GENETIC STRUCTURE OF THE SWORDFISH (*XIPHIAS GLADIUS)* STOCKS IN THE ATLANTIC USING MICROSATELLITE DNA ANALYSIS

P. Kasapidis[*1], J. Mejuto[2], G. Tserpes[3], A. Antoniou[1], B. Garcia-Cortes[2], P. Peristeraki[3], K. Oikonomaki[1], G. Kotoulas[1], A. Magoulas[1]

SUMMARY

The genetic structure of the swordfish (Xiphias gladius) *in the Atlantic Ocean was examined by genotyping 1179 individuals for 4 microsatellite loci. These individuals were collected during the years 1999-2002 from the following regions: south Atlantic (up to 5°N), mid-Atlantic (9°N to 20°N), northeast Atlantic (North of 30°N and East of 31°W), northwest Atlantic (North of 30°N and West of 31°W). Gene diversity was very high (>0.96) for all four loci. Heterogeneity tests and pairwise Fsts showed that the South Atlantic group was significantly different from all the other groups (p<0.000).These results are in agreement with previous mitochondrial DNA data and do not support a need for changing the 5°N latitude as a boundary for fisheries. Some individuals were also genotyped for the Calmodulin intron 4 gene (CaM) to compare with an earlier dataset for this marker. Allele frequencies found for CaM were in agreement with previous observation and showed a different population structure pattern compared to that revealed by microsatellites. However, the pattern reflected in CaM should be treated with caution, since CaM is only a single diallelic locus, possibly under strong selection. The genotyping of the samples for more microsatellite loci, which is in progress, is expected to further elucidate the genetic structure of the swordfish in the Atlantic, by further increasing resolution.*

RÉSUMÉ

*La structure génétique de l'espadon (*Xiphias gladius) *dans l'Océan Atlantique a été étudiée par le génotypage de 1.179 spécimens pour révéler 4 loci microsatellites. Ces spécimens ont été collectés entre 1999 et 2002 dans les régions suivantes: Atlantique Sud (jusqu'à 5°N), Atlantique Centre (9°N à 20°N), Atlantique Nord-Est (au nord de 30°N et à l'est de 31°W), Atlantique Nord-Ouest (au nord de 30°N et à l'ouest de 31°W). La diversité génétique était très élevée (>0.96) pour les quatre loci. Les tests d'hétérogénéité et les Fst montraient que le groupe de l'Atlantique Sud était très différent de tous les autres groupes (p<0.000). Ces résultats coïncident avec les données de l'ADN mitochondrial précédentes et ne renforcent pas le besoin de modifier la latitude de 5°N comme ligne de délimitation des pêcheries. On a également établi le génotype de certains spécimens pour l'intron 4 du gène de la Calmoduline (CaM) aux fins de comparaison avec un jeu de données antérieur pour ce marqueur. Les fréquences alléliques trouvées pour le CaM coïncidaient avec les résultats observés précédemment et révélaient un schéma de structure de population différent de celui révélé par les microsatellites. Toutefois, le schéma reflété par le CaM doit être considéré avec prudence étant donné que le CaM n'est qu'un locus diallélique unique, faisant possiblement l'objet d'une forte sélection. On prévoit que le génotypage des échantillons visant à révéler un nombre plus élevé de loci microsatellites et qui est actuellement en cours permettra d'élucider encore davantage la structure génétique de l'espadon dans l'Atlantique en accroissant davantage la résolution.*

[*] Corresponding author: P. Kasapidis E-mail: kasapidi@her.hcmr.gr
[1] Institute of Marine Biology and Genetics, Hellenic Centre for Marine Research, P.O. Box 2214, 71003 Heraklion, Crete, Greece.
[2] Instituto Español de Oceanografia, P.O. Box 130, 15 080 LaCoruña, Spain.
[3] Institute of Marine Biological Resources, Hellenic Centre for Marine Research, P.O.Box 2214, 71003 Heraklion, Crete, Greece.

RESUMEN

*Se examinó la estructura genética del pez espada (*Xiphias gladius*) en el océano Atlántico estableciendo el genotipo de 1179 ejemplares para revelar 4 loci microsatélite. Estos ejemplares se recogieron durante los años 1999 a 2002 de las siguientes regiones: Atlántico sur (hasta 5ºN), Atlántico medio (9ºN a 20ºN), Atlántico nordeste (norte de 30ºN y al este de 31ºW), Atlántico noroeste (norte de 30ºN y al oeste de 31ºW). La diversidad génica era muy elevada (>0,96) para los cuatro loci. Las pruebas de heterogeneidad y los Fsts mostraron que el grupo del Atlántico sur difería notablemente de todos los demás grupos (p<0,000). Estos resultados están de acuerdo con los datos previos del ADN mitocondrial y no respaldan la necesidad de cambiar la latitud de 5ºN como límite para las pesquerías. Se estableció el genotipo de algunos ejemplares para el intrón 4 del gen de la calmodulina (CaM) con el fin de compararlos con un conjunto de datos anterior para este marcador. Las frecuencias de alelos descubiertas para CaM estaban de acuerdo con la observación previa y mostraban un patrón diferente de estructura de población en comparación con el revelado por los microsatélites. Sin embargo, el patrón reflejado en CaM debería tratarse con precaución, ya que CaM es sólo un locus dialélico único, posiblemente bajo una fuerte selección. Se espera que el establecimiento del genotipo de las muestras para revelar más loci microsatélite, que se encuentra en marcha, arroje más luz sobre la estructura genética del pez espada en el Atlántico, aumentando la resolución.*

KEYWORDS

Stock identification, Atlantic, genetics, microsatellite DNA, calmodulin, swordfish

1. Introduction

Swordfish (*Xiphias gladius*) is a cosmopolitan highly migratory large pelagic species that is heavily exploited in the world seas. In the Mediterranean Sea and the Atlantic Ocean, management advice is provided by the International Commission for the Conservation of Atlantic Tuna (ICCAT), which carries out regular assessments for large pelagic stocks in the above area. Based on different types of available information, ICCAT has considered during the last decades the existence of three different swordfish stocks for assessment and management: one in the Mediterranean and two in the Atlantic (North and South). The straights of Gibraltar define the borders between the Mediterranean and Atlantic stocks, while the 5ºN parallel separates the two assumed Atlantic stocks. The existence of these different stocks of swordfish has been confirmed by studies using different genetic markers. The separation between Mediterranean and Atlantic stocks has been confirmed mainly by mitochondrial DNA analysis (Magoulas *et al*., 1993; Kotoulas *et al.* 1995; Rosel and Block 1996; Alvarado Bremer *et al.* 1999). The existence of two stocks in the Atlantic has been confirmed both by mitochondrial DNA markers (Alvarado Bremer *et al.*, 2005), as well as by single copy nuclear markers (Chow and Takeyama, 2000; Greig *et al.*, 2000). The latter two stocks seem to correspond with the two major spawning regions in the Atlantic, supported by different kinds of evidence, located off the coast of Brazil and in the Caribbean and the Gulf of Mexico (reviewed in Alvarado Bremer *et al.*, 2005). The other areas of the Atlantic are feeding grounds or transitional areas between spawning and feeding regions (Turner *et al*. 1999), where individuals from different stocks may mix. However, there are uncertainties about the structure, mixing and boundaries of the two Atlantic stocks and it has been pointed out that the current position of the border line at 5º N may not be optimal (Chow *et al.*, 2002).

In the present work we provide preliminary results on swordfish genetic structure in the Atlantic based on data from four new microsatellite DNA loci, obtained through the analysis of large samples from different Atlantic areas (total sample size=1179). We also analyse a subset of those samples for the calmodulin gene intron 4 (*CaM*).

2. Materials and methods

2.1 Development of microsatellite DNA markers

Total DNA was extracted from individual fish, using standard methods (Sambrook *et al.*, 1989) and digested with the restriction enzyme *Mbo*I. Restriction fragments were separated on a 1% agarose gel and fragments sized

300-800 bp were recovered from the gel using the Prep-A-Gene Purification kit (BRL). Fragments were ligated to dephosporylated pUC 18 plasmid vectors, using NEB T4 ligase and the ligated product was used to transform *E. coli* DH5a competent cells. Transformed cells were plated on LB/agar/ampicillin medium that contained IPTG and X-gal and left at 37^{o}C overnight. The colonies were transferred to nylon membranes (Hybond N), which were denatured, neutralized, dried, hybridized using $(GT)_{20}$ oligonucleotide end-labeled with ^{32}P-dATP and autoradiographed in order to detect the positive colonies (Batargias *et al.*, 1998). Plasmid DNA was isolated from the positive clones and those clones were sequenced. Four pairs of primers were designed, synthesized (MWG Biotech) and optimized for the polymerase chain reaction (PCR): XgA, Xg47, Xg51, Xg195. Primers' sequences for those loci and PCR conditions (annealing temperature and $MgCl_2$ concentration) are given in **Table 1**.

2.2 Sampling

A total number of 1179 specimens of swordfish were collected all around the Atlantic Ocean during the years 1999-2002. The number and geographic position of the specimens were allocated in 1 x 1 degree squares (**Figure 1**). Specimens were then grouped into four geographic regions as shown in **Figure 1**. The four different groups were defined as: (1) a South Atlantic (South of 5^{o}N), (2) a mid-Atlantic (9^{o}N to 20^{o}N), (3) a northeast Atlantic (North of 30^{o}N and East of 31^{o}W), (4) a northwest Atlantic (North of 30^{o}N and West of 31^{o}W) (**Table 2**).

All specimens were obtained from fish harvested by the Spanish longline fisheries operating in the respective areas. A piece of tissue from each fish was stored in 95% ethanol and sent to Hellenic Centre for Marine Research jointly with its biological information (size, sex, etc.) for genetic analysis.

2.3 Genotyping

Total DNA was extracted from the specimens using standard methods (Sambrook *et al.*, 1989). All samples were genotyped for the four microsatellite loci (XgA, Xg47, Xg51, Xg195). Genotyping of individual fish was performed through PCR amplification. For each locus the forward primer was fluorescently labeled with Texas-Red and the PCR products were electrophoresed on a Vistra automated sequencer. Allele size was determined by eye by comparing with marker-alleles of various sizes from individuals, whose size had been determined by comparison with standard fragments. A subset of 310 individuals from all areas were also genotyped for the calmodulin gene intron 4 (*CaM*), according to Chow (1998).

2.4 Statistical analyses

For the microsatellite data, observed and expected heterozygosity values were computed for each sample (for each locus separately, as well as for all loci together), using GENETIX version 4.05 (Belkhir, 2000). Deviation from Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP version 3.4 (Raymond and Rousset, 1995) with specified Markov chain parameters of 10000 dememorization steps followed by 500 batches of 5000 iterations per batch. Gene diversity and the mean number of alleles per sample per locus were calculated using FSTAT (Goudet, 1995). The analogue of Wright's F_{IS} (Wright, 1969) was estimated in GENEPOP. Significant differences of allelic distributions between pairs of samples were tested using Fisher's exact test as implemented in GENEPOP. Levels of population differentiation were investigated by calculating pairwise Fst values, using ARLEQUIN version 3.0 package (Excoffier *et al.*, 2005), and permutating the data 1023 times to obtain the p-values. Significance levels for HWE tests, Fisher's exact tests and Fst values were adjusted according to the sequential Bonferroni correction (Rice, 1989).

Samples genotyped for CaM were combined with the data from Chow and Takeyama (2000). For all samples, deviation from HWE for each sample and Fisher's exact tests for allelic differences among pairs of samples were calculated using GENEPOP. Fst values were also calculated using ARLEQUIN. All p-values were adjusted according to Bonferroni correction.

3. Results

All four microsatellite loci used were highly polymorphic and the total number of alleles sampled ranged from 52 for locus Xg51 to 74 for locus Xg47 (**Table 1**). Similarly, the gene diversity per locus per population was very high and similar among groups, ranging from 0.953 to 0.972. All groups had an excess of homozygotes in all loci, which was possibly due to the presence of null alleles and/or to population sub-structure.

Fisher's exact tests showed significant differences in allelic frequency distribution for all loci between all pairs of groups except of the pair North Atlantic 9°N to 20°N (NA_9_20) and NE Atlantic (NA_30E). The South Atlantic group (SAtl) was significantly different from all the rest ($p<0.001$), while all other groups did not differ from each other, after a Bonferroni correction. The pairwise Fst values were small (the highest was 0.00178), but were statistically significant only between the South Atlantic group and all others, after a Bonferroni correction (**Table 3**).

Allelic frequencies and sample sizes obtained for calmodulin within the current study are shown in **Table 4** and **Figure 2**. All samples were in Hardy-Weinberg equilibrium. Samples south of 20°N had a frequency of allele A between 0.81-0.92, while samples North of 30°N had a lower allele A frequency between 0.45-0.70. Exact tests and Fst values (**Table 5**) among all pair of samples (of the current study and that of Chow and Takeyama, 2000), after Bonferroni correction, showed that all samples South of 20°N did not differ from each other, and the same was true among samples over 30°N except from the NW Atlantic sample and Tarifa. Samples NWA and Tarifa were different from all the samples South of 20°N, while samples NW Atlantic and NE Atlantic differed from some but not all samples South of 20°N.

4. Discussion

The microsatellite data presented here showed that there is a small but statistically significant genetic differentiation between South and North Atlantic, which is consistent with results from previous studies using mitochondrial DNA (Alvarado Bremer *et al.*, 2005), and are congruent with a 5°N boundary of the Atlantic stocks. These results confirm a north-south reduction in gene flow within the Atlantic. The reduction of the homogenizing force of gene flow leads to divergence at a rate that is a function of the breeding population size; the bigger the size the slower the rate of divergence. Considering the large population size of swordfish it is not surprising that the difference is not profound, even for fast evolving markers such as microsatellites. Since the Atlantic group between latitudes 9 to 20°N (NA_9_20) do not differ from the other North Atlantic groups, while it does differ from the South Atlantic one, these results do not support a need for changing the 5°N latitude as a boundary for fisheries.

All groups had an excess of homozygotes in all microsatellite loci, which was possibly due to the presence of null alleles. An alternative or complementary hypothesis is that the defined groups contain individuals, which belong to different stocks (Whalund effect). Since most of the specimens studied are from areas that have been considered as transitional or feeding regions for swordfish (Turner *et al*., 1999), and since swordfish is a highly migratory fish, our results in these regions are in agreement with a mixed stock situation.

The genetic analysis for the CaM locus has to some degree confirmed previous results on allele frequencies (Chow and Takeyama, 2000). In the present study additional areas were sampled that were not covered by Chow and Takeyama (2000). These included the area between latitudes 9 to 20°N and from the northwest Atlantic between longitudes 45 to 50°W. The current results confirm the higher frequency of allele B in the North Atlantic. Moreover, they show that the sample between latitudes 10 to 20°N does not differ in allele frequencies to the samples of South Atlantic, while it does differ from all samples North of 30°N. While these results may hint to a different boundary between the North and South Atlantic stocks of swordfish, they have to be treated with caution for a number of reasons, as explained below. Moreover, even if we only consider CaM data, the present analysis shows that a 20°N boundary between the North and South swordfish stocks is problematic, since there are some North Atlantic samples that are not significantly different from samples of the South Atlantic (**Table 5**). This means that in certain cases, the CaM locus polymorphism fails to detect a North-South stock difference, even between areas that are found different by means of other markers. This is by itself interesting, but it is weak for addressing stock boundary issues.

The different genetic markers that have been used so far to study the population structure of swordfish (mtDNA, scnDNA, microsatellites) have different attributes and behave differently in terms of selection. MtDNA and microsatellites are considered neutral markers and therefore, their diversity and patterns of distribution reflect the demographic history and time since divergence among populations (Vitalis *et al*., 2001). MtDNA is maternally inherited and thus it reveals the evolutionary and population history of the female component of a species, while microsatellite DNA markers are bi-parentally inherited and can reveal differences in gene flow among sexes (Hansen *et al*., 2001). Microsatellite markers are usually highly polymorphic and are classified among the markers providing the highest resolution power. On the other hand, single copy nuclear DNA markers, such as CaM, are embedded into or close to coding genes and thus may be subjected to strong selection forces. Selection shapes genetic diversity in a locus-specific manner. These markers therefore are good for trying to understand

patterns of adaptive evolution, but not for obtaining estimates of population history and structure parameters, which require the exclusion of loci under selection (Vitalis *et al*., 2001).

Although neutral markers tend to reflect the population history, and, consequently, are more suitable for analysis of population structure, this occurs in a probabilistic way. Therefore, many markers are needed to describe this history accurately, reflected by the average statistics of individual markers. This should be even more the case for coding sequences and therefore we cannot draw conclusions about population structure based on single markers.

CaM is a single locus, actually a Single Nucleotide Polymorphism (SNP), with only two alleles. This limits the ability of CaM to resolve more than two states in the data, which could be a problem if the data are complex, as in the case of two or more stocks mixing. Moreover, CaM marker is on an intron, which is non-coding, but embedded in a gene those codes for a calcium-binding protein, which is essential for muscle contraction, metabolism, and numerous other cellular processes important to cell survival. In other words, this marker is linked to a gene under strong selection and is probably not a neutral marker. In the last few years, SNPs are increasingly being used for population studies. However, a large number of them, larger than those of microsatellite loci, are necessary for adequately resolving genetic population structure (Morin *et al*., 2004). The pattern reflected in CaM should thus be treated with caution, especially when four other neutrally evolving microsatellite loci, as well as the mtDNA, do not support the same pattern.

The genotyping of these present specimens at additional microsatellite loci, which is currently in progress, is expected to further elucidate the genetic structure of the swordfish in the Atlantic. The seasonal movements of swordfish are an additional key element that has to be considered. Given the large scale of those movements, assignment of individuals to stocks by means of several markers seems to be necessary.

Acknowledgments

This work was funded by the EU project "SWORDGEN", FAIR CT 98 3941. We thank Dr. C. Reeb for her useful comments on the manuscript. We thank Dr. Cameron Brown for linguistic improvement of the manuscript.

References

ALVARADO BREMER, J.R., J. Mejuto, J. Gomez-Marquez, J. Viñas and F. Boan, P. Carpintero, J.M. Rodriguez, C. Pla, J.M. de la Serna and B. Ely. 1999. Hierarchical analysis of nucleotide diversity reveals extremely low levels of mitochondrial DNA gene flow between northeast Atlantic and Mediterranean swordfish populations. Col. Vol. Sci. Pap. ICCAT, 49(1): 467-475.

ALVARADO BREMER, J.R., J. Mejuto, J. Gomez-Marquez, F. Boan, P. Carpintero, J.M. Rodriguez, J. Viñas, T.W. Greig and B. Ely. 2005. Hierarchical analyses of genetic variation of samples from breeding and feeding grounds confirm the genetic partitioning of northwest Atlantic and South Atlantic populations of swordfish (*Xiphias gladius* L.). *Journal of Experimental Marine Biology and Ecology*, 327: 167-182.

BATARGIAS, C., E. Dermitzakis, A. Magoulas and E. Zouros. 1998. Characterization of six polymorphic microsatellite markers in gilthead seabream, *Sparus aurata*. *Molecular Ecology*, 8: 897-899.

BELKHIR, K. 2000. GENETIX, Logiciel Sous WindowsTM Pour la Génétique Des Populations. Laboratoire Génome, Populations, Interactions CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

CHOW, S. 1998. Universal PCR primers for calmodulin gene intron in fish. *Fisheries Science,* 64:999-1000.

CHOW, S. and H. Takeyama. 2000. Nuclear and mitochondrial DNA analyses reveal four genetically separated breeding units of swordfish. *Journal of Fish Biology,* 56:1087-1098.

CHOW S., K. Nohara and Y. Takeuchi. 2002. Boundary between North and South Atlantic stocks of swordfish (*Xiphias gladius*): an implication from nuclear DNA analysis**.** Col. Vol. Sci. Pap. ICCAT, 54(5): 1544-1546.

EXCOFFIER, L., G. Laval and S. Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, 1: 47-50.

GOUDET, J. 1995. Fstat version 1.2: a computer program to calculate Fstatistics. *Journal of Heredity*, 86: 485-486.

GREIG, T.W., J.R. Alvarado Bremer and B. Ely. 2000. Nuclear markers provide additional evidence for population subdivision among Atlantic swordfish. Col. Vol. Sci. Pap. ICCAT, 51(5): 1637-1640.

HANSEN, M.M, E. Kenchington and E.E. Nielsen. 2001. Assigning individuals to populations using microsatellite markers. *Fish and Fisheries*, 2:93-112.

KOTOULAS, G., A. Magoulas, N. Tsimenides, E. Zouros. 1995. Marked mitochondrial DNA differences between Mediterranean and Atlantic populations of the swordfish, *Xiphias gladius*. *Molecular Ecology*, 4: 473-481.

MAGOULAS, A., G. Kotoulas, N. Tsimenides, E. Zouros, J.M. de la Serna and G. de Metrio. 1993. Genetic structure of swordfish (*Xiphias gladius*) populations of the Mediterranean and the eastern side of the Atlantic: analysis by mitochondrial DNA markers. Col. Vol. Sci. Pap. ICCAT, 40(1): 126-136.

MORIN, P.A., G. Luikart, R.K. Wayne and the SNP workshop group. 2004. SNPs in ecology, evolution and conservation. *Trends in Ecology and Evolution*, 19(4): 208-216.

RAYMOND, M. and F. Rousset. 1995. GENEPOP (version1.2): population genetics software for exact tests and ecumenism. *Journal of Heredity*, 86: 248-249.

ROSEL, P. and B. A. Block. 1996. Mitochondrial control region variability and global population structure in the swordfish, *Xiphias gladius*. *Marine Biology*, 125:11-22.

RICE, W.R. 1989. Analyzing tables of statistical tests. *Evolution*, 43: 223-225.

SAMBROOK, J., E. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York.

TURNER, S.C, M. Ortiz, J. Mejuto and V.R. Restrepo. 1999. Stratification of swordfish catch at size by sex. Col. Vol. Sci. Pap. ICCAT, 49 (1): 346-380.

VITALIS, R., K. Dawson and P. Boursot. 2001. Interpretation of variation across marker loci as evidence of selection. *Genetics*, 158: 1811-1823.

WRIGHT, S. 1969. The theory of gene frequencies. Vol. 2. Chicago: University of Chicago Press.

Table 1. Primer sequences and PCR conditions (annealing temperature, $MgCl_2$ concentration) for the four microsatellite loci.

Locus	*Primer sequence (5'-3')*	*Range of alleles (bp)*	*No. of alleles*	*Ta (°C)*	*[$MgCl_2$] (mM)*
XgA	F: CCAAAATGACTTAGCCACAAAG R: TCCCTTCATCCTACACAAACC	108-236	61	58	3.5
Xg47	F: ACAGACAGGGAGGCTACAGG R: TCCCTTGTCCTTGCTTGA	116-220	74	59	4.0
Xg51	F: GTAAAGAGCAGCTTACTAGG R: ACACGTACATCTAGCTGAG	118-212	52	54	3.0
Xg195	F: GAGGGAAAGTGTATCAGAGAGCG R: CCATGATGGAGGCATGTTG	194-322	72	62	2.0

Table 2. Information on collection year and sex for the groups of specimens used in microsatellite analysis. F: females, M:males, U: unidentified.

		1999	*2000*	*2001*
SAtl	F:	60	73	29
	M:	11	5	2
NA_9_20	F:	21	0	68
	M:	10	0	17
NA_30-W	F:	172	76	71
	M:	101	31	0
NA_30-E	F:	65	150	26
	M:	54	87	14
	U:		36	

Table 3. Fst values (below diagonal) and Fst P values obtained after 1023 permutations (above diagonal) for the microsatellite data. Fst values significant at 5% level after a Bonferroni correction are in bold.

	SAtl	*NA_9_20*	*NA_30-W*	*NA_30-E*
SAtl		0.00488	0.00488	0.00000
NA_9_20	**0.00178**		0.13867	0.46777
NA_30W	**0.00122**	0.00083		0.03027
NA_30E	**0.00158**	0.00033	0.00050	

Table 4. Sample names, sample size, genotype and allelic frequencies and collection year for the samples analyzed for the CaM gene.

Sample Name	*Sample Size*	*Collection year*	*Coordinates*	*AA*	*AB*	*BB*	*Allele A freq.*	*Allele B freq.*
NW-Atl	36	2000	36-38N, 46-47W	17	17	2	0.708	0.292
NE-Atl	54	2000	36N, 07W	26	20	8	0.667	0.333
10_20N	115	1999, 2001	10-20N, 20-46W	88	26	1	0.874	0.126
Eq	46	2000	00N, 10-30W	32	14	0	0.848	0.152
SAtl	59	1999-2001	11-31S, 10-35W	41	14	4	0.814	0.186

Table 5. Fst values (below diagonal) and Fst P values obtained after 1023 permutations (above diagonal) for the CaM data of the present and previous studies (Chow and Takeyama, 2000). Fst values significant at 5% level after a Bonferroni correction are in **bold**.

	Brazil	*SAtl*	*TSA*	*Eq*	*TNA*	*10_20N*	*NWA*	*NW-Atl*	*NE_Atl*	*Tarifa*
Brazil		0.08496	0.31250	0.34082	0.67773	0.76270	0.00000	0.00000	0.00000	0.00000
SAtl	0.01636		0.74219	0.61523	0.10938	0.11914	0.00000	0.12207	0.04688	0.00000
TSA	0.00245	-0.00712		0.99902	0.25977	0.36914	0.00000	0.06445	0.00977	0.00000
Eq	-0.00043	-0.00560	-0.01066		0.29883	0.55273	0.00000	0.01953	0.00684	0.00000
TNA	-0.00567	0.02822	0.01190	0.00777		0.46875	0.00000	0.00195	0.00098	0.00000
10_20N	-0.00380	0.01052	-0.00130	-0.00356	-0.00324		0.00000	0.00195	0.00000	0.00000
NWA	**0.33004**	**0.19329**	**0.22091**	**0.22962**	**0.30247**	**0.30960**		0.00293	0.00781	0.57520
NW-Atl	**0.10626**	0.02016	0.03828	0.04448	0.11482	0.09143	0.08002		0.62402	0.00000
NE_Atl	**0.14509**	0.04654	0.06762	0.07452	**0.14422**	**0.12891**	0.05182	-0.00766		0.00293
Tarifa	**0.41422**	**0.25377**	**0.28645**	**0.29683**	**0.38135**	**0.39091**	-0.00587	**0.11829**	0.08347	

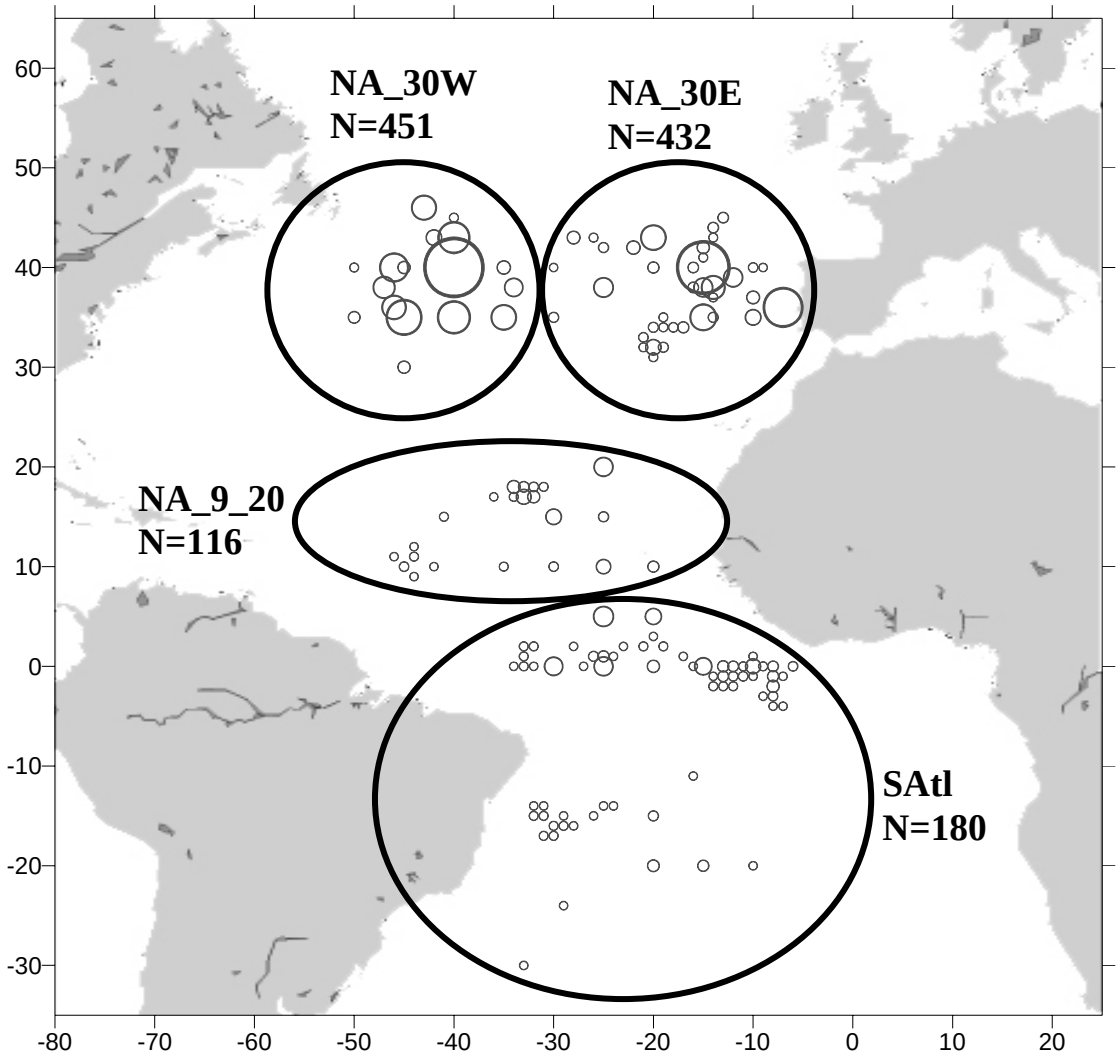

Figure 1. Geographic localities of the samples used for microsatellite analysis. Circle size is proportional to the number of individuals collected in 5 x 5 degrees. The smallest circle corresponds to one sample while the largest to 92.

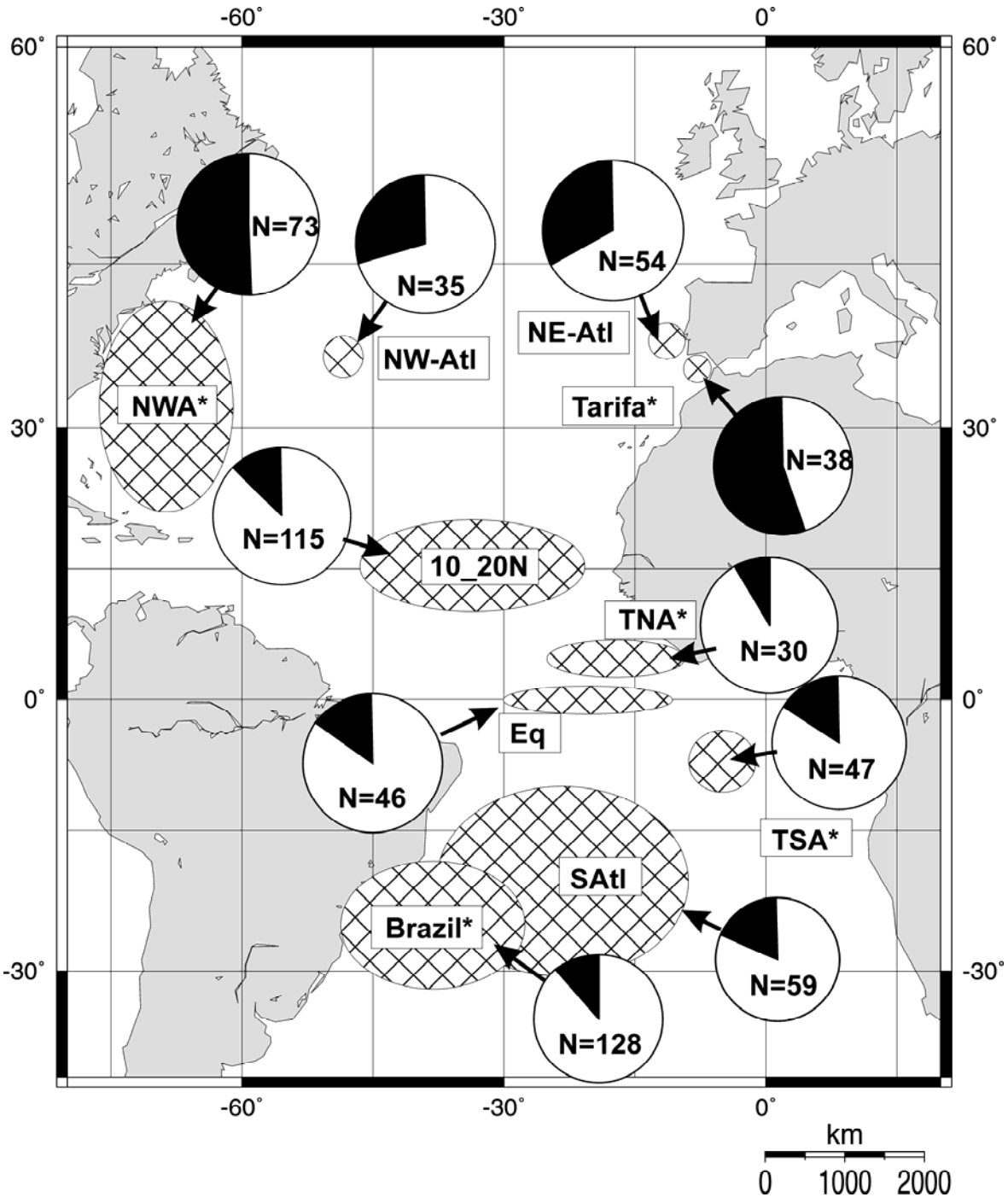

Figure 2. Geographic localities, sample sizes and sample names for the samples used for CaM gene analysis. Frequencies of alleles A (black) and B (white) are given for each sample in pies. Samples analyzed by Chow and Takeyama (2000) are denoted by an asterisk