11
OPB 9 & OPB 20	12	12
Total	67	58

of each dNTP (dATP, dCTP, dGTP and dTTP), 2 μL of 10× AmpliTaq polymerase buffer (Perkin Elmer Cetus), 2.5 μL of 2 mM $MgCl_2$, 2 μL of 12.5 μM primer, 0.5 μL (5 U/μL) AmpliTaq polymerase enzyme (Perkin Elmer Cetus), and 5 ng of flyingfish genomic DNA in a final volume of 25 μL. Reactions were overlaid with an equal volume of mineral oil and DNA amplification was performed in a thermal cycler (Hybaid). The reaction mix was preheated at 94 °C for 4 min followed by 40 cycles of amplification (94 °C for 1 min, 32 °C for 1 min and 72 °C for 2 min). The reaction was then subjected to a final step at 72 °C for 10 min. One negative control (reaction mix with no DNA) and one positive control (reaction mix which contained DNA that is known to give a recognizable PCR–RAPD banding profile) were included in each set of amplifications. Approximately 16 μL of amplification product from each sample was separated electrophoretically at 70 V for ≈ 4 h on 1.4% agarose gels (Sigma Chemical Company) in TAE buffer (40.0 mM Tris–acetate, 1.0 mM EDTA). DNA bands were stained with ethidium bromide (0.5 μg/ml), observed under UV light and photographed.

Population analysis

Following electrophoresis, the sizes of the amplification products were estimated by comparisons of distance travelled by each fragment with distance travelled by known size fragments of a molecular weight marker (lambda DNA digested with *Eco*RI and *Hin*dIII (Sigma Chemical Co.)). All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to size and scored visually on the basis of their presence or absence, separately for each fish for each primer and primer pair. Banding patterns were checked for consistency by performing repeat amplifications on a random subsample of individual fish with all primers and primer pairs using identical PCR conditions and parameters. Band scores for a random subset of RAPD profiles were checked for consistency by comparing results of three independent readers.

The development of statistical techniques for evaluating RAPD data is in a rapidly growing and evolving, but still preliminary, stage. We therefore employed a variety of indices to determine the presence of genetic differentiation among the geographically separated populations.

Band presence and absence data for all fish with all primers and primer pairs were combined into a single presence/absence matrix. This was used to compare the frequencies of all polymorphic RAPD markers among populations through a Monte Carlo simulation with 1000 bootstrapped replicates using the CHIRXC program in the RANDOM software package (Zaykin & Pudovkin 1993). This procedure avoids problems created by the occurrence of any cells with an expected frequency of less than 1.0, and/or by more than 20% of the cells having an expected frequency of less than 5.0, such as occurs with rare polymorphisms and small sample sizes. Probability corrections for multiple tests were carried out using the standard Bonferroni procedure (Lessios 1992). The predetermined significance level, P was adjusted using this method to obtain corrected significance levels, α'.

The presence/absence matrix was also used to calculate two separate indices of genetic relatedness: Nei & Li's (1979) similarity index (S), and the gene diversity index (ϕ) which is analogous to Wright's (1951) F_{ST} statistic. Nei & Li's (1979) indices of similarity between individuals and between populations use the shared presence of bands approach and were calculated using the computer program RAPDPLOT (Black 1994) according to the formulations of Lynch (1990). Interpopulation similarity indices were then clustered by the neighbour-joining algorithm to produce a similarity dendrogram using the NEIGHBOUR program and Nei's genetic distance (Nei 1978) in the PHYLIP software package (version 3.5c; Felsenstein 1993).

Gene diversity indices were estimated using presence and absence data for each RAPD marker for all individuals within each population, among all populations within subregions, and among all subregions using the analysis of molecular variance (AMOVA) software package (Excoffier *et al*. 1992) according to the formulations of Cockerham (1969, 1973). This approach estimates the variance components in a hierarchical fashion and tests the significance of the gene diversity index. ϕ-statistics calculated in a pairwise fashion between subregions were used to estimate the extent of gene flow (Nm) between subregions using $Nm = (1/F_{ST} - 1)/4$ (Wright 1943).

ϕ-statistics obtained from the nuclear DNA RAPD markers were compared with ϕ-statistics for the same populations obtained from mtDNA RFLP markers. The latter were calculated using the composite mitotype frequency data presented in Gomes *et al*. (1998) using the AMOVA software package (Excoffier *et al*. 1992). Estimates of gene flow (Nm) calculated from the nuclear DNA RAPD markers were compared with estimates of Nm_f (an indicator of the extent of maternal gene flow) calculated from the mtDNA RFLP markers by $Nm_f = (1/\phi - 1)/2$ (Takahata & Palumbi 1985).

Results

Amplification of flyingfish genomic DNA from all 360 samples with the three primers and three primer pairs generated 67 distinct bands (RAPD markers), 58 of which were considered polymorphic (either occurring in or absent in less than 95% of all individuals) and therefore used in the analyses (Table 1; Fig. 2). The amplification results were routinely repeatable even after the DNA was stored at – 20 °C for more than 6 months. Furthermore, amplification with primer pairs produced fewer (and different-sized)

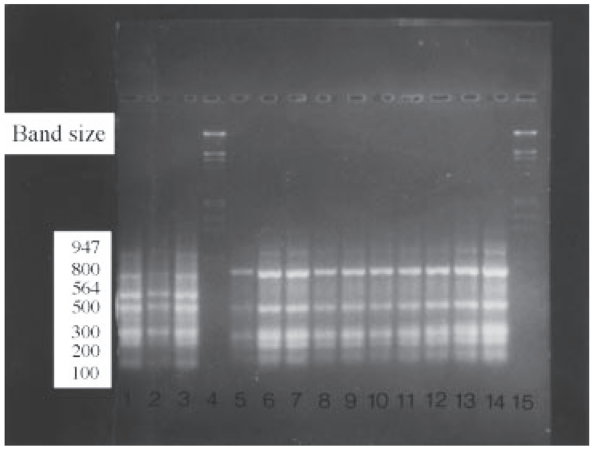

Fig. 2 RAPD profiles for *Hirundichthys affinis* from Brazil using primer OPB9, showing the diagnostic marker for this subregion ($OPB9_{100}$), and polymorphism for marker $OPB9_{564}$. Lanes 1–3 represent samples with band presence for $OPB9_{564}$, lanes 5–14 represent bands with band absence for $OPB9_{564}$ and lanes 4 and 15 are the molecular weight marker lambda phage DNA digested with *Eco*RI and *Hin*dIII.

bands than those produced by both single primers. This suggests that different sections of the genome were being screened by these paired amplifications (Table 1).

Twenty population-specific markers (eight dominant and 12 recessive) and six subregion-specific markers (two dominant and four recessive) were identified (Table 2). Note that three of these markers are definitively diagnostic for Brazil, occurring in 100% of Brazil flyingfish and in no other population (Table 2).

Polymorphic RAPD markers occurred with significantly different frequencies among all populations within the central western Atlantic (Monte Carlo chi-squared 6×58 contingency test; max. $\chi^2_r = 325 < \chi^2_o = 7313$, $P < 0.001$; $\alpha' = 0.054$). Similarly, frequencies of polymorphic RAPD markers occurring within each of the three distinct geographical subregions (eastern Caribbean, southern Netherlands Antilles and Brazil) also revealed significant heterogeneity (Monte Carlo chi-squared 3×58 contingency test; max. $\chi^2_r = 137 < \chi^2_o = 3621$, $P < 0.001$; $\alpha' = 0.054$). Furthermore, a significant difference was also detected in the frequencies of markers among populations

Table 2 Population-specific and subregion-specific RAPD markers for flyingfish (*Hirundichthys affinis*) from the central western Atlantic showing frequency of occurrence in individuals within each population

	EC					
RAPD marker	Dominica	Barbados (Jan)	Barbados (May)	Tobago	SNA Curaçao	Brazil Caiçara
$OPB6_{480}$†	0.00	0.00	0.00	0.00	0.15	0.93
$OPB6_{500}$	0.93	0.35	1.00	0.00	0.67	0.13
$OPB6_{125}$	0.40	0.38	1.00	0.57	0.63	0.00
$OPB9_{200}$	0.68	0.32	0.90	0.20	0.98	0.00
$OPB9_{150}$	0.00	0.90	0.93	1.00	0.98	1.00
$OPB9_{100}$*†	0.00	0.00	0.00	0.00	0.00	1.00
$OPB20_{831}$	0.02	0.08	0.52	0.60	0.83	0.00
$OPB20_{700}$†	0.03	0.65	0.50	0.60	0.00	0.96
$OPB20_{600}$*†	0.00	0.00	0.00	0.00	0.00	1.00
$OPB20_{450}$	0.08	0.75	0.95	0.00	0.95	1.00
$OPB20_{300}$	0.55	0.85	0.70	0.00	0.73	0.96
OPB6 & 9_{600}	0.00	0.47	0.00	0.00	0.00	0.00
OPB6 & 9_{400}	0.87	0.53	1.00	0.60	0.00	1.00
OPB6 & 20_{947}	0.00	0.53	0.00	0.00	0.00	0.00
OPB6 & 20_{700}	0.00	0.53	0.00	0.00	0.00	0.00
OPB6 & 20_{564}	0.00	0.53	0.00	0.00	0.00	0.00
OPB6 & 20_{300}	0.00	0.00	0.00	0.00	0.00	0.47
OPB6 & 20_{200}*†	0.00	0.00	0.00	0.00	0.00	1.00
OPB6 & 20_{100}†	1.00	1.00	1.00	0.80	0.00	0.00
OPB9 & 20_{400}	0.98	0.33	0.55	0.35	0.00	1.00
OPB9 & 20_{350}	0.00	0.22	0.78	0.83	1.00	1.00
OPB9 & 20_{125}	1.00	0.00	0.78	0.40	1.00	0.95

*Indicates diagnostic markers.
†Indicates subregion specificity.
EC, eastern Caribbean; SNA, southern Netherlands Antilles.
Subscripts given with the OPB primer numbers represent the molecular sizes of bands in bp.

within the eastern Caribbean (Monte Carlo chi-squared 4×53 contingency test; max. $\chi^2_r = 186 < \chi^2_o = 3151$, $P < 0.001$; $\alpha' = 0.05$). Further analysis of this heterogeneity within the eastern Caribbean subregion revealed significant differences in marker frequencies between all possible pairs of populations from different locations (max. $\chi^2_r < \chi^2_o$, $P < 0.001$; $\alpha' < 0.05$ in all cases) and between the Barbados January and May populations (Monte Carlo chi-squared 2×51 contingency test; max. $\chi^2_r = 67.50 < \chi^2_o = 1145.89$, $P < 0.001$; $\alpha' = 0.048$).

Cluster analysis of similarity indices indicated segregation of central western Atlantic flyingfish populations into two groups (Brazil and the Caribbean) and further segregation of Caribbean populations into two groups aligning with the southern Netherlands Antilles and the eastern Caribbean subregions (Fig. 3). Brazil and Caribbean populations were separated by a genetic distance of 0.26, populations within the Caribbean region were separated by a distance of 0.21, whilst populations within the eastern Caribbean subregion were separated by distances of ≤ 0.16 (Fig. 3). Interestingly, within this subregion the Barbados (May) population appears more closely related to the Dominica population than to the Barbados (January) population, although this is probably not significant (Fig. 3).

The within-population estimate of gene diversity (ϕ_{ST}) was 0.42 for RAPD markers (0.80 for RFLP markers) indicating significant heterogeneity for flyingfish within the western central Atlantic (Table 3). The ϕ_{CT} estimate (0.49 for RAPD markers; 0.79 for RFLP markers) also indicates significant heterogeneity among subregions (Table 3). However, the estimate of ϕ_{SC} (for both markers) among flyingfish populations within the eastern Caribbean was not significantly different from zero, suggesting a lack of clear population structuring in this subregion (Table 3). Gene flow estimates between all pairs of subregions were < 1 in all cases for both RAPD and RFLP markers, suggesting an exchange of less than one female per generation between them (Smith 1989).

Discussion

Polymorphic RAPD markers occurred with significantly different frequencies across all populations indicating

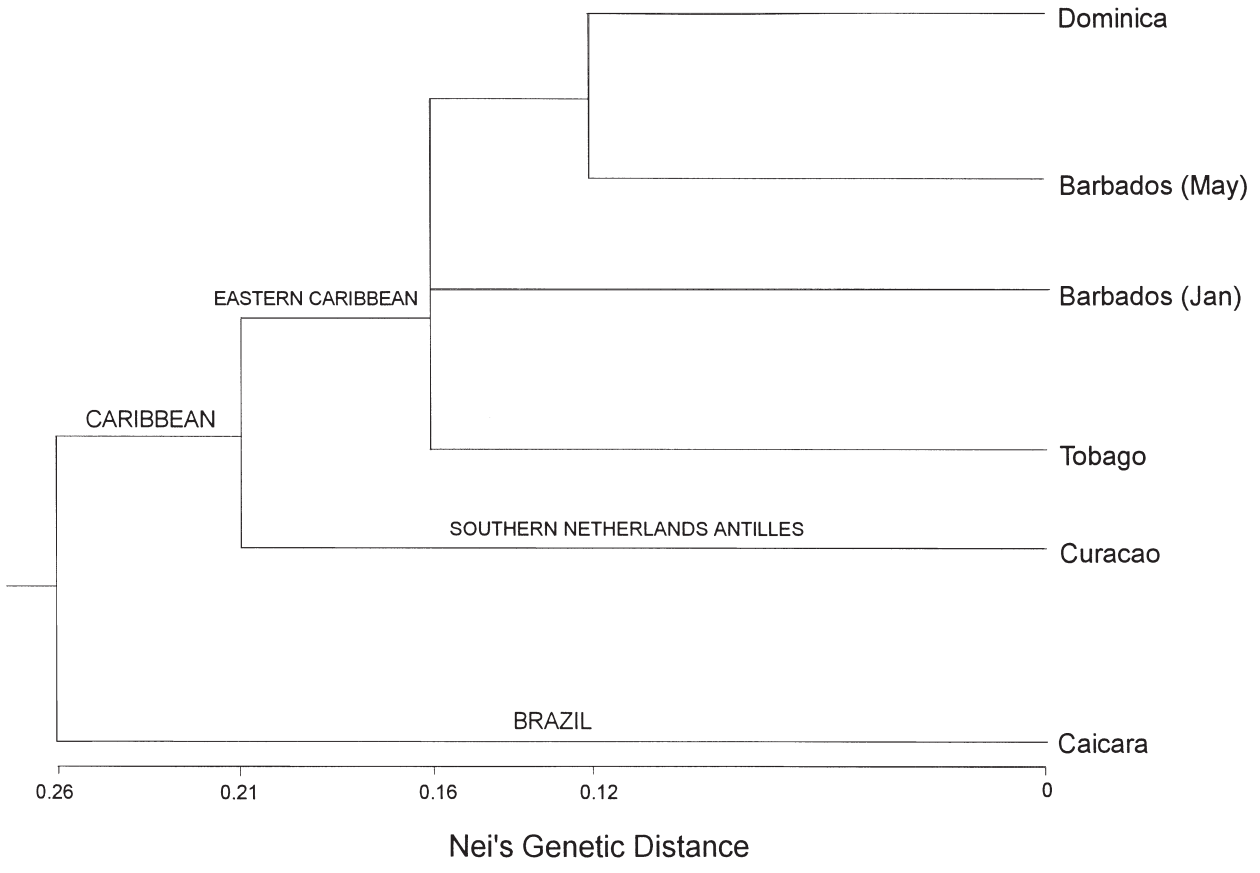

Fig. 3 Nei's genetic distance dendrogram based on similarity indices of the shared presence of fragments from PCR–RAPD analysis of six sampled populations of *Hirundichthys affinis* within the central western Atlantic.

Table 3 Hierarchical analysis of variance in gene diversity based on frequency of RAPD (nuclear DNA) and RFLP (mtDNA) markers for *Hirundichthys affinis* from the central western Atlantic. RFLP frequency data were obtained from Gomes *et al*. (1998)

	Observed partition							
	Variance		Per cent total		*P**		ϕ statistic	
Variance component	RAPD	RFLP	RAPD	RFLP	RAPD	RFLP	RAPD	RFLP
Among subregions	0.45	0.539	71.43	79.67	< 0.001	< 0.001	$\phi_{CT} = 0.49$	$\phi_{CT} = 0.79$
Among EC populations	0.05	0.002	7.94	0.40	0.50	0.06	$\phi_{SC} = 0.14$	$\phi_{SC} = 0.02$
Within populations	0.13	0.135	20.63	19.93	< 0.001	< 0.001	$\phi_{ST} = 0.42$	$\phi_{ST} = 0.80$

*Probability of having a more extreme variance component and ϕ-statistic than the observed values by chance alone.
ϕ_{CT} is tested under random permutation of whole populations across regions. ϕ_{SC} is tested under random permutation of individuals across populations, but within the same region. ϕ_{ST} is tested under random permutation of individuals across populations disregarding either their original populations or regions.

restricted gene flow and population structuring. Cluster analysis of similarity indices among all sampled populations from the central western Atlantic produced three distinct groups which aligned perfectly with geographical origin (eastern Caribbean, southern Netherlands Antilles and Brazil) and indicated greater separation between Caribbean and Brazil populations than between eastern Caribbean and southern Netherlands Antilles populations. This genetic discreteness of flyingfish populations from the three geographical subregions was supported by estimates of gene diversity and gene flow. Results of these analyses are therefore consistent with one another in suggesting the presence of at least three unit stocks of flyingfish within the central western Atlantic. Furthermore, both the marker frequency analysis and cluster analysis indicated significant heterogeneity among sampled populations within the eastern Caribbean subregion.

These results are entirely consistent with the results obtained from analysis of RFLP markers in mtDNA performed on the same samples of flyingfish (Gomes *et al.* 1998). The fact that mtDNA and nuclear DNA display very similar patterns of heterogeneity and population structuring provides evidence that neither males nor females are travelling and interbreeding between subregions, and that barriers are effective for both sexes (Takahata & Palumbi 1985).

The existence of genetically discrete flyingfish stocks within the central western Atlantic was not initially anticipated given the high level of gene flow typical for marine species, particularly oceanic pelagic species (Ovenden 1990; Gold 1994; Graves & McDowell 1994; Ward *et al*. 1994; Ferguson 1995; Shulman & Bermingham 1995). However, population structuring has been reported in migratory pelagic species (e.g. sea bass: Bowen & Avise 1990; blue marlin: Finnerty & Block 1992; king mackerel: Johnson 1993; swordfish: Kotoulas 1995). The within-population gene diversity index estimated for flyingfish falls within the range reported for other migratory species (e.g. 0.64 for hawksbill turtles: Bass 1996; 0.56 for Gulf sturgeon: Stabile 1996; 0.44 for striped bass: Bielawski & Pumo 1997), although it is higher than those reported for other oceanic pelagic species (e.g. 0.02 for yellowfin tuna: Ward *et al*. 1994; 0.15 for swordfish: Alvarado Bremer *et al*. 1994). Note, however, that F_{ST} (ϕ) estimates can vary quite markedly depending on the types of genetic markers used and the geographical scope of the study (Ferguson *et al*. 1995), and have not always been reported in studies of oceanic pelagic species, nor in the few studies using RAPD markers in fish.

The marked genetic variation among flyingfish from within the central western Atlantic indicates the presence of oceanographic barriers and/or limited dispersal capabilities of flyingfish preventing gene flow between populations. The possible mechanisms for genetic isolation of flyingfish between subregions have been discussed in detail by Gomes *et al*. (1998). Essentially, geographical distances between subregions (≈890 km between the eastern Caribbean and southern Netherlands Antilles and 3500 km between the eastern Caribbean and Brazil) appear to be large enough to prevent migration and interbreeding of both males and females. This is supported by a lack of trans-subregion tag returns (Oxenford 1994). Also, the short lifespan (< 2 years; Campana *et al*. 1993) and small maximum size (24.5 cm; Oxenford *et al*. 1994) relative to many oceanic pelagic highly migratory species reduces the probability that this species is capable of long-distance migration. Oceanic characteristics (e.g. biological, physical and chemical properties of surface water) and/or surface-water currents (including the major ocean currents affecting the central western Atlantic such as the South Equatorial Current, North Brazil Current, Guiana Current and the Lesser Antilles Current; and mesoscale currents and gyres) appear to be preventing passive transport of viable eggs, larvae and juveniles between subregions (see Gomes *et al*. 1998).

The apparent heterogeneity (in both the mitochondrial and nuclear genomes) among spatially and temporally separated populations of flyingfish within the eastern Caribbean subregion (although considerably less than among subregions) is also surprising given the relatively small (or lack of) geographical distances between sample locations and the fact that both sexes are known to be travelling freely between them (Oxenford 1994). This situation could arise from spatially distinct selective pressures within an environmentally heterogeneous habitat, and/or strong natal site philopatry by both male and female members of the stock occupying this geographically continuous area (Ehrlich & Raven 1969; Sokal & Wartenberg 1983). This type of localized genetic structure has been reported for fish (e.g. Chapman 1989) and green turtles (Peare & Parker 1996), and sympatric but genetically discrete spawning populations are known for other fish species (e.g. Atlantic herring (Iles & Sinclair 1982), North Sea herring (Cushing 1981), chinook salmon in the lower Columbia River (Miller 1993), chum salmon in Johnstone Strait in Canada (Beacham 1987)). Temporally discrete spawning populations may exhibit temperature preferences thereby preventing mixing of 'families' and giving rise to apparent genetic variation (Ovenden 1990). Add to this scenario larval recruitment that occurs only at a certain water temperature, and a complete reproductive barrier may be maintained within the species, despite its potential for a large amount of dispersal (Ovenden 1990). In this case, there are marked differences in flyingfish fishing intensity and presumably in fishing mortality across the subregion (Oxenford *et al*. 1993; Oxenford 1994), which could provide the mechanism for spatially distinct selection. There is also some evidence for preferred spawning areas based on patterns of regional abundance at the period of peak spawning (Oxenford *et al*. 1995), and possible segregation to spawning sites based on reduced rates of movement during the two spawning periods (Oxenford 1994). The apparent heterogeneity between temporally separated samples in Barbados is consistent with the suggestion of two genetically discrete populations spawning there, although stochastic temporal fluctuation in allele frequencies of the Barbados population could provide an alternative explanation. Interestingly, the two peaks in spawning activity occur off Barbados when oceanographic conditions are different (December/January: mean surface salinity is 33.7‰, mean surface-water temperature is 26.8 °C; April/May: mean surface salinity is 35.6‰, mean surface water temperature is 29.0 °C; Storey 1983; Lao 1989), thereby providing a possible mechanism for maintaining isolation of reproductive populations. Mesoscale currents and gyres have also been reported in the eastern Caribbean (e.g. Molinari 1981; Kinder 1983; Cowen & Castro 1994; Bowman *et al*. 1996) which could provide a mechanism for retention of pelagic eggs, larvae and juveniles in localized areas.

Although we have suggested possible mechanisms for restricted gene flow among sampled populations within the eastern Caribbean, the situation remains unclear. One of the potential problems with interpretation of the genetic variation observed within the subregion is the lack of knowledge regarding the existence and/or locations of discrete flyingfish spawning areas. This increases the possibility that one or more of our samples inadvertently represents a mix of populations.

The use of RAPD markers to examine genetic variation is relatively new, and has many potential advantages over the more-established molecular genetic techniques (Arnold *et al*. 1991). For example, the technique is less labour intensive and faster than other DNA procedures; many samples may be analysed simultaneously and large areas of the nuclear genome scored; the entire procedure from DNA isolation to visualization of amplified DNA on a gel can be performed without the use of hazardous radioactive chemicals and within 24 h; only minute amounts of DNA are required for analysis; and a priori information about the DNA sequence is not required. The technique therefore potentially offers a relatively easy and inexpensive method of examining population genetics. However, to date, it has not been widely used in fish population studies, in part because of a number of drawbacks recognized for this technique. For example, homozygotes cannot be differentiated from heterozygotes, precluding the possibility of allelic frequency analyses (Schierwater & Ender 1993; Williams *et al*. 1993); the PCR–RAPD amplification procedure is particularly sensitive to changes in reaction conditions which may affect reproducibility of amplification products (Williams *et al*. 1990; Arnold *et al*. 1991; Carlson 1991; Klein-Lankhorst 1991); there may be subjectivity in band scoring (Hadrys *et al*. 1992); and analysis of RAPD markers assumes that they are independent and that distinct amplified fragments of similar size do not comigrate (Ferguson *et al*. 1995).

In this study DNA amplification products from all primers and primer pairs were consistently reproducible over a period of 1 year; band scores by three independent readers were highly consistent; and amplifications with pairs of primers demonstrated at least some independence of information. Furthermore, the RAPD profiles in this study displayed a high degree of polymorphism even revealing population-specific, subregion-specific and diagnostic markers which indicated a population structure entirely consistent with that obtained from analysis of RFLP markers in the same fish (Gomes *et al*. 1998). To date, few studies have compared the results of RAPD markers with mtDNA markers (but see Ferguson *et al*. 1995; Ward & Grewe 1995; Bielawski

& Pumo 1997). The consistency in population structuring based on several analyses of both RAPD and RFLP markers, seen in this study, reinforces the reliability of the interpretations (Ferguson *et al*. 1995), and confirms the suitability of RAPD markers for discrimination of flyingfish stocks. Also, the identification of stock-specific nuclear DNA markers identified here and mtDNA markers identified by Gomes *et al*. (1998) provides useful a priori information on the choice of molecular tools for examination of genetic variation in this species (Carvalho & Hauser 1995).

The confirmation of three genetically discrete flyingfish stocks and the identification of stock-specific markers is a significant step towards realizing the goal of stock-based fishery management and conservation of the flyingfish resource in the central western Atlantic (Ferguson *et al*. 1995). The result strengthens the suggested three-stock management approach for flyingfish given in Gomes *et al*. (1998) and confirms that populations damaged by overfishing in one subregion are unlikely to be replenished by immigration of adults or recruitment of juveniles from another subregion. Finally, the mechanisms for restricted gene flow among flyingfish within the eastern Caribbean subregion remain unclear, and will require further investigation of spawning behaviour and genetic variation both temporally and spatially to resolve the localized population structure within the subregion and refine management of this stock.

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Our primary research theme is sustainability of the flyingfish resource in the central western Atlantic. Charmaine Gomes is a postgraduate student registered in the Marine Resource and Environmental Management Programme at the University of the West Indies, Barbados. The research is directed and supervised by Hazel Oxenford and Richard Dales who are both concerned with the application of DNA technology to Caribbean fish species.
