View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Digital.CSIC

Evaluation of a fast method based on the presence of two restriction sites in the mitochondrial ND5 (mtND5) gene for the identification of *Scomber* species

Iciar Martinez[1,2,*], Amaya Velasco[1], Ricardo Pérez-Martín[1], Eskil Forås[3], Michiaki Yamashita[4] and Carmen G. Sotelo[1]

[1]Instituto de Investigaciones Marinas (IIM-CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain.
[2]Norwegian College of Fishery Science, University of Tromsø, NO-9037 Tromsø, Norway.
[3]SINTEF Fisheries and Aquaculture, SINTEF Sealab, Brattørkaia 17C, NO-7010 Trondheim, Norway.
[4]National Research Institute of Fisheries Science, 2 12 4 Fukuura, Yokohama 236 8648, Japan

***Corresponding author**: Iciar Martinez, Instituto de Investigaciones Marinas (IIM-CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain Tel: +34-986231930, Fax: +34-986 292 762, Email: iciarm@iim.csic.es.

KEYWORDS

Species identification, fish, mackerel, Scomber, PCR, FINS, mtDNA, RFLP-PCR, saba, caballa, scombrus, colias, japonicus, australasicus, traceability.

Acknowledgements

We would like to thank Dr Jun Sakai, Chief Researcher of the Food Marketing Research and Information Center (FMRIC), Tokyo, Japan, for his assistance during the work and for providing the products in the Japanese market. Susana Otero from the Instituto de Investigaciones Marinas-CSIC is acknowledged for excellent technical assistance. The work was financed by The Research Council of Norway, project "Traceability control mechanisms in the mackerel food chain between Norway and Japan- TraCtrolMac" (no. 25181-0704011000009).

Evaluation of a fast method based on the presence of two restriction sites in the mitochondrial ND5 (*mt ND5*) gene for the identification of *Scomber* species

KEYWORDS

Species identification, fish, mackerel, Scomber, PCR, FINS, mt DNA, RFLP-PCR, saba, caballa, scombrus, colias, japonicus, australasicus, traceability.

ABSTRACT

The purpose of this work was to evaluate the suitability of a method based on the presence of two restriction sites (for *Hae III* and *Hindf I*) in the mitochondrial NADH dehydrogenase subunit 5 (*mt ND5*) gene to identify *Scomber* species. The evaluation was performed on 144 reference and market samples by sequencing of the entire 505 bp fragment of the *mt ND5* gene and of a 464 bp fragment of the Kocher fragment of the cytochrome b gene (*mt Cytb*). Sequence analysis of any of the two fragments allows the identification of each of the four *Scomber* species, but *S. japonicus* and *S. colias* had the same restriction sites at the ND5 amplicon and would not have been differentiated by this analysis. Similarly, loss of the *Hae III* site in some *S. scombrus* individuals would have misidentified them as not being *Scomber*. All the market products were correctly labeled except one acquired in Spain labeled as originating in the Atlantic and containing *S. japonicus.*

INTRODUCTION

Classically only three species were considered within the world-wide distributed genus *Scomber*: *S. scombrus* found mainly in the North Atlantic, Mediterranean and Black Sea*; S. australasicus* in the Pacific and Southeast Indian Oceans and *S. japonicus*, in warm and temperate waters of the Atlantic, Indian and Pacific Oceans and adjacent seas (Infante, et al., 2007 and references therein). However, significant differences detected between *S. japonicus* from the Atlantic and the Pacific in their morphology (for example *S. japonicus* from the Pacific had very few or no belly spots while *S. japonicus* from the Atlantic had a more prominent spot pattern and different scale size) and parasites (Matsui, 1967) led some taxonomist to propose the recognition of two species: *S. japonicus* in the Pacific and *S. colias*

in the Atlantic. This proposal was been further substantiated by the analysis of the nuclear 5S rDNA (Infante et al., 2007) and of partial (Espiñeira et al., 2009) and entire *mt DNA* (Catanese et al., 2010a). It is therefore nowadays agreed that there are four species in the genus *Scomber*: *S. scombrus* and *S. colias* in the Atlantic Ocean and *S. japonicus* and *S. australasicus* in the Pacific Ocean. Analysis of the *mt DNA* was also used by Nesbø et al., (2000) to study the population structure of *S. scombrus*.

In order to ensure truthful labeling, analytical methods have been developed to identify the species of the ingredients contained in food products. It has been shown that DNA-based methods are usually the most reliable and easier to perform on a great variety of products, since the processing does not alter the diagnostic DNA sequences (Mackie et al., 1999; Martinez et al., 2003; Sotelo and Pérez-Martín, 2007), although it may render them shorter (Quinteiro et al., 1998). Different DNA fragments have been targeted for species identification and many laboratories use forensically informative sequences (Bartlett and Davidson, 1992) often from the *mt DNA* due to its resilience and high copy number in most tissues. In our lab, we use sequencing of a 464 bp (Burgener, 1997), or of a shorter 140 bp fragment for canned products (Quinteiro et al., 1998; Meyer, 1993) from the Kocher fragment of the mt cytochrome b (*mt Cytb* gene (Kocher et al., 1989). Recently published *Scomber* spp identification methods also target the mt DNA (Botti and Guiffra, 2010; Catanese et al., 2010b). However, faster methods not requiring sequencing are usually preferred and have led to the development of a multiplex PCR method targeting a 123 bp fragment of mitochondrial NADH dehydrogenase subunit 5 gene (*mt ND5*) with a positive control amplicon corresponding to the small 12S rRNA subunit, proposed by Catanese et al. (2010b) to differentiate the four *Scomber* species and to a restriction fragment polymorphism (RFLP) analysis of a 505 bp fragment using *Hae* III and *Hinf* I, also from the *mt ND5*, proposed in Japan to differentiate *S. japonicus*, *S. australasicus* and *S. scombrus* (Anon. 2007).

The work presented here is part of the research project TraCtrolMac which deals with the identification of traceability control mechanisms for *S. scombrus* exported from Norway to Japan. Japan is the largest importer of Norwegian mackerel: of a total of 265,000 tons of exported frozen round mackerel in 2010 for a value of 2.8 billion Norwegian kroner, Japan acquired 75,000 tons. Most of this mackerel, about 51,000 tons, was first exported to China were it was filleted and marinated before entering the Japanese market. The production chain of Norwegian mackerel to the Japanese market was mapped by Forås and Thakur (2010) and Thakur et al., (2011). The identification numbers used in the traceability chain could also be used as references for product information if an additional type of identifier, with which it

would not be possible to tamper, such as DNA profiles or diagnostic sequences, accompanied the product. Software based traceability solutions would enable a direct linking of the product to the results of the genetic analysis and to the responsible laboratory. By law, seafood products must be labeled with information regarding the species and geographic origin of capture both in Europe (EU CR No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation EC No 104/2000 which regards informing consumers about fishery and aquaculture products) and in Japan (Law on Standardization and Proper Labeling of Agricultural and Forestry products - JAS Law- of 1999).

It is obviously of the outmost importance that the analyses performed using different methods and in different countries on the same samples produce consistent results.

The aim of this work is to evaluate the suitability of the analysis proposed in Japan based on polymorphisms at two restriction sites in the *mt ND5* gene (Anon. 2007) for *Scomber* species identification purposes.

MATERIALS AND METHODS

Authentic mackerel species and commercial products: 144 samples were analyzed that included authentic reference samples (processed and unprocessed) and products of *S. scombrus, S. colias, S. japonicus* and *S. australasicus*. The samples purchased in the Japanese market were labelled with the generic term "saba" (mackerel) that refers to any *Scomber* species, except for one product labelled as "masaba" that should contain *S. japonicus*. All the products purchased in Japan and labelled as "of Norwegian origin" should contain S. *scombrus*. The products purchased in Norway should all of them contain *S. scombrus.* The correct names allowed to mackerel products in the Spanish market are regulated by law (B.O.E., 2010): one of the three Spanish canned products was labelled as *caballa* (generic term for all four species) from Cabo Verde, and should therefore contain *S. scombrus* or *S. colias*. The other two were labelled with the term *caballa del sur*, that refers to both *S. colias* or *S. japonicus* (BOE, 2010); one was from Perú, and should therefore contain *S. japonicus* and the other from Isla Cristina (Spain) and should contain *S. colias*. The reference samples of *S. colias* belonged to the reference collection of the Instituto de Investigaciones Marinas and had been preserved frozen. All the other samples had been preserved in 96% ethanol until the DNA was extracted. One sample of canned herring was included as a negative control and it was correctly identified as *Clupea harengus*.

DNA extraction, PCR amplification, sequencing and data treatment: DNA was extracted using the standard *Wizard™ DNA Clean-up System* (Promega). PCR amplifications were carried out (1) by the method described by Kocher et al., (1989) as modified Burgener (1997) to amplify a 464 bp fragment of the *mt Cytb* (Kocher fragment) using the primers H15149AD: 5´-GCICCTCARAATGAYATTTGTCCTCA-3' and L14735: 5´-AAAAACCACCGTTGTTATTCAACTA-3´ and (2) as described in the "Manual determination for the fish species *Scomber* (Anon. 2007) to amplify the 505 bp fragment of the *mt ND5* gene using the primers LSs1-LEU: 5´-ATCCGCTGGTCTTAGGAACC-3' and HSs1-ND5: 5-CCTTCTCAGCCGATAAATAGTT-3'. This method is based on the assumption that only three *Scomber* species need to be distinguished. Although the Norwegian canned products amplified satisfactorily both reactions, the three Spanish canned products did not and were analyzed following the protocol described by Quinteiro et al., (1998) using primers H15573- 5'-AAT AGG AAG TAT CAT TCG GGT TTG ATG-3' and L15424-5'-ATC CCA TTC CAC CCA TAC TAC TC-3'; corresponding to those described by Meyer (1993) that amplify a 176 bp suitable for heavily fragmented DNA. PCR reactions were performed in volumes of 25 μL using *illustra TM puReTaq Ready-To-Go PCR beads* (GE Healthcare) and 20 μl of PCR product were sequenced in an ABI PRISM 310 DNA Sequencer (Applied Biosystems). The diagnostic *Hae III* and *Hinf I* restriction sites described by Anon (2007) between the positions 243-244 and between 415-416 respectively of the *mt ND5* amplicon were identified from the sequences. The data were processed using the software BIOEDIT (Hall, 1999) with CLUSTAL used to align the sequences (Thompson et al., 1994) and MEGA 4.0 to construct phylogenetic trees (Tamura et al., 2007) according to Tamura and Nei (1993), Saitou and Nei (1987) and Felsestein (1985).

RESULTS AND DISCUSSION

As already mentioned, the aim of this study was to evaluate the suitability of a fast method proposed in Japan and based on the presence of two polymorphic restriction sites in the *mt ND5* gene for the identification of *Scomber* species. Our results target the food control authorities and research community responsible for providing consumers, the seafood industry and traders of mackerel worldwide with reliable, tested and validated methods for species identification. Mislabelling maybe very serious and it is important to differentiate the species from the Atlantic from those of the Pacific because they carry different hazards and constitute different potential risks. In the absence of food safety considerations however, the consumers´

usual preferences for their local products are reflected in higher market prices. Therefore, it is of the outmost relevance that all the stakeholders involved in the mackerel chain have the same data and validated official methods to refer to in case of disagreements, disputes or mislabellings.

Comparison of the results using Kocher and mt ND5 fragments: Fig. 1 shows the phylogenetic trees constructed with genetic distances among specimens of the 4 mackerel species with Kocher and ND5 sequences. Our results confirm the already reported (Infante et al., 2007) close relatedness of *S. colias*, S. *japonicus* and S. *australasicus* at the two loci tested, but the loci registered different variability depending on the species (see the number of haplotypes identified at each locus in Tables 1 and 2). Thus, even if the number of samples is small to extrapolate to real population variability, the Kocher fragment seemed to be more polymorphic in the species from the Atlantic, i.e., *S. scombrus* and *S. colias* while the *mt ND5* seemed to show a higher number of polymorphisms in the species from the Pacific ocean, *S. japonicus* and *S. australasicus,* (Table 3). However, for each species and locus, there was one widely predominant haplotype with over 50% of the samples belonging to it; which were Kocher haplotypes number 1, 26, 27 and 36 and ND5 haplotypes number 1, 20 25 and 27 for *S. scombrus, S. japonicus, S. colias* and *S. australasicus* respectively (see Tables 1 and 2).

The RFLP analysis described by Anon. (2007) is based on two polymorphic sites in the *mt ND5* sequence: one *Hae III* site between bp 243-244 of the amplicon present in *S. scombrus* and *S. japonicus* and absent in *S. australasicus* and a target site for *Hinf I* (bp 415-416) present only in *S. scombrus*. Sequence analysis of all the samples from this work showed that all the *S. colias* had the same sequence as *S. japonicus* at these two sites and would therefore have been misidentified as *S. japonicus.* In addition 3 haplotypes of *S. scombrus* corresponding to 4 samples would have remained unidentified due to the loss of the *Hae III* site: from the most frequent sequence GGCC to GGCA in haplotype 6 (n=2) to GACC in haplotype 8 (n=1) and to GGGC in haplotype 11 (n=1).

Identification of products from the Japanese, Norwegian and Spanish markets: None of the products purchased was labelled with the name of the species. Most of them used a generic term: *saba* in Japan, *makrell* in Norway and *caballa* or *caballa del sur* in Spain, followed by a region from which one should expect a given species, except in the case of Norway, where *makrell* is commonly understood as being only *S. scombrus.*

Sequence analysis of either the Kocher fragment or the *mt ND5* allowed the easy identification of the mackerel products belonging to either of the four *Scomber* species; except, as expected, for some of the canned products that required the use of a shorter

diagnostic sequence. All the products were correctly labelled according to their claimed origin except the Spanish canned product from Cabo Verde that should have contained *S. colias* for its claimed Atlantic provenance but it was shown to be *S. japonicus*. Regarding the analysis of samples from the Japanese market, two of three products labelled with the generic "saba" term contained *S.australasicus* and the third *S. japonicus*. We could also identify that the three pieces of one of the Japanese products belonged at least to two different *S. australasicus* individuals.

An optimal analysis for species identification should be of high throughput, fast and easy to perform. However, the PCR-RFLP method examined here and proposed by Anon. (2007) is not optimal and would not have identified any *S. colias*. This is not surprising due to the very close genetic relationship between *S. japonicus* and *S. colias* but given the different geographic origin of these two species and the globalization of fisheries and trade, a method that permits the unequivocal identification of all four species should be implemented. An additional problem of RFLP analysis is that intraspecific variability affecting the restriction sites may lead to false positive or negative identifications. In the present work 4 samples of *S. scombrus* would have remained unidentified by the RFLP analysis.

As the methods here used, most of the advance methods published to identify *Scomber* and Scombrid species are based on sequence analysis (Infante et al., 2007; Espiñeira et al., 2009; Catanese et al., 2010a,b; Botti and Giuffra, 2010) and all of them are reliable for this purpose. However, simpler analyses need to be developed to diminish the costs both in terms of time and money that sequencing demands. Of the published fast methods, only the multiplex described by Catanese et al., (2010b) is able to discriminate the four species, but close examination of the results illustrates one of the facts usually encountered in multiplex analysis: in some samples the amplification of either the control of the specific band is weaker than it should have been and in others the presence of weak but detectable shadows may hinder a clear and undoubtful identification, for example in case the shadow represents a mixture of two species where one of them is present in much smaller amounts or due to the close relatedness between the species examined.

CONCLUSION

In conclusion, the two loci tested are suitable for species identification of *Scomber* species using sequence analysis, but an analysis based on the presence of the *Hae III* and *Hinf*

237 *I* restriction sites in the *mt ND5* amplicon would misidentify all *S. colias* and would not be
238 able to identify as *Scomber* some genotypes of true *S. scombrus*. Sequencing remains a
239 demanding and time consuming task, and future improvements should focus in designing fast
240 specific methods for each of the four species that increase the reliability of the analysis and
241 decrease the number of steps involved, such as those based on the use of species-specific
242 probes and/or Real-Time PCR techniques.
243
244
245
246 REFERENCES
247

248 Anon. 2007. *Manual determination of fish species Scomber*. Technical document issued
249 by the Center for Food Quality and the Fisheries Research Agency. 14 pp.
250 http://www.famic.go.jp/technical_information/hinpyou/pdf/saba-manual.pdf.
251 Accessed 23rd June 2011.
252 Bartlett, S.E. and Davidson, W.S. 1992. FINS (Forensically informative nucleotide
253 sequencing): A procedure for identifying the animal origin of biological
254 specimens. Biotechniques. 12: 408–411.
255 B.O.E. 2010. *Resolución de 24 de marzo de 2010, de la Secretaría General del Mar, por*
256 *la que se establece y se publica el listado de denominaciones comerciales de*
257 *especies pesqueras y de acuicultura admitidas en España.* Ministerio de Medio
258 Ambiente, Medio Rural y Marino. Boletín Oficial del Estado Núm. 82 Lunes 5 de
259 abril de 2010 Sec. III. Pág. 31242-31265.
260 Botti, S. and Guiffra E. 2010. Oligonucleotide indexing of DNA barcodes: identification
261 of tuna and other scombrid species in food species. BMC Biotechnology. 10-60
262 (7pp).
263 Burgener, M. 1997. Molecular species differentiation of fish and mammals. PhD
264 dissertation, University of Bern, Switzerland.
265 Catanese, C., Manchado, M. and Infante, C. 2010a. Evolutionary relatedness of mackerels
266 of the genus *Scomber* based on complete mitochondrial genomes: Strong support
267 to the recognition of Atlantic *Scomber colias* and Pacific *Scomber japonicus* as
268 distinct species. Gene. 45: 35-43.

269 Catanese, G., Manchado, M., Fernandez-Trujillo, A. and Infante, C. 2010b. A multiplex-
270 PCR assay for the authentication of mackerels of teh genus Scomber in processed
271 fish products. Food Chem. 122: 319-326.
272 Espiñeira, M., González-Lavín, N., Vieites, J. M. and Santaclara, F.J. 2009. Development
273 of a method for the identification of scombroid and common substitute species in
274 seafood products by FINS. Food Chem. 117: 698–704.
275 Felsestein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap.
276 Evolution. 39*:* 783-791
277 Forås, E. and Thakur, M., 2010. Documentation of Norwegian mackerel cold chain during
278 storage and transport between Norway and Japan. SINTEF report A18062.
279 ISBN978-82-14-04948-0.
280 Hall, T.A. 1999. BIOEDIT: a user-friendly biological sequence alignment editor and
281 analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41:
282 95-98.
283 Infante, C., Blanco, E., Zuasti, E., Crespo, A. and Manchado, M. 2007. Phylogenetic
284 differentiation between Atlantic *Scomber colias* and Pacific *Scomber japonicus*
285 based on nuclear DNA sequences. Genetica. 130: 1–8.
286 Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paado, S., Villablanca, F.X. and
287 Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals:
288 Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA.
289 86: 6196-6200.
290 Mackie, I.M., Pryde, S.E., González Sotelo, C., Medina, I., Pérez-Martín, R.I., Quinteiro,
291 J., Rey-Méndez, M. and Rehbein, H. 1999. Challenges in the identification of
292 species of canned fish. Trends Food Sci Technol. 10: 9-14.
293 Martinez, I., Aursand, M., Erikson, U., Singstad, T.E., Veliyulin, E. and van der Zwaag,
294 C. 2003. Destructive and non-destructive analytical techniques for authentication
295 and composition analyses of foodstuffs. Trends Food Sci Technol. 14: 489-498.
296 Matsui, T. 1967. Review of the mackerel genera *Scomber* and *Rastrelliger* with
297 description of a new species of *Rastrelliger*. Copeia. 7: 1–83.
298 Meyer, A. 1993. Evolution of mitochondrial DNA in fishes. In Biochemistry and
299 Molecular Biology of Fishes. Hochachka, P.W. and Mommsem, T.P. (Eds.)
300 Amsterdam, Elsevier Science (Vol 2). pp. 1-38.
301 Nesbø, C.L., Rueness, E.K., Iversen, S.A., Skagen, D.W. and Jakobsen, K. S. 2000.
302 Phylogeography and population history of Atlantic mackerel (*Scomber scombrus*

303 L.): a genealogical approach reveals genetic structuring among the eastern Atlantic
304 stocks. Proc Royal Soc London, B, 267, 281-292.
305 Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Perez-Martin, R.I., Rey-
306 Mendez, M. and Mackie, I.M. 1998. Use of mtDNA direct polymerase chain
307 reaction (PCR) sequencing and PCR-restriction fragment length polymorphism
308 methodologies in species identification of canned tuna. J Agric Food Chem. 46:
309 1662-1669.
310 Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for
311 reconstruction of phylogenetic trees. Mol Biol Evol. 4: 406-425
312 Sotelo, C.G., and Pérez-Martín, R.I. 2007 Fish and seafood authentication. In
313 Authentication of Food and Wine. Ebeler, S.E., Takeoka, G., and Winterhalter, P.
314 (Eds.) ACS Symposium Series 952, Oxford University Press, USA. pp. 126-137.
315 Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the
316 control region of mitochondrial DNA in humans and chimpazees. Mol Biol Evol.
317 10: 512-526
318 Tamura, K, Dudley, J, Nei, M and Kumar, S. 2007. MEGA4: Molecular Evolutionary
319 Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596-1599.
320 Thakur, M., Sørensen, C.F., Bjørnson, F.O., Forås, E. and Hurburgh, C.R. 2011.
321 Managing food traceability information using EPCIS framework. J Food Eng. 103:
322 417-433.
323 Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the
324 sensitivity of progressive multiple sequence alignment through sequence
325 weighting, position specific gap penalties and weight matrix choice. NAR. 22:
326 4673-4680.
327
328

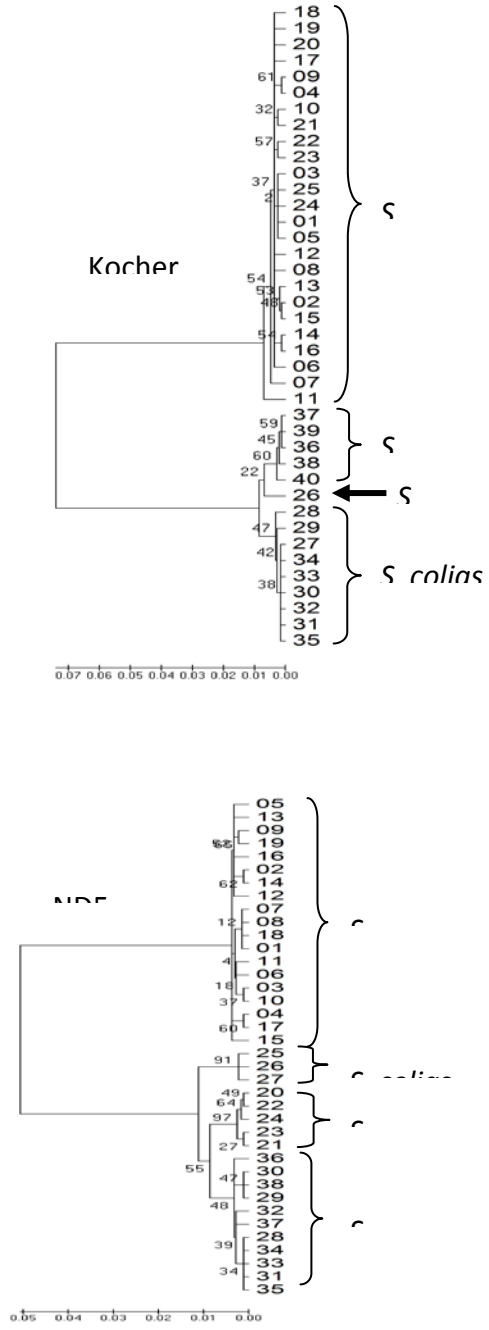

Figure 1.- Dendograms obtained by Neighbor-Joining of the 40 haplotypes identified at the Kocher (upper) and the 38 haplotypes at the ND5 (bottom) loci by the Tamura-Nei distance measurement method.

TABLE 1. Haplotypes identified by the Kocher fragment. 138 samples were analyzed by this method.

Species	Haplotype number	n of individuals in the haplotype
S.scombrus	1	48
S.scombrus	2	5
S.scombrus	3 to 5	2
S.scombrus	6 to 25	1
S. japonicus	26	27
S. colias	27	4
S. colias	28	2
S. colias	29 to 35	1
S. australasicus	36	13
S. australasicus	37	3
S. australasicus	38 to 40	1

TABLE 2. Haplotypes identified by the ND5 fragment. 132 samples were analyzed by this method.

Species	Haplotype number	n of individuals in the haplotype
S.scombrus	1	41
S.scombrus	2	7
S.scombrus	3 and 4	5
S.scombrus	5 and 6	2
S.scombrus	7 to 19	1
S. japonicus	20	16
S. japonicus	21	6
S. japonicus	22	2
S. japonicus	23 and 24	1
S. colias	25	10
S. colias	26 and 27	1
S. australasicus	28	8
S. australasicus	29	2
S. australasicus	30 to 38	1

TABLE 3. Comparison of the number of haplotypes identified in the four *Scomber* species at the Kocher and ND5 loci

	Kocher		ND5	
Species	n fish	n haplotypes	n fish	n haplotypes
S. scombrus	79	25	75	19
S. japonicus	27	1	26	5
S. colias	13	9	12	3
S. australasicus	19	5	19	11