www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18: 729-738 (2018) DOI: 10.4194/1303-2712-v18_5_08

RESEARCH PAPER

A Report on Ecotypes of *Setipinna phasa* (Hamilton-Buchanan, 1822) from Indian Waters

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Received 07 February 2017
Accepted 18 September 2017

Abstract

Species of genus *Setipinna* are major contributors in total landings of anchovies. Under the genus, four species have been reported from Indian waters. Among them, *Setipinna phasa* is a dominant species, distributed in Ganga river system from Allahabad, Uttar Pradesh to West Bengal coast of India. The identity of *Setipinna* species was found to be ambiguous that needed validation; hence, in the present study, *Setipinna* species were collected from Diamond harbour and Digha Mohana, West Bengal, India for differentiation based on morphological, meristic and molecular traits. ANOSIM, SIMPER and principal component analysis of twenty three morphometric characters revealed difference among the three variants of *S. phasa*. While DNA barcoding using mitochondrial cytochrome *c* oxidase subunit I and 16S ribosomal RNA gene showed very less divergence values (0.00-0.008) among these variants. Further phylogenetic analysis using combined DNA sequences (COI and 16S rRNA) displayed clustering of these variants of *S. phasa* with significant bootstrap values. Hence the study concludes that even though variants of *Setipinna* displayed considerable morphological variations, they are not distinct genetically from *S. phasa* species. Thus the variants of the species seem to be ecotypes of *S. phasa* and a base in the evolution of others *Setipinna* species.

Keywords: Ecotype, *Setipinna*, DNA barcoding, India.

Introduction

Species of genus *Setipinna* Swainson, 1839 (Order: Clupeiformes, family: Engraulidae) are distributed throughout Indo-West Pacific from eastern coast of India to Papua, New Guinea. These pelagic fishes are amphidromus / potamodromus and form one of the major fisheries along the North-East coast of India (Saigal, Mitra, & Karmarkar, 1987). Among eight species of *Setipinna* reported globally, four species viz., *S. brevifilis* (Valenciennes, 1848), *S. phasa* (Hamilton-Buchanan, 1822), *S. taty* (Valenciennes, 1848) and *S. tenuifilis* (Valenciennes, 1848) have been reported from India (Whitehead, Nelson & Wongratana, 1988). Even though contribution of *Setipinna* species to total fish landings is relatively smaller (8507 tonnes), their fishery (artisanal fishery) is important for livelihood of the local fisherman (CMFRI, 2014). Among species of *Setipinna*, *S. phasa* has restricted distribution from the Ganges riverine system to the coastal waters of West Bengal (Jones & Menon, 1952a; Whitehead, 1972; Whitehead et al., 1988).

Correct identification of different fish species is essential for assessment and formulation of management measures. The constraints in this regards include confusions created due to adaptations by the species to different environment and ecological factors, altering the morphology of conspecific individuals. Often these individuals of a species are called as "ecotypes" or "ecospecies" which have adapted to a specific locale or set of environmental conditions (Turesson, 1922). Different environmental factors, such as temperature, salinity and oxygen may control the rate of development (Barlow, 1961). Several fishes have been reported to exist as locally adapted populations with gene flow between them (Dionne, Caron, Dodson, & Bernatchez, 2008). Further, several pelagic fishes have been reported to exhibit phenotypic plasticity against changing environmental conditions (Baumann & Conover, 2011). Such morphological variations have been reported in many pelagic fishes, including species of Engraulidae (Cheng, 2010). These morphotypes or ecotypes could be mistakenly identified or labeled as different species. In the present study, species status of different morphological variants of *S. phasa* collected from estuaries of Bay of Bengal were

identified, separated based on morphometric and meristic variables and also subjected to multigene barcoding for confirmation.

Materials and Methods

Sample Collection and Morphometric Analysis

A total of thirty specimens of each variant of *S. phasa* were collected from Diamond harbour (22.1987° N, 88.2023° E) and Digha Mohana (21.6302° N, 87.5432° E), West Bengal, India during October – December 2014 (Figure 1). The specimens were identified using the description given by Whitehead (1972), Wongratana (1983), Whitehead et al. (1988), Talwar and Jhingran (1991) and FishBase (Froese & Pauly, 2015). Based on the meristic characters, the specimens were classified into three variants *i.e* pure *S. phasa* (P), variant 1 (V1) and variant 2 (V2) (Figure 2). A total of twenty three morphometric and thirteen meristic characters were measured, of which, seventeen morphometric variables were scaled to standard length (SL) and six to head length (HL). All the morphometric ratios of the three variants were converted to percentage of SL and HL, and the means of the twenty three morphometric ratios thus obtained were subjected to ANOVA for testing any significant variation among

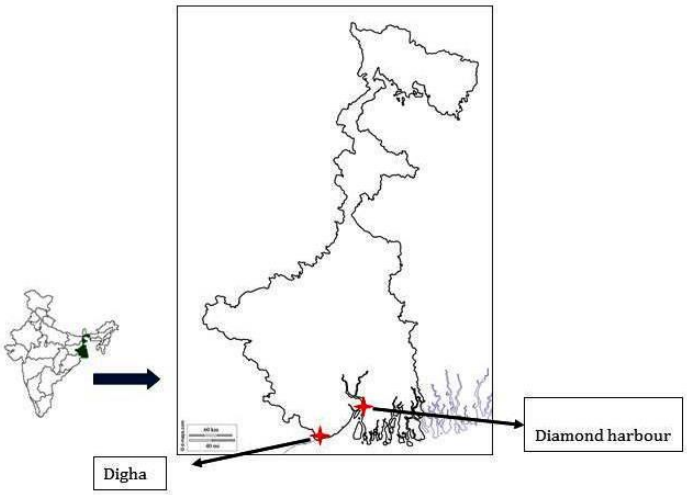

Figure 1. Study area map Diamond harbor and Digha landing centres of West Bengal.

Figure 2. *Setipinna phasa* (Pure) = SP (P), *Setipinna phasa* (Variant 1) = SP (V1), *Setipinna phasa* (Variant 2) = SP (V2).

them. Furthermore, principal component analysis was performed on standardized and natural log-transformed data of ratios of morphometric variables using PRIMER 6 v6.1.7, which separated morphological variations into linear combinations of variables that describe body shapes. In addition, analysis of similarities (ANOSIM) and similarity of percentage analysis (SIMPER) were also carried out on log-transformed morphometric ratios employing PRIMER 6 v6.1.7 to assess the percentage contribution of morphometric ratios to the overall variations in body shapes.

Molecular Analysis

Total genomic DNA was isolated from five specimens of each variant by salting out method (Miller, Dykes, & Polesky, 1998). Mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA partial genes were amplified using reported primers (Ward, Zemlak, Innes, Last & Hebert, 2005; Shekhar, Natarajan, & Vinay, 2011). PCR was performed in the 25 µl reaction volume containing 100 ng template DNA, 10 pmol of each specific primer, 200 µM of each dNTPs, 1.0 units of Taq DNA polymerase and 1xTaq buffer containing 1.5 mM $MgCl_2$. The thermocycler was programmed for initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec for denaturation, annealing and extension, with final extension at 72°C for 10 min. The amplicons were purified and sequenced commercially using PCR primers (Xcelris lab, Ahemadabad, India).

The sequences of each specimen were manually assembled using Gene Runner V 3.0 software. Assembled sequences were end-trimmed to homologous region to avoid sequencing errors. Open Reading frame of COI gene was predicted using NCBI ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence quality of 16S rRNA gene was observed by distinct and evenly spaced peaks in chromatograms. Intra and inter specific genetic divergence values were calculated using Kimura two parameters (K2P) distance model implemented in MEGA 7(Kumar, Stecher, & Tamura, 2016) software. A Neighbour-joining (NJ) tree was constructed from evolutionary distance data representing divergence pattern among the species with 100 bootstrap replications (Saitou & Nei, 1987).

Results

Analysis of morphological characters of *Setipnna* variants revealed overlapping of meristic characters of variant 1 (V1) within the range of *Setipinna wheeleri*, while, variant 2 (V2) indicated similarity in few meristic characters of *S. phasa* (P). The analysis of ANOVA for 23 morphometric ratios revealed significant difference in 17 ratios, among three ecotypes (Table. 1). The meristic characters of variant 1 differed significantly from the remaining variants. The morpho and meristic characters of variant 1 includes laterally compressed and silvery body, silvery grey eyes with the black pupil; upper caudal lobe truncated; 14 or 15 pre-pelvic scutes, 6 or 7 post-pelvic scutes; total 20 or 22 keeled scutes from the isthmus to anus. Maxilla tip pointed, 2nd supra-maxilla narrow and tapering anteriorly; 18 or 19 gillrakers on the lower limbs; 70 to 75 anal fin rays. The pectoral filament long, reaching to the 46 or 47th ray of the anal fin; 2nd pectoral fin ray reaching to anal fin origin (Figure 3). The numbers of dorsal, pectoral and pelvic fin rays recorded were 12, 1+12 and 7, respectively. Scales with very few anterior striae in comparison of *S. phasa* (P) (Figure 4A and Figure 4B).

Principal component analysis (PCA) using the twenty-three morphometric ratios revealed morphometric differences among the ecotypes. The first five principal components accounted for 87.2% of the overall variance (Table 2). PC1 was associated with the eye diameter, length of pectoral filament, distance between origin of pectoral fin to origin of anal fin and distance between origin of pelvic fin to origin of anal fin. PC2 was associated with snout length, Inter orbital width, distance between origin of dorsal fin to origin of anal fin and maximum body depth. The PC1 summarizes variations between length of pectoral filament and eye diameter, while second PC summarizes the variations between inter orbital width and maximum body depth. The PCA plot displayed three clusters, where pure *S.phasa* (p) has been clearly separated out from other two clusters while variant 1 and variant 2 showed some percentage overlapping (Figure 5).

Analysis of similarities (ANOSIM) of morphometric ratios showed well-differentiated clusters ($R = 0.743$; $P < 0.1$). Results of SIMPER analysis pointed out differences in length of pectoral filament, eye diameter, distance between origin of pectoral fin to origin of anal fin, distance between origin of pelvic fin to origin of anal fin and distance between origin of pectoral fin to origin of pelvic fin which accounted for 51.43% of variation between *S. phasa* (P) and variant 1. Similarly differences in length of pectoral filament, distance between origin of pelvic fin to origin of anal fin, eye diameter, distance between origin of pectoral fin to origin of anal fin, maximum body depth, distance between origin of pectoral fin to origin of pelvic fin and snout length accounted for 54.56% of the variation between *S. phasa* (P) and variant 2. Differences in length of pectoral filament, inter orbital width, pelvic fin length, distance between origin of pectoral fin to origin of pelvic fin, distance between origin of pelvic fin to origin of anal fin, distance between origin of pectoral fin to origin of anal fin and eye diameter accounted for 50.91% of the variations between variant 1 and 2 (Table 3). DNA sequence analysis of the variants revealed a total of 557 (COI) and 520

Table 1. The descriptive statistics of morphometric ratios in *S. phasa (*pure) and *S. phasa* variant 1 and 2

		Setipinna phasa (Pure)	*Setipinna phasa* (Variant 1)	*Setipinna phasa* (Variant 2)	F-ratio	*p* value
SNL/HL	Mean	0.157	0.166	0.170	27.497	0.000
	SD	0.010	0.002	0.006		
HL/SL	Mean	0.166	0.171	0.168	10.798	0.000
	SD	0.005	0.006	0.002		
POHL/HL	Mean	0.652	0.648	0.649	0.926	0.400
	SD	0.012	0.010	0.013		
IOW/HL	Mean	0.296	0.292	0.317	42.571	0.000
	SD	0.008	0.013	0.013		
ED/HL	Mean	0.200	0.230	0.220	130.273	0.000
	SD	0.010	0.005	0.006		
UJL/HL	Mean	0.851	0.843	0.834	3.883	0.024
	SD	0.022	0.022	0.025		
LJL/HL	Mean	0.764	0.727	0.743	21.021	0.000
	SD	0.025	0.019	0.021		
DFBL/SL	Mean	0.077	0.080	0.079	7.608	0.001
	SD	0.003	0.003	0.003		
AFBL/SL	Mean	0.546	0.548	0.543	2.293	0.107
	SD	0.011	0.009	0.010		
PFBL/SL	Mean	0.014	0.012	0.011	70.084	0.000
	SD	0.001	0.000	0.001		
PLFL/SL	Mean	0.066	0.073	0.066	70.962	0.000
	SD	0.002	0.002	0.004		
PTBL/SL	Mean	0.046	0.045	0.045	2.109	0.128
	SD	0.002	0.002	0.002		
PTFL/SL	Mean	0.462	0.590	0.530	183.265	0.000
	SD	0.022	0.028	0.027		
PTFLS/SL	Mean	0.194	0.193	0.200	11.842	0.000
	SD	0.008	0.004	0.006		
TSDF/SL	Mean	0.444	0.442	0.444	0.747	0.477
	SD	0.008	0.007	0.007		
TSAF/SL	Mean	0.415	0.400	0.404	16.772	0.000
	SD	0.012	0.005	0.013		
TSPF/SL	Mean	0.314	0.307	0.309	4.290	0.017
	SD	0.011	0.006	0.012		
TSPTF/SL	Mean	0.176	0.182	0.177	9.314	0.000
	SD	0.005	0.005	0.007		
AFDL/SL	Mean	0.276	0.262	0.261	35.125	0.000
	SD	0.011	0.006	0.006		
MBD/SL	Mean	0.269	0.256	0.249	51.574	0.000
	SD	0.010	0.006	0.007		
BPTFPL/SL	Mean	0.150	0.134	0.138	36.724	0.000
	SD	0.007	0.006	0.010		
BPTFAF/SL	Mean	0.263	0.232	0.240	67.591	0.000
	SD	0.010	0.004	0.015		
BPLFAF/SL	Mean	0.117	0.101	0.103	30.062	0.000
	SD	0.011	0.006	0.008		

Note: Standard length or SL, Snout length SNL (1), Head length HL (2), Postorbital head length POHL (3), Interorbital width IOW (4), Eye diameter ED (5), Upper jaw length UJL (6), Lower jaw length LJL (7), Dorsal fin base Length DFBL (8), Anal fin base length AFBL (9), Pelvic fin base length PFBL (10), Pelvic fin length PLFL (11), Pectoral fin base length PTBL (12), Pectoral filament length PTFL (13), Length of second ray of Pectoral fin PTFLS (14), Distance between tip of snout to of dorsal fin TSDF (15), Distance between tip of snout to origin of anal fin TSAF (16), Distance between tip of snout to origin of pelvic fin TSPF (17), Distance between tip of snout to origin of pectoral fin TSPTF (18), Distance between origin of dorsal fin to origin of anal fin AFDL (19), Maximum body depth MBD (20), Distance between origin of pectoral fin to origin of pelvic fin BPTFPL (21), Distance between origin of pectoral fin to origin of anal fin BPTFAL (22), Distance between origin of pelvic fin to origin of anal fin BPLFAF (23)

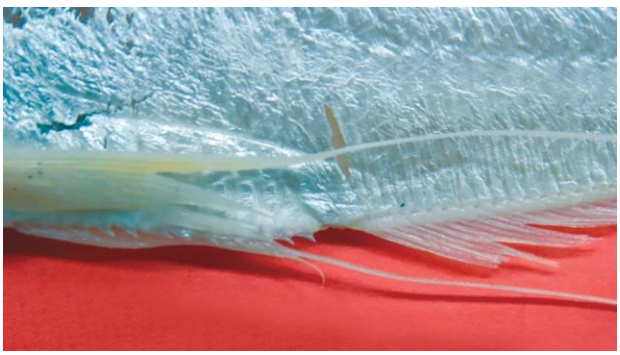

Figure 3. Second pectoral fin ray reaching to anal fin origin.

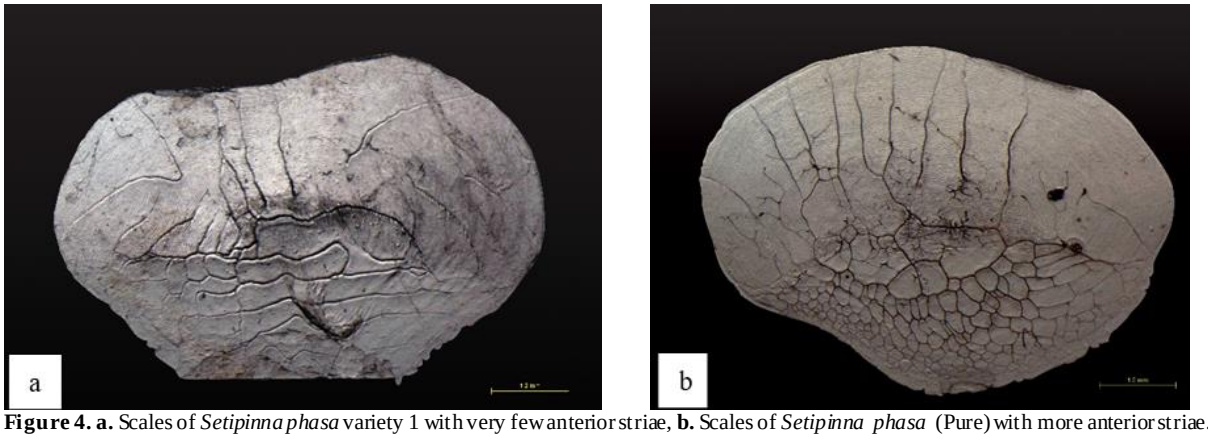

Figure 4. a. Scales of *Setipinna phasa* variety 1 with very few anterior striae, **b.** Scales of *Setipinna phasa* (Pure) with more anterior striae.

Table 2. Summary of principal component analysis (eigen values and eigenvectors) calculated from 23 morphometric ratios

Variable	PC1	PC2	PC3	PC4	PC5
Eigen Value	60.50	10.00	8.70	4.60	3.40
% variance Eigen vector	0.0194	0.0032	0.0028	0.0015	0.0011
Snout length	0.137	-0.328	0.080	-0.251	-0.272
Head length	0.082	0.169	0.039	-0.180	-0.318
Post orbital head length	-0.023	-0.028	0.026	-0.025	-0.354
Inter orbital width	-0.034	-0.625	0.309	0.015	-0.161
Eye diameter	0.343	-0.029	-0.047	0.106	-0.048
Upper jaw length	-0.048	0.064	0.096	0.419	0.270
Lower jaw length	-0.149	-0.085	0.046	0.340	0.225
Dorsal fin base length	0.064	0.060	0.112	0.151	0.119
Anal fin base length	0.001	0.087	0.094	-0.183	-0.025
Pelvic fin base length	-0.078	0.158	-0.074	-0.058	0.131
Pelvic fin length	0.125	0.129	-0.255	-0.194	-0.089
Pectoral fin base length	-0.011	-0.019	0.094	0.012	0.124
Pectoral filament Length	0.672	-0.028	-0.385	-0.081	0.218
Length of second ray of pectoral fin	-0.001	-0.166	0.256	-0.204	0.199
Distance between tip of snout to origin of dorsal fin	0.004	0.100	0.130	0.022	-0.272
Distance between tip of snout to origin of anal fin	-0.110	-0.029	-0.127	-0.076	0.003
Distance between tip of snout to origin of pelvic fin	-0.066	-0.045	-0.107	-0.262	0.087
Distance between tip of snout to origin of pectoral fin	0.079	0.199	0.087	-0.115	-0.142
Distance between origin of dorsal fin to origin of anal fin	-0.135	0.332	0.192	-0.066	-0.169
Maximum body depth	-0.144	0.422	0.129	-0.102	0.020
Distance between origin of pectoral fin to origin of pelvic fin	-0.275	-0.116	-0.113	-0.461	0.424
Distance between origin of pectoral fin to origin of anal fin	-0.327	-0.093	-0.258	-0.236	0.015
Distance between origin of pelvic fin to origin of anal fin	-0.330	-0.127	-0.619	0.290	-0.307

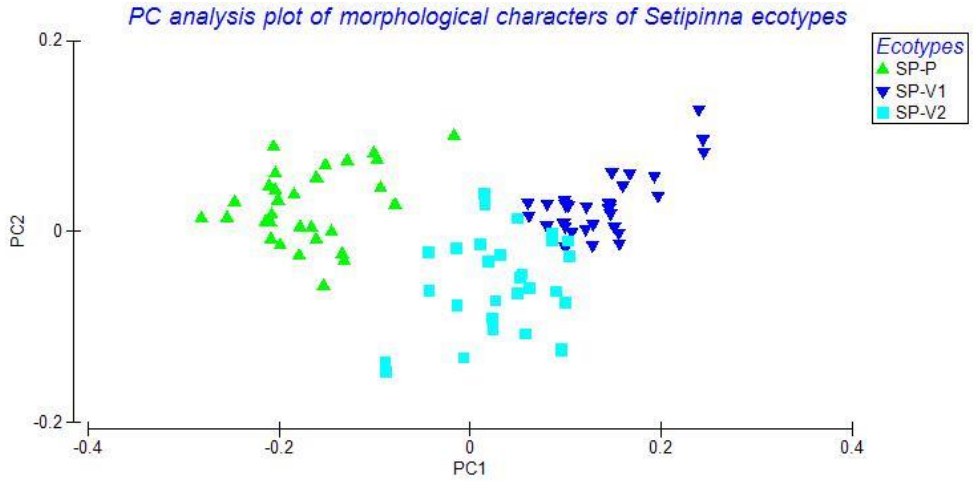

Figure 5. PC analysis plot for morphological characters *Setipinna phasa* variants.

Table 3. Results of SIMPER within three ecotypes of *S. phasa*

Within SP(P) and SP(V1)			Within SP(P) and SP(V2)			Within SP(V1) and SP(V2)		
Variables	Contri %	Cumm %	Variables	Contri %	Cumm %	Variables	Contri %	Cumm %
Length of pectoral filament	18.42	18.42	Length of pectoral filament,	12.34	12.34	Length of pectoral filament	12.66	12.66
Eye diameter	9.18	27.60	Distance between origin of pelvic fin to origin of anal fin	8.75	21.09	Inter orbital width	9.75	22.41
Distance between origin of pectoral fin to origin of anal fin	8.53	36.13	Eye diameter	7.52	28.61	Pelvic fin length	6.97	29.38
Distance between origin of pelvic fin to origin of anal fin	8.34	44.47	Distance between origin of pectoral fin to origin of anal fin	7.22	35.83	Distance between origin of pectoral fin to origin of pelvic fin	5.69	35.07
Distance between origin of pectoral fin to origin of pelvic fin	6.96	51.43	Maximum body depth	6.31	42.14	Distance between origin of pelvic fin to origin of anal fin	5.68	40.75
Pelvic fin length	4.31	55.74	Distance between origin of pectoral fin to origin of pelvic fin	6.22	48.36	Distance between origin of pectoral fin to origin of anal fin	5.60	46.35
Lower jaw length	4.17	59.91	Snout length	6.19	54.55	Eye diameter	4.54	50.99

(16S rRNA) characters which were observed after aligning all sequences to homologous position. The numbers of variable characters for COI and 16S rRNA were 105 and 48, respectively. The nucleotide frequencies for both COI and 16S rRNA genes were A: 26.8, T: 30, G: 19.0, C: 24.3% and A: 32.7, T: 23.0, G: 22.1, C: 22.3%, respectively. The AT content for both the genes were high (55%) than GC content (45%). DNA sequences were submitted to NCBI with GenBank accession no, KU871015-28, 35 (COI) and KU904316-27(16S rRNA). Average genetic distance values for COI and 16S rRNA genes were 0.2% and 0.1%, respectively (Table 4 and Table 5). Neighbour-joining tree revealed clustering of different variants in to a single clade with significant bootstrap value (Figure 6). These results indicate existence of ecotypes of same species (*S. phasa*) in the regions of their distribution in Indian waters.

Discussion

The findings of present study on morphometric analysis indicate towards the occurrence of two variants (morphotypes / ecotypes) of *S. phasa* in the Indian waters, apart from pure. The species has been reported to migrate from freshwater to brackish water and vice versa (Jones & Menon, 1952b). Accordingly, salinity levels might also have an impact on distribution of these variants. The variant 1 and variant 2 also seems to have broad (distant) migrational range while *S. phasa* (pure) has restricted migration. This variation in migration pattern coupled with influences of changing environmental on biological behaviour could be responsible for difference in morphometric differentiation among these variants. Several studies have reported environment induced morphological differences in fishes (Hedgecock, 1986; Kinsey, Orsoy, Bert, & Mahmoudi, 1994). Furthermore, habitat and population associated morphological differences have also been reported in fishes including clupeids (Nelson, Tang, & Boutilier, 1994; Tudela, 1999; Cheng & Han, 2004; Thomas, Willette, Carpenter, & Santos, 2014). Sukumaran et al. (2016) have also observed different morphotypes in *Sardinella longiceps* and attributed this variation to divergent selection and adaptive variation. In the present study also the attributes may be similar but, in view of absence of population structure information about these morphotypes, we refrained to ascribe this variation to adaptive variation.

In the current study, meristic characters of variant 1 of *S. phasa* were within the range of *S. wheeleri* (Wongratana, 1983). However, *S. wheeleri* was reported to have restricted distribution in Mynamar and Thailand (Nelson, 1970; Vidthayanon, Termvidchakorn, & Pe, 2005). Hence hypothesize that *S. wheeleri* might be an ecotype / morphotype of *S. phasa*. However, further studies on habitat, dietary preferences and depth preferences of these morphotypes need to be studied.

Mitochondrial cytochrome *c* oxidase subunit I gene has been standardized as a barcoding gene to delimit the metazoans (Hebert, Ratnasingham, & de Ward, 2003). This approach has been successfully used to discriminate fish species and more than 80% of clupeiform fishes were barcoded as on September 2016 (Rathnasingham & Hebert, 2007; Ward, Hanner & Hebert, 2009). Multigene barcoding approach has been implied to resolve the sibling and cryptic species

Table 4. Genetic divergence values (K2P model) of COI gene across variants of *Setipinna phasa*

	SPV1	SPV1	SPV1	SPV1	SPV1	SPV1	SPV2	SPV2	SPV2	SPV2	SPV2	SPP	SPP	SPP	SPP	SPP	STF
S.phasa variant1		0.003	0.003	0.000	0.003	0.003	0.003	0.003	0.004	0.004	0.003	0.003	0.003	0.003	0.002	0.003	0.022
S.phasa variant1	0.005		0.000	0.003	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant1	0.005	0.000		0.003	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant1	0.000	0.005	0.005		0.003	0.003	0.003	0.003	0.004	0.004	0.003	0.003	0.003	0.003	0.002	0.003	0.022
S.phasa variant1	0.005	0.000	0.000	0.005		0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant1	0.005	0.000	0.000	0.005	0.000		0.000	0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant2	0.005	0.000	0.000	0.005	0.000	0.000		0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant2	0.005	0.000	0.000	0.005	0.000	0.000	0.000		0.002	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.022
S.phasa variant2	0.007	0.002	0.002	0.007	0.002	0.002	0.002	0.002		0.003	0.002	0.002	0.002	0.002	0.004	0.002	0.022
S.phasa variant2	0.007	0.002	0.002	0.007	0.002	0.002	0.002	0.002	0.004		0.002	0.002	0.002	0.002	0.004	0.002	0.022
S.phasa variant2	0.005	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.002	0.002		0.000	0.000	0.000	0.004	0.000	0.022
S.phasa pure	0.005	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.002	0.002	0.000		0.000	0.000	0.004	0.000	0.022
S.phasa pure	0.005	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000		0.000	0.004	0.000	0.022
S.phasa pure	0.005	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.000		0.004	0.000	0.022
S.phasa pure	0.002	0.007	0.007	0.002	0.007	0.007	0.007	0.007	0.009	0.009	0.007	0.007	0.007	0.007		0.004	0.022
S.phasa pure	0.005	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.000	0.000	0.007		0.022
Setipinna tenuifilis	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.204	0.205	0.202	0.202	0.202	0.202	0.205	0.202	

Table 5. Genetic divergence values (K2P model) of 16SrRNA gene across variants of *Setipinna phasa*. Below the diagonal distance values and above the diagonal standard deviation values

	SPV1	SPV1	SPV1	SPV1	SPV1	SPV2	SPV2	SPV2	SPV2	SPV2	SPP	SPP	SPP	SPP	SPP	STF
S.phasa variant 1		0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.015
S.phasa variant 1	0.002		0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 1	0.002	0.000		0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 1	0.002	0.000	0.000		0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 1	0.002	0.000	0.000	0.000		0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 2	0.002	0.000	0.000	0.000	0.000		0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 2	0.004	0.002	0.002	0.002	0.002	0.002		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.015
S.phasa variant 2	0.002	0.000	0.000	0.000	0.000	0.000	0.002		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 2	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.015
S.phasa variant 2	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.015
S.phasa	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.015
S.phasa	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.015
S.phasa	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.015
S.phasa	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.015
S.phasa	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.015
S.teneuiphillis	0.100	0.098	0.098	0.098	0.098	0.098	0.096	0.098	0.098	0.098	0.098	0.098	0.098	0.098	0.098	

Figure 6. NJ tree of COI gene of *Setipinna phasa* variants.

(Krück, Innes, & Ovenden, 2013). In the present study, genetic distance values for 16S rRNA genes varied from 0.000 to 0.004, whereas for COI gene, the values were in the range of 0.000 – 0.007. These two genes have not shown sufficient genetic distance among *S. phasa* variants to assign them to different species. Lack of DNA barcoding gap among variants suggests that all these specimens indeed belong to single species despite morphological difference. By using mitochondrial and nuclear markers, several studies have proved that morphotypes of certain fishes (for instance species of *Macroramphus*) were conspecific individuals of single species (Robalo, Sousa-Santos, Cabral, Castilho, & Almada, 2009; Noguchi et al., 2015).

The study concludes that *Setipinna phasa* exists as three ecotypes in the type locality of the species. In the absence of DNA barcoding, the three variants could have been separated as three species. Further phylogeny studies including nuclear markers would be required to rule out the possibility of hybridization / introgression.

Acknowledgements

This work was carried out under the Ph. D. study of the main author, at ICAR-Central Institute of Fisheries Education, Andheri, Mumbai, India. The authors would like to thank and Dr. Gopal Krishna, Director, ICAR-CIFE for providing support and facilities for carrying out this work.

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