Recognizing Species from Commercial Catches: Molecular and Morphometric Analyses of *Scomberomorus* spp. off the Mexican Pacific Coast

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Abstract

This study addresses variations of mtDNA and otolith morphometrics in *Scomberomorus concolor* and *Scomberomorus sierra* collected along the Mexican Pacific Coast and Gulf of California (GC) in 2002, 2007 and 2008. The catches of GC are reported without distinguishing species, serious problem account that *S. concolor* is included in the red list by the IUCN. For molecular identification a 592 bp fragment of the 16SrRNA gene was used, and a 410 bp fragment from the mtDNA control region was analysed. The 16SrRNA analysis gene generated two clades, corresponding to each species, which could only be distinguished from two nucleotide sites. The mtDNA sequences maintained the detected lineages, even when both species presented elevated polymorphism in relation to their levels of divergence. For morphometric analysis, a first analysis included samples of fish obtained in 2007 that were genetically identified. A second analysis included fish captured in the central GC that were identified using meristic criteria. Both shape analysis, generated statistically significant classification, the allocation per species was 100%, whereas the allocation of the fish identified using meristic characters was 95%. The deformation grids identified the dorsal zone of the otolith as the origin of the discrimination between species and the criterion for separation.

Keywords: Gulf of California, taxonomic classification, otoliths, morphometrics, *16SrRNA*, mtDNA control region.

Introduction

The fishes of the genus *Scomberomorus* Lacepède, 1801 epipelagic fishes are distributed in tropical and subtropical waters along the North, Central and South American Pacific coast (Collette and Nauen, 1983). Currently, 18 valid species are recognised, two of which are located in the eastern Pacific and Gulf of California: the Monterey Spanish mackerel *Scomberomorus concolor* (Lockington, 1879) with a distribution zone restricted to the Gulf of California, and the Pacific sierra *Scomberomorus sierra* Jordan and Starks in Jordan (1895) which is distributed from La Jolla, USA to Paíta, Perú, including the Gulf of California and the Galápagos Islands (Collette and Nauen, 1983).

In Mexico, both species are subject to exploitation by artisanal fisheries, and their catch is commercialised in the national market. However, official reports do not separate by species. The availability of these species is seasonal and represents an important part of the coastal fishery finfish catch. The historical trend of this resource´s catches (1964-2010) in the eastern tropical Pacific indicates an average production of 6,156 tonnes, with 72% corresponding to catches made in Mexico (Froese and Pauly, 2014). This resource currently supports a well-established fishery in the Gulf of California; from an analysis of its biology, ecology and fishery, Montemayor-López and Cisneros-Mata (2000) classified *S. concolor* and *S. sierra* as a fishery management unit (FMU), based on the fact that both have a habitat affinity. Valdovinos-Jacobo (2006) mentioned that the southern distribution limit of *S. concolor* along the eastern part of the Gulf of California is found in Guaymas, Sonora, whereas along the western coast there are records up to Bahía Concepción (Rodríguez *et al.*, 1992, 1994) and even to Bahía de La Paz, Baja California Sur (ichthyological collection CICIMAR-IPN: records 386, 552, 3111, 3373, 2257 and 6650. In the Guaymas region, in the central part of the Gulf of California, the distributions of *S. concolor* and *S. sierra* overlap; both species are exploited by artisanal fisheries and their catch represents approximately 40-50% of the yearly catches of Mexico. However, the two species are not differentiated in landing reports. Furthermore, a population of *S. concolor* assumed to have existed in the north-eastern Pacific Ocean based on reports of a well-developed fishery at Monterey Bay in 1870s

and 1880s, followed by its disappearance from the area (Fitch and Flesching, 1949). As a result, *S. concolor* has been included in the IUCN red list as a vulnerable species due to the reduction of its distribution (Collete *et al.*, 2011).This calls for the identification of catches, and for the evaluation of life parameters for each species, since the current management plan does not allow the evaluation of the effect of the fishery on each species.

Problems in the identification of species arise when the populations are made up of cryptic species that are morphologically similar but genetically different, or when identical populations are reproductively isolated (Carvalho and Nigmatullin, 1998). The main diagnostic characteristic distinguishing *S. concolor* from *S. sierra* is the number of gill rakers on the first gill arch; while *S. concolor* presents 5-9 gill rakers on the upper arch and 15-20 on the lower arch, *S. sierra* presents 3-5 and 10-13 on the upper and lower arches respectively (Miller and Lea, 1972). The only noticeable difference between the two species is the lack of gold spots on the sides of the *S. concolor* male, which in most instances would not be sufficient to recognise the species, because once they are out of the water the gold spots fade.

Molecular markers, specifically from mitochondrial DNA (mtDNA) sequences, are a useful tool for discriminating species (Kocher *et al.*, 1989). The importance of mtDNA lies mainly in the fact that it is of uniparental origin and as a consequence provides relevant information on evolutionary lineages. In particular, the genes 16SrRNA, Cytochrome b and the subunit I of the oxidase cytochrome (COI) have been used to resolve species identification problems (Grant and Utter, 1984; Patarnello *et al.*, 1994; Di Finizzio *et al.*, 2007; Imaizumi *et al.*, 2007; Lakra *et al.*, 2009). The use of COI as a useful marker for the identification and definition of species under a known concept such as DNA barcode has gained much interest (Hebert *et al.*, 2003). However, despite COI being a robust evolutionary marker, it has been shown to have a higher mutation rate in several organisms. We therefore selected the mitochondrial 16SrRNA gene, which has also been shown to be a good marker for species differentiation, and has been of great use in studies of intergeneric and interspecific comparisons in several Perciform families (Ritchie *et al.*, 1997; Bernardi *et al.*, 2000; Streelman *et al.*, 2002).

Another useful and statistically robust tool for the discrimination of species is morphometric analysis, using the body shape or some other body structure. In fish, the *sagittal* otoliths have been used both for the discrimination of populations or stocks (Campana and Casselman, 1993), and for species identification (Monteiro *et al.*, 2005). Otoliths are calcium carbonate structures located in the inner ear of fish that act as sound transducers and play an important role in the hearing capacity of fish

(Gauldie, 1988; Popper and Lu, 2000; Rodríguez-Mendoza, 2006). The size and shape of otoliths are considered characteristic of each species and phylogenetic patterns can be deduced from their morphology. Ramírez-Pérez *et al.* (2010) evaluated the power of discrimination of *sagittal* otoliths for distinguishing phenotypic stocks of *S. sierra* in three different locations of the Gulf of California. In the present study genetic variations of mtDNA sequences and morphometric variations of otolith shape in the gulf sierra *S. concolor* and the Pacific sierra *S. sierra* were addressed in order to provide elements to discriminate between both species and assess potential biases in the traditional identification based on meristic characters.

Materials and Methods

Data Collection

Otolith and tissue samples of *Scomberomorus* spp. were obtained at nine sites in the northern part of the Gulf of California (both coasts) and western coast of the Baja California Peninsula, south to Chiapas State, in 2002, 2007 and 2008 (Table 1; Figure 1). The otoliths were stored dry in plastic tubes and the tissue samples were stored in 96% ethanol at room temperature until processing.

Fish identification at species level was based on the number of gill rakers on the first gill arch as described by Miller and Lea (1972).

Molecular Identification

Genomic DNA was extracted using a commercial kit (DNeasy®Tissue-Qiagen), according to the manufacturer´s specifications. Quality of the DNA was verified using 1% agarose gels stained with SybrGold and visualised in a BioDoc-itTM (UVP) ultraviolet light photo-documentation system.

For molecular identification of the species the 16SrRNA gene was used, which is widely employed in phylogenetic studies since it is highly conserved; hence the analysis can be performed with few individuals. This situation occurred in the present study, since there were only three *S. concolor* individuals collected at San Felipe (SF), a location where *S. sierra* specimens have never been recorded. Ten individuals from six locations (BM, LRT, GYM, COL, ACA and CHI) and three individuals from one location (SF) identified by meristic characters as *S. sierra* and as *S. concolor*, respectively, were selected for this analysis (Table 1). A fragment (~592 bp) was amplified using the primers 16Sar-L (5' CGCCTGTTTATCAAAAACAT) and 16Sar-H (5' CCGGTCTGAACTCAGATCACGT) reported by Palumbi *et al.* (1991). The reactions were carried out in 35 µl volumes containing: PCR 1X Buffer (Invitrogene), 0.2 mM of dNTP mix, 0.48 µM of each primer, 4.0 mM of $MgCl_2$ and 2.5 U of Taq DNA

Table 1. Sites and dates of sampling of *Scomberomorus concolor* and *S. sierra* along the Mexican Pacific coast

Sampling sites			Number of samples			
			mtDNA		Morphometrics	
	Sampling date		16SrRNA	Control region	Genetically identified	Meristic
S. concolor (Gulf sierra)						
San Felipe, B.C. (SF)	Mar	2007	3			
Guaymas, Son. (GYM)	Oct	2007			12	
	Jan	2002				17
	Feb	2002				32
	Mar	2002				
	Apr	2002				21
	May	2002				15
	Jun	2002				15
S. sierra (Pacific sierra)						
Bahía Magdalena, B.C.S. (BM)	Jun	2007	1	8		
	Aug	2007		6		
	Nov	2007		16	15	
Loreto, B.C.S. (LRT)	Oct	2007	1	30	10	
Guaymas, Son. (GYM)	Mar	2007	5	12		
	Oct	2007		18		
	Jan	2002				10
	Feb	2002				13
	Mar	2002				10
	Apr	2002				17
	May	2002				26
	Jun	2002				22
Mazatlán, Sin. (MZT)	Dec	2007		30	10	
Chacala, Nay. (CH)	Oct	2007		30	5	
Manzanillo, Col. (COL)	Nov	2007	1	20		
	Dec	2007		10		
Acapulco, Gue. (ACA)	Jul	2008	1	30		
Puerto Arista, Chi. (CHI)	Mar	2008	1	30		
Total			13	240	52	198
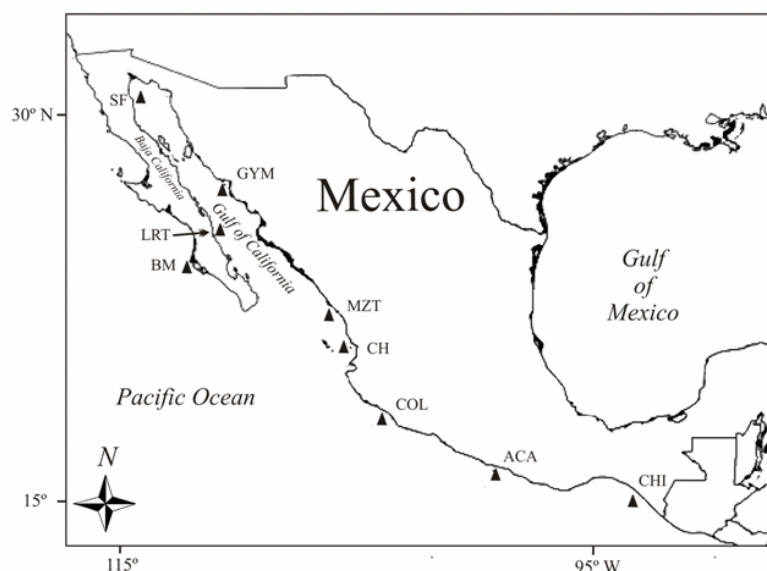
Figure 1. Geographic location of the sampling sites along the Mexican Pacific coast. The full names of the abbreviations of the sampling sites are presented in Table 1. In San Felipe (SF), north of the Gulf of California, only one sampling trip was conducted to collect gulf sierra *S. concolor*; Pacific sierra *S. sierra* has not been reported in this region.

Polymerase (Invitrogene). The thermocycler parameters were: 2 minutes at 94ºC for denaturation of mixtures, followed by 35 cycles with three temperature segments (94°C, 58°C and 72°C during 1 minute each), and a final extension of 4 minutes at 72ºC. In addition, 240 sequences of mtDNA control region were obtained from individuals identified as *S. sierra* using meristic criteria, in order to genetically validate the taxonomic identification (Table 1). The clades found were associated with the clades obtained via the 16SrRNA gene. These results allowed the confirmation of whether the genetic variations found

were caused by the polymorphism of *S. sierra* or by the divergence between the two species (finding clades associated with each species). The fragment of the control region (~410 bp) was amplified using primers designed in this study (ScomRc: 5' ATTGAAGGTGAGGGACAA and ScomPhe: 5' CGGAGCTTTCTAGGGCTCAT) based on a complete sequence of the mtDNA control region of *Scomberomorus cavalla* (access in GenBank # NC_008109). The amplification of the control region was carried out using the same concentrations and thermocycler parameters used in the amplification of the 16SrRNA gene.

The products of the amplifications (gene 16SrRNA and control region) were confirmed by electrophoresis in 1% agarose gels. The gels were stained with SybrGold and visualised in an ultraviolet light photo-documentation system. Once the success of the amplification was confirmed, the products were sequenced (MacroGen Inc., Korea) in both directions using the same primers used in the amplification.

All sequences were aligned and arranged using Sequencher 4.5 (GeneCode Inc.). The sequences, both of the 16SrRNA gene and of the control region, were used to construct a Neighbor-Joining (NJ) phylogram based on the Kimura-2 parameter model using MEGA 3.0 (Kumar *et al*., 2004), and to estimate the intra and inter-specific genetic distances.

Morphometric Analysis

In order to validate the use of otolith shape to distinguish between *S. concolor* and *S. sierra,* a sample collected in 2007 of up to 40 randomly selected otoliths per species that were genetically identified (via the mtDNA control region), were used for a preliminary morphometric analysis (Table 1). Fish otoliths from GYM (fishing site where both species overlap in their distribution and are caught by fisheries) identified via gill raker counts (n=100 of *S. concolor*, n=98 of *S. sierra*) were used in a posterior morphometric analysis (Table 1). In order to reduce the potential interannual variation in otolith shape, the fish selected for this analysis (GYM) came from the 2002 fishing season.

Images of the otoliths were taken using a Sony digital camera connected to an Olympus stereoscope. All images were digitalised at 10x with their respective measurement scale. To provide guidelines of equal angular spacing, a reference was constructed on the digital image of each otolith using the MakeFan program (Sheets, 2003-2005) to identify points in the same position in all otoliths. In accordance with Ramírez-Pérez *et al.* (2010), 32 landmarks were established, one on the otolith focus and the rest on the posterior dorsoventral contour (Figure 2).

A superimposition method based on generalized Procrustes analysis (GPA) was used to remove differences attributed to the position, orientation, and scale between configurations. (Rohlf and Bookstein, 1990; Rohlf and Slice, 1990). These analyses were performed using the CoordGen6 program (Sheets, 2003-2005). A Canonical Variable Analysis (CVA)
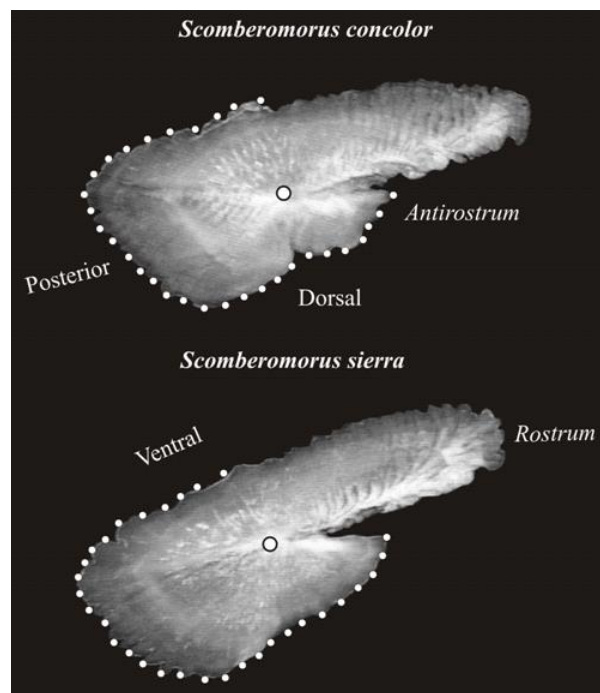
Figure 2. Digital images of sagittal otoliths of the gulf sierra *S. concolor* and of the Pacific sierra *S. sierra*. The references on the outer edge of the posterior dorso-ventral region of the otoliths are homologous points selected according to the criteria established for the Pacific sierra by Ramírez-Pérez *et al.* (2010).

was performed to evaluate the variation of the otolith shape of each species using CVAGen6 (Sheets, 2003-2005). The statistical significance was evaluated through Wilks´ Lambda value (Λ). Values closer to zero mean that there is perfect discrimination, while values closer to one represent a lack of discrimination. An allocation matrix was obtained using Mahalanobis distances of the scores produced by the CVA. The allocation is based on the distance between each individual and the centroid of the closest group. Finally, in order to observe the trend in shape variation, the average shape of the otolith and its variation in each species was obtained through deformation grids (Thin Plate Spline).

## Results

Molecular Identification

The gene tree generated from the 16SrRNA gene sequences of the species identified using meristic characters (*S. sierra n*=10, and *S. concolor n*=3) generated two clades (Figure 3). Two individuals of those identified as *S. sierra* (GYM 2 and GYM 4) were included in the *S. concolor* clade, indicating an incorrect identification based on meristic criteria. None of the five *S. concolor* sequences had nucleotide changes, thus its genetic distance was zero. In *S. sierra* the genetic distance was 0.13%, and between the two species it was 0.34%. The confirmation of the identification error was not possible due to the gill rakers being discarded after the preliminary count.

The GYM 2 and GYM 4 sequences were used as a reference for recognition of *S. concolor,* using the control region, and for the identification of the genetic differences produced by divergence between species with respect to the variations produced by intraspecific variability, using mtDNA.

For the analysis of the mtDNA control region, only 66.3% (159) of the 240 samples were properly amplified. Of the 159 sequences of the control region, 12 (7.5%, including GYM 2 and GYM 4) were recognised as *S. concolor*; all were from Guaymas (GYM). The 147 remaining sequences were included in the *S. sierra* clade. For representation purposes, only 6 of the 147 sequences of *S. sierra* and the 12 sequences identified as *S. concolor* were included in Figure 4. Using these data, we found a genetic distance of 2.58% for *S. sierra* and 1.75 % for *S. concolor*. The percentage of divergence between these species was 4.69%. The nucleotide variations present in the control region allow the lineages associated with each species to be clearly characterised.

Morphometric Analysis

Based on the differences detected in the gene tree of the control region, the first morphometric analysis that included the otolith shape of the 12 individuals classified as Monterey Spanish mackerel *S. concolor* was compared with 40 otoliths of the Pacific sierra *S. sierra.* The latter were selected at random from Bahía Magdalena (BM, *n*=15), Loreto (LRT, *n*=10), Mazatlán (MZT, *n*=10), and Chacala
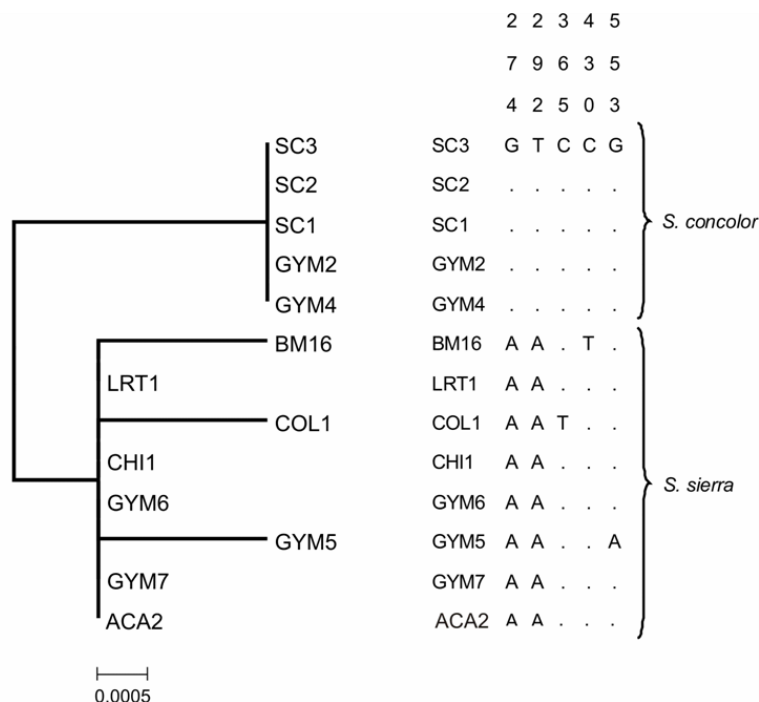
Figure 3. Gene tree based on the sequences of a 592 bp fragment of the 16SrRNA gene, showing two clades that are associated with the gulf sierra *S. concolor* and the Pacific sierra *S. sierra* along the Mexican Pacific coast. The 2-parameter Kimura nucleotide substitution model and the Neighbor-Joining (NJ) construction method were used.
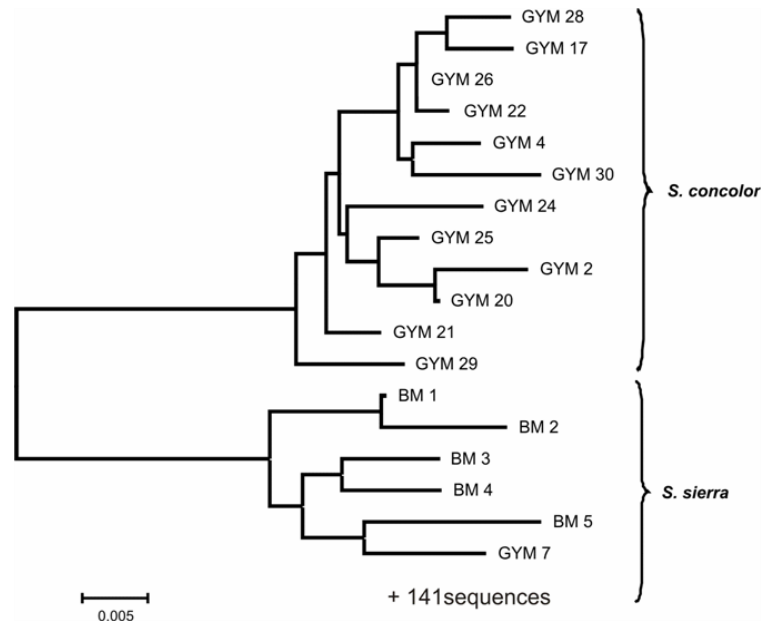
Figure 4. Gene tree variations based on the sequences of a 410 bp fragment from the mtDNA control region of the gulf sierra *S. concolor* and the Pacific sierra *S. sierra* along the Mexican Pacific coast. The scale bar is equal to 0.005 and represents genetic distances.

(CH, *n*=5).

The CVA detected significant differences (CV1, Λ= 0.10, P<0.05). The frequency of the CV1 indicated a clear separation of the shape of the otoliths (Figure. 5), which suggests a morphological differentiation between the species. According to the Mahalanobis distances, the average percentage of correct allocation was 100% for both species (Table 2). By observing the deformation grids that represent the average shape of the posterior part of the otolith of each species, it can be seen that the difference in otolith shape between *S. concolor* and *S. sierra* originates in the otolith dorsal zone (Figure 5).

Based on the results of the above analysis and in order to assess the degree of discrimination between species, a second morphometric analysis of the otolith shape of organisms sampled in 2002 (identified only from gill raker counts) was carried out. The CVA detected significant differences in the shape of the otoliths (CV1, Λ= 0.26, P<0.05). The frequency of the CV1 scores show two groups (Figure 6), suggesting that a morphological difference exists between the otoliths of the two species. According to the Mahalanobis distances, the average percentage of correct allocation for *S. concolor* was 95% and 94% for *S. sierra* (Table 2). The deformation grids were consistent in demonstrating that the otolith dorsal zone generates the discrimination between *S. concolor* and *S. sierra*.

Discussion

The reduction in fishery resources around the world has stimulated research into the structure and genetic diversity of the populations of commercially important fish (Gauldie, 1991; Sotelo *et al.*, 1993; Mackie, 1996). In many cases, the diagnostic characters (size, shape and appearance) for the identification of species are difficult to obtain, and the catch is reported generically (encompassing various species), which affects fishery management strategies. The comparison of mtDNA sequences represents a powerful alternative for taxonomic identification. Due to the fact that it includes a high rate of mutation and generally presents matrilineal inheritance, mtDNA enables the evolutionary lineages of closely related species to be distinguished.

In the Gulf of California, species of the *Scomberomorus* genus are found to be partially sympatric and due to their external similarity, catch records do not distinguish one species from the other. The results of the present study, based on molecular markers, allowed the detection of two lineages, which were associated with *S. concolor* and *S. sierra* (defined *a priori* based on meristic characters and using specimens with a typical and presumably exclusive distribution). It was possible to distinguish between species from two nucleotide sites of the amplified fragment of the 16SrRNA gene. Polymorphism was also identified in *S. sierra* (BM16, COL1 and GYM5) but this variability occurred in fish from sampling sites where no overlap occurred in the distribution of the species, hence the two detected lineages were maintained. The two clades found by means of the mtDNA control region were also clearly associated with each species. The above allows the differentiation of genetic differences attributed to divergences between species from those related to intra-population polymorphism.

There were relatively few nucleotide differences
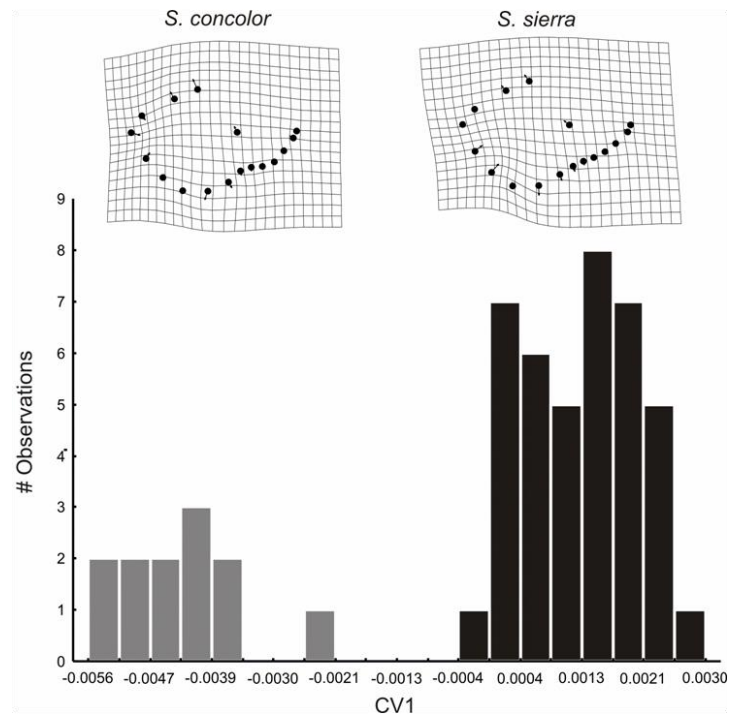
Figure 5. CV1 score frequencies of the canonical analysis (CVA) of two groups identified genetically and by deformation grids. The grey bars represent gulf sierra *S. concolor* otoliths and black bars represent Pacific sierra *S. sierra* otoliths.
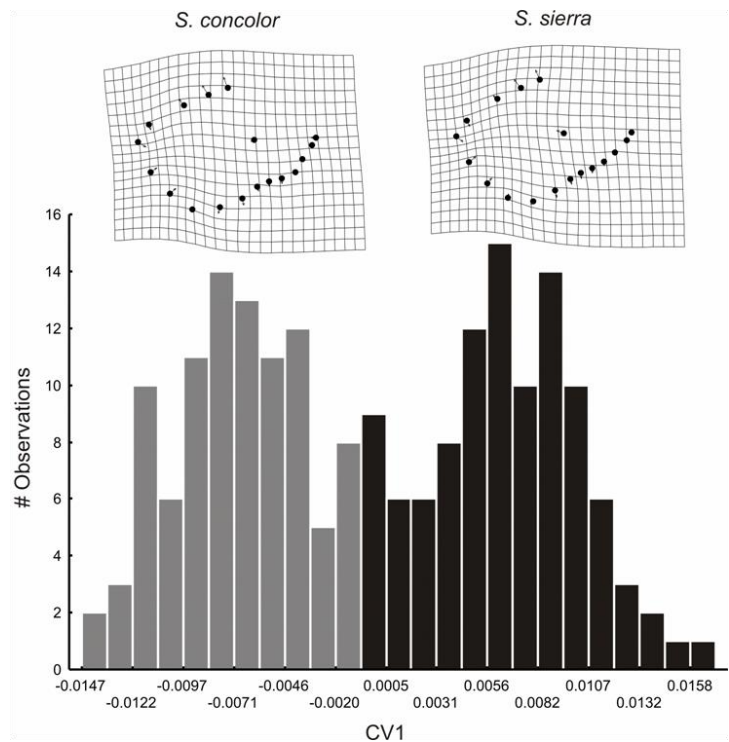
Figure 6. CV1 score frequencies of the canonical analysis (CVA) of two groups identified by meristic characters and deformation grids. The grey bars represent gulf sierra S. concolor otoliths and black bars represent Pacific sierra S. sierra otoliths.

between the two species in the sequences of the 16SrRNA gene (only at two nucleotide sites: 274 and 292), taking into account that in *S. sierra* polymorphism was detected in three of the five variable sites (Figure 3). The results from the mtDNA control region did not indicate a strong genetic divergence between the two species as a function of the elevated intraspecific polymorphism. The mean interspecies distance was very low, 0.34% for the 16SrRNA and 4.69% for the control region. For two

Table 2. Results of the classification by number and percentages based on Mahalanobis distances. The rows represent the correct allocation

	S. concolor	*S. sierra*	N	% Correct Allocation
S. concolor[1]	12	0	12	100
S. sierra[1]	0	40	40	100
S. concolor[2]	95	5	100	95
S. sierra[2]	6	92	98	94

mtDNA fragments, the inter-specific distance was only around two-fold higher than the intra-specific distance. The relatively high genetic similarity between *S. sierra* and *S. concolor* can also be noticed from the little differentiation of ATPase 8, ATPase 6, ND2 and 12SrRNA mitochondrial genes and the absence of differences in the nuclear Aldolase intron 5 reported by Banford *et al.* (1999). These authors suggested that within the eastern Pacific, the separation of *S. concolor* and *S. sierra* could have occurred around 2.3 Ma, as a combination of physical factors most likely played a role in eastern Pacific speciation. Considering the molecular information reported by Banford *et al*. (1999) and that obtained in the present study, the forces driving evolution have not apparently been strong enough to generate highly divergent lineages. In addition, the coalescence time has been relatively short. In fact, the separation of *S. concolor* and *S. sierra* could have occurred later than 2.3 Ma. Considering a mutation rate of 3.6%/Ma for the control region (Donaldson and Wilson, 1999; Dominguez Lopez *et al*., 2010) and the genetic distance of 4.69%, a rough estimate of the coalescence time would be around 0.65 Ma. Thus, the speciation of *S. concolor* and *S. sierra* could had been influenced by the Pleistocene glaciations, isolating two populations that evolved into the actual species, as Banford *et al*. (1999) suggested for the species in the tropical western Atlantic.

The molecular analyses in the present study indicate that there was greater confusion concerning the recognition of each species through meristic characters in organisms from the Guaymas region. This can be explained by the fact that in this region both species coincide geographically, increasing the probability of finding one of the two species and identifying it incorrectly. Another possibility would be that during the evolutionary process, the species presented secondary contact, which generated a genetic exchange; such a hybridisation event should be addressed in greater detail through the use of molecular tools based on nuclear DNA.

In order to evaluate an alternative approach to genetic analysis for the identification of species a morphometric analysis was used, which has proved to be a robust statistical tool in the recognition of species and the detection of populations/stocks of different commercially important resources (Begg *et al*., 2001; Jayasankar *et al*., 2004; Hossain *et al*., 2010). The implementation of morphometric analysis has become increasingly common due to the notable and growing development of image processing techniques, favoured by the improvement of computer equipment and digital cameras (Cadrin and Friedland, 1999; Cadrin, 2000). In the present study, the shape of the otolith was used to distinguish between species. The results obtained from the analysis of the genetically identified individuals made it possible to detect that the shape of the otolith differentiated the individuals of both species by 100% (Table 2). When the shape of the otoliths of individuals identified meristically was compared, approximately 6% of the allocation was incorrect (Table 2). Considering that the percentage error in the identification using meristic characters was 7.5% (inferred from the control region), it is suggested that the incorrect allocation of individuals in the morphometric analysis was probably due to poorly identified individuals. Hence the present results support: a) the power of otoliths for the discrimination between species and the detection of dubiously identified individuals; and b) the need for genetic markers to confirm the *Scomberomorus* species in the Gulf of California.

For fishing purposes, the identification of the stock structure of exploited populations represents an important task for resource management (Allendorf *et al*., 1987; Ferris and Berg, 1987). The results of the present study show that it is possible to differentiate the Pacific sierra *S. sierra* from the gulf sierra *S. concolor* based on otolith shape. This method has also been used to identify stocks in the population of *S. cavalla* exploited in the Gulf of Mexico (DeVries *et al*., 2002) and in the population of *S. sierra* in the Gulf of California (Ramírez-Pérez *et al*., 2010). Given that otoliths are structures that store information and allow the history of the fish´s life to be understood (Campana and Thorrold, 2001; Elsdon *et al*., 2008), the implementation of additional analyses concerning their chemical composition would enable studies that address the ecology, migration, mixing of stocks, feeding and spawning zones (Rooker *et al*., 2003; Petterson *et al*., 2004).

At present, the fishery of species of the *Scomberomorus* genus in the Gulf of California is managed by fishing permit criteria, generally for finfish and minimum catch volumes. In order to specify biological reference points for sustainable exploitation, the population parameters of each species should be evaluated. For this, the taxonomic identification must be reliable. Considering the results obtained in the present study, a greater effort should be made based on the use of molecular markers in

order to improve the recognition of species. Genetic analyses and morphometrics should be applied together in order to identify the existence of stocks throughout the distribution area of each of the two species where they are exploited by fisheries of different magnitudes.

Acknowledgements

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