GENETIC TRACEABILITY OF SELECTED POPULATIONS OF THE YELLOWSTRIPE SCAD, *SELAROIDES LEPTOLEPIS* (ACTINOPTERYGII: PERCIFORMES: CARANGIDAE), BASED ON THE ANALYSIS OF MICROSATELLITE DNA—CELFISH PROJECT—PART 3

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Background. Monitoring the genetic diversity of fish populations can provide information necessary to determine fishing quotas for the commercially caught species. One of the species subjected to intensive exploitation is the yellowstripe scad, *Selaroides leptolepis* (Cuvier, 1833), commonly occurring in the coastal waters of Vietnam, Cambodia, Malaysia, and Indonesia. This paper is the third part of a larger project: "Development of a genetic-based system for identification of food products from fisheries and aquaculture introduced to the European Union customs area".

Materials and methods. Fin fragments of the yellowstripe scad, *Selaroides leptolepis*, were obtained in 2012 and 2013 from local markets in Vietnam (SVN), Cambodia (SKH), Malaysia (MMY), and Indonesia (MID). The analyses focused on the amplification and sequencing of a fragment of the nuclear rhodopsin gene which utilized as identification label. Subsequently, in the obtained SVN, SKH, MMY, and MID samples, 11 microsatellite regions (SRR) were analysed. Sequence analyses were performed using the BioEdit and BLAST software, while the analysis of the obtained SRR data was conducted using the GenAlEx software.

Results. The analysis of the obtained loci variants divided the investigated populations into two genetically homogeneous groups: the Vietnamese–Indonesian group and the Malaysian–Cambodian group. The analysis of genetic diversity revealed statistically significant deviations from the Hardy–Weinberg equilibrium in the investigated populations and indicated the Vietnamese population as the most stable, while the Malaysian population as the least stable.

Conclusions. The study provides information on the genetic diversity of the investigated populations and allows assignment of the captured fish to the geographical regions specified. Moreover, indicate that among analysed populations the safest populations were those caught in the local fisheries (continental shelf) of Vietnam and Cambodia due to their high mean genetic diversity.

Keywords: microsatellite, genetic variation, seafood authentication, seafood counterfeiting

INTRODUCTION

Genetic identification of food ingredients is an important method that enables the traceability and confirmation of authenticity of fish products. With regard to common cases in which valuable fish species are replaced by low-value equivalents, it is necessary to provide reliable methods which would precisely identify those fish species that are valuable for the economy. These methods help to eliminate counterfeit products from markets but also provide tools that might be used by governmental units which control food safety, including custom services. Population identification using molecular methods also provides information about catch region (Anonymous 2015a), which is highly useful in protection strategies of overfished populations, or catches of the IUU group (Illegal, Unreported, and Unregulated).

The results presented in this paper are part of the work carried out within the scientific research project "Development of a genetic-based system for identification of food products form fisheries and aquaculture introduced to the European Union customs area" funded by the European Union within 2011–2013.

Species of the family Carangidae are the fifth most intensively exploited fish group, following gadoids (Gadidae) (Anonymous 2015b). The yellowstripe scad, *Selaroides leptolepis* (Cuvier, 1833), is a marine pelagic species occurring in the waters of the Indian Ocean and Pacific, from the Persian Gulf to the Philippines, northern Japan, as well as the south coast of the Arafura Sea and Australia. The maximum body size is 22 cm and the weight does not exceed 625 g (Froese and Pauly 2014). The species is the only representative of the genus *Selaroides*, (family Carangidae), that feeds mainly on mussels and snails, which classifies the species as predatory. In terms of human consumption, it is a species of an average taste value of the meat, consumed mainly as dried fish or used for the preparation of stuffing for burgers, but also sold locally as whole fish, either fresh or frozen. Studies have shown that due to the high content of red meat combined with the high content of fat and mioglobin, the species is used for the production of high-quality surimi in Asia (Arfat and Benjakul 2012).

Selaroides leptolepis is known in Malaysia and Indonesia under the local name of "kuning" and its market price is within the average values for fish for human consumption (Sumaila et al. 2007). In the European Union, the yellowstripe scad was imported as part of the scad fish group without a clear indication of a particular species. Within 5 years, there were 57 reports of this species mainly from New Zealand and Sri Lanka (Skrzypczak unpublished). Global catches of the species in 2011 were approximately 200 000 t, while in 2012 the value decreased to 198 600 t (Anonymous 2015b).The natural resources of the yellowstripe scad are most intensively exploited in Malaysia (41 341 t) and United Arab Emirates (3196 t). According to Tan and Yap (2006), the species is the third most intensively captured species after *Rastrelliger kanagurta* (Cuvier, 1816) and *Decapterus maruadsi* (Temminck et Schlegel, 1843). It is also reported as trawling bycatch in Australia (Dell et al. 2009). Among the nets used for fishing are mainly seines, trawls, trapping devices, and nets with a mesh size of up to 20 mm (typically 15 mm). The species status according to IUCN is *not evaluated*, i.e., not assessed according to the IUCN criteria. The population of that species is rather poorly characterized which is probably due to underestimation of the nutritional value of the *S. leptolepis* meat, as well as the limited activities for the protection of stocks in countries such as Indonesia, Malaysia, and Vietnam, where the species constitutes native ichthyofauna. These countries are mainly focused on aquaculture, whereas the commonly fished wild species are not a subject of population studies. In the Philippines, the yellowstripe scad is defined as one of the most often caught species, apart from *Decapterus macrosoma* Bleeker, 1851; *Selaroides leptolepis* (Cuvier, 1833); and *Rastrelliger kanagurta*, although a population analysis allowed the conclusion that the species is exploited too heavily (Rueca et al. 2009). According to Tandon (1964) "overfishing of marine species and ineffective reintroduction necessitate scientific research aimed at species identification, a well as the spatial and temporal modes of spreading of ichthyofauna stocks." The economic significance of the species is high as it is inexpensive and although it is captured using nets with too large mesh (no selectivity of fishing), it still covers the bulk of the protein demand of local populations. The potential for confusion with other species (Gunn 1990) is primarily due to the morphological similarity and overlapping of the areas of occurrence of 2 species: *Selar boops* (Cuvier, 1833) and *Selar crumenophthalmus* (Bloch, 1793). The latter is particularly valued in northern Australia, reaching very high prices. Visual identification of such products is very difficult and often impossible. Past experience and controls carried out by various institutions indicate that, for a variety of reasons, the declared fish species does not always correspond to the contents of the package (Jacquet and Pauly 2008).

The lack of information necessary to assess the genetic stability of the yellowstripe scad, a relevant species from both economic and ecological point of view, convinced the authors to perform genetic analysis of selected populations of the species.

MATERIAL AND METHODS

The study material consisted of fin sections from juvenile individuals of the yellowstripe scad, *Selaroides leptolepis*, obtained in 2012 and 2013 from local markets in Phan Thiet, Vietnam (SVN), Phnom Penh, Cambodia (SKH), Malacca, Malaysia (MMY), and Jakarta, Indonesia (MID).

The collected fin fragments of the SVN ($n = 8$), SKH ($n = 7$), MMY ($n = 11$), and MID ($n = 8$) populations were dried for transport and then conserved in 5 mL tubes containing silicone medium. DNA isolation from fin sections was performed using the peqGOLD Tissue DNA Mini Kit (peqlab) following the attached protocol. The qualitative and quantitative assessment of the isolates was carried out by electrophoresis in 1.5% agarose gel followed by spectrophotometric measurements using the NanoDrop 2000 instrument (Thermo Scientific). For all investigated samples, PCR amplification of a fragment of the nuclear rhodopsin gene (RH1) was conducted using the primers Rod-F2W and Rod-R4n in accordance with the methodology proposed by Sevilla et al. (2007). Subsequently, in the obtained SVN, SKH, MMY, and MID samples, 11 microsatellite regions (SRR) were analysed: Orla 21-231, Orla 16-32, Orla 2-91, Orla 22-135, Orla 5-131, Orla 12-160, Orla 16-185, Orla 8-113, Orla 9-204, Orla 9-38, and Orla 20-134. The amplification of the regions was conducted in accordance with the methodology used by Gotoh et al. (2013). The standard PCR profile was modified to that of touchdown PCR: 94°C for 5 min, then 30 cycles of denaturation (94°C, 45 s), primer annealing (62°C, 30 s × 7 and 59°C, 30 s × 27), and elongation (72°C, 20 s), followed by final elongation at 72°C for 7 min. The reaction mix was prepared based on the GoTaq®G2 Hot Start polymerase (Promega), Green Master Mix 12.5 µL, F-primer 0.5 µL (10 pmol · µL^{-1}), R-primer

0.5 µL (pmol · µL^{-1}), H$_2$O DEPC 10.5 µL, DNA 1 µL. All PCR amplifications were carried out on a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) and the results were evaluated by amplicon electrophoresis in 2% agarose gel. Sequencing of the obtained RH1 PCR products was ordered from Genomed, Warsaw, Poland. Analysis of thus obtained sequences was performed using the BioEdit software and BLAST (Altschul et al. 1990, Hall 1999). The separation of SRR fragments was carried out using the SEQ 8000 sequencer (Beckman Coulter), while the analysis of the obtained SRR data was conducted using the GenAlEx software (Peakall and Smouse 2012).

RESULTS

Comparing the nucleotide sequences of the rhodopsin gene from the SVN, SKH, MMY, and MID samples in the BioEdit software revealed no differences between SVN and MID, and between MMY and SKH. Differences were observed between: SVN and SKH, SVN and MMY, MID and SKH, and MID and MMY. The RH1 sequences were compared with those submitted to GenBank using BLAST and it was found that there was no 100% overlap between the compared sequences and those from GenBank. No sequences originating from this species were also found. A preliminary analysis of the SSR markers demonstrated that all investigated loci in the four populations were polymorphic and, therefore, qualified for further analysis. The total number of alleles for the SVN, SKH, MMY, and MID populations was 89, 85, 114, and 70, respectively, while the total number of alleles of the investigated loci was 4–14 (mean 8.09) for the SVN population, 2–10 (mean 7.73) for the SKH population, 2–17 (mean: 10.36) for the MMY population, and 2–9 (mean 6.36) for the MID population. The mean values of allele distribution with the expected heterozygosity (He) marked for all populations are shown in Fig. 1. The observed heterozygosity (Ho) which determines the ratio between the heterozygous genotypes and 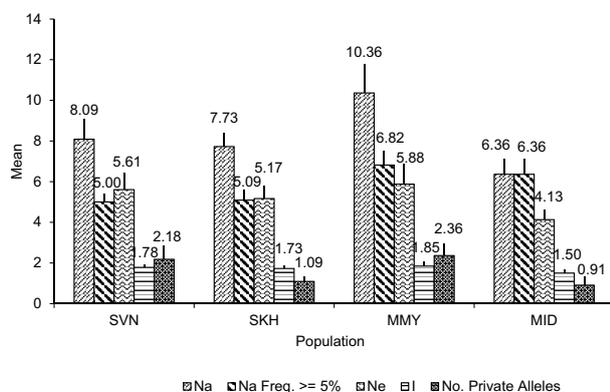the total number of genotypes per locus was between 0.36 and 0.91 (mean 0.65) for the SVN population, between 0.33 and 1.00 (mean 0.65) for the SKH population, between 0.00 and 0.95 (mean 0.70) for the MMY population, and between 0.11 and 0.79 (mean 0.63) for the MID population. Genetic diversity (He) was between 0.44 and 0.85 (mean 0.77) for the SVN population, between 0.48 and 0.88 (mean 0.76) for the SKH population, between 0.1 and 0.92 (mean 0.47) for the MMY population, and between 0.11 and 0.82 (mean 0.69) for the MID population (Table 1). There were statistically significant deviations from the Hardy–Weinberg equilibrium: in the SVN population for loci Orla 16-32 and Orla 12-160 at $P < 0.001$ and $P < 0.01$, respectively; in the SKH population for loci Orla 9-38, Orla 16-32 and Orla 20-134 at $P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively; in the MMY population for loci Orla 2-91, Orla 12-160, Orla 9-38 and Orla 20-134 at $P < 0.001$, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; in the MID population for loci Orla 12-160 at $P < 0.05$. Average genetic diversity of the SVN, SKH, MMY, and MID populations of yellowstripe scad, *Selaroides leptolepis* was presented in the Table 2. The process of assigning randomly selected individuals to the baseline populations, based on the results of the species identification test, correctly indicated the origin of 75% of the analysed samples. The test was carried out using the significance level of 0.05 (Figs. 2 and 3).
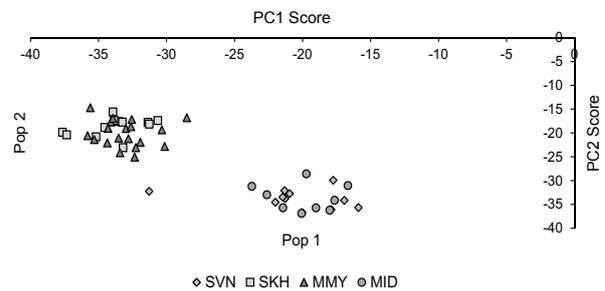

Fig. 2. Principal component analysis based on the genetic distances between individuals from the SVN, SKH, MMY, and MID populations of yellowstripe scad, *Selaroides leptolepis*; SVN = Vietnam, SKH = Cambodia, MMY = Malaysia, MID = Indonesia

DISCUSSION

Selaroides leptolepis is a species commonly occurring in the waters of the Indo–Pacific, quite extensively exploited in such countries as Malaysia (Chong et al. 2010) and the Philippines (Bacalso and Wolff 2014). In general, in countries with access to fresh fish of this species, they are consumed directly after catching, however, due to their affordable price, they have also been seen in Europe. In Germany, available is a "snack" product that is produced by smoking (E150), salting, and drying fillets of the yellowstripe scad. The price is 26.39 EUR per kg. However, the quality of the meat and its sanitary and parasitological evaluation pose a problem. According to Agusa et al. (2007), in the fish of the family Carangidae, originating from the coastal waters of Malaysia, high concentrations

Fig. 1. Distribution of alleles in 11 microsatellite loci for the SVN, SKH, MMY, and MID populations of yellowstripe scad, *Selaroides leptolepis*; SVN = Vietnam, SKH = Cambodia, MMY = Malaysia, MID = Indonesia; Na = number of different alleles, Ne = number of effective alleles, I = Shannon's Information Index

Summary of Chi-square test for the assessment of the Hardy–Weinberg equilibrium in the SVN, SKH, MMY and MID populations of yellowstripe scad, *Selaroides leptolepis*

Locus	Population	df	χ^2	P	Population	df	χ^2	P
Orla 21-231	SVN	78	59.041	0.946	MMY	45	38.317	0.749
Orla 16-32	SVN	21	47.972	0.001***	MMY	28	34.130	0.197
Orla 2-91	SVN	36	47.300	0.098	MMY	120	175.201	0.001***
Orla 22-135	SVN	28	31.005	0.317	MMY	136	113.531	0.920
Orla 5-131	SVN	91	91.056	0.479	MMY	136	137.222	0.455
Orla 12-160	SVN	45	77.000	0.002**	MMY	55	78.796	0.019*
Orla 16-185	SVN	36	36.667	0.438	MMY	28	14.711	0.981
Orla 8-113	SVN	10	10.368	0.409	MMY	45	27.431	0.982
Orla 9-204	SVN	15	9.625	0.843	MMY	28	26.061	0.570
Orla 9-38	SVN	6	11.688	0.069	MMY	21	42.708	0.003**
Orla 20-134	SVN	6	7.639	0.266	MMY	1	19.000	0.000***
Orla 21-231	SKH	28	21.000	0.825	MID	21	18.163	0.639
Orla 16-32	SKH	28	56.328	0.001**	MID	6	8.796	0.185
Orla 2-91	SKH	45	44.833	0.479	MID	28	25.000	0.628
Orla 22-135	SKH	45	33.387	0.899	MID	36	38.000	0.378
Orla 5-131	SKH	45	55.053	0.145	MID	36	31.867	0.666
Orla 12-160	SKH	28	37.083	0.117	MID	36	56.000	0.018*
Orla 16-185	SKH	21	6.188	0.999	MID	15	17.438	0.293
Orla 8-113	SKH	28	30.080	0.359	MID	10	9.610	0.475
Orla 9-204	SKH	21	23.429	0.323	MID	28	25.240	0.615
Orla 9-38	SKH	21	34.720	0.03*	MID	1	0.031	0.860
Orla 20-134	SKH	1	7.252	0.007**	MID	3	1.331	0.722

df = degrees of freedom, calculated as df = [Number of alleles(Number of alleles -1)]/2, χ^2 = Chi-squared statistic χ^2, P = probability of obtaining the observed Chi-squared value (*P <0.05, **P < 0.01, ***P < 0.001).

Average genetic diversity of the SVN, SKH, MMY, and MID populations of yellowstripe scad, *Selaroides leptolepis* based on the analysis of 11 SSR loci

Population	n	Na	Ne	I	Ho	He	uHe	F
SVN	11	8.091 ± 1.013	5.607 ± 0.829	1.785 ± 0.147	0.653 ± 0.072	0.772 ± 0.040	0.809 ± 0.042	0.159 ± 0.077
SKH	12	7.727 ± 0.675	5.170 ± 0.612	1.729 ± 0.135	0.645 ± 0.084	0.763 ± 0.040	0.796 ± 0.042	0.175 ± 0.098
MMY	19	10.364 ± 1.410	5.877 ± 1.014	1.846 ± 0.207	0.697 ± 0.081	0.741 ± 0.070	0.761 ± 0.072	0.134 ± 0.095
MID	9	6.364 ± 0.766	4.131 ± 0.479	1.501 ± 0.169	0.626 ± 0.062	0.687 ± 0.067	0.728 ± 0.071	0.064 ± 0.053

Values are mean ± standard error; n = number of analysed fish, Na = number of alleles, Ne = number of effective alleles, I = Shannon's Information Index, Ho observed heterozygosity, He = expected heterozygosity, uHe = unbiased expected heterozygosity, F = Fixation Index (Inbreeding Coefficient).

of heavy metals, such as V, Cr, Zn, Pb, and Bi, are noted. Representatives of the same family from Indonesian fisheries were characterised by the highest mean concentration of Sn and Hg. The authors suggested a negative impact of the consumption of those fish on the health of the general population of Southeast Asia. Taking into account the occurrence of the product on the EU market, registered under the number EA8550, its safety should be a matter of particular attention. However, in the Rapid Alert System for Food and Feed of the European Union (RASFF), the only time when the quality of products from this species imported to Germany from Vietnam was questioned was in 2011 (as reported by the Border Veterinary Surgeon in Szczecin). Questionable is also the quality of the meat from *S. leptolepis* as feedstock for the production of surimi or other low-processed products commonly consumed in Asian countries. The study by Peng et al. (2011) confirmed that the species is a host for muscle larvae of *Anisakis* sp., *Contracaecum* sp., and *Porrocaecum* sp. These parasites are particularly dangerous to people consuming raw fish
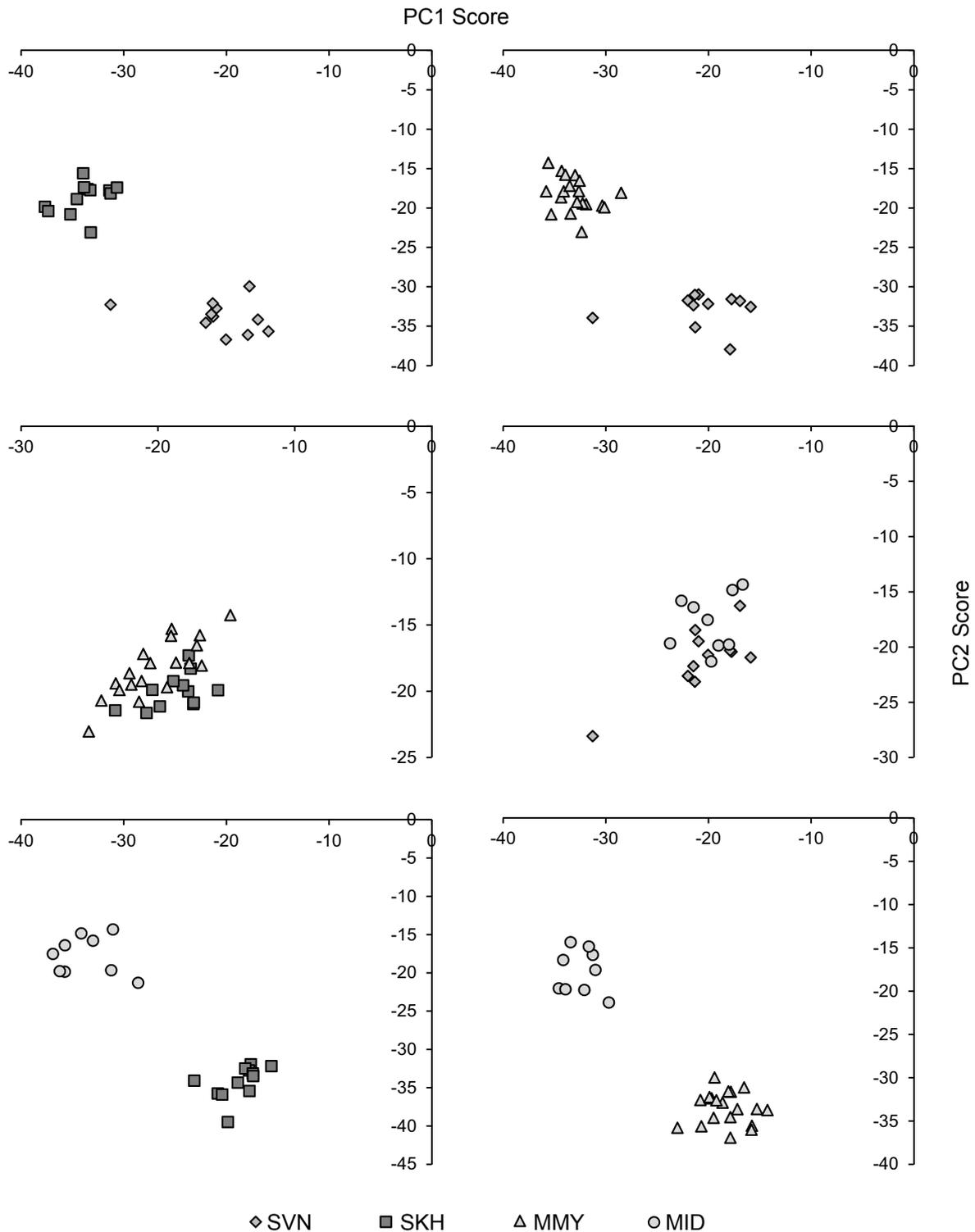
Fig. 3. Principal component analysis of inter-population genetic distances of yellowstripe scad, *Selaroides leptolepis*; SVN = Vietnam, SKH = Cambodia, MMY = Malaysia, MID = Indonesia

meat as they may cause a life-threatening parasitic disease known as anisakiosis (herring-worm disease, cod-worm disease). Studies indicate that it is not a species whose meat can be considered as of high quality, however, despite poor toxicological parameters, it is one of the main sources of proteins in the countries of Southeast Asia.

In the case of a species so intensively exploited, it is necessary to determine the characteristics of the population and locate safe places for fishing. This study demonstrated that populations caught in Vietnam and Indonesia, as well as Malaysia and Cambodia, are genetically homologous. Since the Malaysian population is in the worst condition and its consumption is harmful, a temporary restriction of fishing or additional monitoring should be introduced. The species is described as migratory between freshwater and ocean in both directions, its migrations are cyclic and its

migration range is more than 100 km. It appears, however, that the analysis of microsatellite loci confirms the hypothesis of isolation of schools/populations that do not mix genetically, which may be associated with the spatial or temporal separation of spawning of specific schools, or the existence of another mechanism of isolation (Figs. 2 and 3). In turn, the analysis of the mean genetic diversity of the populations, referred to as intraspecific variability, revealed the highest stability in the population caught in the coastal waters of Vietnam and the lowest stability in that of the coast of Malaysia. It is probable that the populations of this species are undergoing genetic regression due to overfishing and poor environmental conditions of the waters in the coastal zone of Indo–Pacific countries, especially near metropolitan areas, such as Jakarta, that do not have proper sewer systems. As was observed in this study, among the investigated fishing locations, the safest populations were those caught in the local fisheries (continental shelf) of Vietnam and Cambodia due to their high mean genetic diversity.

The exploitation of fisheries in the zone of continental shelf without respecting catch limits or permitted and safe catching methods causes degradation of unique ecosystems, such as the coastal coral reefs in the Philippines, or destabilizes the entire fish populations. In the case of *Selaroides leptolepis* of the Indo–Pacific region, there are still strong and genetically stable populations which should be particularly monitored by fishing law, while populations undergoing genetic degradation or constituting a part of diet dangerous to human health should be subject to separate fishing regulations and increased attention of veterinary services.

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