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Restriction Fragment Length Analysis of the Cytochrome *b* Gene and Muscle Fatty Acid Composition Differentiate the Cryptic Flatfish Species *Solea solea* and *Solea aegyptiaca*

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Supporting Information

ABSTRACT: Overlapping external morphometric characters easily confound the flatfishes *Solea aegyptiaca* and *Solea solea* (Soleidae) in areas of the Mediterranean Sea where both species live in sympatry. This leads to uncertainties in the fisheries and marketing of the species, in addition to misinterpretations in biogeography and conservation studies. This paper describes a simple restriction fragment length-based diagnostic test that differentiates *S. solea* from *S. aegyptiaca*, as well as from other species of the Soleidae family. Furthermore, the two species living in sympatry in the Gulf of Kavala (North Aegean Sea, Greece) present significant qualitative differences in muscle fatty acid composition, a property that can also be used to distinguish the two cryptic species.

KEYWORDS: *Solea solea, Solea aegyptiaca, species diagnosis, cytochrome b gene, EcoR V restriction fragment length, white muscle fatty acid composition*

INTRODUCTION

The common sole (*Solea solea* Linnaeus, 1758), is an important fisheries resource in the northeastern Atlantic Ocean and Mediterranean Sea.[1] Due to its high commercial value, its market has the potential for fraudulent (deliberate) substitution with fish of lesser value.[2] This practice is facilitated in processed products, such as fresh or frozen filets, where species identification by morphological characters is not feasible. However, the unintentional substitution of *S. solea* can also occur when it is fished along with other Soleidae species sharing many common morphometric characters.

In the Mediterranean Sea *S. solea* co-occurs with two other *Solea* species, the Egyptian sole (*Solea aegyptiaca* Chabanaud, 1927) and the Senegalese sole (*S. senegalensis* Kaup, 1858).[3] These species are morphologically similar and share the same habitat, that is, sandy/muddy bottoms in marine or brackish waters,[4] resulting in identification issues, especially in areas where they live in sympatry. Hence, solely on the basis of morphometric data, the species status of *S. aegyptiaca* has been contested and synonomy with *S. solea* has been proposed.[5] However, early biochemical genetic studies,[6−8] as well as more recent cytochrome *b* (cyt *b*) and cytochrome oxidase subunit I (COI) gene-based phylogenies,[2,9] along with the latest taxonomic revision of the genus *Solea*,[10] all argue for maintaining *S. aegyptiaca* as a distinct species.

In contrast to *S. solea*, which has a Mediterranean-wide distribution,[11] reports of the occurrence of *S. aegyptiaca* limit the range of the species along the Mediterranean North African coast and the Gulf of Lion, in the northwestern Mediterranean Sea,[6,8] as well as in the southernmost extremity of the Adriatic Sea.[3] However, older sources report the presence of the species also in the upper part of the Adriatic Sea.[12] Furthermore, the species does not appear in the fisheries statistics of any Mediterranean country with the exception of Egypt, where it constitutes an important fisheries resource.[13]

On the basis of the study of the cyt *b* gene sequence variation of *Solea* spp. specimens, we report herein the presence of *S. aegyptiaca* in sympatry with *S. solea* in the Gulf of Kavala (North Aegean Sea, Greece). The identification of *S. aegyptiaca* in the northeastern part of the Mediterranean Sea, where it is confounded with *S. solea* and is being marketed as such, implies

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a potentially Mediterranean-wide distribution of the species. This in turn raises concerns about the fisheries of the species at the local and regional levels and traceability in the food market.[14] Furthermore, it also emphasizes the lack of knowledge on ecological aspects of both species, such as habitat preference, resilience to anthropogenic impacts, and reproductive isolation. Indeed, hybridization between *S. aegyptiaca* and *S. solea* is possible, as recently documented between *S. aegyptiaca* and *Solea senegalensis* in the Gulf of Tunis, where the two species live in sympatry.[8,15] Therefore, it is important that simple control tools are available to scientists and control authorities to differentiate unambiguously *S. solea* from *S. aegyptiaca*, especially in cases when identification by morphometric characters is not possible. Furthermore, in the case of unintentional species substitution, the qualitative traits of *S. aegyptiaca* of interest to consumers have to be evaluated and compared to those of *S. solea*.

We propose a simple and rapid diagnostic test, based on the PCR amplification of the cyt *b* gene followed by restriction fragment length analysis, to differentiate *S. solea* from *S. aegyptiaca* as well as from other Mediterranean Soleidae. Furthermore, we examine and compare the fatty acid composition, that is, an important indicator of nutritional quality, in the white muscle tissue of both species. We demonstrate that tissue fatty acid composition also differentiates the two species living in sympatry in the Gulf of Kavala.

MATERIALS AND METHODS

Fish and Tissue Samples. Fish samples were collected from the Gulf of Kavala, North Aegean Sea, Greece, in 2008, 2009, and 2010 within the framework of the FishPopTrace research project (http://fishpoptrace.jrc.ec.europa.eu/). Specifically, the 2008 sample included 3 *S. aegyptiaca* specimens, of which 2 were female; the 2009 sample included 16 *S. solea* specimens (8 female and 8 male) and 11 *S. aegyptiaca* (4 female and 7 male); the 2010 sample consisted of 12 *S. solea* fish, of which 6 were female and 6, male. Coordinates of sample collection localities are available in the FishPopTrace database (GIS-based monitoring, http://fishpoptrace.jrc.ec.europa.eu/map/geobrowser.html). Additional specimen details are summarized in Table S1 of the Supporting Information. Fin clips and muscle tissue were obtained for DNA extraction and fatty acid analysis, respectively, and were stored appropriately (fin clips in ethanol, muscle deep frozen) until analyses.

DNA Extraction and Analyses. Total DNA was extracted from fin clips and/or muscle tissue with the DNeasy tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The 5′ end of the *S. aegyptiaca* cyt *b* gene was amplified from 0.5 μg of genomic DNA using 200 nM of each Sol-CYTBF1 (5′-ACA ATG ACT AGT CTA CGA AAA TCC) and CytBI-5R (5′-GGT CTT TGT AGG AGA AGT ATG GGT GGA A) primer and 1 unit of Phusion DNA polymerase (Finnzymes, Vantaa, Finland) in a 50 μL reaction volume containing 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Reaction conditions involved an initial denaturation step at 98 °C (30 s), followed by 35 cycles of 98 °C (10 s)−57 °C (30 s)−72 °C (35 s) and a final elongation step of 7 min at 72 °C. The 3′ end of the cyt *b* gene was amplified with 200 nM of each of the Cytb3-814bp-IG (5′-CTT CGT AGG ATA CGT CCT CCC-3′) and Sol-CYTBR1 (5′-GGC GCT CTA ACA CTG AGC TAC) primers and 1 unit of *Taq* DNA polymerase (Invitrogen, Paisley, UK) in a 40 μL reaction volume containing 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Reaction conditions involved an initial denaturation step for 3 min at 94 °C, followed by 35 cycles of 94 °C (45 s)−59 °C (30 s)−72 °C (90 s) and a final extension step of 7 min at 72 °C. The cyt *b* gene from *S. solea*, *Pegusa lascaris* (Risso, 1810), and *Synapturichthis kleinii* (Risso, 1827) was PCR-amplified as described in the corresponding entries in the FishTrace database (www.fishtrace.org).[16] PCR products were column purified (PCR purification kit, QIAGEN) before sequencing and/or *Eco*RV (Minotech, Heraklion, Greece) restriction analysis. All sequencing was performed by a commercial sequence service provider (Macrogen Europe, Amsterdam, The Netherlands) employing the same primers used for the amplification of the cyt *b* gene. PCR products and their *Eco*RV fragments were analyzed by agarose gel electrophoresis (1.5%) and visualized by ethidium bromide staining.

Sequence alignment and restriction enzyme cleavage site searches were performed with the OMIGA software package (Accelerys, Cambridge, UK). All reference cyt *b* sequences of Soleidae (a total of 120 sequences) were derived within the framework of the FishTrace project (www.fishtrace.org) and correspond to taxonomically validated specimens. These included 96 *S. solea* sequences, from fish originating from the North Aegean Sea (20 sequences, GENBANK accession nos. JF509431−JF509450), the western Mediterranean (15 sequences, GENBANK accession nos. JN561668−JN561682), the Bay of Biscay (18 sequences, GENBANK accession nos. JF969239−JF969252), the North Sea (20 sequences, GENBANK accession nos. JN561627−JN561646), and the Skagerrak (21 sequences, GENBANK accession nos. JN561647−JN561661). The remaining 24 sequences included four Senegalese sole sequences (*S. senegalensis*, two from the western Mediterranean and two from the Canary Islands, GENBANK accession nos. EF439590−1 and EF427601−2, respectively), eight sand sole sequences (*P. lascaris*, two from the North Aegean, two from the western Mediterranean, two from the Canary Islands, and two from the Bay of Biscay with GENBANK accession nos. EU036472-3, EF439565−6, EF392603−4, and EU224070−1, respectively), six Klein's sole sequences (*S. kleinii*, two from the North Aegean, two from the western Mediterranean, and two from the Canary Islands with GENBANK accession nos. EU036510−1, EF439603−4, and EF392623−4, respectively), and six thickback sole sequences (*Microchirus variegatus* Donovan, 1808, two from the Western Mediterranean, two from the Cantabric Sea, and two from the Bay of Biscay with GENBANK accession nos. EF439550−1, EF427582−3, and EU224062−3, respectively).

For the amplification of the *S. aegyptiaca* rhodopsin gene nested PCR was used. The primer set for the first amplification step was Rod F2B (5′-CTC TGC AAG CCC ATC AGC AAC TTC CG) and Rod 5R (5′-GGT GGT GAT CAT GCA GTG GCG GAA) and for the second Rod F2 (5′-AGC AAC TTC CGC TTC GGA GAG AA) and Rod 4R (5′-CTG CTT GTT CAT GCA GAT GTA GAT). The PCR conditions for both steps were as previously described by Sevilla et al. (protocol 30).[16] The *S. aegyptiaca* rhodopsin sequences were deposited in GENBANK under accession nos. JX292784−5.

Distance divergence between Soleidae sequences at the cyt *b* and the rhodopsin loci was estimated with the *p*-distance method (bootstrap validated, 1000 replications) using the MEGA5 software program.[17]

Chemical Analysis. From each fish, the skin from the eyed side was removed and a piece of white muscle tissue (5−8 g), above the lateral line and near the caudal fin, was obtained and lyophilized overnight in a Christ alpha 2-4 freeze-dryer (Martin Christ GmbH, Osterode am Harz, Germany). Dehydrated tissue (0.2 g) was ground to fine powder, and lipids were extracted according to the Folch method.[18] One milligram of total lipid was used for the transesterification of fatty acids, as described by Falch et al.,[19] and tissue fatty acid composition was determined by gas chromatography on a Hewlett-Packard 5809 series II gas chromatograph, equipped with a flame ionization detector (FID) and a 60 m, 0.25 mm internal diameter, BPX-70 capillary column (SGE Analytical Science, Ringwood, Australia). The inlet was set at the split mode (split 1:60) and the inlet temperature at 320 °C. The detector temperature was set at 300 °C. Helium was used as carrier gas and nitrogen as auxiliary gas (flow rate at 36 mL min^{-1}). FID gases were hydrogen (flow rate = 30 mL min^{-1}) and compressed air (flow rate = 330 mL min^{-1}). The program used to separate the fatty acid methyl esters included a 2 min hold time at 50 °C, temperature increase from 50 to 150 °C at a rate of 20 °C min^{-1} and from 150 to 230 °C at a rate of 1.5 °C min^{-1}, followed by a 3 min hold time at 230 °C. For peak identification, solutions of reference substances (37 Component FAME Mix, Sigma-Aldrich, St. Louis, MO, USA) were analyzed under the same

conditions, and their retention times and chromatograms were compared to those of the target sample. In each sample, heptadecanoic acid (C 17:0, Sigma-Aldrich) was used as internal standard. The contribution of each identified compound was expressed as the percentage of its peak area to the total area of all peaks eluted in each chromatogram. Peak areas were calculated with Hewlett-Packard GC-Chem Station software. A total of 28 *S. solea* specimens (14 male and 14 female, corresponding to the 2009 and 2010 samples) and 11 *S. aegyptiaca* specimens (7 male and 4 female, corresponding to the 2009 sample) were analyzed.

Statistical Analysis. Significant differences in muscle fatty acid content and composition were determined at the individual fatty acid level between the two species by the *t* test for independent variables by group. Homogeneity of variances was tested by the Levene and/or the Brown−Forsyth tests. For fatty acids exhibiting not normal distribution, the nonparametric Kolmogorov−Smirnov two-sample test was used. Sex-dependent differences within or between the two species were determined by factorial ANOVA, followed by Tukey's post test. Homogeneity of variances was tested with Levene's test for ANOVA. The nonparametric Kruskal−Wallis ANOVA test was applied to those fatty acids not conforming to the homogeneity of variance assumption. For species differentiation based on muscle fatty acid composition discriminant function analysis (DA) was applied. This analysis is used to determine the variables or set of characters that discriminate between naturally occurring groups. For forward-step DA, the fatty acids for which means were affected by extreme values, rendering their statistical significance unreliable, were excluded. *K*-fold cross-validation and repeated random subsampling validation were performed on the data set that resulted from forward step DA. *K*-fold cross-validation allows prediction for a given observation once left out of the estimation sample. In repeated random subsampling validation, after random splitting of the data set to "training" and "validation" samples, the training sample is used to build the model and the validation sample to assess its predictive accuracy. Validation samples ranging from 20 to 50% of total observations were used in this analysis. All analyses were performed with the Statistica 7 software package (StatSoft, Tulsa, OK, USA), except for the cross-validation for which the XLSTAT application (Addinsoft, Paris, France) was used.

RESULTS AND DISCUSSION

Presence of *S. aegyptiaca* in the North Aegean Sea. Initial evidence for the presence of *S. aegyptiaca* in the North Aegean Sea came from sequencing of the 5′-end of the cyt *b* gene of presumed *S. solea* specimens, collected in the Gulf of Kavala in 2008. Three sequences (GENBANK accession nos. JN225430−JN225432) diverged significantly, that is, >11%, from the sequence of validated *S. solea* specimens from the same geographic area (GENBANK accession nos. JF509431−JF509450). In contrast, they were 100% identical to the published *S. aegyptiaca* sequence[2,9] (GENBANK accession nos. AF289718 and EU513872−74, respectively). An additional 11 fish collected in 2009 were identified at the cyt *b* locus as *S. aegyptiaca* (GENBANK accession nos. JN225426−JN225429 and JN248266−JN24871), providing strong support for an established population of this species in the Gulf of Kavala, where it co-occurs with *S. solea*.

Development of a Molecular Diagnostic Test for *S. solea*. The presence of *S. aegyptiaca* co-occurring with *S. solea* in the North Aegean Sea, and potentially in other Mediterranean areas where it is currently confounded with *S. solea*, highlights the need for the development of a rapid, cost-efficient, and readily applicable diagnostic tool to reliably discriminate between the two species. To achieve this, the cyt *b* gene of *S. solea* was scanned, along with the newly generated *S. aegyptiaca* cyt *b* sequences for the presence/absence of cleavage sites of common restriction endonucleases. To account for intraspecific variability at the cyt *b* gene, we included the sequences of 96 additional *S. solea* individuals in this analysis, originating from different Mediterranean and Atlantic areas (see Materials and Methods for the geographic origin of sequences used and corresponding GENBANK accession nos.). This in silico restriction approach revealed that all *S. solea* sequences, irrespective of geographic origin, contained a single *Eco*RV cleavage site at position 413 of the gene, which was absent in the *S. aegyptiaca* sequences analyzed (Figure 1A). We further

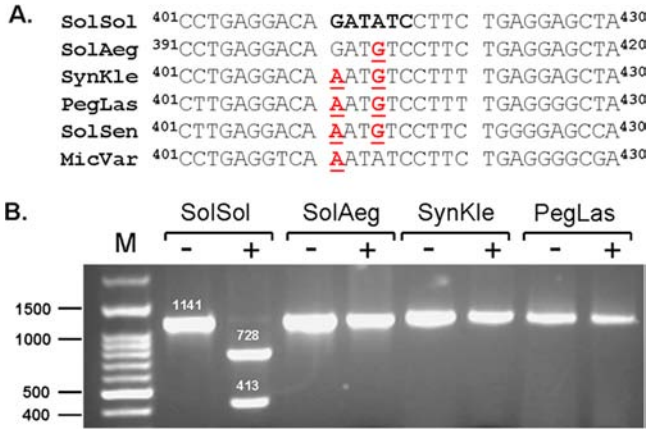

Figure 1. The presence of an *Eco*RV restriction endonuclease site in the cyt *b* gene differentiates *S. solea* from other Soleidae species. (A) Sequence comparison of the cyt *b* gene of *S. solea* (SolSol), *S. aegyptiaca* (SolAeg), *S. kleinii* (SynKle), *P. lascaris* (PegLas), *S. senegalensis* (SolSen), and *M. variegatus* (MicVar). Base numbering is according to GENBANK entries EU264020, JN225426, EU036510, EU036472, EF439591, and EF439551 for SolSol, SolAeg, SynKle, PegLas, SolSen, and MicVar, respectively. The *Eco*RV site in the SolSol sequence is indicated by bold letters, and diverging nucleotides in the sequences of the other species are underlined. (B) Agarose gel electrophoresis of the entire PCR-amplified cyt *b* gene (1141 nt) of different Soleidae species in the absence (−) or presence (+) of the *Eco*RV restriction endonuclease. Species names are as in panel A. The size of the SolSol fragments is indicated. M is the molecular size marker (100 bp DNA Ladder, Nippon Genetics Europe, Düren, Germany).

examined whether this diagnostic feature could also be used to differentiate *S. solea* from other Soleidae species, in addition to *S. aegyptiaca*, that are, or could be, used as commercial substitutes of *S. solea*. Thus, the analysis was expanded to include the cyt *b* gene sequence of four *S. senegalensis*, of eight *P. lascaris*, of six *S. kleinii*, and of six *M. variegates* (see Materials and Methods for the geographic origin of sequences used and corresponding GENBANK accession nos). As was the case for *S. aegyptiaca*, none of the sequences of the above species contained the *Eco*RV cleavage site (Figure 1A).

To confirm the results of the in silico analysis and, thus, to validate the potential of this unique *S. solea* feature as a diagnostic tool, the sequences encoding the cyt *b* gene of *S. solea*, *S. aegyptiaca*, *S. kleinii*, and *P. lascaris* were PCR-amplified and subjected to *Eco*RV digestion. As expected, the restriction endonuclease cleaved only the *S. solea* cyt *b* gene, resulting in two fragments of 413 and 728 bp (Figure 1B). Hence, the above results demonstrate that this rapid and cost-efficient test, which eliminates the need for extensive sequencing effort, can be effectively used to differentiate *S. solea* from *S. aegyptiaca*, as well as from other Soleidae species, in cases when identification is not possible through morphological characters. This in turn

Table 1. White Muscle Tissue Fatty Acid Composition (Percent Total Fatty Acids) in the *S. solea* (SolSol) and *S. aegyptiaca* (SolAeg) Samples[a]

FA	% FA SolSol (n = 28)	% FA SolAeg (n = 11)	p	FA	% FA SolSol (n = 28)	% FA SolAeg (n = 11)	p
C14:0	1.42 ± 0.45	0.96 ± 0.17	<0.03	C22:0	0.32 ± 0.20	0.18 ± 0.06	<0.05
C14:1	0.26 ± 0.10	0.11 ± 0.03	<0.01	C22:1 n-11	0.34 ± 0.20	0.25 ± 0.07	NS
C15:0	0.77 ± 0.27	0.79 ± 0.11	NS	C20:5 n-3	4.68 ± 1.24	2.92 ± 0.77	<0.01
C15:1	0.21 ± 0.05	0.24 ± 0.07	NS	C23:0	0.28 ± 0.16	0.13 ± 0.04	<0.01
C16:0	19.55 ± 1.72	22.73 ± 1.57	<0.01	C22:3 n-3	2.50 ± 0.46	2.87 ± 1.13	NS
C16:1 n-7	3.44 ± 1.05	2.01 ± 0.41	<0.01	C22:4 n-6	3.02 ± 0.87	3.96 ± 0.84	<0.01
C17:1	1.15 ± 0.28	1.05 ± 0.23	NS	C24:1 n-9	0.92 ± 0.39	0.36 ± 0.11	<0.01
C18:0	6.16 ± 0.73	8.07 ± 0.63	<0.01	C22:5 n-3	7.12 ± 1.17	4.41 ± 0.56	<0.01
C18:1 n-9t	1.96 ± 1.68	0.75 ± 0.99	<0.05	C22:6 n-3	19.76 ± 4.81	21.29 ± 3.70	NS
C18:1 n-9c	8.99 ± 0.89	11.56 ± 1.02	<0.01				
C18:1 n-7	2.25 ± 0.39	3.60 ± 0.71	<0.01	total SAEA	29.25 ± 1.72	33.14 ± 1.42	<0.01
C18:2 n-6t	0.24 ± 0.08	0.13 ± 0.07	<0.01	total MUFA	19.93 ± 2.34	20.32 ± 1.30	NS
C18:2 n-6c	0.61 ± 0.10	1.00 ± 0.10	<0.01	total PUFA	49.93 ± 2.66	45.76 ± 2.21	<0.01
C18:3 n-6	0.44 ± 0.25	0.20 ± 0.14	<0.01	total n-3	35.23 ± 3.96	32.25 ± 2.41	<0.05
C20:0	0.77 ± 0.37	0.29 ± 0.08	<0.01	total n-6	14.70 ± 2.37	13.51 ± 1.87	NS
C18:4 n-3	0.80 ± 0.42	0.51 ± 0.22	<0.05	n-3/n-6	2.50 ± 0.73	2.44 ± 0.45	NS
C20:1 n-9	0.41 ± 0.14	0.41 ± 0.18	NS				
C20:2 n-6	0.17 ± 0.10	0.10 ± 0.17	<0.01	total fat (%)	3.60 ± 0.97	3.39 ± 0.44	NS
C20:3 n-6	0.26 ± 0.08	0.42 ± 0.21	<0.01				
C20:3 n-3	0.41 ± 0.17	0.25 ± 0.05	<0.01				
C20:4 n-6	9.98 ± 2.30	7.76 ± 1.59	<0.01				

[a]Values are the mean ± SD. Column "p" presents the significant differences between species (t test). NS indicates nonsignificant differences.

can contribute to the control of potential fraudulent and unintentional substitutions in *S. solea* fisheries and marketing and, consequently, protect both consumers and businesses. Furthermore, the identification of *S. aegyptiaca* in the North Aegean Sea in sympatry with *S. solea* suggests that the geographical distribution of the species in the Mediterranean Sea needs reappraisal. Moreover, it underscores the need for improved understanding of the ecological and biological aspects of the two species, such as their resilience to anthropogenic impacts and their potential for interspecific hybridization. With respect to the interspecific hybridization issue, the limitations of diagnostic tests based on mitochondrial markers, which identify only the maternal lineage, are obvious. Therefore, development and use of nuclear markers is necessary to unambiguously address this question. Relevant to this, our preliminary analysis of the rhodopsin gene in a limited number of the above-described *S. aegyptiaca* specimens revealed a modest degree of sequence divergence (2%) from the *S. solea* gene, that is, within the levels expected for closely related congeneric species.[16] Thus, the rhodopsin gene and the *S. solea*-specific SNP markers that we are currently developing represent tools that can be applied toward resolving this issue.

Fatty Acid Profile Analysis of White Muscle Tissue of the Two Species. The fatty acid composition of Mediterranean *S. solea* has been determined before,[20−22] but to our knowledge this has not been done for *S. aegyptiaca*. Therefore, to evaluate and compare the nutritional value of the two sympatric species in the North Aegean Sea, the fatty acid composition of the white muscle tissue was determined in a total of 28 *S. solea* specimens and 11 *S. aegyptiaca* specimens. In each species a total of 30 fatty acids (FA) were identified and quantified (Table 1). Species-specific differences at the individual FA level were determined by the t test for independent samples. Significant between-species differences were observed in 23 FA. Of the seven FA that did not present differences in the two species, five were minor components of total FA, that is, each representing <1% of total FA, with the remaining two being docosahexaenoic acid (C22:6 n-3, DHA) and docosatrienoic acid (C22:3 n-3) (Table 1). This comparison also revealed that *S. aegyptiaca* muscle contained significantly higher levels of total saturated FA (SAFA), whereas *S. solea* was richer in total polyunsaturated FA (PUFA), as well as in total n-3 FA. Accordingly, the n-3/n-6 FA ratio, an important indicator for human nutrition,[23] was higher in the *S. solea* fish as compared to the *S. aegyptiaca* sample. No significant differences were observed in the muscle total fat content of the two species (Table 1).

In addition to the unbalanced sample size between the two species (n = 28 and n = 11 for *S. solea* and *S. aegyptiaca*, respectively), the *S. solea* sample consisted of 14 female and 14 male fish, whereas only 4 *S. aegyptiaca* specimens were female. Furthermore, in contrast to the female *S. solea* fish, which were spawning at sampling time, the *S. aegyptiaca* sample and the male *S. solea* fish were all nonspawning (Supporting Information, Table S1). Thus, to evaluate potential effects of sex and/or reproductive stage on the tissue FA composition, the analysis was repeated by considering both species and sex as categorical predictors in factorial ANOVA. As shown in Table 2, between-species differences in tissue content of several fatty acids were directly dependent on sex/reproductive stage in the *S. solea* sample. Specifically, the observed species differences in tissue content for myristic (C14:0), palmitoleic (C16:1 n-7), eicosanoic (C20:0), arachidonic (C20:4 n-6, ArA), and eicosapentaenoic (C20:5 n-3, EPA) acids were solely due to the higher level of these FA in the female *S. solea* fish. In addition, the significantly lower DHA content in female *S. solea* fish, as compared to both *S. aegyptiaca* and male *S. solea*, was masked in the between-species comparative analysis. Further-

Table 2. White Muscle Tissue Fatty Acid Composition (Percent Total Fatty Acids) in the Female (F) and Male (M) Specimens of *S. solea* (SolSol) and *S. aegyptiaca* (SolAeg)[a]

	% FA				
	SolSol-F (n = 14)	SolSol-M (n = 14)	SolAeg-F (n = 4)	SolAeg-M (n = 7)	Spp.& sex $p < 0.01$
C14:0	1.75 ± 0.40	1.08 ± 0.13	0.83 ± 0.12	1.04 ± 0.15	a, b, c
C14:1	0.33 ± 0.09	0.19 ± 0.07	0.09 ± 0.02	0.11 ± 0.03	a, b, c
C15:0	0.68 ± 0.25	0.86 ± 0.26	0.83 ± 0.12	0.76 ± 0.11	NS
C15:1	0.21 ± 0.05	0.21 ± 0.06	0.19 ± 0.05	0.26 ± 0.07	NS
C16:0	18.90 ± 1.66	20.19 ± 1.57	22.04 ± 1.60	23.12 ± 1.52	b, c, e
Cc16:1 n-7	4.29 ± 0.77	2.59 ± 0.37	1.81 ± 0.23	2.10 ± 0.47	a, b, c
C17:1	1.23 ± 0.34	1.08 ± 0.18	1.03 ± 0.29	1.06 ± 0.21	NS
C18:0	5.67 ± 0.57	6.64 ± 0.51	8.28 ± 0.19	7.95 ± 0.78	a, b, c, d, e
C18:1 n-9t	1.75 ± 1.45	2.17 ± 1.91	1.25 ± 1.64	0.46 ± 0.17	NS
C18:1 n-9c	9.17 ± 0.83	8.80 ± 0.95	11.75 ± 0.92	11.46 ± 1.13	b, c, d, e
C18:1 n-7	2.21 ± 0.32	2.29 ± 0.45	3.63 ± 0.77	3.59 ± 0.73	b, c, d*, e
C18:2 n-6t	0.26 ± 0.10	0.22 ± 0.05	0.12 ± 0.08	0.14 ± 0.07	b, c, d, e
C18:2 n-6c	0.61 ± 0.10	0.61 ± 0.10	1.07 ± 0.06	0.95 ± 0.09	b, c, d, e
C18:3 n-6	0.45 ± 0.27	0.44 ± 0.23	0.13 ± 0.05	0.24 ± 0.16	NS
C20:0	1.10 ± 0.14	0.45 ± 0.19	0.30 ± 0.11	0.29 ± 0.07	a, b, c
C18:4 n-3	0.89 ± 0.53	0.72 ± 0.28	0.47 ± 0.28	0.54 ± 0.19	NS
C20:1 n-9	0.47 ± 0.16	0.35 ± 0.09	0.40 ± 0.21	0.41 ± 0.18	NS
C20:2 n-6	0.23 ± 0.08	0.11 ± 0.08	0.05 ± 0.03	0.05 ± 0.04	a*, b, c
C20:3 n-6	0.29 ± 0.07	0.23 ± 0.07	0.46 ± 0.25	0.39 ± 0.19	NS
C20:3 n-3	0.51 ± 0.13	0.31 ± 0.15	0.25 ± 0.05	0.24 ± 0.05	a, c
C20:4 n-6	11.77 ± 1.38	8.19 ± 1.49	9.22 ± 1.25	6.93 ± 1.10	a, b, c
C22:0	0.44 ± 0.20	0.20 ± 0.13	0.18 ± 0.04	0.17 ± 0.07	a,, b*, c
C22:1 n-11	0.45 ± 0.20	0.22 ± 0.12	0.22 ± 0.08	0.26 ± 0.07	a, b*, c*
C20:5 n-3	5.39 ± 0.75	3.97 ± 1.25	3.16 ± 0.83	2.78 ± 0.77	a, b, c
C23:0	0.29 ± 0.18	0.26 ± 0.14	0.13 ± 0.04	0.13 ± 0.04	b*, c, e*
C22:3 n-3	2.40 ± 0.56	2.60 ± 0.33	3.25 ± 1.79	2.66 ± 0.61	NS
C22:4 n-6	2.67 ± 0.73	3.37 ± 0.89	3.96 ± 0.78	3.95 ± 0.93	b*, c*
C24:1 n-9	1.14 ± 0.15	0.70 ± 0.30	0.34 ± 0.10	0.37 ± 0.12	a, b, c, d*, e*
C22:5 n-3	7.68 ± 0.92	6.56 ± 1.14	4.56 ± 0.84	4.33 ± 0.39	a*, b, c, d, e
C22:6 n-3	15.69 ± 2.86	23.84 ± 2.05	20.74 ± 5.40	21.61 ± 2.81	a, b*, c
total SAEA	29.12 ± 1.17	29.80 ± 1.75	32.00 ± 1.16	34.06 ± 2.37	b, c, d, e
total MUFA	22.06 ± 3.84	18.62 ± 1.97	19.92 ± 2.92	20.32 ± 2.34	a
total PUFA	48.35 ± 3.90	51.17 ± 2.10	47.83 ± 2.56	45.38 ± 1.74	e
total n-3	32.56 ± 2.50	38.00 ± 3.07	32.43 ± 3.42	32.15 ± 1.96	a, d, e
total n-6	15.78 ± 3.23	13.17 ± 2.16	15.40 ± 2.61	13.23 ± 2.26	NS
n-3/n-6	2.37 ± 1.14	2.99 ± 0.74	2.18 ± 0.62	2.49 ± 0.46	NS
total fat	3.65 ± 1.34	3,55 ± 0,40	3,47 ± 0,39	3,32 ± 0,50	NS

[a]Values are the mean ± SD. Column "Spp. & sex" presents significant differences between SolSol-M and SolSol-F (a), between SolSol-F and SolAeg-F (b), between SolSol-F and SolAeg-M (c), between SolSol-M and SolAeg-F (d), and between SolSol-M and SolAeg-M (e) at $p < 0.01$, except in the cases indicated by an asterisk ($p < 0.05$). NS indicates nonsignificant differences. Fatty acids in bold were used in discriminant analysis.

more, stearic (C18:0), nervonic (C24:1 n-9), and docosapentaenoic (C22:5 n-3, DPA) acids were found to exhibit significant differences at both the species level and the sex/reproductive stage in *S. solea*. A third group of FA included those that exhibited exclusively species-specific differences, with major representatives being palmitic (C16:0), oleic (C18:1 n-9c), vaccenic (C18:1 n-7), and linoleic (C18:2 n-6c) acids. No differences were observed in any FA between male and female *S. aegyptiaca* fish. However, the significance of this finding cannot be evaluated at this point because, as noted above, the female *S. aegyptiaca* sample was limited to only four fish.

The above analysis also revealed that the differences in PUFA and n-3 FA content observed at the species level (Table 1) were due to higher values in these two FA classes in the male *S. solea* fish, as compared to the female fish of the same species as well as to the *S. aegyptiaca* fish, irrespective of sex (Table 2). Thus, from the human nutrition perspective, the higher level of total PUFA and the lower level of total SAFA in *S. solea* white muscle, as determined in the present study, may render this species more desirable. However, in terms of total fat and n-3/n-6 FA ratio (Tables 1 and 2) the two species could be considered of equal value.

As mentioned above, sex/reproductive stage-dependent differences of important fatty acids were observed in the *S. solea* sample (Table 2). Of these, the higher concentration of ArA in the female specimens is in accordance with its documented importance in fish reproduction and egg quality[24] and its accumulation in tissues of spawning fish.[25] Similarly, the

Table 3. Summary of the Results of the Forward Step (Step 6) Discriminant Analysis

				standardized coefficients	
variable	Wilks' λ	p	tolerance	root 1	root 2
C20:0	0.026998	0.000001	0.668299	0.44911	0.932660
C18:2 n-6c	0.017978	0.000645	0.740109	−0.30636	0.731265
C18:1 n-9c	0.016578	0.002266	0.750750	−0.67010	0.096680
C16:1 n-7	0.015349	0.007478	0.831833	0.58318	0.065626
C18:1 n-7	0.017006	0.001527	0.842815	−0.55507	0.380078
C16:0	0.014127	0.027059	0.849294	−0.37843	0.371972
		eigenvalue		17.99382	3.704125
		proportion (%)		83	17

lower levels of DHA in the spawning female fish may reflect the selective transfer of this FA to the eggs, as suggested for other fish species.[25,26] However, detailed comparative analyses in spawning and nonspawning *S. solea* of both sexes are required to evaluate the biological significance of these differences, as well as those concerning the higher EPA and DPA levels in the female spawning fish (Table 2).

Tissue Fatty Acid Composition as a Predictor of Species Identity. In addition to its use as a nutritional quality indicator, tissue FA composition analysis has been applied in recent years to various studies of interest to fisheries and aquaculture, ranging from species discrimination to fish stock identification.[19,27,28] As clear differences at individual FA levels were established in the two *Solea* species, we examined whether specific pattern(s) in tissue FA composition could be identified and applied to species and sex/reproductive stage discrimination. To that end, the FA data set was used in discriminant function analysis (DA), a multivariate analysis that is used to determine which continuous variables discriminate between two or more naturally occurring groups.[29] From this analysis we excluded those FA that exhibited high within-group variances, rendering their statistical significance unreliable. This concerned 10 FA, all minor constituents of total FA (cumulative <6% of total FA in both species). The remaining 20 FA (bold in Table 2) were employed in forward stepwise DA. In this analysis, all variables were evaluated in a sequential manner to determine which ones contributed most to the discrimination between groups. At each step a variable was selected to be included in the model, and the process was repeated until the addition of a variable violated the significance criterion ($p <$ 0.5) for the relationship (canonical correlation) between the variables. Through this process, significant discrimination between the two species and sex/reproductive stage in *S. solea* was reached with the inclusion in the model of six FA, namely, C20:0, C18:2 n-6c, C18:1 n-9c, C16:1 n-7, C18:1 n-7, and C16:0 (Table 2). The Wilk's λ and standardized coefficients values presented in Table 3 indicated that C20:0 provided the highest discriminatory power and C16:0 the lowest, with canonical root 1 (eigenvalue associated with the canonical function, Table 3) representing 83% of the variance. Furthermore, the plot of the canonical scores (Figure 2A) revealed that discrimination between species was along root 1, whereas root 2 discriminated the *S. solea* specimens according to sex/reproductive stage. Projection of the standardized coefficients for each FA on the plane of discriminant functions (Figure 2B) indicated that the tissue content for C18:2 n-6c, C18:1 n-9c, C18:1 n-7, and C16:0 discriminated between the two species, whereas that for C20:0 and C16:1 n-7 discriminated between sex/reproductive stage in *S. solea* in addition to discriminating between species. Cross-validation of the DA results for the six FA model identified only a single female *S. solea* (indicated by an arrow in Figure 2A) misclassified as male of the same species (Table 4). The discriminatory power of the six FA model was further tested by random subsampling validation. Irrespective of the size of the validation sample used, which ranged from 20 to 50% of total

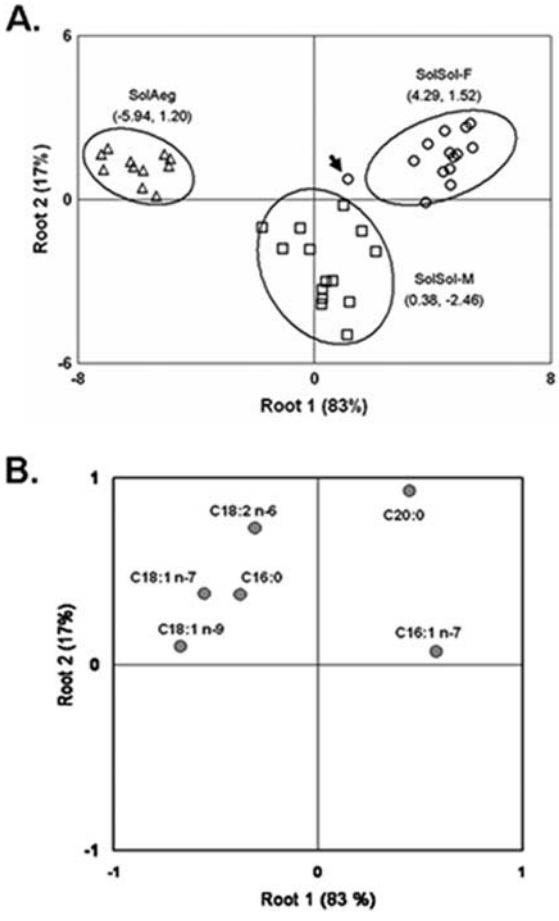

Figure 2. White muscle tissue content for six fatty acids discriminates *S. solea* from *S. aegyptiaca*.. (A) Plot of canonical scores from discriminant function analysis. *S. solea* female (SolSol-F) and male (SolSol-M) specimens are represented by circles and squares, respectively; triangles represent the *S. aegyptiaca* (SolAeg) specimens. The means of canonical variables (centroids) are given in parentheses. The arrow indicates the SolSol-F specimen misclassified in cross-validation. (B) Projection of the standardized coefficients of the six fatty acids along the discriminant functions plane.

Table 4. Classification of Individuals According to Species and Sex/Reproductive Stage Prior to and after Cross-Validation of the Discriminant Analysis Results of the Six Fatty Acid Data Set[a]

from/to	SolAeg	SolSol-F	SolSol-M	total	% correct
		Estimtion Sample			
SolAeg	11	0	0	11	100
SolSol-F	0	14	0	14	100
SolSol-M	0	0	14	14	100
total	11	14	14	39	100
		Cross-Validation Results			
SolAeg	11	0	0	11	100
SolSol-F	0	13	1	14	92.86
SolSol-M	0	0	14	14	100
total	11	13	15	39	97.44

[a]Abbreviations are as in Table 1.

cases, this analysis also resulted in >90% correct classification according to species and sex/reproductive stage (not shown).

A prerequisite for the utility of phenotypic markers, such as the tissue FA composition, as well as of genetic markers in species discrimination and/or population structure studies, is that they exhibit temporal and spatial stability. Our results provide preliminary evidence for temporal stability in the tissue content of the six FA in *S. solea* at the local scale. The *S. solea* sample consisted of individuals collected in two consecutive years (2009 and 2010) with equal numbers of female and male fish in each yearly sample (Supporting Information, Table S1). The values for the tissue content of the six FA in individuals of the same sex did not present significant differences between the two years (Table 5). This is also reflected by the close clustering of the *S. solea* individuals according to sex/reproductive stage along the discriminant functions of Figure 2. However, it is also important to note that seasonal as well as geographical differences in FA composition have been observed in several fish species including *S. solea*.[19,21,30−33] Specifically, for the study concerning seasonal FA variation in female *S. solea*,[21] differences were observed in all FA examined, including four of the six FA used in our model. Therefore, it is evident that analysis of additional samples, including seasonal samples of both species as well as samples originating from other geographical areas where the two species live in sympatry, is essential to establish the spatial and temporal stability of the six FA model and, consequently, its potential as a tool for discriminating between the two sibling flatfish species.

■ ASSOCIATED CONTENT

Supporting Information

Details of the samples used in this study (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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▲Member of the FishPopTrace Consortium (http://fishpoptrace.jrc.ec.europa.eu/).

▼Member of the FishTrace Consortium (www.fishtrace.org).

■ ABBREVIATIONS USED

Cyt *b*, cytochrome *b*; bp, base pairs; FA, fatty acids; ArA, arachidonic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DA, discriminant function analysis.

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Table 5. Interannual Comparisons of White Muscle Tissue Content for the Six Fatty Acids in Female and Male *S. solea*[a]

sex	year	C16:0	C16:1 n-7	C18:1 n-7	C18:1 n-9c	C18:2 n-6c	C20:0
F	2009 (n = 8)	19.33 ± 1.46	4.49 ± 0.98	2.25 ± 0.34	8.97 ± 0.93	0.82 ± 0.38	1.03 ± 0.28
F	2010 (n = 6)	18.31 ± 1.87	4.02 ± 0.44	2.42 ± 0.81	10.29 ± 2.23	0.69 ± 0.36	1.05 ± 0.29
	p	0.272	0.287	0.612	0.154	0.515	0.895
M	2009 (n = 8)	20.12 ± 1.96	2.52 ± 0.37	2.30 ± 0.43	8.42 ± 0.99	0.61 ± 0.10	0.38 ± 0.15
M	2010 (n = 6)	20.30 ± 1.87	2.69 ± 0.38	2.28 ± 0.53	9.31 ± 0.66	0.61 ± 0.25	0.54 ± 0.19
	p	0.837	0.412	0.914	0.081	0.988	0.124

[a]Values are the mean ± SD.

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