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Larvae Identification and Development of the only Freshwater Sardinella, *Sardinella tawilis* Endemic to Taal Lake, Philippines

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The only freshwater Sardinella, *Sardinella tawilis* (Herre 1927), endemic to Taal Lake, Philippines, continues to be an important food commodity and serves as a piece of cultural heritage within the country. However, the early life history of this species is unknown. In the present study, identification of the *S. tawilis* larvae has been finally confirmed through the utilization of the DNA barcode marker *cytochrome c oxidase I* (CO1) gene and the Kimura 2-parameter (K2P) distance model. Results showed 100% identity of three larvae samples with *S. tawilis* GenBank reference sequences based on clustering analysis, which was supported by mean genetic distance of 1%, suggesting accurate identification of the larvae samples. Subsequently, the larval developmental stages in pre-flexion, flexion, post-flexion and juvenile stage of *S. tawilis* were described and illustrated. Morphological analyses revealed that the larvae had elongated and straight gut with budding pectoral fin during pre-flexion stage, disappearance of fin-fold on the flexion stage, growth of fin rays during post-flexion stage and well developed fins in juvenile stage. This study serves as the first report on the identification of *S. tawilis* larvae as well as its morphological description during larval development.

Key words: DNA barcoding, larval development, *Sardinella aurita, Sardinella tawilis* larvae

INTRODUCTION

Sardines are a very important group of fish in the Philippines from an economic as well as cultural standpoint (Willette et al. 2011a). To date, there are at least twelve species recorded in the country under family Clupeidae, some of which have just been discovered in the past five years (Stern et al. 2016; Thomas et al. 2014; Willette & Santos 2012; Willette et al. 2011b; Quilang et al. 2011).

Among these sardine species is the *Sardinella tawilis*, also regarded as the only freshwater *Sardinella* species known worldwide (Whitehead 1985). It is endemic to Taal Lake, the third largest lake in the Philippines. Recently, it was confirmed that it indeed evolved from the marine environment with the discovery of its marine sister species, Taiwan sardine *Sardinella hualiensis*, found in marine waters of Northern Philippines (Willette et al. 2011; Willette et al. 2014). This commercially important species had peak abundance in 1998 yielding 1,120 tons of harvest but declined in 2009 to only 132 tons, an 82% reduction in ten years (Mamaril 2001; Mutia et al 2011; Fernandez 2011). Hence, effective resource management strategies should be identified and implemented to sustain the *S. tawilis* population. The reproductive biology and life cycle from juvenile to mature stages of *S. tawilis* have been previously studied (Aypa et al. 1991; SM Aypa, personal

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communication, April 4, 2017; Mutia 2015). However, the early life history of *S. tawilis* is still unknown.

Morphology and ecology of marine *Sardinella* spp. including Spanish sardine *S. aurita* and Brazilian sardine *S. brasiliensis*, have been previously studied (Ditty et al. 1994; Kurt & Matsuura 2001). These studies have been used to establish a reference for their normal developmental organogenesis, provide initial information for possible reproduction and cultivation experiments, and reveal potential aquaculture products (Padros et al. 2011; Conides & Glamuzina 2001).

Identifying species in its early life stages, particularly during its larval period using conventional techniques is at times problematic. In recent years, DNA barcoding has been successful in utilizing a genetic marker, a region in a species' genome conserved enough to be amplified with universal primers and divergent enough to allow discrimination between species, to identify an organism to its species level (Hebert et al. 2003). In the Philippines, DNA barcoding using *cytochrome c oxidase I* (COI) has been applied to identifying species composition in fry fisheries such as milkfish (*Chanos chanos)* fry (Asis et al. 2014); siganid fry (Agmata et al. 2013), small pelagic fry locally named "dulong" (Thomas et al. 2014) and in discriminating small-sized yellowfin tuna (*Thunnus albacares)* and bigeye tuna (*Thunnus obesus)* in landed catches (Pedrosa-Gerasmio et al. 2012). This has also been found effective in identifying adult specimens of *S. tawilis* (Quilang et al. 2011).

This study aims to confirm the identity of *S. tawilis* larvae through DNA-based analysis by the amplification of COI gene, subsequently describe its four developmental stages namely: pre-flexion, flexion, post-flexion and juvenile, and compare against its marine relative, Spanish sardine *S. aurita*.

MATERIALS AND METHODS

Sample Collection

Larvae samples were collected monthly from 2013 to 2015 for 36 months to identify and to validate the spawning season and distribution of *S. tawilis*. Samples were collected from seventeen sites in Taal Lake, Batangas (Figure 1 and Table 1), using a bongo net sampler with 50 cm diameter frames fitted with 330 µm mesh size, 2 m conical plankton net. The bongo net was deployed horizontally below the surface of the water with an average speed of 1.5 knots lasting for 10 minutes of each tow (Smith & Richardson 1977). Vertical tows were also done at 20 m depth. The bongo net was slowly pulled up and the nets were washed with the lake water to get all the plankton into the cod ends. The collected larvae were placed in a 1-L sampling bottle and fixed with

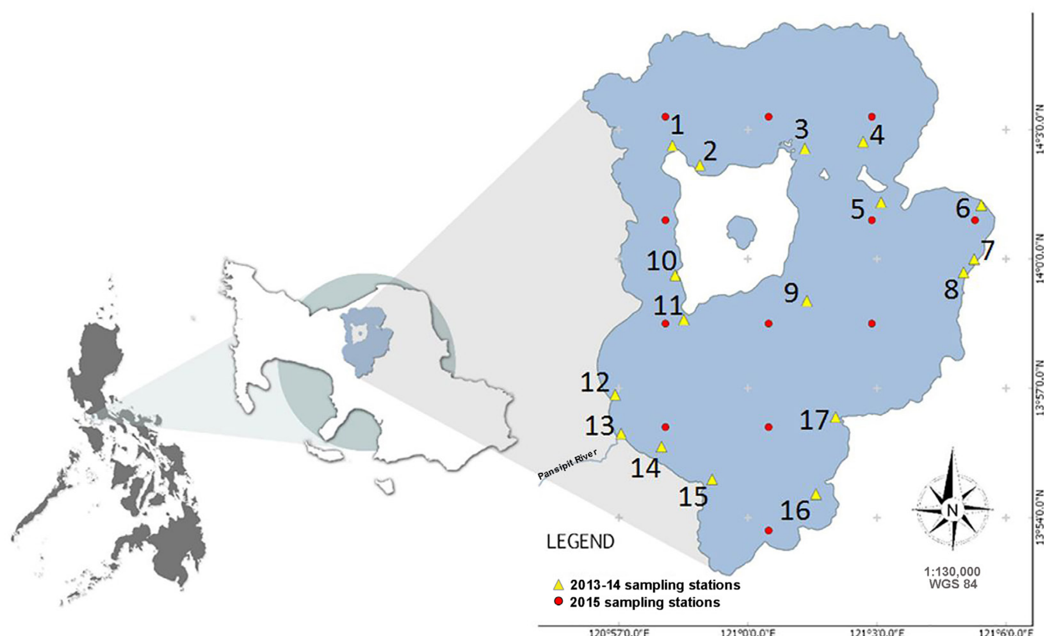

Figure 1. Sampling sites (17) of larvae collection from different areas of Taal lake, Batangas, Philippines: (1) Binintiangmalaki; (2) Panikihan; (3) Pira-piraso; (4) Mahabangbuhangin; (5) Napayong; (6) Sala; (7) Kinalaglagan; (8) Nangkaan; (9) Puntor; (10); Bird sanctuary; (11) Pulangbato; (12) Subic; (13) Pansipit; (14) Tagudtod; (15) Saimsim; (16) Don Juan; and (17) Napapanayan.

Table 1. Sampling sites (17) in Lake Taal with corresponding coordinates.

Station No.	Station Name	Latitude	Longitude
1	Binintiang Malaki	N 14° 02’ 34.9”	E 120° 58’ 11.8”
2	Panikihan	N 14º 02' 10.7"	E 120º 58' 52.8"
3	Pira-piraso	N 14° 02’ 29.5”	E 121° 01’ 31.5”
4	Mahabang Buhangin	N 14° 02’ 18.4”	E 121° 02’ 57.4”
5	Napayong	N 14° 01’ 46.3”	E 121° 02’ 40.8”
6	Sala	N 14° 01’ 12.5”	E 121° 05’ 28.1”
7	Kinalaglagan	N 13° 59’ 55.8”	E 121° 05’ 12.1”
8	Nangkaan	N 13° 59’ 36.3”	E 121° 05’ 00.3”
9	Puntor	N 13° 58’ 30.8”	E 121° 00’ 28.2”
10	Bird Sanctuary	N 13º 59' 36.7"	E 120º 58' 20.1"
11	Pulang Bato	N 13° 58’ 32.0”	E 120° 58’ 39.7”
12	Subic	N 13º 56' 51.0"	E 120º 56' 53.2"
13	Pansipit	N 13º 55' 58.5"	E 120º 56' 59.7"
14	Tagudtod	N 13° 56’ 09.3”	E 120° 58’ 07.1”
15	Saimsim	N 13º 54' 55.0"	E 120º 59' 04.4"
16	Don Juan	N 13° 54’ 56.9”	E 121° 00’ 39.2”
17	Napapanayan	N 13° 56’ 05.2”	E 121° 01’ 49.3”

95% ethanol for genetic analysis or with 4% buffered formaldehyde solution for quantitative sorting. All sites were geo-referenced using a Global Positioning System (GPS) receiver (Garmin GPS 76CSX).

Sorting and Pre-identification

A total of sixty larval specimens were sorted using dissecting microscope (Nikon™Binocular Microscope) and placed in separate labeled vials containing 70% ethanol. Fish larvae were identified up to family level using fish larval identification guide (SEAFDEC 2008; Leis & Carson-Ewart 2000). Morphological characteristics such as body length (BL), pre-anal length (PAL) and body depth (BD) were measured with an ocular micrometer. Since *S. tawilis* is a member of the family Clupeidae, larvae with characteristics similar to Clupeid larvae were pre-identified as “*S. tawilis*” and were separately stored from other groups by sampling area and random samples were subjected to DNA barcoding analysis for further confirmation.

Identification using DNA Barcoding

Three pre-identified “*S. tawilis*“ larvae were subjected to DNA barcoding analysis to confirm its identity. Whole larval samples collected from Sites Pulang Bato, Kinalaglagan and Sala, were used for DNA extraction using QIAGEN DNeasy® Blood and Tissue Kit. Approximately 600 bp of the *Cytochrome oxidase I* (COI) gene were amplified using the following primers developed by Ward et al. (2005): FishF2 (5’-TCGACTAATCATAAAGATATCGGCAC-3’) and FishR1 (5’-TAGACTTCTGGGTGGCCAAAGAATCA-3’). The 26-µl PCR reactions consisted of 4.8 µl ultrapure water and reagents with volume and concentration as follows: 5 µl 0.5x PCR buffer, 2.5 µl of 2 mM dNTPs, 2.5µl 25 mM $MgCl_2$, 2.5 µl of 10 mM of each primers, 4 µl of 5x BSA, 0.2 µl of 5 units/µl *Taq* polymerase and 2 µl of template. The PCR cocktails were subjected to the following conditions: initial denaturation at 95°C for 2 min, 38 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min, then final extension at 72°C for 10 min. Product amplicons were electrophoresed in 1% agarose gel stained with ethidium bromide and submerged in 1x TAE buffer. Standard sequencing and DNA purification were outsourced to Macrogen Inc., Korea.

Consensus sequences were generated using Geneious software (version 6.1.8). The generated consensus sequences were run as query in Basic Local Alignment Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and results with similarity >84% were used as voucher sequences. MEGA 6 (Tamura et al. 2013) was used for aligning consensus sequences together with the voucher sequences. Species identification was inferred using Neighbour-Joining (NJ) tree based on the Kimura 2-parameter (K2P) model with 500 bootstrap replications (Tamura et al. 2013). Mean genetic distances between voucher sequences of species under the genus *Sardinella* and *Hilsa* were analysed using K2P model (Kimura 1980).

Morphological Description and Larvae Illustration

Four larval stages, specifically: pre-flexion; flexion; post-flexion; and juvenile, pre-identified *S. tawilis* were further examined microscopically (Biological System Microscope Olympus™ CX41) and illustrated using graphite sketch pencils (HB and 3B). Larval sketches were then scanned and graphically enhanced using Adobe Photoshop CS6. All developmental stages were illustrated based on their larval diagnostic characteristics following the larval description of Spanish sardine, *S. aurita* (Valenciennes 1847), in the paper of Ditty et al. (1994).

RESULTS AND DISCUSSION

Larval Density Collected

A total of 25,680 fish larvae were collected within the duration of the study. Of these, only 0.93% were morphologically identified as belonging to family Clupeidae. In 2014 and 2015, the percentage composition of larvae identified as *S. tawilis* was 0.12% and 7.78%

of the total larval samples collected, respectively. No *S. tawilis* larvae were identified in 2013. Most of the larvae identified belonged to families Gobiidae, Blenniidae, Atherinidae, Syngnathidae, among others.

Identification of *S. tawilis* Larvae

Mitochondrial DNA *cytochrome oxidase I* (COI) sequences of the three pre-identified "*S. tawilis*" larvae (GenBank Accession numbers KU184506, KU184507 and KU184508) were analyzed. The analysis involved 22 nucleotide sequences with a total of 361 positions in the final dataset. All positions containing gaps and missing data were eliminated. The results confirm that the larvae are indeed *S. tawilis* based on the distinct monophyletic clade formed, supported by a bootstrap value of 100% (Figure 2). Furthermore, computation of mean genetic distances between the larval samples and voucher sequences revealed 1% mean genetic distance between larval samples in the study and voucher sequences of *S. tawilis* falling below the threshold value set at 3.0 – 3.5% for species delineation (Ward et al. 2009). The region of mtDNA COI showed high accuracy of distinguishing *S. tawilis* larvae among other sardine species.

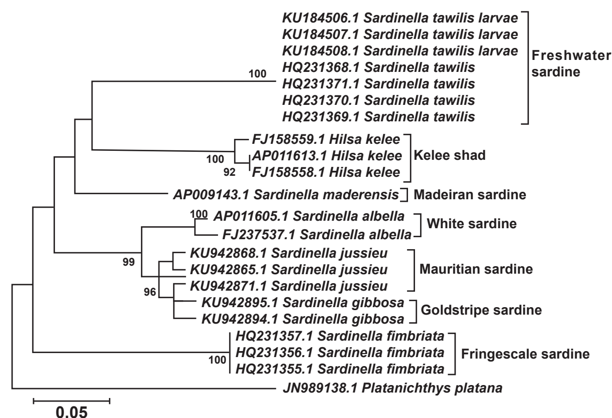

Figure 2. Neighbour-Joining tree generated using Kimura 2-parameter model of mtDNA COI sequences from three pre-identified *S. tawilis* larvae with voucher sequences of species under the genus *Sardinella* and *Hilsa*. GENBANK accession numbers are shown next to species name. (Outgroup: *Platanichthys platana*)

Morphology of Larval Development Stages

The larval developmental stages of *S. tawilis* from pre-flexion (Figure 3), flexion (Figure 4), post-flexion (Figure 5) larvae and juvenile (Figure 6) were described. Table 3 shows the summary of larvae body measurements in each of their developmental stage.

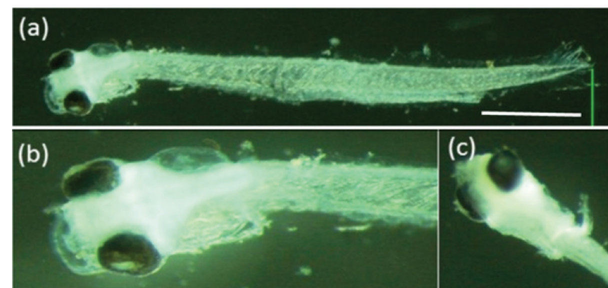

Figure 3. Pre-flexion stage of pre-identified *S. tawilis* larvae. (a) Elongate and slender body of a larvae having a continuous fin-fold; (b) lower jaw is longer than the upper jaw and the head showing the small depression above the optic region; (c) developing pectoral fin (scale is equivalent to 1mm).

Pre-flexion

In the flexion stage, the notochord tip bend completely in an upward position with BL of 8.3 - 12.8mm, with BD of 6.6 - 8.10% and PAL of 75.52 - 88.6% of the BL (Table 3). The yolk-sac was absorbed and the eyes were pigmented. Initially, the head of the larvae had a globular shape, which eventually became elongated. The body was elongated and slender (Figure 3a), similar to *S. aurita* and distinct from *S. pilchardus*, which has a tube-like body shape (Ditty et al. 1994; Ré & Meneses 2009). The mouth was formed and the lower jaw was longer than the upper jaw

Table 2. Computed mean genetic distances between larvae samples and voucher sequences of *Sardinella* and *Hilsa* species using Kimura 2-parameter model. The standard errors are reflected as blue.

		1	2	3	4	5	6	7	8	9
1	Larvae		0.001	0.030	0.029	0.030	0.030	0.033	0.038	0.046
2	*Sardinella tawilis*	0.001		0.030	0.029	0.030	0.030	0.033	0.038	0.046
3	*Sardinella maderensis*	0.185	0.184		0.027	0.028	0.028	0.026	0.033	0.038
4	*Sardinella jussieu*	0.196	0.195	0.170		0.004	0.028	0.014	0.029	0.039
5	*Sardinella gibbosa*	0.201	0.200	0.175	0.009		0.028	0.013	0.030	0.039
6	*Hilsa kelee*	0.204	0.202	0.185	0.199	0.198		0.028	0.036	0.036
7	*Sardinella albella*	0.226	0.225	0.160	0.067	0.067	0.190		0.032	0.039
8	*Sardinella fimbriata*	0.263	0.261	0.221	0.208	0.215	0.256	0.230		0.042
9	*Platanichthys platana*	0.342	0.341	0.271	0.308	0.308	0.275	0.297	0.306	

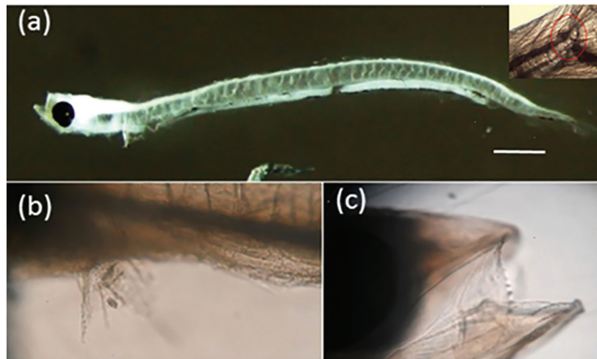

Figure 4. Flexion stage of pre-identified *S. tawilis* larvae. (a) Disappearance of fin-fold; (b) developing pelvic fin with soft rays; (c) minute conical teeth in the upper jaw (scale is equivalent to 1mm).

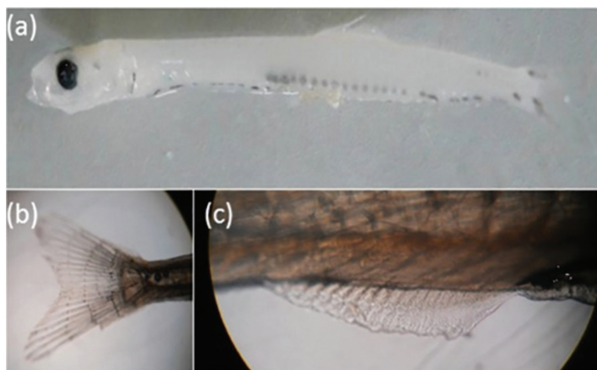

Figure 5. Post-flexion stage of pre-identified *S. tawilis* larvae. (a) Melanophores at the ventral part of the body; (b) four vertical lines at the caudal fin; (c) has few melanophores along the base of the anal fin (scale is equivalent to 2 mm).

and a small depression was observed to develop above the optic region (Figure 3b). At this stage, the melanophores of larvae appeared along the ventro-lateral aspect of the gut and a small group above the mid-gut and a few black melanophores below the tail (Table 3). Continuous fin-fold from nape up to the ventral midgut and pectoral fin bud were observed (Figure 3c, Table 4).

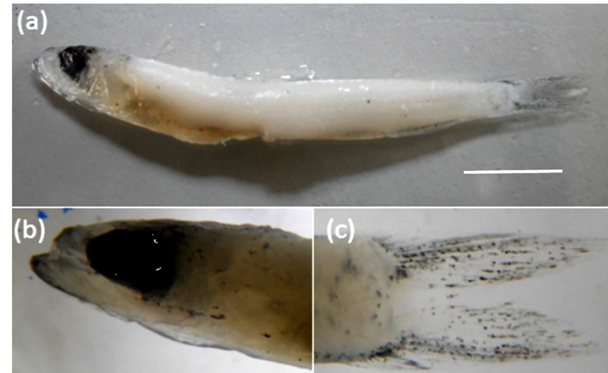

Figure 6. Juvenile pre-identified *S. tawilis* larvae. (a) Almost fish-like features; (b) melanophores on the side of the dorso-median line posterior to the eye and on the tip of the snout; (c) denser melanophores on the caudal fin (scale is equivalent to 1 cm).

Flexion

In the flexion stage, the notochord tip bend completely in an upward position with BL of 8.3 - 12.8mm, with BD of 6.6 - 8.10% and PAL of 75.52 - 88.6% of the BL (Table 3). In addition to these, the snout became longer with the length almost equal to the eye diameter and with minute conical teeth present in the upper jaw (Figure 4c). Few melanophores were observed to appear along the dorsal aspects of the fore and hind gut which is different with *S. aurita* observed with 12 different nape pigmentation patterns (Table 3). Fin-fold was absent (Figure 4a) and the pectoral fin was with few soft rays (Figure 4b) while the caudal fin was rounded (Figure 4c).

Post-flexion

In the post-flexion stage, morphological observations revealed that it had a BL of 16.10 – 20.50 mm, with BD of 7.19 – 10.98% and PAL of 70.30 – 78.57% of the BL (Table 3). In this stage, the body became wider and the head became larger. The mouth is in superior position and the angle of the pigmented lower jaw was seen prominent. There was also a black patch in the supra occipital part of the head. About ten melanophores at the mid-lateral part

Table 3. Morphological data for *S. tawilis* larvae in the pre-flexion (PL), flexion (FL), post-flexion (PFL) stages and juvenile period (JV). PL, FL, PFL and JV are expressed in mm as minimum-maximum values ± sd (standard deviation).

Measurement	Larval Stage						JV (n=21)	
	PF (n=43)		FL (n=7)		PFL (n=10)			
	min-max±sd	Proportions (%)	min-max±sd	Proportions (%)	min-max±sd	Proportions (%)	min-max±sd	Proportions (%)
Body Length (BL)	3.7-7.30±1.01	-	8.30-12.85±1.63	-	16.10-20.50±1.57	-	20.5-40.1±4.9	-
Body Depth (BD)	0.20-0.60±0.08	5.37-13.33	0.55-0.95±0.144	6.63-8.10	1.20-2.25±0.43	7.19-10.98	5.0-8.0±1.0	18.2-26.2
Pre-Anal Length (PAL)	2.0-5.70±0.83	70.65-89.06	6.76-10.5±1.51	75.52-x88.60	12.65-14.65±0.74	70.30-78.57	17.0-29.0±3.4	67.8-90.6

of the body and fourteen melanophores at the ventral part (Figure 5a) while seven melanophores along the base of the anal fin (Figure 5c) were observed. Moreover, two black patches were visible at the base of the operculum and heavily pigmented on the lower part of the operculum (Table 3). Four vertical lines are seen at the caudal fin (Figure 5b). Six pelvic fin rays and sixteen dorsal fin rays were likewise observed. There were seventeen anal fin rays and thirty caudal fin rays (Table 4).

Table 4. Comparison of meristic and pigmentation characters of *S. tawilis* with the data of *S. aurita* from Ditty et al. (1994).

Character	Freshwater sardine, *Sardinella tawilis*	Spanish sardine, *Sardinella aurita*
Habitat	Freshwater	Marine
Juvenile meristics		
Dorsal rays	13-17	16-19
Anal rays	17-23	16-20
Pectoral rays	11-17	15-16
Pre-anal length (%)	69	≥85
Pigmentation		
Body		
Dorsal	No	No
Ventral	Yes	Yes
Nape	Yes	Yes
Caudal	Yes	Yes

Juvenile

At juvenile stage, the BL were recorded at 20.5 – 40.1 mm with BD of 18.2 – 26.2% and PAL of 67.8 – 90.6% of the BL (Table 3). Teeth were present in the lower jaw and the angle of the lower jaw was more prominent. Body was elongated and cycloid scales started to develop. Eye diameter ranged from 0.35 –0.7 mm. The dorsal part of the body was grayish while the lower body had silvery color. The mouth shape had a superior type. Pigmentation was seen on the tip of the snout and on the inner side of the tip of the lower jaw as well as on the sides of the dorso-median line posterior to the eyes. A few melanophores were seen behind the dorsal fin and pigmentation of the caudal fin were denser. Melanophores of twenty-three, fifteen and five were present in the ventral part of the body, base of the anal fin and posterior to the anal fin, respectively (Table 3). A black spot at the base and tip of the dorsal and caudal fin was also observed.

Morphological illustration

Illustration of larval development of freshwater sardine, *S. tawilis* from Taal Lake (Figure 7) shows its distinct morphological characters in each of its life stage. Fin-fold

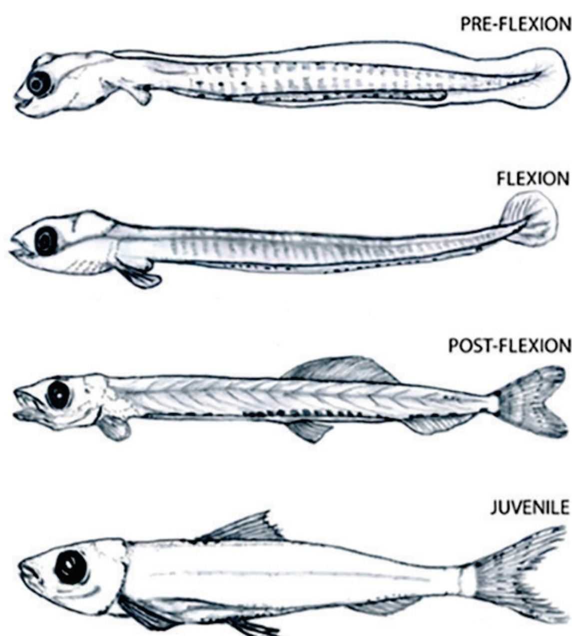

Figure 7. Illustration of *S. tawilis* larvae at different stages of early development, from pre-flexion to juvenile stage showing prominent changes based on fin development and pigmentation.

was present at the pre-flexion stage and the pectoral fin started to bud while few melanophores were seen on the ventral side of the caudal fin. The pectoral fin developed with soft rays on the flexion stage and there were a number of melanophores seen in the larvae gut and ventral side of the body. In addition to this, disappearance of fin-fold was evident. The post-flexion stage revealed the developed pelvic, dorsal, anal and caudal fins along with melanophores distributed on the lower jar, body and anal fin of the larvae. Juvenile stage shows well developed dorsal fin containing few melanophores, pectoral fin, caudal fin with dense melanophores, and anal fin having pigments in the base and posterior end.

Comparison of S. tawilis and S. aurita larvae

Development of *S. tawilis* larvae was found similar with that of Spanish sardine, *S. aurita*, which lives in a marine environment (Table 5). Among the characters considered, pigmentation differences was said to be a more useful taxonomic character because it can be used on different ranges of larval sizes (Ditty et al. 1994). Distinct pigmented areas in all clupeids found in the Gulf of Mexico has been established based on a number of studies wherein a row of melanophores above the foregut and below the hindgut; and pigment above the anus were specifically observed (Ditty et al. 1994; Houde et al. 1974; Powles 1977). In *S. tawilis*, melanophores were present along the dorsal of foregut and hindgut during the flexion

stage and in the base of anal fin near the anus. Given this, patterns of pigmentation may appear as conserved characteristic during larval development of Clupeids.

Taken together, this study serves as the first report of the identification of *S. tawilis* larvae through genetic analysis and the first to describe its early life development stages. This should provide more information on the biology of *S. tawilis* larvae for the Taal Volcano Protected Landscape (TVPL) management plan particularly in the proposed resolution of the Unified Rules and Regulations for Fisheries (URRF) designating a Tawilis Reserve Area by the Protected Area Management Board (PAMB). Moreover, this could also aid in endorsing area closure in Taal Lake where there is high abundance of *S. tawilis* larvae to promote undisturbed reproduction and eventually increase population of this species.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

AUTHORS' CONTRIBUTIONS

Mutia, Muyot, and Tordecilla carried out collection, morphological analyses as well as the partial writing of the manuscript specifically the larval development. Sarmiento conducted genetic analyses particularly DNA extraction, PCR amplification, and analysis of DNA sequences; and wrote the manuscript including the abstract, introduction, collated the tables, figures, references and appendices, and applied necessary revisions on results and discussion. Mendiola provided the schematic drawing of the larval development and wrote the methods of illustration. Santos partly conceptualized the study and helped in the structure, review and revision of the manuscript.

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