Acta Biologica Hungarica 65(2), pp. 132–143 (2014)
DOI: 10.1556/ABiol.65.2014.2.2

PCR-BASED IDENTIFICATION OF ADRIATIC SPECIMEN OF THREE SCORPIONFISH SPECIES *(SCORPAENIDAE, TELEOSTEI)*

J. M. Saju,[1]* Sz. Németh,[2*#] Réka Szűcs,[2]* Rashmi Sukumaran,[1,3##] Z. Lim,[1,4###] L. Wong,[3] L. Orbán[1,2,4]** and M. Bercsényi[2]**

[1]Reproductive Genomics Group, Strategic Research Program, Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore 117604
[2]Department of Animal Sciences and Breeding, Georgikon Faculty, University of Pannonia, Deák Ferenc u. 16, H-8230 Keszthely, Hungary
[3]School of Computing, National University of Singapore, Computing 1, 13 Computing Drive, Singapore 117417
[4]Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543

(Received: April 2, 2013; accepted: October 30, 2013)

The identification of three scorpionfish species, the black scorpionfish (*Scorpaena porcus* Linnaeus, 1758), the large-scaled scorpionfish (*S. scrofa* Linnaeus, 1758) and the small red scorpionfish (*S. notata* Rafinesque, 1810) is possible in adults by morphometry, but often problematic in juveniles due to their similar phenotypes. To develop a molecular species identification tool, first, we have analyzed the genetic similarity of the three species by a PCR-based 'blind method' that amplified bands from various locations of the genome. We found high levels of nucleotide similarity between *S. porcus* and *S. scrofa*, whereas *S. notata* showed a higher level of divergence from the other two species. Then, we have searched these patterns for differences between the genomes of Adriatic specimen of these three species and identified several species-specific products in two of them. For the third one a species-specific primer pair amplifying from the 16S ribosomal DNA was designed. One marker for each species was cloned, sequenced and converted into Sequence Characterized Amplified Region (SCAR) markers amplified by specific primer pairs. The SCAR markers amplified robust bands of limited variability from the target species, while no or only occasional weak products were obtained from the other two, proving that they can be used for molecular identification of these three species. These markers can help the conservation and future analysis of these three species as well as their possible selection programs for aquaculture purposes.

Keywords: Scorpaena – molecular species ID – 16S rDNA – fluoMEP – SCAR – molecular aquaculture

*These authors have contributed to the results presented in this paper equally.
**Corresponding authors; e-mail addresses: laszlo@tll.org.sg and bm@georgikon.hu
Present addresses: #Neptun Dive Center, Budapest, Hungary; ##Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, 1E, Kent Ridge Road, NUHS Tower Block Level 10, Singapore 119228; ###Department of Biological Sciences, National University of Singapore, Blk S1A, #06-20, 14 Science Drive 4, Singapore 117543.

INTRODUCTION

The monophyletic group of Scorpaeniformes contains a large number of marine teleost species [1], many with disputed taxonomic positions [20]. Members of the genus *Scorpaena* are quite similar morphologically and only a few morphological [3] or cytogenetic studies [2, 10] have been carried out to investigate their taxonomic or genetic relationships. The black scorpionfish (*Scorpaena porcus* Linnaeus, 1758), large-scaled scorpionfish (*S. scrofa* Linnaeus, 1758) and small red scorpionfish (*S. notata* Rafinesque, 1810) belong to this genus, together with over five dozens of other, mostly venomous species. All three of them are non-migratory and they live in the Mediterranean Sea and the European regions of the Atlantic ocean [6]. These are bottom-dwelling, solitaire predators, that are typically hiding in holes of the rocky bottom or among larger fields of seaweed or algae [5].

The black scorpionfish grows to a maximal total length (TL) of 25 cm [12]. Its body is drop-shaped, the head is large and the color might range from bright red to brownish with brown spots on the fins. The ctenoid scales of black scorpionfish are quite small and they are firmly attached to the epidermis. The scale count along the lateral line is higher than 55, usually 65–70. The dorsal fin contains 12 spines and 9 fin rays, whereas the pectoral one has 16–18 rays that reach to the second anal spine [12]. Typically, several dermal flaps can be found on the body and head of black scorpionfish, but never on the lower jaw [12].

The body shape and proportions of large-scaled scorpionfish are similar to those of the black one, however, its adult body color ranges from brick red to light pink with darker blotches on the body and fins. It grows to larger size than its relative: its maximal TL is 50 cm [13]. The number of ctenoid scales along its lateral line is about 45. The dorsal fin contains 12 spines and 9 soft fin rays, with a distinct notch between these two sets of rays. The pectoral fin contains 18–20 rays, none of them reaches the first anal spine [13]. Most individuals have several dermal flaps on the jaw; they can be used for their differentiation from the black scorpionfish.

The small red scorpionfish also looks very similar to the black one, but they differ in the length of supraorbital tentacles (they are usually shorter in the former). Its size is the smallest among the three (maximal TL: 20 cm, typical TL: 15 cm; [14]). The general color pattern of this species is red-brown and often there is a large black spot between spines 6–8 and 10–11. It has 43–46 ctenoid scales along the lateral line. The dorsal fin contains 12 spines and 9 soft fin rays, with a distinct notch between the two sets. The pectoral fin contains 17–19 rays, and they reach over the first anal fin [14].

Although adults of the three species could be separated by morphometry [19], the identification of their juveniles is often problematic due to their overlapping phenotypes. Therefore, the applicability of molecular approaches was considered, since they have proven to be useful for resolving problematic issues of marine fish biology (see e.g. [1, 7]). A recent publication described the systematics of five scorpionfish species, including the three analyzed here, based on morphological data and mitochondrial sequences [19]. The authors have analyzed a 783-bp long fragment of the 16S mitochondrial gene and found 0.7–7.6% differences among them in pair wise

comparisons, whereas the morphometric differences ranged between 10.9–11.2% [19]. They have noted that one of the major challenges for these marine species is to understand patterns of their genetic differentiation, because the absence of physical barriers and high dispersal potential provide little opportunity for allopatric speciation [19]. Improved knowledge of the reproduction of different scorpionfish species that live in a common habitat could contribute to the understanding of that process.

Since there is a growing demand for these scorpionfish species, especially for the large-scaled scorpionfish, their aquaculture is expected to develop in the upcoming years. The artificial propagation of the black scorpionfish has recently been developed [17]. Future selection programs of scorpionfishes for their aquaculture, including their interspecific hybridization, may benefit from their molecular identification.

The fluoMEP method [4] is a genotyping tool based on the Random Amplified Polymorphic DNA assay (RAPD; [15–17]), but combines its advantages with those of Amplified Fragment Length Polymorphism (AFLP; [21]). By using a fluorescently labeled "common primer" and a series of RAPD primers, DNA templates can be screened quickly and effectively for polymorphisms. Earlier, we have demonstrated the potential advantage of this technique for the identification of sex-associated DNA markers from guppy and rosy barb [8]. The present paper describes i) the analysis of intra- and interspecific genetic diversity of the three scorpionfish species described above; ii) isolation and characterization of species-specific DNA markers from their genome; and iii) their conversion into SCAR markers that allow for their molecular identification without sequencing.

MATERIALS AND METHODS

We have used 15, 17 and 5 individuals from black, large-scaled and small red scorpionfishes, respectively. All were from the vicinity of Pula (Croatia): some were collected from the Adriatic sea by dip net snorkeling (GPS coordinates 44.83335,13.83222 at Verudela/Pula or 44.77599,13.91453 Premantura, both Croatia), whereas the rest were purchased at the local market in Pula, Croatia, where they were sold as wild-caught specimen. Fin clip samples were collected from all individuals and stored in 75% ethanol until use.

Genomic DNA (gDNA) was extracted from the fin clips by phenol-chloroform extraction method. The resulting DNA was subsequently quantitated using 0.6% agarose gel and Quantity One software (BioRad). The DNA samples were aliquoted and stored at –80 °C until use. The identity of the samples were verified by sequencing of PCR-amplified 16S rDNA sequences (Genbank IDs: EU747076.1, EU747070.1 and EU747073.1) and by fluoMEP analysis [11]. Neither of the two methods has yielded any indication for potential hybrids among the specimen sampled.

For the analysis of 16S rDNAs, their PCR-amplification was performed essentially as described by [13]. For the first phase of fluoMEP analysis, species-specific pools were created by combining equal amounts of the gDNA samples diluted to 10 ng/µl from all the individuals in that species. Fluorescently labeled 'common

Table 1
Primers used in the fluoMEP assays

Primer	Primer sequence	GC content (%)
C117F*	NNGGAGTTTGCA	41.7
C118F	TCCTCTTGCAGA	50.0
C131F	AAACTCCTCCAAG	46.2
R-OPR-09**	TGAGCACGAG	60.0
R-OPY-15	AGTCGCCCTT	60.0
R-OPT-06	CAAGGGCAGA	60.0
R-OPT-07	GGCAGGCTGT	70.0
R-OP-M17	TCAGTCCGGG	60.0
R-OP-C18	TGAGTGGGTG	60.0
R-OP-AV06	CCCGAGATCC	70.0
R-OP-AT07	ACTGCGACCA	60.0

*C – common primer; F – FAM-labeled.
**R – RAPD primer (commercial 10mer).

primers' (C-prefix; Table 1) were custom-synthetized by 1stBase (Singapore), whereas 10mer RAPD primers (R-prefix; Table 1) were from Operon Biotechnologies (Cologne, Germany). The conditions of FluoMEP and analysis of labeled PCR products on a 3730xl DNA Analyzer were performed as described in [11]. The products were visualized as peaks using GeneMapper v3.5 software (ABI). Peak profiles were analyzed by the FluoMEP Marker Finder v1.0 software [18]. At first, pooled gDNA samples from the three species were screened with seven different fluorescently labeled common primers only (i.e. without RAPD primers). The best three common primers (C117F, C118F and C131F) with a large number of stable peaks were selected and tested on the DNA pools together with eight 10mer RAPD primers (the total of 24 combinations: see Table 1 for primer sequences). The six best combinations, which showed stable peak profiles with a large number of new peaks, were chosen for the analysis of the genetic diversity of the individual samples (see Fig. 1 for typical examples).

FluoMEP profiles were analyzed by the FluoMEP Marker Finder (FMF; [18]) software designed for this purpose. For the source code and software description please see https://sourceforge.net/projects/fmf/. The relatedness between the three scorpionfish species, was determined through calculation of Genetic Similarity (GS) Index based on the formula given by [21]:

$$GS_{ij} = 2N_{ij}/(N_i + N_j) \quad (\#1)$$

where N_{ij} is the number of peaks common in individuals i and j, and N_i and N_j are the total number of peaks in the individual profiles of i and j, respectively. FMF was

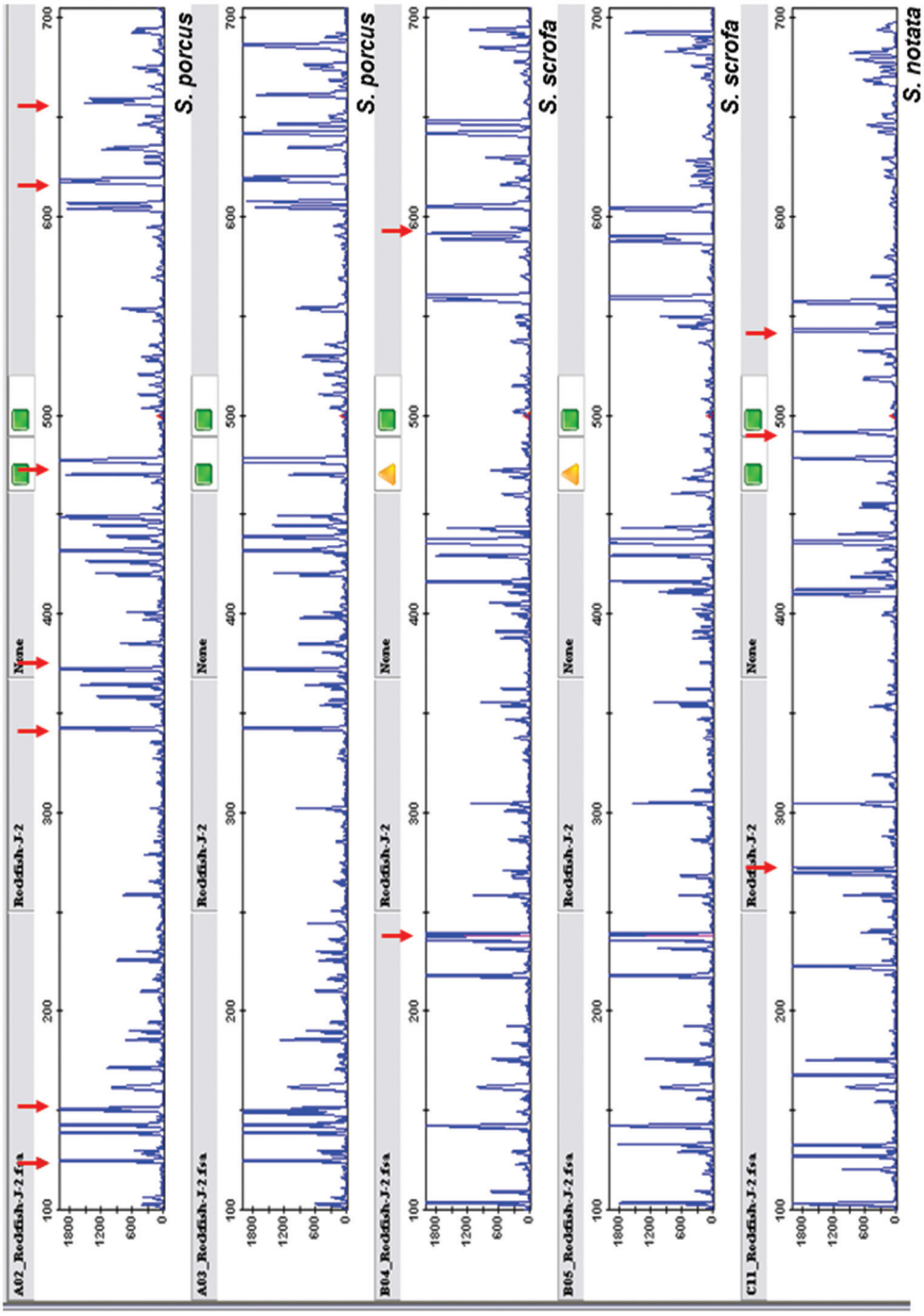

Fig. 1. Typical examples for the FluoMEP peak profiles of the genomic DNA of three scorpionfish species showing several differential peaks in every pattern. Peak profiles generated using primer combination C117F+OPR 09 are shown. The Y-axis indicates the intensity of peaks, whereas the X-axis shows their size. Arrowheads indicate differential peaks present in one species, but missing from the other two

modified to calculate the GS Index in a pairwise fashion for all profiles (see the above website for details).

Genetic dissimilarity indices were calculated from the fluoMEP profiles produced with six primer combinations according to Nei and Li [15]. The six sets of genetic similarity indices were converted to one PCA plot using R programming language (version 2.15.2). Firstly, each set of similarity indices were converted to a set of dissimilarity indices (1 – similarity index). Secondly, each set was converted to a set of points in space using the cmdscale package in R. Given a set of dissimilarities, the function *cmdscale* in the cmdscale package does multidimensional scaling on the dissimilarities, and returns a set of points in space such that the distances between the points are approximately equal to the dissimilarities given. Thirdly, as each set of points has a different scaling, they need to be aligned to each other to ensure there is no bias. Thus, five sets of points were aligned with the sixth (a randomly chosen reference set), using the vegan package in R. Given a reference set and a set of points, the function *procrustes* in the vegan package, rotates and scales the points such that it has maximum similarity (i.e., minimum sum of the squared differences between the points) with the reference set. Lastly, principal component analysis was performed on the set of aligned points using the prcomp package in R.

Species-specific DNA markers were identified by comparing peak patterns amplified from individual samples. For their isolation, FluoMEP products were amplified from pooled DNA samples from each species with the unlabeled version of selected common primers and RAPD primers. Products specific for each species were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Purified PCR products were cloned into pGEM-T Easy cloning vector (Promega), and sequenced from both sides with M13F&R primers by the BigDye assay kit (v3.1; Applied Biosystems) according to the manufacturer's recommendations. Sequences of the same marker were clustered by the Sequencher software (GeneCodes, Ann Arbor, MI, USA), their consensuses were submitted to GenBank (Scp1 – JM170466, Scn1 – JM170465). Specific primers were designed by the Primer3 software. Scs1 and Sc.cntr1 primers were designed based on 16s rDNA sequences downloaded from GenBank. The specificity of primers was confirmed by attempting PCR-amplification of the fragments from the other species.

RESULTS AND DISCUSSION

We have performed molecular analysis of a total of 37 specimens belonging to three different scorpionfish species (black, large-scaled and small red). The identity of the fish – that was originally determined by their overall phenotype – was verified by sequencing their 16S rDNA and screening their genome with a 'blind PCR method' that amplified several dozen bands through the consecutive use of six primer pairs. The mtDNA sequences have shown the expected results for all individuals analyzed (data not shown). Principal component analysis of the fluoMEP results has not indi-

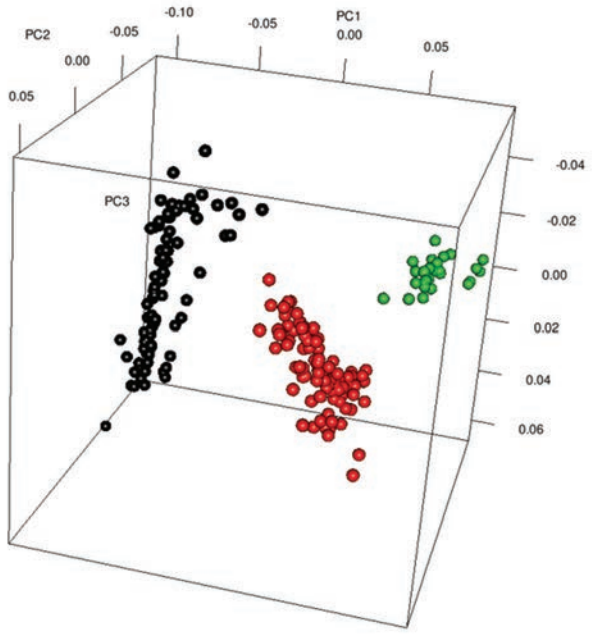

Fig. 2. PCA analysis of the genetic similarity data generated by FluoMEP. Genomic DNA was isolated from all 37 scorpionfish individuals and amplified by six different primer combinations. Genetic dissimilarity indices were calculated from the fluoMEP profiles according to Nei and Li [15] and converted to a PCA plot using a combination of cmdscale, vegan and prcomp packages in R. Labels: Black – black scorpionfish *(Scorpaena porcus);* red – large-scaled scorpionfish *(S. scrofa)*, and green – small red scorpionfish *(S. notata)*

cated any sign of hybridization, as the data points have formed three separate groups without intermediates (Fig. 2).

When the genetic relationships were calculated based on fluoMEP data from a single primer combination (C117/R09) using five representatives each of the three species, the average 'within-species' genetic similarity (GS) indices were as follows: 0.90 (range: 0.87–0.94) for black scorpionfish, 0.93 (0.87–0.98) for large-scaled scorpionfish and 0.90 (0.84–0.95) for small red scorpionfish. The 'between-species' average GS values were 0.85 (range: 0.80–0.94) between the black and large-scaled, 0.78 (range: 0.7–0.84) between the large-scaled and small red, as well as 0.76 (range: 0.7–0.83) between the black and small red scorpionfish species. (Calculations made with additional five different primer combinations showed similar results; data not shown.) Interestingly, these data differ from the GS values obtained earlier by others through the analysis of mitochondrial 16S rDNA sequences, where the corresponding values were 0.93, 0.99 and 0.93, respectively [19]. The difference might be due either to the different sample numbers (a total of 37 specimen here vs. 8 in [13]) or to the use of different DNA markers (multiple, mostly genomic DNA regions here vs. a single mitochondrial locus in [19]) or to potential genetic variations across scorpion-

Fig. 3. The nucleotide sequence of amplicons specific to A) black scorpionfish *(Scorpaena porcus; Scp1)*, B) large-scaled scorpionfish *(S. scrofa; Scs1)*, and C) small red scorpionfish *(S. notata; Scn1)*. Panel D shows the sequence of the internal control *(Sc.cmn1)*. Nucleotides underlined indicate the SCAR primers

fish populations of the three species sampled at different, far-away locations (Croatia vs. Turkey).

We have selected a species-specific peak from each of the following two fluoMEP profiles: C117/OPR09 – 610 bp, black scorpionfish; and C117/OPY15 – 510 bp for small red scorpionfish. A unique region from the mitochondrial 16S rDNA sequence was used to design primers specific for large scaled scorpionfish and from the same gene another region conserved in all three species was used for positive control. These four products were cloned and sequenced (Fig. 3). When compared against known sequences deposited in the GenBank nr database, the species-specific marker from black scorpionfish (Scp01 – GenBank ID: JM 170466) did not result in any BLASTn hits. As for the marker from the small red scorpionfish (Scn01 – JM 70465), the best BLASTn hit was a zebrafish genomic DNA sequence showing 85% identity at the nucleotide level for about a quarter of the query only, but not for the rest of the sequence, suggesting that this was also a novel sequence. As the marker from

Table 2
The sequence, product size and annealing temperature of species-specific and internal control SCAR primers for the three scorpionfish species analyzed

Species	SCAR primer	Sequence (5'-3')	Product size	Annealing temp. (°C)
Scorpaena porcus	*Scp1*	F- CTTATGATGTCAATATAGTGGC R- GGAGTAATCTTCATACTTCTGG	262 bp	55
Scorpaena scrofa	*Scs1*	F- AACCCTACACAGGAGTGTTG R- AAGACATTAGGGAAGGCAA	395 bp	55
Scorpaena notata	*Scn1*	F- CTCTTGTACCCGCGATGT R- AGAGCTTGTGGAAGTTTGG	330 bp	60
Control	*Sc.cntrl*	F- CGCGAAGGTAGCGCAATCAC R- TATCCCCGCTTCTGCACGG	130 bp	55

S. scrofa showed 100% identity with *S. elongata* 16s ribosomal RNA sequence, when compared against the sequences in the GenBank nr database, the advantage of this marker might be restricted to the separation of the three species analyzed here. Additional testing would be required before it could be utilized in a broader context among additional Scorpaena species, especially *S. elongata*. Specific primer pairs designed to amplify the putative markers (Table 2) were then tested on representatives of the three different Scorpaena species. Marker Scp1 produced a 262 bp band from all black scorpionfish individuals, but not from the other two species. Similarly, Scs1 and Scn1 both produced species-specific bands of 395 bp and 330 bp length from large-scaled and small red scorpionfish, respectively (Fig. 4). A very weak product of entirely different size was observed in a few individuals of the other species when these two markers were tested for cross-species amplification.

In summary, our data reveal the presence of distinct genetic differences between these three morphologically similar species, and identify markers that will make it possible to identify and differentiate their representatives with rapid PCR-based tests. It was shown earlier, that information about genetic diversity can provide insights into the demographic history and origin of the analyzed population or taxa [11] contributing to the understanding of evolving processes of diversity within and among populations. However, at the moment, the applicability of these tools is proven only for specimen collected from the Pula region of the Adriatic Sea. Samples from other locations must be collected and analyzed to find out, whether they can be extended for more general use.

Earlier, a study has shown that the 16S rDNA gene is suitable to design oligonucleotide probes that are able to differentiate eleven fish species, including the black and large-scaled scorpionfish from European locations through a microarray-based approach [7]. Our method offers a more cost-effective and faster option for the differentiation of these scorpionfish species.

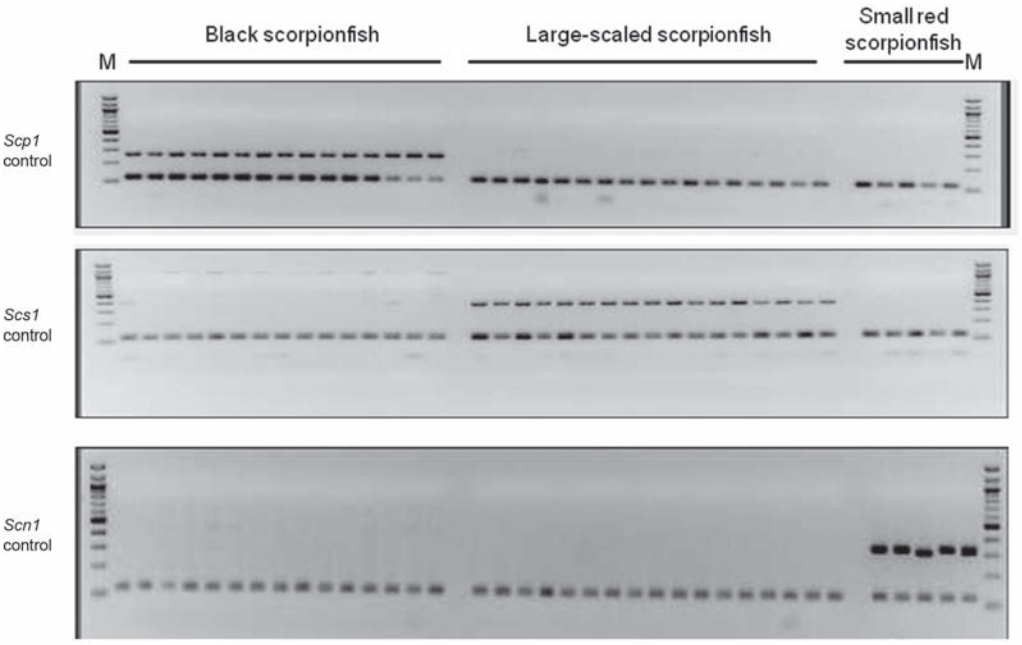

Fig. 4. Each species-specific primer pair amplified a distinct product from the target species, but not from the other two species. The internal control band *(Sc.cmn1)* was amplified from all representatives of every species. M: 100 bp DNA molecular marker

Additional molecular markers (e.g. microsatellite and SNPs) would also be needed to design effective conservation strategies for these species, and possibly for their close relatives as well. It would be quite important, since due to the increasing interest towards them as table fish, some of these scorpionfish species appear to be overharvested [9]. Future aquaculture of these species might also promote preservation of their natural stock by easing the fishing pressure on them. The methods developed here for the molecular identification of these species may contribute to their efficient culture and breeding in the future. Since the phenotypic differences observed in adults can hardly be used in juveniles, their molecular identification can help the early identification of these species (and even some of their potential hybrids) and therefore aid their protection as well. These results indicate that fluoMEP might be a useful tool for molecular identification of species that cannot be differentiated easily based on their phenotypes.

Earlier, we have also developed *in vitro* fertilization methods for the black scorpionfish [9]. We hope that our efforts on the front of propagation and protection will eventually help the stabilization of natural populations of scorpionfishes in the Adriatic Sea and they may contribute to the development and improvement of aquaculture technologies for these species in the future.

ACKNOWLEDGEMENTS

The authors would like to thank the help of Ivesa Neven during sample collection. Financial support from the following organizations is gracefully acknowledged: Hungarian Scientific Research Fund (OTKA) grant 77681 (for MB) and the Strategic Research Program of the Temasek Life Sciences Laboratory (for LO).

REFERENCES

1. Borsa, P., Collet, A., Durand, J. D. (2004) Nuclear-DNA markers confirm the presence of two anchovy species in the Mediterranean. *C. R. Biol. 327,* 1113–1123.
2. Caputo, V., Sorice, M. (1999) Electrophoretic studies on some species of Scorpaeniformes (Teleostei, Percomorpha). *Italian J. Zool. 66,* 79–85.
3. Caputo, V., Sorice, M., Vitturi, R., Magistrelli, R., Olmo, E. (1998) Cytogenetic studies in some species of Scorpaeniformes (Teleostei: Percomorpha). *Chromosome Res. 6,* 255–262.
4. Chang, A., Liew, W. C., Chuah, A., Lim, Z., Lin, Q. et al. (2007) FluoMEP: a new genotyping method combining the advantages of randomly amplified polymorphic DNA and amplified fragment length polymorphism. *Electrophoresis 28,* 525–534.
5. Hureau, J. C., Litvinenko, N. I. (1986) Scorpaenidae. In: Whitehead, P. J. P., Bauchot, M. L., Hureau, J. C., Nielsen, J., Tortonese, E. (eds) *Fishes of the North-Eastern Atlantic and the Mediterranean.* UNESCO, Paris, France, pp. 1211–1229.
6. Knowlton, N. (2000) Molecular genetic analyses of species boundaries in the sea. *Hybdrobiologia 420,* 73–90.
7. Kochzius, M., Nölte, M., Weber, H., Silkenbeumer, N., Hjörleifsdottir, S. et al. (2008) DNA microarrays for identifying fishes. *Mar. Biotechnol. 10,* 207–217.
8. Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R. et al. (2012) Polygenic sex determination system in zebrafish. *PLoS ONE 7,* e34397.
9. Matic-Skoko, S., Dulcic, J., Krajevic, M., Tutman, P., Pallaoro, A. (2008) Recent status of coastal ichthyocommunities along the Croatian coast. In: *43rd Croatian and 3rd International Symposium on Agriculture, 18/02/2008–21/02/2008, Opatija, Croatia,* pp. 737–741.
10. Mesa, G. (2005) A revised description of *Scorpaena maderensis (Scorpaenidae)* by means of meristic and morphometric analysis. *Mar. Biol. Assoc. UK 85,* 1263–1270.
11. Milligan, B. G., Leebens-Mack, J., Strand, A. E. (1994) Conservation genetics: beyond the maintenance of marker diversity. *Mol. Ecol. Notes 3,* 423–435.
12. MSIP (2013) Marine Species Identification Portal: *Scorpaena porcus* http://species-identification.org/species.php?species_group=fnam&id=2129&menuentry=soorten (accessed on 25th of March, 2013).
13. MSIP (2013) Marine Species Identification Portal: *Scorpaena scrofa* http://species-identification.org/species.php?species_group=fnam&id=2130&menuentry=soorten (accessed on 25th of March, 2013).
14. MSIP (2013) Marine Species Identification Portal: *Scorpaena notata* http://species-identification.org/species.php?species_group=fnam&id=2128&menuentry=soorten (accessed on 25th of March, 2013).
15. Nei, M., Li, W.-H. (1979) Mathematical modelling for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA 76,* 5269–5273.
16. Nelson, J. (2006) *Fishes of the World.* Wiley and Sons, New York, NY, USA, 624 p.
17. Németh, S., Budaházi, A., Szűcs, R., Bercsényi, M. (2008) Out of the season artificial propagation of the black scorpionfish *(Scorpaena porcus)* in captivity. *Mediterr. Aqua J. 1,* 1–8.
18. Sukumaran, R. (2008) *Computational analysis of genotypes produced by fluoMEP. Honours Year Project Report.* National University of Singapore, Singapore, 88 p.
19. Turan, C., Gunduz, I., Gurlek, M., Yaglioglu, D., Erguden, D. (2009) Systematics of Scorpaeniformes species in the Mediterranean Sea inferred from mitochondrial 16S rDNA sequence and morphological data. *Folia Biol. (Krakow) 57,* 219–226.

20. van der Land, J., Costello, M. J., Zavodnik, D., Santos, R. S., Porteiro, F. M. et al. (2001) Pisces. In: Costello, M. J., Emblow, C., White, R. (eds) *European Register of Marine Species: A Check-List of the Marine Species in Europe and a Bibliography of Guides to their Identification. Collection Patrimoines Naturels, 50.* Muséum national d'Histoire naturelle, Paris, France, pp. 357–374.
21. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res. 23,* 4407–4414.
22. Welsh, J., McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res. 18,* 7213–7218.
23. Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res. 18,* 6531–6535.
24. Williams, J. G., Reiter, R. S., Young, R. M., Scolnik, P. A. (1993) Genetic mapping of mutations using phenotypic pools and mapped RAPD markers. *Nucleic Acids Res. 21,* 2697–2702.