Gene 452 (2010) 35–43

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Evolutionary relatedness of mackerels of the genus *Scomber* based on complete mitochondrial genomes: Strong support to the recognition of Atlantic *Scomber colias* and Pacific *Scomber japonicus* as distinct species

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ARTICLE INFO

Article history:
Received 5 November 2009
Received in revised form 11 December 2009
Accepted 12 December 2009
Available online 24 December 2009

Received by M. Di Giulio

Keywords:
Scomber
Scomber colias
Scomber japonicus
Mitochondrial genome
Phylogeny

ABSTRACT

Mackerels of the genus *Scomber* are commercially important species, but their taxonomic status is still controversial. Although previous phylogenetic data support the recognition of Atlantic *Scomber colias* and Pacific *Scomber japonicus* as separate species, it is only based on the analysis of partial mitochondrial and nuclear DNA sequences. In an attempt to shed light on this relevant issue, we have determined the complete mitochondrial DNA sequence of *S. colias*, *S. japonicus*, and *Scomber australasicus*. The total length of the mitogenomes was 16,568 bp for *S. colias* and 16,570 bp for both *S. japonicus* and *S. australasicus*. All mitogenomes had a gene content (13 protein-coding, 2 rRNAs, and 22 tRNAs) and organization similar to that observed in *Scomber scombrus* and most other vertebrates. The major noncoding region (control region) ranged between 865 and 866 bp in length and showed the typical conserved blocks. Phylogenetic analyses revealed a monophyletic origin of *Scomber* species with regard to other scombrid fish. The major finding of this study is that *S. colias* and *S. japonicus* were significantly grouped in distinct lineages within *Scomber* cluster, which phylogenetically constitutes evidence that they may be considered as separate species. Additionally, molecular data here presented provide a useful tool for evolutionary as well as population genetic studies.

1. Introduction

The family Scombridae contains 15 genera and about 51 species of epipelagic and generally migratory marine fish, characterized by an elongate and fusiform body although moderately compressed in some genera (Collette et al., 2001; Collette, 2003). It includes species with a high commercial interest such as mackerels, bonitos, and tunas, which in 2007 reached nearly 9 million tons of global captures in the world (FAO, 2007). Particularly, mackerels of the genus *Scomber* represented the fourth-part of these captures being extremely appreciated by consumers for the excellent properties and quality of the meat. From a taxonomic point of view, three different species were classically recognized inside this genus based on multiple morphological characters: *Scomber scombrus* (Linnaeus, 1758), *Scomber australasicus* (Cuvier, 1832), and *Scomber japonicus* (Houttuyn, 1782). The chub mackerel *S. japonicus* was traditionally considered the most geographically widespread species in the genus, being found antitropically in warm and temperate waters of the Atlantic, Indian, and Pacific Oceans and adjacent seas. However, the systematic status of this species was first called into question by Matsui (1967) owing to the considerable morphological variability found between Pacific and Atlantic specimens in pigmentation, tooth crenulation, scale size, and gill-raker counts on the lower first arch. More recently, the presence of highly specific parasites (Oliva et al., 2008) as well as a high level of genetic divergence at mitochondrial (Scoles et al., 1998; Collette, 2003; Espiñeira et al., 2009) and nuclear (Infante et al., 2007) DNA between specimens occurring in the Atlantic and Pacific Oceans have been reported. Such new findings are in agreement with morphological data and suggest the recognition of two different species: *S. japonicus* in the Indo-Pacific, and *Scomber colias* (Gmelin, 1789) in the Atlantic. Nevertheless, genetic support to this proposed taxonomic status is based on the analysis of limited sequence data, which may cause important errors in estimates of evolutionary relatedness due to a large variation in substitution rate (Martin et al., 1990). Hence, analysis of longer sequences appears necessary to shed a conclusive light on *Scomber* phylogeny.

Abbreviations: 5S rDNA, 5S ribosomal DNA; A + T, adenine + thymine; *ATP6* and *ATP8*, ATPase subunits 6 and 8; bp, base pairs; *COI–COIII*, cytochrome oxidase subunits 1–3; CSB-X, conserved sequence blocks 1–3 and D; cyt *b*, cytochrome *b*; DHU, dihydrouridine; G + C, guanine + cytosine; kb, kilobase; mitogenome, mitochondrial genome; mtDNA, mitochondrial DNA; *ND1–6* and *ND4L*, NADH dehydrogenase subunits 1–6 and 4L; nt, nucleotide; O_L, replication origin of the mitochondrial light-strand; PCR, polymerase chain reaction; rRNA, ribosomal RNA; T + C, thymine + cytosine; TAS, termination associated sequence; $tRNA^{XXX}$, genes encoding for transfer RNA molecules with the corresponding amino acids denoted with a three-letter code and anticodon indicated in parenthesis (*XXX*) when necessary; WANCY, cluster of tRNA genes corresponding to amino acids tryptophan, alanine, asparagine, cysteine, and tyrosine.

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doi:10.1016/j.gene.2009.12.004

In vertebrates, the mitochondrial DNA (mtDNA) is a small and double-stranded circular molecule ranging in size from 15 to 17 kb. It is commonly used in population genetic studies, species identification assays, and molecular phylogenetic analyses due to its high abundance in the cell, high mutation rate, and maternal inheritance (Curole and Kocher, 1999). The gene content of vertebrate mtDNA is a nearly identical set of 13 proteins, 22 transfer RNAs (tRNAs), and 2 (12S and 16S) ribosomal RNAs (rRNAs). In addition, the mtDNA possesses two main noncoding regions involved in replication and transcription processes: the control region or D-loop, and the light-strand replication origin or O_L (Clayton, 1992; Shadel and Clayton, 1997; Boore, 1999). In bony fish, a considerable progress in the sequencing of complete mtDNA genomes (mitogenomes) has been observed during the past years. In this sense, they have become useful genetic tools in resolving persistent controversies over phylogenetic relationships of teleosts at different taxonomic levels (Inoue et al., 2001a; Miya et al., 2003; Saitoh et al., 2006; Lavoué et al., 2007; Kawahara et al., 2008; Lavoué et al., 2008; Setiamarga et al., 2008; Yamanoue et al., 2008).

In the present study, we report the complete mitogenome sequence of *S. japonicus*, *S. colias*, and *S. australasicus*; that of *S. scombrus* was previously obtained (Takashima et al., 2006). Main features, gene structure and codon usage are described and compared with those of other teleosts. We performed a maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) phylogenetic analysis using mitogenomes from *Scomber* species and other scombrids. Overall, our phylogenetic data suggest that *S. colias* and *S. japonicus* actually need to be recognized as separate species.

2. Materials and methods

2.1. Fish sampling

The three *Scomber* specimens analyzed in this study were provided by the UBAGO Group Mare, S.L. (the Spanish group is a more-than-80-year-old family-run company specialized in canned fished products). These were collected in the Eastern Atlantic (*S. colias*; Area FAO 34) and North-Western Pacific oceans (*S. australasicus* and *S. japonicus*; Area FAO 61). Taxonomic classification of *Scomber* examples was carried out in basis to morphologic and meristic features as described by Collette and Nauen (1983).

2.2. DNA isolation, amplification, and sequencing

Total genomic DNA was isolated from 150 mg of tissue using the FastDNA kit for 40 s and speed setting 5 in the FastPrep FG120 instrument (Bio101 Inc.). All DNA isolation procedures were performed following the manufacturer's protocol.

PCRs and mitogenome sequencing strategies were as previously described (Manchado et al., 2004; Catanese et al., 2008; Ponce et al., 2008). Briefly, the mitochondrial genome was amplified by long PCR in two fragments of ~10 and ~7 kb, respectively. Reactions were carried out using the Elongase Enzyme Mix (Invitrogen) according to supplier's recommendations. Long PCR products were purified with the CONCERT Rapid PCR Purification System (Invitrogen) and then cloned using the TOPO XL PCR cloning kit (Invitrogen). Plasmids were purified using the CONCERT Rapid Plasmid Purification System (Invitrogen). Complete sequencing was accomplished by primer walking. Only one clone of each of the two amplified fragments was sequenced.

Sequencing reactions were carried out with the BigDye Terminator v3.1 kit (Applied Biosystems) on the ABI3130 genetic analyzer. DNA sequences were analyzed using the Sequencing Analysis v3.4.1 (Applied Biosystems) and the Seqman v5.51 (DNASTAR) programs. Mitogenome sequences of *S. japonicus*, *S. colias* and *S. australasicus* have been deposited in GenBank/EMBL/DDBJ under Accession Nos. AB488405, AB488406, and AB488407, respectively.

2.3. Sequence and phylogenetic analyses

Sequences were aligned using the Megalign v5.51 software (DNASTAR). DnaSP v5.0 (Librado and Rozas, 2009) was used to estimate the number of polymorphic sites and among *Scomber* sequences. Uncorrected *p*- and Tamura–Nei genetic distances (Tamura and Nei, 1993) were calculated with the PAUP 4.0*b*10 (Swofford, 2000) software.

The Modeltest v3.7 software (Posada and Crandall, 1998) was used as a guide to determine the best-fit ML model. The General Time Reversible model + Invariable sites + Gamma model (GTR + I + G) for sequence evolution was selected as the most appropriate with the following parameters: base frequency A = 0.2934, C = 0.3103, G = 0.1551, T = 0.2412; proportion of invariable sites was 0.5546, and gamma distribution (α) = 0.7434. In addition, ML, NJ, and MP analyses were performed using the PAUP 4.0*b*10 software to construct phylogenetic trees. The degree of confidence assigned to nodes in trees was determined by bootstrapping with 2000 replicates (Felsenstein, 1985). ML analysis was performed using the fast stepwise-addition search with random addition sequence. For NJ, the ML distance settings were employed. The MP tree was found by using the tree-bisection-reconnection (TBR) branch-swapping algorithm with randomized stepwise addition of taxa under the heuristic search method. Gaps were treated as fifth state.

The entire mtDNA sequences of *S. scombrus* (Accession No. AB120717), *Thunnus thynnus* (AY302574), *Thunnus orientalis* (AB185022), *Thunnus alalunga* (AB101291), *Auxis rochei* Mit I (AB103467), *A. rochei* Pac-Mit II (AB105165), *A. rochei* MA-Mit II (AB103468), *Auxis thazard* (AB105447), *Katsuwonus pelamis* (AB101290), *Euthynnus alletteratus* (AB099716), and *Scomberomorus cavalla* (DQ536428) were retrieved from databases and used for comparative analysis and phylogeny. *S. cavalla* sequence was employed as an out-group to root trees.

3. Results and discussion

3.1. Genome organization and base composition

The complete mtDNA sequences of the L-strand were determined to be 16,568 bp in length for *S. japonicus,* and 16,570 bp for both *S. colias* and *S. australasicus*. These values were similar to those reported for other Scombridae fish (Manchado et al., 2004; Manchado et al., 2005; Broughton and Reneau, 2006; Catanese et al., 2008) as well as for other teleosts (Zardoya et al., 1995; Hurst et al., 1999; Broughton et al., 2001; Inoue et al., 2001b; Takashima et al., 2006; Wang et al., 2008; Zhu and Yue, 2008). The structural organization and location of different features in the three genomes conformed to the common vertebrate mitogenome model and consisted of 13 protein-coding genes, 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes, and 1 putative noncoding control region (Boore, 1999; Miya et al., 2001) (Table 1). As in other vertebrates (Miya et al., 2003), most of the genes were encoded on the H-strand; only the NADH dehydrogenase subunit 6 (*ND6*) and eight tRNA (Gln, Ala, Asn, Cys, Tyr, Ser(UCN), Glu, and Pro) genes were encoded on the L-strand. Mitochondrial genes overlapped by a total of 52 bp in 13 different locations from 1 to 10 bp (Table 1).

The overall base composition values for the L-strand of the three *Scomber* mitogenomes were as follows: A (28%), C (30%), T (25%), and G (17%). As described for other teleosts, two main features were found. First, the most represented base in all cases was C; secondly, a bias against G was observed. The G + C content of the *Scomber* mitogenomes was 47.0% for both *S. colias* and *S. japonicus*, and 46.8% for *S. australasicus*, which are in the range found in other Scombridae species (Manchado et al., 2004; Manchado et al., 2005; Catanese et al., 2008). Nevertheless, these values were higher than in other teleosts (Hurst et al., 1999; Broughton et al., 2001; Inoue et al., 2001b; Oh et