

Testing species boundaries between Atlantic and Pacific lineages of the Patagonian rockfish *Sebastes oculatus* (Teleostei: Scorpaenidae) through mitochondrial DNA sequences

Evaluando límites específicos entre los linajes Atlántico y Pacífico de *Sebastes oculatus* (Teleostei: Scorpaenidae) a través de secuencias de ADN mitocondrial

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Resumen.- Se investigó la diferenciación genética y límites de especies entre los linajes Atlántico y Pacífico de *Sebastes oculatus* a través de secuencias de la región D-loop del ADN mitocondrial (541 bp). Las secuencias se obtuvieron de 47 individuos de siete localidades de las costas del Pacífico y Atlántico de Sudamérica (*S. oculatus*) y una localidad de Sudáfrica (*S. capensis*). Adicionalmente, se obtuvieron dos secuencias de *Helicolenus lengerichi* que fueron utilizadas como grupo externo. Estos datos fueron combinados con secuencias disponibles en GenBank correspondientes a 21 especies de *Sebastes*. Las aproximaciones de máxima verosimilitud y análisis Bayesiano mostraron distintividad topológica entre las poblaciones sudamericanas y africanas de *Sebastes*, apoyando la existencia de dos especies filogenéticas: *S. oculatus* y *S. capensis*. Sin embargo, las poblaciones del Pacífico y del Atlántico de *S. oculatus* no formaron grupos monofiléticos recíprocos. La aplicación del protocolo de Wiens & Penkrot para evaluar límites entre especies no apoyó la existencia de dos especies en las costas de Sudamérica. El flujo génico entre las poblaciones de *S. oculatus* podría ser explicado por una extensa dispersión larval favorecida tanto por la corriente de Humboldt y la Corriente de la Deriva del Oeste a lo largo de las costas de Sudamérica.

Palabras clave: Hemisferio sur, cabrilla, mtDNA, especiación

Abstract.- Genetic differentiation and species boundaries between Atlantic and Pacific lineages of the Patagonian rockfish *Sebastes oculatus* was investigated using mtDNA D-loop partial sequences (541 bp). Sequences were obtained for 47 individuals from seven locations off the Pacific and Atlantic coasts of South America (*S. oculatus*) and one off the coast of South Africa (*S. capensis*), and for two specimens of *Helicolenus lengerichi* (outgroup). These data were then combined with sequences from GenBank corresponding to 21 *Sebastes* species. Maximum likelihood and Bayesian phylogenetic approaches showed topological distinctiveness between South American and South African *Sebastes* populations, supporting the existence of two phylogenetic species: *S. oculatus* and *S. capensis*. However, Atlantic and Pacific populations of *S. oculatus*, did not form reciprocal monophyletic assemblages. Application of the Wiens & Penkrot's protocol to test species boundaries within this species did not support the existence of two different phylogenetic taxa. Gene flow between Atlantic and Pacific populations of *S. oculatus* could be explained by extensive larval dispersal, favored by both the Humboldt current and the West Wind Drift current along the South American coast.

Key words: Southern hemisphere, rockfishes, mtDNA, speciation

INTRODUCTION

Rockfishes of the genus *Sebastes* Cuvier, 1829 comprise over 110 species, constituting one of the most diverse genera of marine fishes (Nelson 1994, Kendall 2000). Most of this species diversity is explained by allopatric speciation (Narum *et al.* 2004), although a few cases, mainly those described at local scale, are explained by reproductive

isolation mechanisms such as complex mating behavior (Gingras *et al.* 1998) and pronounced color polymorphism (Orr & Blackburn 2004). *Sebastes* species are mostly distributed across the continental shelf and shoreline waters (less than 500 m deep) of the northern hemisphere (Chen 1971, Johns & Avise 1998, Hyde & Vetter 2007),

but some rockfish species also occur in the western and eastern Atlantic and eastern Pacific basins of the southern hemisphere (Barsukov 1988, Stransky & MacLellan 2005). In fact, two closely related species of *Sebastes* are currently recognized in the southern hemisphere: *Sebastes capensis* (Gmelin, 1789) distributed from the Tristan da Cunha Island to the southwestern coast of South Africa, and *Sebastes oculatus* (Valenciennes, 1833) distributed from the Pacific coast of Peru and Chile to the Falkland Islands in the Atlantic (Stransky & MacLellan 2005).

Despite the recognition of both entities, the specific boundaries and geographic limits of *S. oculatus* remain unclear and controversial. Rocha-Olivares *et al.* (1999a), using mitochondrial DNA sequence genetic analysis, proposed that the populations from off South Africa waters should be considered *S. capensis*, and those populations from off western and southeastern South America waters should be named *S. oculatus*. Posteriorly, Rocha-Olivares *et al.* (1999b) performed a geographically more extensive genetic analysis and recognized three distinctive lineages as possible cryptic species: a *S. capensis* lineage found around Tristan da Cunha and off the coast of southwestern South Africa, a southwestern Atlantic *oculatus* lineage from the Falkland Islands and Argentina, and a southeastern Pacific *oculatus* lineage from Chile and Peru. However, due to their limited sampling along the southeastern Pacific coast, Rocha-Olivares *et al.* (1999b) concluded that these results must be interpreted with caution and would require further comparative studies.

Recent development of theoretical and operational approaches to species delimitation (Wiens & Penkrot 2002, Sites & Marshall 2004, Wiens 2007, Bond & Stockman

2008, Ross *et al.* 2008) coupled to sequence data analyses, have allowed to test the validity of taxonomic nomenclature against phylogenetic hypotheses. In this sense, due that mitochondrial DNA (mtDNA) coalesce faster than nuclear DNA (Palumbi *et al.* 2001), mtDNA markers offer diagnostic characters that satisfy the requirement of lineage-based species definition (Sites & Marshall 2004, Pons *et al.* 2006), showing to be an useful tool to assist traditional taxonomy in separating species where there is little discriminatory morphological variation (Hillis & Wiens 2000, Ferguson 2002, Blaxter & Floyd 2003). Thus several studies have used these markers, particularly control region sequence data, to gain insight into molecular evolution of *Sebastes* speciation (Rocha-Olivares *et al.* 1999a, Kai *et al.* 2002, Cope 2004, Gharrett *et al.* 2005, Hyde & Vetter 2007).

The present study analyzes mitochondrial control region sequences under traditional phylogenetic methods (maximum likelihood and Bayesian analyses) adopting the concept of species as independent evolutionary lineages (De Queiroz 1998) and apply the Wiens & Penkrot protocol (Wiens & Penkrot 2002, Sites & Marshall 2003), to assess boundaries between Pacific and Atlantic *S. oculatus*.

MATERIAL AND METHODS

FISH SAMPLING

We collected mitochondrial D-loop partial sequences for 47 specimens of *Sebastes* belonging to six localities from the southeastern Pacific coast, one locality from Peru (Huacho) and five localities from Chile (Iquique, Antofagasta, Coquimbo, Aysen channels, and Punta Arenas); one locality from the southwestern Atlantic

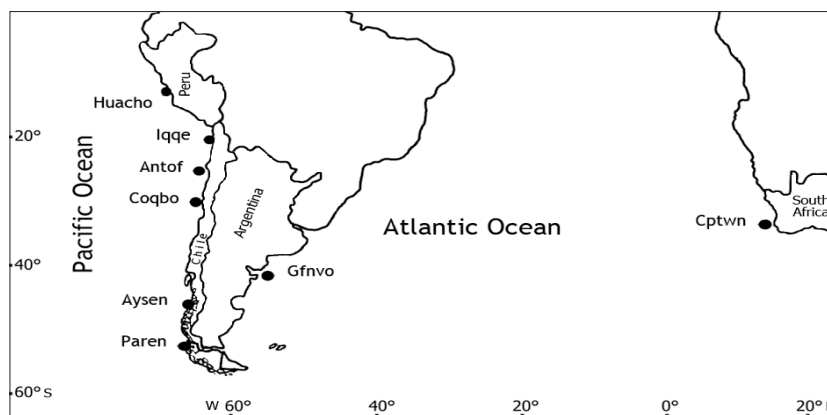


Figure 1. Map of the study area indicating the localities (solid circles). Sample codes as indicated in Table 1 / Mapa del área de estudio indicando las localidades (círculos sólidos). Los códigos de las muestras se indican en la Tabla 1

Table 1. Locality, haplotype code, number of haplotypes, and genetic diversity parameters for each analyzed locality / Localidad, código del haplotipo, número de haplotipos y parámetros genéticos para cada localidad analizada

Locality	Code	Haplotypes/N	Hd (±SD)	π	S	Mean pairwise divergence (±SD)	Tajima's D statistic
Huacho, Peru (11°00'S)	Huacho	5/6	0.93±0.12	0.024	29	0.0249±0.005	-0.227
Iquique, Chile (20°13'S)	Iqqe	5/5	1.00±0.12	0.013	13	0.0134±0.004	0.195
Antofagasta, Chile (23°38'S)	Antof	4/5	0.90±0.16	0.019	17	0.0198±0.006	1.657
Coquimbo, Chile (29°58'S)	Coqbo	9/9	1.00±0.05	0.012	17	0.0117±0.003	-0.457
Aysén, Chile (45°34'S)	Aysen	5/5	1.00±0.12	0.012	14	0.0128±0.004	-0.609
Punta Arenas, Chile (53°10'S)	Paren	1/1	-	-	-	-	-
Golfo Nuevo, Argentina (43°45'S)	Gfnvo	7/7	1.00±0.08	0.064	61	0.0680±0.010	1.360
Cape Town, South Africa (33°51'S)	Cptwn	6/9	0.92±0.09	0.018	18	0.0186±0.005	0.282
Total		42/47	0.994±0.06	0.036	88	0.0394±0.007	-1.200

(Golfo Nuevo, Argentina); and one from the South African (Cape Town) coast (Table 1, Fig. 1). Fishes were captured with hand lines from artisanal boats or by divers and were morphologically identified. We included D-loop partial sequence data from GenBank for *S. capensis* (AF031503), *S. oculatus* (AF031502), *S. constellatus* (AF031505), *S. notius* (AF031510), *S. lentiginosus* (AF031509), *S. umbrosus* (AF031516), *S. exsul* (AF031514), *S. spinorbis* (AF031515), *S. chlorostictus* (AF031504), *S. eos* (AF031506), *S. rosenblatti* (AF031511), *S. helvomaculatus* (AF031508), *S. simulator* (AF031513), *S. ensifer* (AF031507), *S. rosaceus* (AF031512), *S. serranoides* (AF031498), *S. inermis* (ab071260), *S. maliger* (AF031500), *S. schlegelii* (NC005450), *S. paucispinis* (AF031499), and *S. ruberrimus* (AF031501) (Rocha-Olivares *et al.* 1999a, Kai *et al.* 2002, Kim & Lee 2004). Additionally, two specimens of *Helicolenus lengerichi* (a member of the subfamily Sebastinae where *Sebastes* is also included) were also obtained from Valdivia (Chile) to be used as outgroup.

MOLECULAR PROTOCOLS AND SEQUENCE ALIGNMENT

Whole genomic DNA was extracted from tissue samples (muscle preserved in 98% ethanol) using standard protocols of chemical digestion (0.1M Tris-HCl pH 8.0, 0.2M EDTA, 1% SDS, 100 $\mu\text{g mL}^{-1}$ proteinase K) followed by phenol/chloroform extraction (Sambrook *et al.* 1989). Air-dried DNA pellets were eluted in TE pH 8.0. mtDNA PCR amplifications were performed using 50 - 100 ng of genomic DNA and 0.5 units of DNA polymerase (Invitrogen) per 50 μL of reaction volume. Polymerase-chain-reaction (PCR) amplification and sequencing of the D-loop region was performed using the primers L15926 (Kocher *et al.* 1989) and H16498 (Meyer *et al.* 1990). The PCR cycling procedure was as follows: an initial denaturation at 94°C for 3 min followed by 35 cycles of 92°C for 50 sec, 50°C for

1 min, and 72°C for 1 min, followed by chain extension at 72°C for 7 min. The size of the PCR products was checked against a 100-bp DNA ladder on 1.5% agarose gels stained with 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide. Amplified fragments were purified using Qiaquick Purification kits (QIAGEN) following the manufacturer's instructions. Sequences were generated in both directions on an ABI 377 automatic sequencer according to manufacturer protocols. Sequences were edited with Bioedit 7.0 and then aligned using MAFFT v5.7 (Kato *et al.* 2005) under the iterative method of global pairwise alignment (G-INS-i). Default settings were chosen for all the parameters involved. Sequences were submitted to GenBank under Accession Numbers GU136675-GU136720.

NUCLEOTIDE DIVERSITY OF MT DNA SEQUENCES

Haplotype (*Hd*) and nucleotide diversity (π , Nei 1987), number of segregating sites (*S*) for *S. oculatus* and *S. capensis*, and Tajima's *D* (Tajima 1989) were estimated using DnaSP 5.0 (Librado & Rozas 2009). Additionally, to evaluate whether sufficient samples for each locality were collected to characterize genetic diversity (*S*), was plotted against number of sequences for each population.

PHYLOGENETIC ANALYSES

Maximum likelihood (ML) trees (Felsenstein 1981) were estimated using Garli v0.94 (Zwickl 2006) under the HKY+ Γ model (Hasegawa *et al.* 1985), as suggested by Modeltest v3.7 under the Akaike Information Criterion (Posada & Crandall 1998, Posada & Buckley 2004). Likelihood topology tests were conducted using our molecular data and the Shimodaira & Hasegawa (1999) test as implemented in Paup* v4b10 (Swofford 2002). Confidence in the resulting relationships was assessed using the nonparametric bootstrap procedure (Felsenstein

1985) with 100 bootstrap replicates. Bayesian phylogeny estimation was performed using MrBayes v3.0 (Ronquist & Huelsenbeck 2003). Each Markov chain was started from a random tree and run for 1.0×10^7 cycles with every 1,000th cycle sampled from the chain. Model parameters were treated as unknown variables with uniform priors and were estimated as part of the analysis. We ran four chains simultaneously and each analysis was repeated three times. Stationarity was checked using Tracer v1.3 (Rambaut & Drummond 2003). After initial inspection, the first 200 trees from each chain were discarded (burn-in phase).

We used Wiens & Penkrot (2002) protocol to test species boundaries between Pacific and Atlantic *oculatus* lineages. This method is based on a sampling design that includes: (a) focal species (the species of interest in the study, *S. oculatus*) and nonfocal species (closely related reference species, *S. capensis*) to apply the exclusivity test (*i.e.*, all members of a group share a more recent common ancestor with every other member of that group than any of them does with any non-member) for the focal species, and (b) at least two individuals per locality to make the between-population gene flow inferences. It requires a phylogeny of haplotypes of known locality and taxonomic designation. A topology that fails to recover haplotypes from a given locality as a clade is taken as evidence for potential gene flow between populations (*i.e.*, focal species = single species). The method is implemented using dichotomous flow charts that lead to several alternatives for making species-level decisions.

RESULTS

NUCLEOTIDE SEQUENCE ANALYSES

Multiple sequence alignments of the amplified gene region consisted in part of the threonine transfer RNA (tRNAThr) gene, the proline tRNA (tRNAPro) gene and part of the control region. Exclusion of sites with missing data at both ends of the alignment resulted in 541 aligned positions. A total of 42 unique mtDNA haplotypes (Table 1) were found among the 47 specimens examined. Only one sequence from individuals with identical haplotypes was included in the phylogenetic analyses. Within *S. capensis* and *S. oculatus*, 88 sites were polymorphic. Tandem repeats or heteroplasmy were not detected, however a deletion of 75 nucleotides near to the 5' end of the D-loop region was observed in a specimen of the South African population. Results of the estimations of haplotype and nucleotide diversity and segregating sites, as well as results of Tajima tests, are summarized in Table 1. Sequence divergence was similar to other intraspecific comparisons (Cope 2004) for the same mtDNA region, except for the population of Golfo Nuevo (6.8%, SD=1.0%, Table 1). The segregating sites plotted against number of sequences for each population (Fig. 2) showed a moderated leveling indicating that possibly the localities were under sampled, situation that is more evident for the Golfo Nuevo locality.

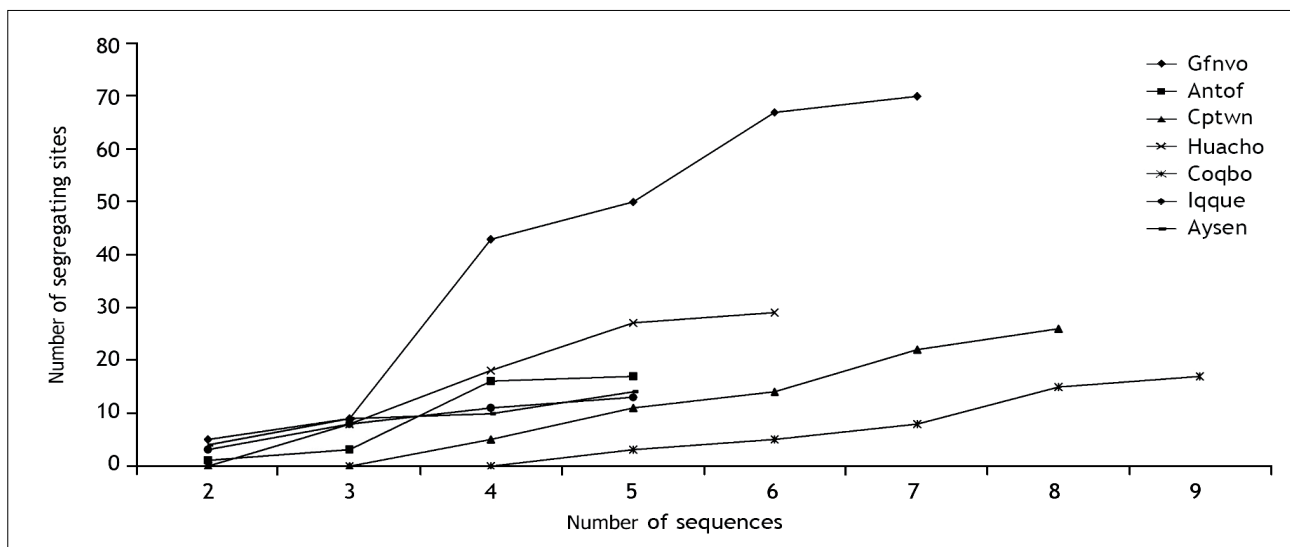


Figure 2. Cumulative segregating sites curves for six localities of *S. oculatus* and one locality of *S. capensis*. Punta Arenas locality was not included because only one sample was available. Sample codes as indicated in Table 1 / Curvas acumulativas de sitios segregantes de seis localidades de *S. oculatus* y una localidad de *S. capensis*. La localidad de Punta Arenas no fue incluida, dado que solo una muestra fue disponible. Los códigos de las muestras se indican en la Tabla 1

ML AND BAYESIAN PHYLOGENETIC ANALYSES

The best-fit model of evolution for the phylogenetic analyses as indicated by Modeltest was the HKY+ Γ model (Hasegawa *et al.* 1985): base frequencies = 0.3818, 0.1720, 0.1460, 0.3002; TRatio = 2.7544, gamma shape = 0.3840. One optimal ML topology was found (lnL = -3814.87). All phylogenetic trees constructed by both methods showed similar topologies. The inconsistency between both methodological approaches was in clades supported by low bootstraps and posterior probabilities. South African and South American populations formed separated lineages supported by bootstrap proportions $\geq 60\%$ and Bayesian posterior probabilities ≥ 0.9 (Fig. 3). Alternative paraphyletic hypotheses clustering South African and South American populations were rejected by the SH topological test ($P < 0.05$). Within the *S. oculatus* clade there was no complete separation of haplotypes into Pacific and Atlantic basins, with most terminals showing short branch lengths and low clade supports. Alternative monophyletic hypotheses separating Pacific and Atlantic populations were not rejected by the Shimodaira-Hasegawa test ($P > 0.05$). Additionally, the Wiens & Penkrot's protocol indicates that *S. oculatus* and *S. capensis* are two different phylogenetic species. On the contrary, according to the same procedure Atlantic and Pacific *S. oculatus* lineages do not qualify as different *Sebastes* species.

DISCUSSION

Sebastes sibling species have been often the center of taxonomic and nomenclature revisions (Seeb 1998, Kendall 2000) and molecular evolutionary studies have revealed recent speciation processes (Johns & Avise 1998, Kai *et al.* 2002, Hyde & Vetter 2007). In the southern hemisphere, *S. capensis* was the only recognized species, distributed from the Pacific coast of Peru and Chile to the Falkland Islands, and to the southwestern coast of South Africa (Chen 1971, Kong 1985). However, Eschmeyer & Hureau (1971) considered that the *Sebastes* populations inhabiting the West coast of South America was *S. oculatus*, and only those populations from the southcentral Atlantic islands and South Africa were *S. capensis*. Rocha-Olivares *et al.* (1999b) using DNA sequences from hypervariable control region found that the southern hemisphere *Sebastes* is represented by *S. capensis* and two lineages of *S. oculatus* inhabiting the coasts of South America: one from the Pacific coasts and the other from the Atlantic coast. In their study, Rocha-Olivares *et al.* (1999b) used specimens from four sampling sites containing one locality from the southeastern Pacific coast (Valparaíso) and one from the southwestern Atlantic (Falkland Islands). Our analyses including more

southeastern Pacific samples are partially concordant with those of Rocha-Olivares *et al.* (1999b) and previous hypotheses from Eschmeyer & Hureau (1971) in the sense that specimens from South Africa and South America form two different phylogenetic species. Moreover, the SH test significantly rejected alternative topologies where these two lineages were presented as paraphyletic. However, neither Atlantic nor Pacific *S. oculatus* samples constituted reciprocal monophyletic assemblages in our tree (Fig. 3). Same result was also found by Rocha-Olivares *et al.* (1999b), where some specimens from the Falkland Islands were included within the Pacific *S. oculatus* and vice versa. The Shimodaira-Hasegawa test did not rejected alternative topologies separating Atlantic and Pacific specimens; hence the existence of two *S. oculatus* lineages in South American waters cannot be ruled out completely based on this topological test. The inadequate resolution of these internal nodes was likely due to the combination of small sample size and a single gene for phylogenetic information, fact that often creates long branches and polytomies (Hyde & Vetter 2007).

According to Wiens & Penkrot (2002), failure of haplotypes from the same locality to cluster together is potential evidence for gene flow between localities (*i.e.*, focal species = single species). Marine organisms usually exhibit high levels of gene flow (*i.e.*, lack of population structuring) due to extensive larvae dispersal associated with planktonic life stages, the potential high vagility among adults, and high fecundity (Seeb 1998). *Sebastes* species have pelagic stages, but most species in this genus, including *S. oculatus*, show adult morphology concordant with sedentary habits (Alvarado 1985, Rocha-Olivares *et al.* 1999a, Gharrett *et al.* 2005). Females of this species retain the eggs until maturation. Pelagic larvae, that have been reported in coastal waters all along the Chilean coast are among the most abundant taxa in ichthyoplankton at the southern tip of South America (Bernal & Balbontin 1999, Rodríguez-Graña & Castro 2003, Landaeta & Castro 2006), where fjords and channels dominate the coastline. Smaller larvae are principally above the halocline in the first 50 m of the water column throughout the channels and fjords, in contrast to the post-flexion larval stage that is more abundant seaward (Landaeta & Castro 2006). This life history could suggest widespread larval dispersal and gene flow such as has been reported in other rockfish species (Buonaccorsi *et al.* 2002, Gilbert-Horvath *et al.* 2006). In addition, the inter-connectivity and larval dispersal of *S. oculatus* along the South American coast could be favored by both the Humboldt current and West Wind Drift current.

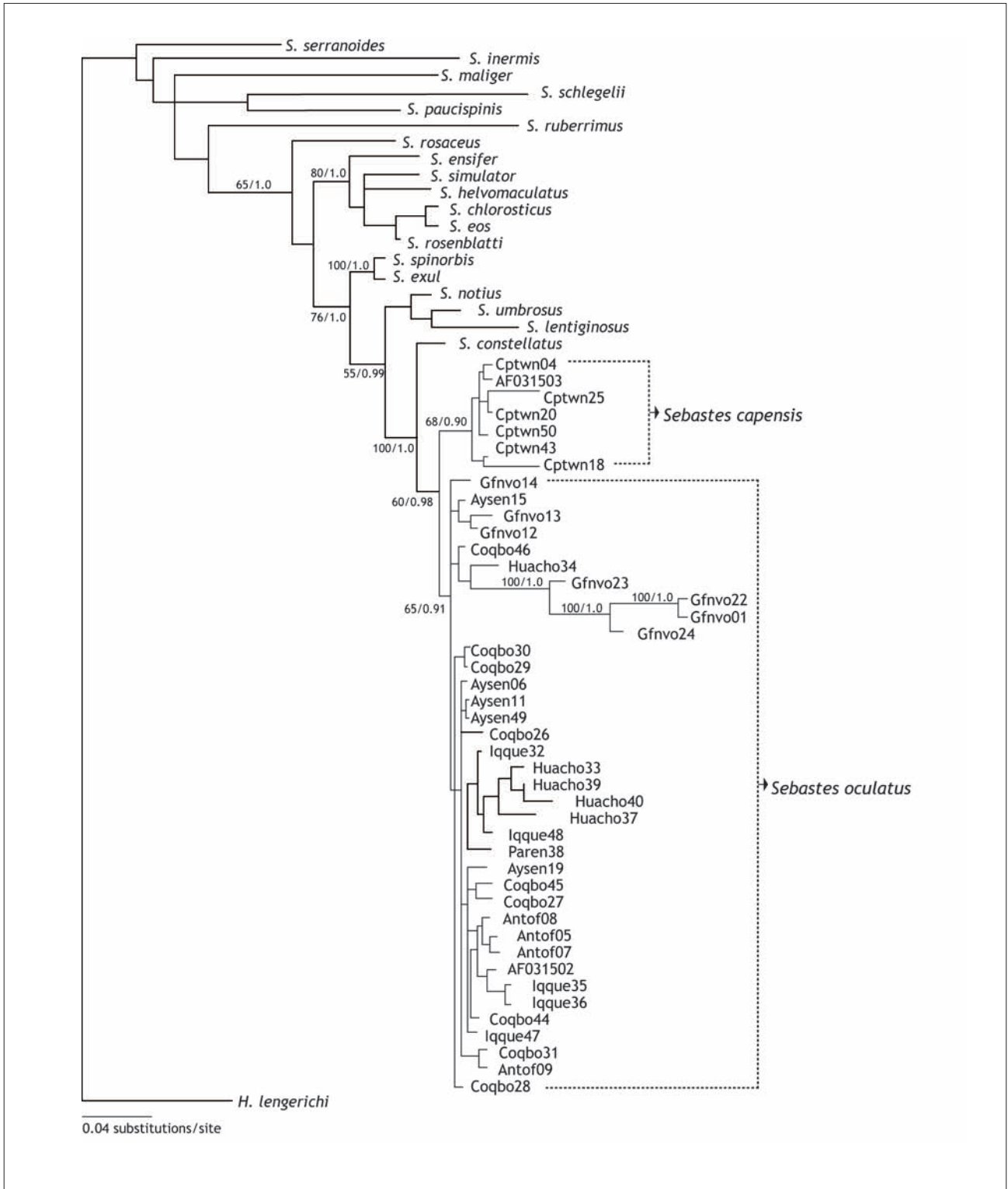


Figure 3. Maximum likelihood phylogenetic tree of *S. capensis* and *S. oculatus* mtDNA control region haplotypes under the HKY+ Γ model. Bayesian analysis generated similar topologies. Clade supports (bootstrap proportions/Bayesian posterior probability) if > 50% are indicated for each node / Árbol filogenético de máxima verosimilitud de los haplotipos mitocondriales de *S. capensis* y *S. oculatus* bajo el modelo HKY+ Γ . El análisis Bayesiano generó similar topología. El soporte de los clados (proporción de bootstrap/ probabilidad *a posteriori* Bayesiana) se indican sobre el 50% en cada nodo

Finally, if Atlantic and Pacific *S. ocellatus* lineages are accepted as valid species alternative interpretations of our phylogenetic results would include: (1) overlapping distributional ranges of both taxa, (2) introgressive hybridization among the lineages or (3) incomplete lineage-sorting of ancestral polymorphisms due to recent processes of speciation. The first and second alternatives can be tested upon a reliable identification and description of both sibling species and the posterior establishment of their distributional range. A molecular phylogeny using nuclear genes and fast-evolving markers, such as microsatellites, as have been used in other studies (Roques *et al.* 1999, An *et al.* 2009) could also give clues about the potential of interbreeding between these. The nature of microsatellite DNA loci has made them particularly amenable for genetic analyses of natural marine populations, and the applications of these markers are increasing in evaluating genetic structure of rockfishes (Sekino *et al.* 2001, Gómez-Uchida & Banks 2005, Sotka *et al.* 2005), and species identification problems (Pampoulie & Daniélsdóttir 2008). No doubt, these analyses can be strongly recommended to address the genetic divergence and speciation of Southern rockfishes in more detail. Nevertheless, until new evidence is presented, we conclude that, based on our results, *Sebastes ocellatus* constitutes a single species in both the Atlantic and Pacific waters and is phylogenetically distinct from its sister species, *S. capensis*, from southern South Africa.

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