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U1 and U2 Small Nuclear RNA Genetic Linkage: A Novel Molecular Tool for Identification of Six Sole Species (Soleidae, Pleuronectiformes)

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We evaluated the usefulness of a genetic linkage between the U1 and U2 small nuclear RNAs for species identification. Six soles belonging to the genera *Solea*, *Dicologlossa*, and *Microchirus* were studied. A simple methodology based on two single PCRs is described. Reproducible band profiles were generated for all samples. This rapid and discriminatory molecular method is highly promising for determining the authenticity of sole fillets in the food industry.

KEYWORDS: Soleidae; snRNAs; genetic linkage; species identification; *Solea*; *Dicologlossa*; *Microchirus*

INTRODUCTION

Flatfish (order Pleuronectiformes) is a common name for over 500 species worldwide (*1*). Particularly, the family Soleidae (soles) comprises 7 genera with 17 different species in the North-Eastern Atlantic and Mediterranean (*2*). Some sole species such as *Solea solea, Solea senegalensis, Solea lascaris, Dicologlossa cuneata,* or *Microchirus azevia* are highly appreciated in southern European markets. In recent years, reduction in the wild catches has motivated the development of a *S. senegalensis* and *D. cuneata* aquaculture industry. The similarity among sole species makes it so that some less valuable species can be mislabeled and sold under the names of more expensive ones. In addition, commercialization as fresh or frozen fillets opens the possibility of fraud substitutions.

U1 and U2 are abundant small nuclear RNAs (snRNAs) found in both plant and animal cells. U1 and U2 as well as the U4, U5, and U6 snRNAs are components of the small nuclear ribonucleoprotein particles that participate in the nuclear splicing of mRNA precursors. It has been estimated that multigene families encoding the U1 and U2 snRNA genes and the 45S and 5S rRNAs together account for ∼2% of the total genome (*3*). The main characteristic of these tandemly repeated multigene families is that they evolve concertedly. Gene conversion and unequal crossing-over mechanisms play an important role in the maintenance of sequence homogeneity within each species and also spread the mutations to all individuals in the population. Recently, a genetic linkage between the 5S rRNA gene and the U1, U2, and U5 snRNAs has been reported in *S. senegalensis* (*4*). These linkages among tandemly repeated multigene families are usually modified by chromosomal rearrangements. Concerted evolution mechanisms produce the homogenization and fixation of that new arrangements and their loss in other related species (*5*).

In this work we investigate the suitability of a genetic linkage between the U2 and U1 snRNAs as a useful marker for the identification of six commercially important sole species. For this purpose, the U2−U1 and U1−U2 snRNA spacer regions were amplified. The linkage was not detected in species of Pleuronectidae and Scophthalmidae families. The results suggest this linkage as a new potential marker for species identification.

MATERIAL AND METHODS

Fish Sampling. Genetic analyses were conducted on 8 individuals of *S. solea*, 10 individuals of *S. senegalensis*, 2 individuals of *Solea kleinii*, 8 specimens of *S. lascaris*, 8 specimens of *D. cuneata*, and 8 specimens of *M.* azevia. Samples of these six species were collected in the Gulf of Cádiz (Spain; northeast Atlantic) during a fish sampling performed throughout September 2004 as a part of the scientific project "Fisheries Resources of the Gulf of Cádiz", supported by the "Consejería de Agricultura y Pesca" of the "Junta de Andalucía" (Spain). *Scophthalmus maximus* and *Platichthys flesus* were purchased at a local market. A muscular portion of each of the specimens was excised and kept at −80 °C.

DNA Isolation, Amplification, and Sequencing. Total genomic DNA was isolated from 150 mg of frozen muscle sections using a FastDNA kit for 40 s at speed setting 5 in the Fastprep FG120 instrument (Bio101, Inc., Vista, CA). All DNA isolation procedures were performed in accordance with the manufacturer's protocol.

Two primer pairs were designed according to the linkage sequences described previously for *S. senegalensis* (*4*). The first primer pair (SseU2·2, 5′-ACCGTTCCTGGTGGCACTGCAAT-3′; SseU1·2, 5′-TTACCTGGCAGGGGAGATACCATGATCAAG-3′) amplified the spacer region between adjacent U2 and U1 snRNA units (**Figure 1**). The second primer pair (SseU2·1, 5′-TTTGAGCAGTGGGAGGT-GAAAACTGG-3′; SseU1·1, 5′-CGTTTGGGGAATTTGCAGGGGT-CAACA-3′) amplified the DNA spacer between adjacent U1 and U2

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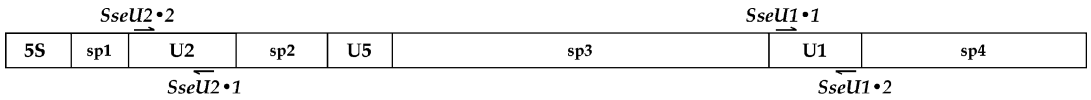

Figure 1. Schematic representation of the U1 and U2 snRNA genetic linkage in *S. senegalensis*. The 5S rRNA gene and the U1, U2, and U5 snRNAs are indicated. DNA spacers are referred to as sp1–sp4. Primers location is shown.

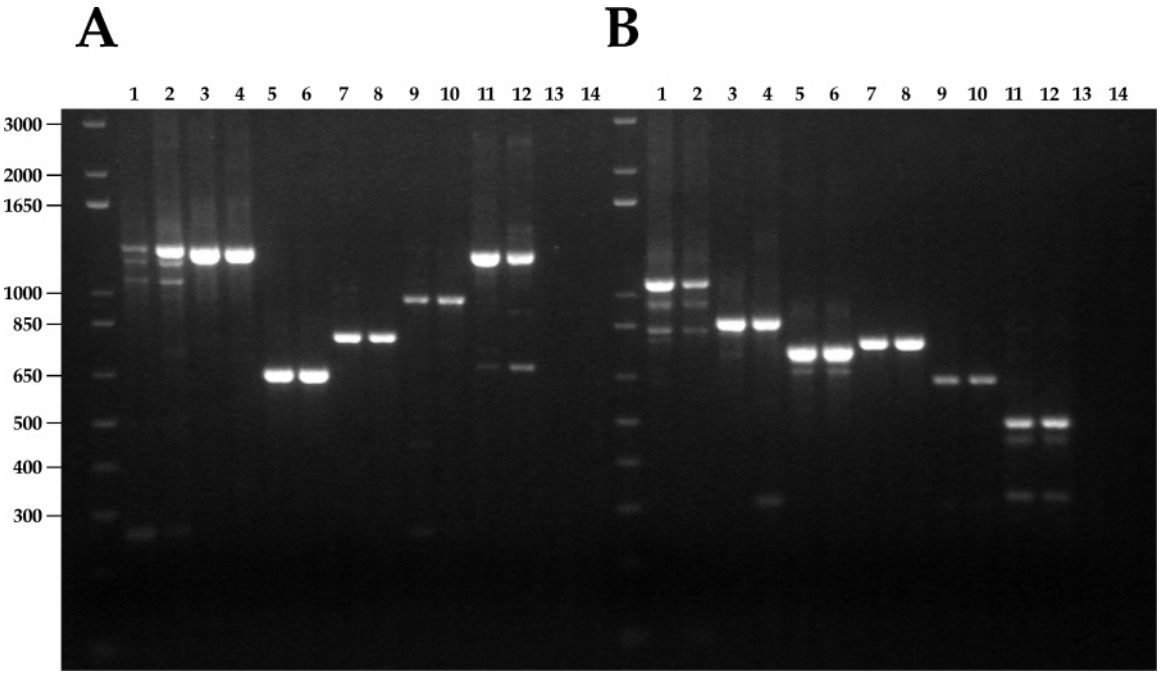

Figure 2. PCR amplification of the U1 and U2 snRNA genetic linkage: (A) U2–U1 spacer region amplification, (B) U1–U2 spacer region amplification; (1–2) *S. senegalensis*, (3–4) *S. solea*, (5–6) *S. kleinii,* (7–8) *S. lascaris,* (9–10) *D. cuneata*, (11–12) *M. azevia*, (13) *S. maximus*, (14) *P. flesus*. DNA ladder band sizes are indicated on the left.

snRNA units. Both primers were designed using the software Oligo 6.89 (Medprobe).

The PCR reactions for each primer pair were carried out in 25 μL of reaction volume: 1 μL of DNA template (~30−50 ng) was added to 24 μL of PCR mix consisting of 17.25 μL of sterile distilled water, 2.5 μL of dNTP mix (10 mM), 2.5 μL of 10× buffer, 1 μL of $MgCl_2$ (50 mM), 0.25 μL (1.25 units) of BioTaq DNA polymerase (Bioline, London, U.K.), and 0.5 μL of each primer (10 μM). The thermal cycle profile was identical for all sole species. An initial denaturation step of 96 °C for 2 min was followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. A final extension for 10 min at 72 °C was also included. PCR products were examined by electrophoresis on ethidium bromide-stained 2.0% agarose (Ecogen, Barcelona, Spain) gel and visualized via ultraviolet trans-illumination. The 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) was used as the molecular weight standard. Fragment lengths were determined using Gel-doc and Quantity One one-dimensional analysis software (Bio-Rad, Hercules, CA).

RESULTS

PCR Amplification of the U2−U1 snRNA Spacer Region. The U2−U1 spacer region was amplified using the primers SseU2•2 and SseU1•2 (Figure 2A). In *S. senegalensis*, three bands of 1252−1284, 1182−1202, and 1061−1078 bp in length were identified. On the other hand, a single PCR product of 1222−1232 bp was detected in the closely related species *S. solea*. A PCR product of 641−646, 786−792, and 960−967 bp was amplified in the species *S. kleinii*, *S. lascaris*, and *D. cuneata*, respectively. Two bands of 1203−1213 and 680−685 bp were obtained in *M. azevia*. Although some additional secondary PCR products could be observed, they did not affect the main pattern described for each species. No DNA amplification was detected in *S. maximus* (Scophthalmidae) and *P. flesus* (Pleuronectidae).

PCR Amplification of the U1−U2 snRNA Spacer Region. For comparison purposes, the DNA spacer region between the U1 and U2 snRNAs was amplified using the primers SseU1•1 and SseU2•1 (Figure 2B). A clear specific band pattern for each species was found. A three-band profile of 1041−1066, 950−963, and 830−836 bp was detected in *S. senegalensis*. On the contrary, a PCR product of 856−867 bp was identified in *S. solea*. Two bands of 731−746 and 670−677 bp were amplified in *S. kleinii*. A single PCR band was detected in *S. lascaris* (767−780 bp) and *D.* cuneata (633−636 bp). A three-band pattern of 496−512, 452−456, and 322−325 bp in length was obtained in *M. azevia*. No DNA amplification was detected in *S. maximus* and *P. flesus*.

To investigate the reproducibility and reliability of this methodology, both spacer regions were amplified in eight new individuals of *S. senegalensis* and six specimens of *S. solea*, *S. lascaris*, *D. cuneata*, and *M. azevia*. Similar band patterns to those described above were obtained in all cases (data not shown).

DISCUSSION

Tandemly repeated multigene families evolve according to a concerted evolution model. This characteristic makes it so that the different units become homogenized in sequence within a species rather than evolving independently among repeats. This feature has led to several authors to consider the usefulness of these tandemly repeated families as species-specific markers

(*6*−*8*). In this study, we employed a linkage between the U1 and U2 snRNAs for identification of six soles. To our knowledge, this is the first report of a genetic linkage being used for species identification.

Several surveys have focused on the authentication of flatfish products. However, most of them differentiated species from Pleuronectidae and Scophthalmidae families. Thus, a PCR−RFLP analysis of a cytochrome *b* mitochondrial gene fragment allowed discrimination among nine commercial species belonging to the Pleuronectidae, Scophthalmidae, and Soleidae families (*9*−*11*). However, only the sole *S. solea* was considered. A databank of IEF protein patterns of sarcoplasmic proteins was also developed for flatfish identification. Although 17 species could be authenticated by this procedure, only 4 sole species were included (*12*). Other authors differentiated *Reinhardtius hippoglossoides* and *S. solea* on the basis of the 5S rDNA (*7*). However, they did not consider closely related species of Soleidae family.

The genus *Solea* includes two subgroups: Solea (*S. solea* and *S. senegalensis*) and Pegusa (*S. kleinii* and *S. lascaris*). Our results revealed the U1−U2 snRNA spacer region as the most suitable marker to identify these four species due to higher PCR product size differences. This region also allowed identification of the more distant species, *D. cuneata* and *M. azevia*. Although smaller size differences among these six sole species were detected in the U2−U1 snRNA spacer region, development of both single PCRs as a dual-validation system in sole species identification is encouraged.

It has been stated that the analytical methods employed in species identification should be rapid and easy to perform without being cost prohibitive (*13*). The methodology described here seems to fulfill all these criteria. In addition, identification of species by two independent DNA analysis techniques is recommended. The method here described constitutes a powerful tool for the detection of mislabeling or fraudulent substitution of sole species and represents a valuable addition to the range of methodologies currently available for flatfish identification.

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