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Direct Sequencing Method for Species Identification of Canned Sardine and Sardine-Type Products

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A direct sequencing method based on a 103 bp diagnostic sequence derived from a species-specific mitochondrial DNA cytochrome *b* sequence of 150 bp obtained by Polymerase Chain Reaction was tested for the identification of 47 commercial canned sardine and sardine-type products from various countries. Multiple alignment of 14 analyzed reference samples belonging to Clupeomorpha species was performed versus the canned samples. Low intraspecific variability was observed for canned sardine (≤0.03), whereas mean interspecific variability was 0.23. A phylogenetic tree was constructed, and the calculated bootstrap values (BP, 88−99%) were used as indicators of the correct assignment of unknown canned samples to reference species. According to this methodology, the 26 commercial canned sardines analyzed were grouped in the same clade as the *Sardina pilchardus* reference and identified unequivocally. These assignments were confirmed by the high BP value of 99%.

KEYWORDS: Species identification; canned product; Clupeomorpha; sardine; cytochrome *b* gene; PCR; genetic divergence; DNA sequencing; Forensically Informative Nucleotide Sequencing (FINS)

INTRODUCTION

Codex Stan 94 defines a positive list of small pelagic fish (21 species) for use in the preparation of canned sardines or sardine-type products (**Table 1**). A product is labeled "sardines" when *Sardina pilchardus* is used exclusively. When other species are processed, the "sardines" label must be completed by a distinctive designation, that is, the name of a country, a geographical area, the species, or the common name of the fish, in accordance with the laws and customs of the country in which the products are sold. As the World Trade Organization applies Codex Alimentarius standards, precise analytical tools are increasingly needed for species identification in sardine-type products to ensure that regulations are enforced.

As the external features allowing morphological identification of whole fish are not apparent after the canning process, analytical methods are needed to detect mislabeling. Traditional methods of fish species identification, such as isoelectric focusing (IEF) of proteins (*1*, *2*) or high-performance liquid chromatography (*3*, *4*), are applicable only to raw fish. Other electrophoretic methods, such as SDS-PAGE or urea IEF (*5*−*10*), can be used to identify processed fish (cooked, smoked, prefried, or breaded) but cannot be applied when proteins have been highly denatured, especially in canning.

As an alternative to protein analysis, techniques based on a more stable molecule (DNA) provide a useful authentication tool (*11*, *12*). Polymerase Chain Reaction−restriction fragment length polymorphism (PCR-RFLP) methods have proved successful in authenticating thermally processed foods such as hake

Table 1. List of the 21 Small Pelagic Fish Authorized by the *Codex Alimentarius* for Preparation of Canned Sardines or Sardine-Type Products

	common name	scientific name	world production[a] (tons)
sardines	European pilchard	*Sardina pilchardus*	1,126,832[c]
sardine-type products	South American pilchard	*Sardinops sagax*[d]	338,131[b]
	Californian pilchard	*Sardinops caeruleus*[d]	685,497[c]
	Japanese pilchard	*Sardinops melanostictus*[d]	339,377[c]
	Southern African pilchard	*Sardinops ocellatus*[d]	196,534
	Australian pilchard	*Sardinops neopilchardus*[d]	not indexed
	Round sardinella	*Sardinella aurita*	300,445[c]
	Indian oil sardine	*Sardinella longiceps*	437,328[c]
	Goldstripe sardinella	*Sardinella gibbosa*	161,200
	Madeiran sardinella	*Sardinella maderensis*	123,674
	Brazilian sardinella	*Sardinella brasiliensis*	82,283
	European sprat	*Sprattus sprattus*	647,417[c]
	Sandy sprat	*Hyperlophus vittatus*	not indexed
	Anchoveta	*Engraulis ringens*	7,213,077[c]
	Argentine anchoita	*Engraulis anchoita*	13,417
	Californian anchoita	*Engraulis mordax*	2,335
	Atlantic herring	*Clupea harengus*	1,952,975[c]
	Round herring	*Etrumeus teres*	58,569
	Atlantic thread herring	*Opisthonema oglinum*	18,752
	Pacific menhaden	*Ethmidium maculatum*	40,845
	Western Australian gizzard shad	*Nematalosa vlaminghi*	not indexed

[a] World production figures obtained from FAO: 1998, 2000,[b] 2001[c]. [d] The traditional species named *Sardinops* spp. are grouped together under a single designation, *Sardinops sagax*.

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baby foods (*13*), smoked salmon (*14*), or canned tuna (*15*, *16*). Bartlett and Davidson showed that segments of the mitochondrial DNA (mtDNA) genome are particularly useful for identification of the animal origin of various meat or fish preparations and that direct sequence analysis is a powerful method for identifying *Thunnus* tuna species (*17*, *18*). Davidson (*19*), in reviewing the problems encountered with species identification of raw and processed meat, emphasized the advantage of using PCR direct sequence analysis, as in the Forensically Informative Nucleotide Sequencing (FINS) method. The gene sequence from a large number of individuals can be determined in a short period of time with a reproducible procedure.

Chapela et al. (*20*), using a 200 bp fragment of DNA, found that the FINS technique is suitable for authenticating commercial seafood products such as processed cephalopods. Terol et al. (*21*), using short sequences of mtDNA cytochrome *b* gene (171 bp), identified commercial canned tuna by direct sequencing and showed the great advantage of calculating bootstrap values as an indicator of the correct assignment of analyzed samples in relation to reference species (*21*).

In a phylogenetic study, Jérôme et al. (*22*) found that the mitochondrial DNA gene encoding cytochrome *b* allowed the discrimination of nine species belonging to Clupeomorpha. Analysis on complete cytochrome *b* indicated that sequence analysis is a powerful tool for authenticating these related species. As the heat sterilization process strongly hydrolyzes DNA, these authors used PCR to amplify a short fragment of ~150 bp for testing on some canned fish. A PCR-RFLP approach with only two restriction enzymes was proposed for the simple and rapid differentiation of *S. pilchardus* from other species. However, this technique did not provide a typical profile for each species tested, and readable profiles were more expensive and complicated to obtain when a large number of reference species was involved. These difficulties indicated that PCR direct sequence analysis would be more appropriate for canned sardines.

The main purpose of the present study was to propose a suitable method (FINS) for authenticating Clupeomorpha species in commercial canned products and providing unambiguous diagnosis. This method is useful for various sardine-type products (preparation, processing, and origin) and allows species identification according to the Clupeomorpha reference collection of our laboratory.

MATERIALS AND METHODS

Sample Collection. Some specimens included in the present study (**Table 2**) were collected for a previous study (*22*). All were frozen, except *Sardinops caeruleus*, which was sampled in ethanol (80%). Additional specimens (a) on the list of 21 small pelagic fish were collected in whole animal or tissue forms of various sizes that were frozen, formalin-fixed, or preserved in 70% (v/v) ethanol. Two additional cytochrome *b* sequences derived from a data bank were used to consider nucleotide divergences on partial cytochrome *b* sequences, namely, *Sardinops melanostictus* (Genbank accession no. AB032554) and *Engraulis japonicus* (AB040676).

Canned products were purchased at local supermarkets or supplied by collaborators. A total of 47 canned products of various origins were collected and analyzed. Twenty-six canned products were labeled as sardiness and 21 as sardine-type products. The origins of these samples are indicated in **Table 2**.

DNA Isolation, Amplification, and Sequencing. Because of its simplicity and rapidity, the Chelex method (Bio-Rad, Hercules, CA) was used preferentially for DNA nucleic acid extraction in reference samples and commercial sardine-type products. A small section of muscle was placed on filter paper for oil and liquid removal and washed with distilled water. The dried muscle was then vortexed in 300 μL of a 5% Chelex water solution (Chelex 100 resin) with 20 μL of proteinase K (Qiagen, 20 mg/mL) and 30 μL of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The mixture was incubated at 56 °C for 4 h to break down all tissue and then heated at 100 °C for at least 15 min to denature and precipitate resin-bound protein. The DNA suspension was stored at 4 °C until use for PCR amplification. When the Chelex DNA extract did not allow correct PCR amplification, total genomic DNA extraction was performed according to a previously described procedure (*16*). Nucleic acid extractions for formalin-fixed tissues were done according to the Chelex method of Söller et al. (*23*).

PCR amplifications were carried out using Hybaid PCR Express (Hybaid, Ashford, U.K.). They were set up in a 100 μL reaction volume containing PCR buffer [75 mM Tris-HCl, pH 9.0; 50 mM KCI; 20 mM $(NH_4)_2SO_4$, 2 mM $MgCI_2$]; 200 μM dNTP mix; 0.4−0.8 μM of each primer; 2.5 units of UptiTherm DNA polymerase (Uptima-Interchim, Montluçon, France); and 1−2 μL of template DNA (phenol extracts) or 1−10 μL of DNA solution (Chelex supernatant). Cycling conditions (30/35 cycles) were 95 °C for 30 s, 50 °C for 40 s, and 72 °C for 30 s, followed by a final extension for 7 min at 72 °C.

Three percent agarose gels (Agarose HR, Uptima-Interchim) were employed to check DNA amplification, using TAE buffer (2 mM EDTA, 40 mM Tris acetate, pH 8.5) with ethidium bromide for band characterization via ultraviolet transillumination (Image Master VDS-CL, Amersham Pharmacia Biotech, Freiburg, Germany).

In some cases, particularly with a few canned products, annealing temperature or the number of cycles (30−35) was slightly modified. If a weak single band was obtained with the expected size, it was used as a template for secondary PCR. This enhanced the yield of PCR product for direct sequencing. Three sets of primers were employed for PCR amplification of an overlapping short fragment. The first and second sets (C-CB285dF/C-CB431R; C-CB284dF/C-CB425dR) were designed in the previous study (*22*), whereas the third set was constituted by the forward primer C-CB280F (TGCATTTACGCCCACATTGGC-CGAGG) used jointly with C-CB431R as reverse primer.

Table 2. Specimens of Reference Species and Canned Products

Specimens			
species	location	date collected	collectors[a]
Sardinella aurita	Ivory Coast	October 2000	IRD, Ivory Coast
Sardinella maderensis	Ivory Coast	October 2000	IRD, Ivory Coast
Sardinella longiceps (a)	The Philippines	2003	Max-Planck-Institut für Biologie
Sardina pilchardus	Bay of Biscay	May 2000	Ifremer (Thalassa ship), France
Sardinops sagax	Chilean coast	September 1996	I.F.O.P, Chile
Sardinops caeruleus	Pacific	Mars 2000	IPN, Mexico
Engraulis encrasicolus	Bay of Biscay	May 2000	Ifremer (Thalassa ship), France
Engraulis anchoita (a)	Argentina	2002	INIDEP, Argentina
Engraulis ringens (a)	Chile	1996	Ifremer
Clupea harengus	North Sea	February 1997	Ifremer (Thalassa ship), France
Sprattus sprattus	Bay of Biscay	May 2000	Ifremer (Thalassa ship), France
Etrumeus teres (a)	Israeli coast	2003	Daniel Galané

Canned Products			
canned product	no.	abbrev	origin
canned sardines	26	C1–C25, C30	Morocco, Spain, Portugal, France
sardine-type products	21	C29, C48, C49, C50, C51	Japan
		C31, C33, C34, C35, C43, C46	Chile, Peru
		C28, C40	Norway
		C37, C38	United States, Canada
		C41	Venezuela
		C26, C36, C39, C47	Thailand
		C27	France

[a] IRD, Institut de Recherche pour le Développement, X. Bard; I.F.O.P, Instituto de Fomento Pesquero; IPN, Instituto Poltécnico Nacional, H. V. Ortiz; INIDEP, Dr. Jorge E. Hansen, Mar del Plata, Argentina; Max-Planck-Institut für Biologie, Dr. Werner E. Mayer.

Table 3. Genetic Distances between Sequences from the 14 Clupeidae and Engraulidae Reference Species As Estimated by the Tamura–Nei Method[a]

	S.pil	C1	C9	C12	C15	C16	C21	C25	S.cae	S.sag	S.mel	S.mad	S.aur	S.lon	S.spr	C.har	E.ter	E.enc	E.jap	E.anc	E.rin
S.pil																					
C1	**0.00**																				
C9	**0.01**	**0.01**																			
C12	**0.01**	**0.01**	**0.02**																		
C15	**0.02**	**0.02**	**0.02**	**0.03**																	
C16	**0.01**	**0.01**	**0.02**	**0.02**	**0.03**																
C21	**0.01**	**0.01**	**0.02**	**0.02**	**0.03**	**0.02**															
C25	**0.01**	**0.01**	**0.02**	**0.02**	**0.03**	**0.02**	**0.02**														
S.cae	0.26	0.26	0.25	0.28	0.24	0.28	0.25	0.26													
S.sag	0.28	0.28	0.26	0.26	0.26	0.29	0.26	0.28	0.01												
S.mel	0.25	0.25	0.23	0.26	0.23	0.26	0.23	0.25	0.01	0.02											
S.mad	0.31	0.31	0.29	0.33	0.29	0.33	0.33	0.33	0.29	0.31	0.27										
S. aur	0.25	0.25	0.23	0.27	0.23	0.27	0.27	0.25	0.23	0.24	0.25	0.21									
S. lon	0.29	0.29	0.27	0.30	0.26	0.31	0.31	0.29	0.23	0.25	0.25	0.19	0.03								
S. spr	0.36	0.36	0.36	0.34	0.32	0.38	0.38	0.37	0.30	0.29	0.32	0.23	0.15	0.17							
C. har	0.26	0.26	0.26	0.27	0.23	0.28	0.28	0.27	0.21	0.23	0.23	0.23	0.23	0.24	0.19						
E. ter	0.29	0.29	0.27	0.31	0.27	0.31	0.27	0.29	0.21	0.22	0.22	0.22	0.18	0.18	0.24	0.23					
E. enc	0.28	0.28	0.28	0.26	0.29	0.29	0.28	0.28	0.24	0.23	0.23	0.28	0.27	0.29	0.27	0.23	0.20				
E. jap	0.26	0.26	0.26	0.25	0.28	0.26	0.26	0.26	0.26	0.24	0.24	0.28	0.26	0.27	0.25	0.24	0.19	0.01			
E. anc	0.25	0.25	0.24	0.24	0.27	0.27	0.27	0.25	0.23	0.22	0.21	0.26	0.25	0.25	0.29	0.29	0.24	0.23	0.22		
E. rin	0.26	0.26	0.24	0.24	0.27	0.27	0.27	0.26	0.28	0.26	0.26	0.27	0.24	0.24	0.27	0.23	0.20	0.17	0.16	0.11	

[a] Names of reference species are indicated by a capital letter representing the first letter of the genus followed by the first three letters of the species. The seven sequence genotypes obtained for canned sardines identified as *Sardina pilchardus* are indicated by C designations. Their genetic distances are reported in boldface. The other 19 sequences from canned sardines identified as *S. pilchardus* were strictly the same as the C1 sequence.

Prior to sequencing, double-stranded PCR products were purified by filtration through an Qiagen QIAquick column according to the manufacturer's protocol. PCR fragments were used for direct cycle sequencing with the ABI Big Dye Terminator cycle sequencing kit. Sequencing reactions were performed (MilleGen, Toulouse, France) on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA) in both directions with the primers used for PCR amplification.

Genetic Distances and Phylogenetic Analyses. All alignments were performed on BioEdit software (*24*), and phylogenetic treatments were computed on MEGA 2.0 software (*25*). Nucleotide divergences were computed using the Tamura−Nei model (*26*), which takes substitutional rate biases and the inequality of base frequencies into account. Phylogenetic trees were constructed using the neighbor-joining method (*27*), and the robustness of topology nodes was tested by the bootstrap method with 1000 iterations.

RESULTS AND DISCUSSION

This study involving a direct sequencing method shows that a short diagnostic sequence selected from mtDNA cytochrome *b* can be used to authenticate the corresponding processed commercial Clupeomorpha food species. As unambiguous differentiation of all species tested was obtained from various heat-sterilized samples, this method appears to be of practical value for all laboratories concerned with the authentication of seafood products.

DNA Extraction and PCR Product Yield. The quality of DNA extract obtained according to the Chelex method was not always sufficient to perform correct PCR amplification. This was the case for almost 25% of the commercial canned samples analyzed. It is likely that severe degradation of template DNA, associated with the absence of any DNA material and the probable presence of inhibitors, resulted in low PCR yield or failure. When the Chelex method failed, the more selective phenol/chloroform/isoamyl alcohol (PCI) method allowed DNA extract to be obtained with correct PCR amplification and sequencing. However, the results for two sardine-type products of the same origin are not reported here because of the poor quality of PCR amplification and the sequence obtained. Visual observation of the contents of some cans indicated that the quality of the samples collected would be highly variable and that recovery of analyzable DNA would be difficult in some cases. The thermal treatment and pressure involved in the canning process were more or less drastic according to the process used. Moreover, the quality of additives such as vegetable oil and seasoning might have affected the integrity of template DNA in the final product. Ram et al. (*15*) reported similar problems with damaged DNA from canned tuna and emphasized that additives or the effects of the canning process might result in the production of PCR inhibitors or a lack of PCR product. For the two samples that provided poor results, a weak band (the band of interest) was observed on the PCR control gel plus an artifact (probably coprecipitated DNA) in the agarose gel well.

Sequence Comparisons of Clupeomorpha Reference Species and Commercial Canned Samples. For each reference sample, a mitochondrial DNA sequence was obtained from overlapping fragments of 142/147/152 bp amplified by PCR. Regions of primers were discarded, and a short region of 103 bp (the diagnostic fragment) was obtained. After multiple alignments with the sequences of the 14 reference species, nucleotide distances were estimated according to the method of Tamura and Nei (*26*). Analysis of the alignment showed 42 variable positions. Sequence differences between species of different genera ranged from 15 to 36% ($d = 0.36$ between *S. pilchardus* and *Sprattus sprattus*) (**Table 3**). The nucleotide divergence between the Clupeidae and Engraulidae families was 19−29%.

PCR was performed with commercial canned samples in the same way as with reference specimens. The samples consisted of traditional French, Moroccan, Spanish, or Portuguese canned sardines (*S. pilchardus* labeled "sardines") prepared in vegetable oil (groundnut or olive oil) or tomato sauce, sometimes with seasoning such as lemon or red chili. Some samples from South American countries such as Peru, Chile, and Venezuela were labeled "sardinas" or imported as "sardinen filets". Another series of canned products was from Thailand and Japan. Canned herring from the United States or Canada was also analyzed, as well as Norwegian canned products probably prepared with

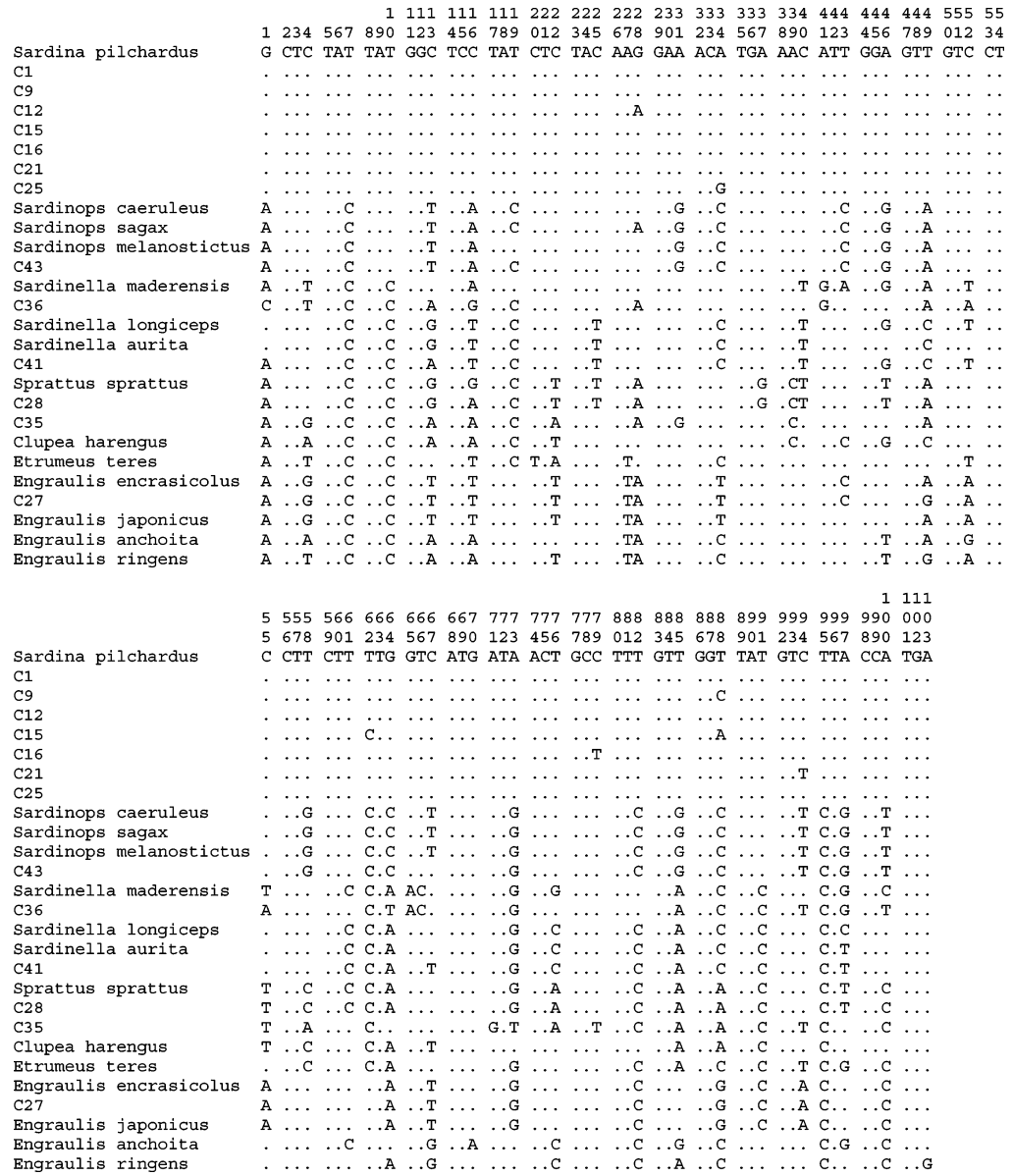

Figure 1. Multiple alignment of the 103 bp cytochrome *b* gene diagnostic fragment from the 14 reference species and 13 sardine type-products (C). Among the 26 sequences identified as belonging to the species *Sardina pilchardus*, only the 7 with defined sequence genotypes are shown (C1, C9, C12, C15, C16, C21, and C25). The C1 sequence was identical to that of our reference *S. pilchardus*, as were the others (*19*) not included in the figure. C27, C28, C35, C36 (a representative specimen of the four identical sequences of Thai sardines-type products), C41, and C43 were the 6 sardine-type products sequences that were not strictly identical to any references species. Dots indicate identity with the *S. pilchardus* sequence.

Sprattus sprattus (Brisling sardines from Norway) and a French canned product labeled "anchovy fillets." The resulting PCR fragments (~150 bp) from the cytochrome *b* gene were double-stranded sequences. As in the case of reference species, a diagnostic sequence of 103 bp was used for phylogenetic analysis.

Among the 47 canned samples, 26 were identified as sardines, all of which were prepared from the species *S. pilchardus* according to European Union regulations. Slight variations involving six sites were found among the different individuals of the 26 *S. pilchardus* sequences (**Figure 1**). Five sites at the third position in their respective codons were silent. The remaining site was at the first position and did not result in amino acid substitution. The variable positions defined seven sequence genotypes. The intraspecific variability observed for canned sardines was 0.00−0.03, whereas the variability between *S. pilchardus* and all other species tested was >0.25.

A neighbor-joining tree was inferred from Tamura and Nei distances between the cytochrome *b* sequences of the numerous specimens and canned samples (**Figure 2**). Bootstrap analysis provided strong support for clades associated with each reference species. All individuals from commercial canned sardines (C1−C25 and C30) and the *S. pilchardus* reference were grouped in the same cluster, with a bootstrap value of 98%. The five Japanese sardine-type products (C29 and C48−C51) were located with the Asian *Sardinops* reference (*S. melanostictus*), and four of the five other samples regarded as South American *Sardinops* (C31, C33, C34, and C46) were *Sardinops caeruleus* individuals. The four sardine-type products of Thai origin (C26, C36, C39, and C47) were grouped together in a separate branch near the *Sardinella* species. C37 and C38 (North American canned herring) were clustered with the *Clupea harengus* species. The C35 sample was a South American canned herring prepared with *Strangomera bentincki* (ex-*Clupea bentincki*),

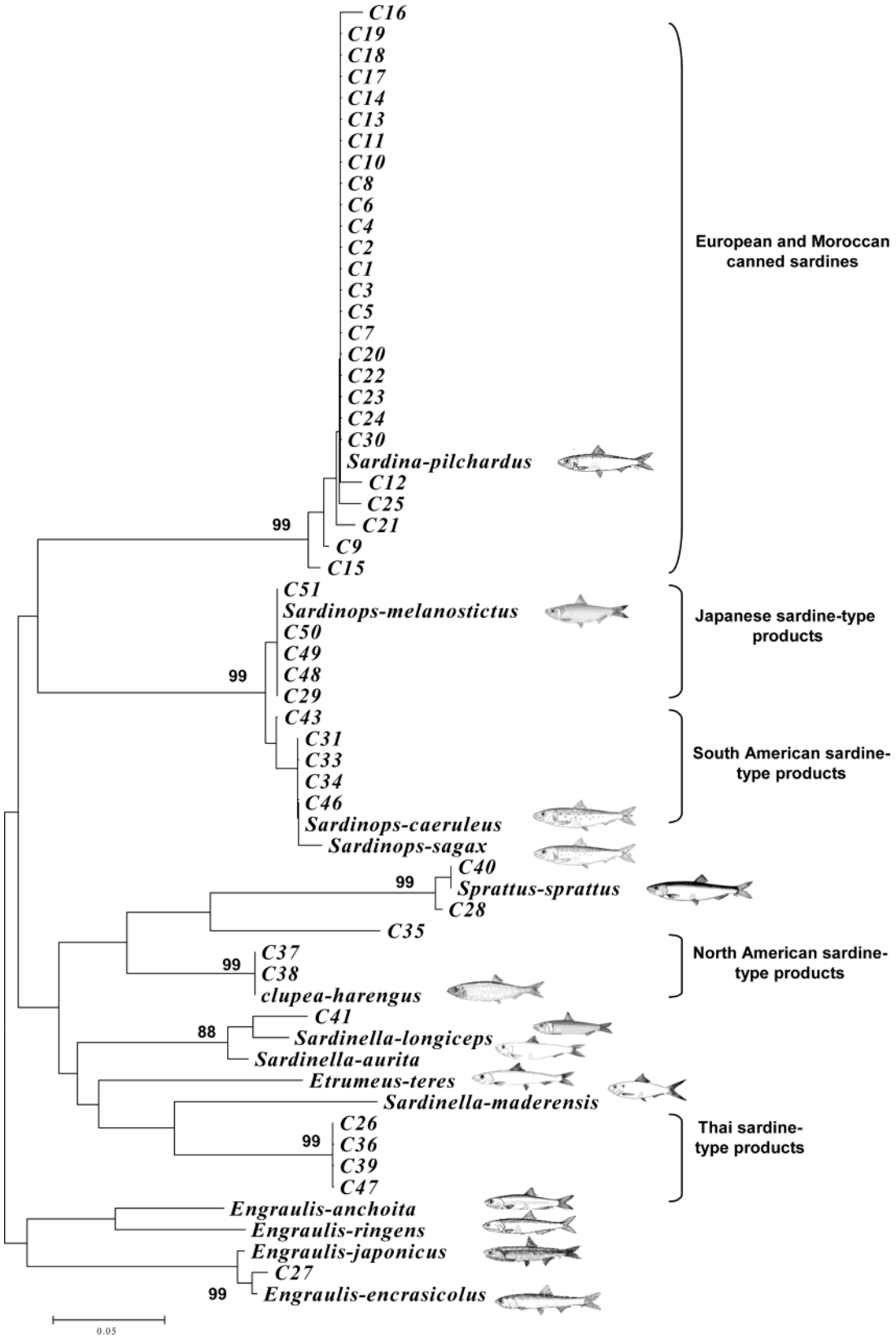

Figure 2. Identification of canned products by the FINS method and phylogenetic relationships among 47 canned samples based on a 103 bp diagnostic sequence of mitochondrial cytochrome *b* gene. Most commercial canned samples are clearly identified. All canned samples labeled as "sardines" and regarded as *Sardina pilchardus* are grouped in the same cluster with our reference *S. pilchardus*. Bootstrap values >70% are reported on the tree. DNA sequences from sardine-types haplotypes and new reference species were submitted to the GenBank database. Accession numbers are as follows: for canned sardine identified as *Sardina pilchardus* (C1, AY394038; C9, AY394039; C12, AY394040; C15, AY394041; C16, AY394042; C21, AY394043; C25, AY394044); for sardine-type products (C27, AY394054; C28, AY394048; C29, AY394045; C31, AY394047; C35, AY394050; C36, AY394053; C37, AY394051; C40, AY394049; C41, AY394052; C43, AY394046); and for the reference species (*Sardinella longiceps*, AY394034; *Engraulis anchoita*, AY394035; *Engraulis ringens*, AY394036; *Etrumeus teres*, AY394037).

which was located in the same group as the other *Clupea* species. C28 and C40 (sprat canned sardine-type products) were close to the reference *Sprattus sprattus*, and C41, a Venezuelan canned product, was situated in the same group as the reference species *Sardinella aurita* and *Sardinella longiceps*. With regard to the Engraulidae family, the French canned anchovy product (sample C27) clustered with reference *Engraulis encrasicolus*. For all canned products, assignments with reference species or specific clustering were confirmed by high bootstrap values (BP 88−99), in accordance with the results of Quinteiro et al. for hake baby foods (*13*) and those of Chapela et al. for processed cephalopod products (*20*). For canned tuna, Terol et al. (*21*) concluded that genetic distances coupled with high BP values (>70%) could be used to evaluate the similarity of unknown sequences to a pool of determined species samples.

The diagnostic 103 bp fragment allowed us to classify most of the commercial samples collected for this study and test the FINS method with Clupeomorpha canned products. No confused relationship with the *S. pilchardus* clade was observed. The clustering of unknown canned products with sequences from the same species, which was confirmed by the high bootstrap values obtained, indicates that the method is reliable. This was particularly true for the various canned sardine samples analyzed, which were assigned a high BP value of 99% in the same group as the reference species *S. pilchardus*. However, a major difficulty with this study was the collection of reference samples that could be identified absolutely as specimens of a single determined species. The lack of some reference species did not allow us to assign a species name to some canned samples. For example, samples of canned sardine-type products of Thai origin clustered in the same unassigned branch. Thus, future research should include investigations about intraspecies variations among the main species other than *S. pilchardus* on the Codex Stan 94 positive list of small pelagic fish. This would involve broad sampling of several specimens of the same reference species from different locations.

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