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research paper

Evolution of the freshwater sardinella, *Sardinella tawilis* (Clupeiformes: Clupeidae), in Taal Lake, Philippines and identification of its marine sister-species, *Sardinella hualiensis*

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ABSTRACT.—We identify the sister species of the world's only freshwater sardinella, *Sardinella tawilis* (Herre, 1927) of Taal Lake, Philippines as the morphologically-similar marine Taiwanese sardinella *Sardinella hualiensis* (Chu and Tsai, 1958). Evidence of incomplete lineage sorting and a species tree derived from three mitochondrial genes and one nuclear gene indicate that *S. tawilis* diverged from *S. hualiensis* in the late Pleistocene. Neutrality tests, mismatch distribution analysis, sequence diversity indices, and species tree analysis indicate populations of both species have long been stable and that the divergence between these two lineages occurred prior to the putative 18th century formation of Taal Lake.

The freshwater sardinella *Sardinella tawilis* (Herre, 1927) is endemic to Taal Lake, Philippines, a crater lake formed by the highly active Taal Volcano (Fig. 1; Herre 1927, Ramos 2002). The sardines and other Clupeiformes are predominantly marine but with many peripheral freshwater representatives (Whitehead 1985). *Sardinella tawilis* is the only freshwater representative of the 22 species in its genus. The origins of this species are enigmatic since Taal Lake putatively formed only as recently as the 18th century after a series of large eruptions of Taal Volcano (Ramos 1986, Hargrove 1991). Having erupted over 30 times since the 16th century, including several large and highly destructive events, Taal Volcano is one of the world's most active volcanoes (Torres et al. 1995, Newhall 1996). The caldera itself formed sometime from a few hundred thousand to a few tens of thousands of years ago (Ramos 2002), but Taal Lake was broadly connected to Balayan Bay until 1754 when a series of violent eruptions constricted and diverted the Pansipit River increasing the depth of the lake to its current level, 3 m above sea level (Wolfe and Self 1983, Hargrove 1991). The history of the hydrography of the lake is not well recorded although sailing ships were reported as navigating between Balayan Bay and Taal Lake prior to the 1754 eruptions (Hargrove 1991). It is also not known if the population of *S. tawilis* that now exists in the lake was previously restricted to the lake or the vicinity of the Taal caldera prior to 1754.

Several studies have sought to identify the evolutionary origin of *S. tawilis*, the most important commercial fishery of Taal Lake and a valued culinary delicacy of Filipino

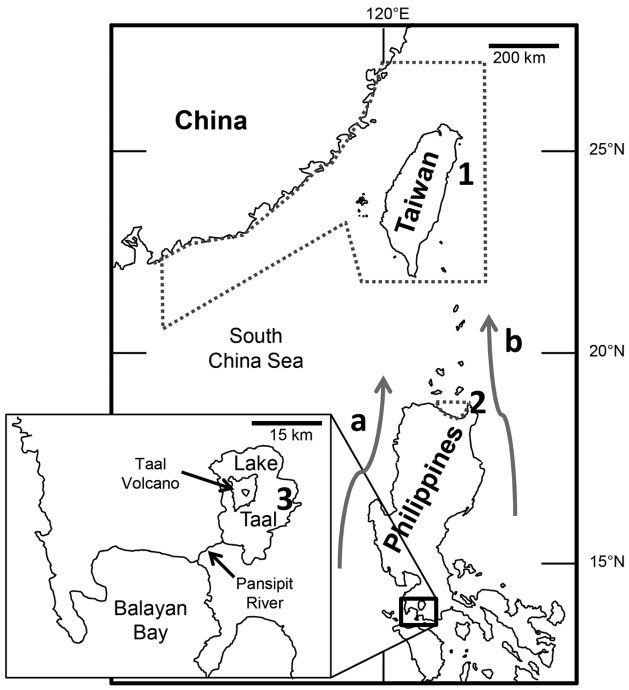

Figure 1. Map of species' ranges and sampling sites. *Sardinella hualiensis* species range is designated by the dotted line surrounding Taiwan and along the Chinese coast per Whitehead (1985). *Sardinella hualiensis's* known distribution is also noted by the dotted line near the northern most tip of the Philippine island of Luzon based on Willette et al (2011). *Sardinella tawilis* geographic range is restricted to the freshwater Taal Lake (insert). The Pansipit River connects Taal Lake to Balayan Bay, the nearest marine environment. Sampling sites are number: (1) Yilan County, Taiwan, (2) Cagayan Province, Philippines, and (3) Cuenca, Batangas Province, Philippines. The direction of prevailing flow for the South China Sea Gyre (a) and Kuroshio Current (b) are indicated along Luzon Island (Hu et al. 2000, Metzger and Hurlbert 2001).

peoples (Mutia et al. 2001). Biometric and molecular evidence originally suggested *S. tawilis* was a descendant of the white sardinella, *Sardinella albella* (Valenciennes, 1847), a broad ranging marine species found in the nearby South China Sea (Samonte et al. 2000, 2009). However, the use of a single exemplar of *S. albella* in the molecular phylogeny and the omission of diagnostic meristic and morphological features in the biometric assessment leave room for additional analysis. A subsequent molecular phylogeny based on Cytochrome *c* oxidase subunit I (COI) indicated *S. albella* as the sister taxon to *Sardinella gibbosa* (Bleeker, 1849) and did not identify a known Philippine *Sardinella* as sister to *S. tawilis* (Quilang et al. 2011). The study postulated that the descendants of the *S. tawilis* ancestral lineage may "still be roaming in the South China Sea waiting to be discovered" (Quilang et al. 2011).

The Taiwanese sardinella, *Sardinella hualiensis* (Chu and Tsai, 1958), a subtropical species previously thought to be restricted to Taiwan and the adjacent mainland coast of China (Fig. 1), was reported as a range extension to the Philippines and as a potential sister species of *S. tawilis* (Willette et al. 2011). The original type descriptions of *S. tawilis* and *S. hualiensis* describe their morphological similarity, though a shared ancestry was dismissed due to geographic and ecological marine-freshwater separation. This may be why *S. hualiensis* was not considered in previous phylogenies

(Samonte et al. 2000, Quilang et al. 2011). Chu and Tsai (1958) stated about *S. hualiensis* (both species formerly placed in the genus *Harengula*): "The present species is so closely related to *Harengula tawilis* that it is difficult to separate them. However, the former [*S. hualiensis*]…is found in the eastern coastal waters of Taiwan; while the latter [*S. tawilis*]…is found in the fresh waters of the Philippines." Subsequent taxonomic treatments recognized *S. tawilis* as a distinct species (Whitehead 1985, Munroe et al. 1999).

The purpose of the present study was to test the potential sister-species relationship of *S. hualiensis* and *S. tawilis* based on their reported morphological similarity and with regard to the restricted, disjunct range of these species. We are confident these ranges are disjunct because the identification of *S. hualiensis* and *S. tawilis* is straightforward based on external morphology and the distribution of members of this genus are well known in the Philippines because of their economic importance (Willette et al. 2011). In the Philippines, *S. hualiensis* is restricted to northern Luzon in isolation from *S. tawilis* (Fig. 1) both proximally and because of differences between marine and freshwater ecology. The disjunct nature of these ranges could be related to the unstable and ephemeral nature of sardine populations that go through sequential range expansions and contractions tied to underlying fluctuations in environmental conditions (Bowen and Grant 1997, Chavez et al. 2003, Takasuka et al. 2007). We use several molecular genetic analyses to test if the divergence of *S. tawilis* from a previously more widely distributed *S. hualiensis* parent population is consistent with the timing of Pleistocene environmental fluctuations. Alternatively, this divergence could have been caused by the isolation and divergence of *S. hualiensis* in the lake because of the 1754 eruption event that gave rise to the present-day configuration of Taal Lake.

Methods

Twenty-five *S. hualiensis* were obtained from fishermen near Santa Ana in Cagayan Province, Philippines (18°30′N, 122°8′E) and 25 *S. hualiensis* were sampled from Nantang-Ao fish market in Yilan County, Taiwan (24°34′N, 121°52′E) (Fig. 1). Forty-eight specimens of *S. tawilis* were purchased from a municipal fish port in Cuenca in Batangas Province, Philippines (13°54′26″N, 121°1′59″E; Fig. 1). Basic measurements (Table 1) and photographs were taken from each fish. Specimens of congeners *Sardinella lemuru* (Bleeker, 1853), *Sardinella fimbriata* (Valenciennes, 1847), S. *gibbosa*, and *S. albella*, and confamilial species *Amblygaster sirm* (Walbaum, 1792) were sampled from Philippine provincial fish markets and used as out-groups in the phylogenetic analysis. Muscle tissue samples from the right flank of each fish were taken and preserved in 95% ethanol. Representative whole specimens from each location were vouchered in 95% ethanol and are stored at the National Fisheries Research and Development Institute, Quezon City, Philippines.

To assess genetic relationships, DNA was extracted by placing a small amount of tissue (approximately 25 mg) in 300 µl of 10% Chelex solution (BioRad) in a 1.7-ml micro-tube, vortexing, and heating to 96 °C for 60 min, then centrifuging at 10,000 rpm for 90 s. DNA was amplified for the following four gene regions:

- mtDNA control region (CRA 5′–TTCCACCTCTAACTCCCAAAGCTAG–3′, CRE 5′–CCTGAAGTAGGAACCAGATG–3′, sequence fragment size of 435 bp),

Table 1. Morphological features and meristic count averages for 10 specimens of each *Sardinella tawilis* Taal Lake, *Sardinella hualiensis* Philippines (PH), and *S. hualiensis* Taiwan (TW). Quantitative values reported as mean (SE). Lengths are listed in millimeters and ratios are listed as percentages. Qualitative feature "Yes" indicates all 10 specimens possessed feature.

Morphology/meristics/coloration	*S. tawilis*	*S. hualiensis* PH	*S. hualiensis* TW
Standard length (SL) in mm	80.4 (1.4)	109.7 (1.1)	170.2 (1.8)
Body depth / SL	30.0 (1.0)	31.0 (1.0)	35.0 (0.3)
Pectoral-fin length / SL	18.7 (0.2)	19.1 (0.2)	18.8 (0.8)
Head length (HL) in mm	20.6 (0.4)	28.3 (0.4)	37.8 (0.6)
Snout / HL	24.2 (0.3)	28.6 (0.7)	27.8 (0.4)
Eye diameter / HL	28.7 (0.4)	27.9 (0.4)	26.5 (0.3)
Post orbital length / HL	47.1 (0.5)	44.2 (0.8)	45.8 (0.3)
Number of lower gillrakers	64.8 (2.0)	81.2 (2.9)	70.0 (1.3)
Number of scutes	28.9 (0.3)	30.4 (0.2)	31.2 (0.2)
Number of dorsal-fin rays	16.8 (0.2)	17.8 (0.1)	17.3 (0.1)
Number of pelvic-fin rays	8.0 (0.0)	8.0 (0.0)	7.9 (0.1)
Number of pectoral-fin rays	14.2 (0.2)	14.2 (0.1)	14.5 (0.2)
Number of anal-fin rays	18.8 (0.4)	18.2 (0.1)	18.6 (0.2)
The n^{th} dorsal-fin ray parallel to the ventral fin's origin	7.6 (0.4)	No data	6.8 (0.3)
Enlarged last 2 anal-fin rays	Yes	Yes	Yes
Scales with overlapping striae	Yes	Yes	Yes
Few or Many perforations on scales	Few	Many	Many
Black spot at dorsal-fin origin	Yes	Yes	Yes
Tips of caudal fin black	Yes	Yes	Yes
Dorsal fin blackish	Yes	Yes	Yes
Black spot at posterior margin of operculum	Yes	Yes	Yes

- mtDNA Cytochrome oxidase *b* (Cyt *b*) (H 5′–GTGACTTGAAAAACCACCGTTG–3′, L 5′–AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA–3′, 407 bp) (Lecomte et al. 2004)
- rRNA 16S (531 bp) (Palumbi 1996), and
- nDNA S7 (16Sar 5′–CGCCTGTTTATCAAAAACAT–3′, 16Sbr 5′–CCGGTCTGAACTCAGATCACGT–3′, 719 bp) (Chow and Hazama 1998) using a polymerase chain reaction (PCR).

Amplification of the nDNA S7 gene region was initially problematic, but successful amplification was obtained by (1) using a 10-fold concentration of template DNA, and (2) applying a nested-PCR method with the primer pairs S7RPEX1f (5′–TGGCCTCTTCCTTGGCCGTC–3′) and S7PEX2R (5′–GCCTTCAGGTCAGAGTTCAT–3′) (Chow and Hazama 1998) in the first reaction, and 1 µl of PCR product with the primers 1F.2 (5′–CTCTTCCTTGGCCGTCGTTG–3′) and 2R.67 (5′–TACTGGGARATTCCAGACTC–3′) (Unmack et al. 2011) in the second reaction. All PCR reactions consisted of 13 µl of 10× PCR Buffer, 2.0 µl of 25 mM $MgCl_2$, 2.5 µl of each 10mM dNTP, 1.25 µl of each primer, 1 µl of BSA (10 µg μl^{-1}), 0.2 units of Taq DNA Polymerase, and 1 µl template DNA in a final volume of 25 µl. PCR parameters were an initial denaturation at 94 °C for 10 min, 38 cycles of 94 °C for 30 s, 45 °C (53 °C for S7) for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min. PCR product was sent to either Macrogen, Inc. (Korea) or the UC Berkeley DNA Sequencing Facility (United States) for purification and sequencing. Sequences were

proofread, assembled and aligned in Sequencher v4.8 (GeneCode, Ann Arbor, MI) and MUSCLE v3.8 (Edgar 2004). All sequences were submitted to the public domain database GenBank (Accession numbers KC951469–KC951538).

Phylogenetic Analysis.—A phylogeny of *S. hualiensis*, *S. tawilis,* and outgroups were inferred using maximum likelihood analysis for the mtDNA 16S and Cyt *b* and nDNA S7 genes. Divergence was estimated using the Kimura 2-Parameter distance model (Kimura 1980) and support for nodes estimated with 1000 bootstrap replications in MEGA v5 (Tamura et al. 2011). The Kimura 2-Parameter distance model was identified as the best-fit model in MEGA v5. Available 16S *Sardinella* sequences (*S. aurita, S. madrensis,* and *S. zunasi*) from GenBank and unpublished S7 *S. gibbosa* sequence (R Thomas unpubl data) were included in the phylogenetic analysis.

Genetic Population Structure.—Relationships between mtDNA control region sequences were visualized with a maximum likelihood tree in DNasp v5.1 (Librado and Rozas 2009). Sequences were pooled by site and calculated for nucleotide diversity (π), haplotype diversity (h), and number of haplotypes in DNasp v5.1 to assess population level variation. Divergence among sequences between and within sites was estimated using Kimura 2-Parameter distance model and mean sequence diversity was calculated in MEGA v5. Population structure was evaluated using Phi-statistics (Φ_{ST}) calculated from pairwise differences in Arlequin v3.5 (Excoffier et al. 2005) with sampling locations as separate populations. To determine if sardine populations had recently expanded, sequences were grouped by (a) location, (b) inferred clades in the maximum likelihood tree, and (c) globally, and assessed in a mismatch distribution in NETWORK v4.6.1.1 (Fluxus Technology). To estimate evolutionary relationships between haplotypes, an un-rooted median-joining parsimony network was constructed in NETWORK v4.6.1.1 using the default settings. The Tajima's *D* and Fu and Li's (1993) *D* statistical tests were also calculated in DNasp v5.1.

Timing of Population/lineage Divergence.—Divergence times between species have frequently been estimated using a single gene region; however, a recently developed method, *BEAST, permits the use of multiple genes from multiple individuals per species to obtain a co-estimated divergence time between the species or non-interbreeding populations (Heled and Drummond 2010). The use of multiple loci to infer a species tree reduces uncertainty and increases confidence in the estimated divergence time (Edwards and Beerli 2000). The *BEAST method outperforms the supermatrix method that concatenates multiple gene region sequences together (Heled and Drummond 2010). Population divergence time of *S. tawilis* and *S. hualiensis* was estimated using the species tree approach in BEAST v1.6.2 (Drummond and Rambaut 2007). Sequence of S7, Cyt *b*, 16S and control region from six representative individuals from each sampling site were prepared in BEAUti v1.62, run for three replicate runs in *BEAST, combined in LogCombiner v1.6.2, and annotated in TreeAnnotator v1.6.2. A burn-in of 10,000 trees was applied when combining runs, and the minimum posterior probability limit of 0.8, maximum clade credibility tree setting, and mean node height setting were used during annotation. A mutation rate of 2%/MY for the Cyt *b* gene region has been applied in past sardine phylogeny work (Grant and Bowen 1998) and was used here. Additionally, rates of 1%/MY, 3%/MY, 5%/MY, and 10%/MY for Cyt *b* were applied for a broader assessment of potential population divergence time. Mutation rates in the other gene regions were estimated

by *BEAST based on the Cyt *b* rate and sequence data. The default settings were used, except for the use of the strict clock model and the following priors: LogNormal for kappa, Uniform for frequencies, and the coalescent constant tree size. The species tree was visualized in FigTree v1.4 and illustrated with error bars representing 95% HPD intervals.

Morphological and Meristic Data.—All fish used in our study were identified to species as described in Whitehead (1985). For a robust comparison between the fish, twenty diagnostic morphological and meristic features were taken from 10 representatives from each site (Table 1). Features assessed in Primer-5 (Primer-E Ltd, Plymouth, UK) in two ways: (1) Bray-Curtis dissimilarity indexes from combined morphological and meristic features were assessed in a dendogram to illustrate overall similarity; and (2) a principle component analysis (PCA) to assign the source of the observed variation.

Results

Six to sixteen 16S rRNA, Cyt *b* mtDNA, and S7 nDNA sequences were obtained from specimens from each of the three sampling sites (Table 2). A single, dominant haplotype was shared by two-thirds of individuals at all sites (*S. hualiensis* from Taiwan and Philippines and *S. tawilis*) for 16S and Cyt *b* sequence data (Fig. 2), whereas one-third of individuals shared a common S7 haplotype across all sites. One to four unique haplotypes (haplotypes restricted to a single sampling site) were found at all sites for all markers (Table 2) and were typically separated by three or fewer mutational steps from shared haplotypes. Overall haplotype diversity was 0.44 for 16S, 0.55 for Cyt *b*, and 0.89 for S7 sequence data. Haplotype diversity varied from site to site, whereas nucleotide diversity was moderate to low (Table 2). Intraspecific genetic distances for Taiwanese and Philippine *S. hualiensis* were 0.1%, 0.1%, and 0.3% for the 16S, Cyt *b*, and S7 gene regions, respectively, and 0.2%, 0.1%, and 0.4% for *S. tawilis*. Nearly identical, interspecific genetic distances between *S. hualiensis* and *S. tawilis* were 0.1% for 16S, 0.1% for Cyt *b,* and 0.3% for S7. Additionally, *S. tawilis* and *S. hualiensis* had identical interspecific genetic distances when compared to *S. albella* for 16S (9.4%) and Cyt *b* (18.1%). Genetic distance between *S. hualiensis* and *S. tawilis* and other out-groups ranged from 9.2% to 21.9%. Similar to COI sequence data by Quilang et al. (2011), our study found moderate to no interspecific genetic distance between *S. albella* and *S. gibbosa* (5.5% for 16S, 0.0% for Cyt *b*). Maximum likelihood phylogenetic analysis inferred a single monophyletic clade consisting of *S. hualiensis* and *S. tawilis* supported by high bootstrap probability value for Cyt *b* (bootstrap probability of monophyletic clade = 100%), 16S (bootstrap probability = 94%), and S7 (bootstrap probability = 62%) sequence data (Fig. 3). Partitioning of sub-clades indicating the two *S. hualiensis* locations and sub-clades inferring the two species were unresolved or supported by low bootstrap probability values in the 16S tree and S7 tree (Fig. 3). This ambiguous result was similar in the Cyt *b* tree (not shown). The 16S sequences from as many *Sardinella* species as we had available were used to identify an expanded taxonomic outgroup to the *S. tawilis* and *S. hualiensis* clade. The closest sister clade contained the three species *S. gibbosa*, *S. albella*, and *S. fimbriata* (Fig. 3).

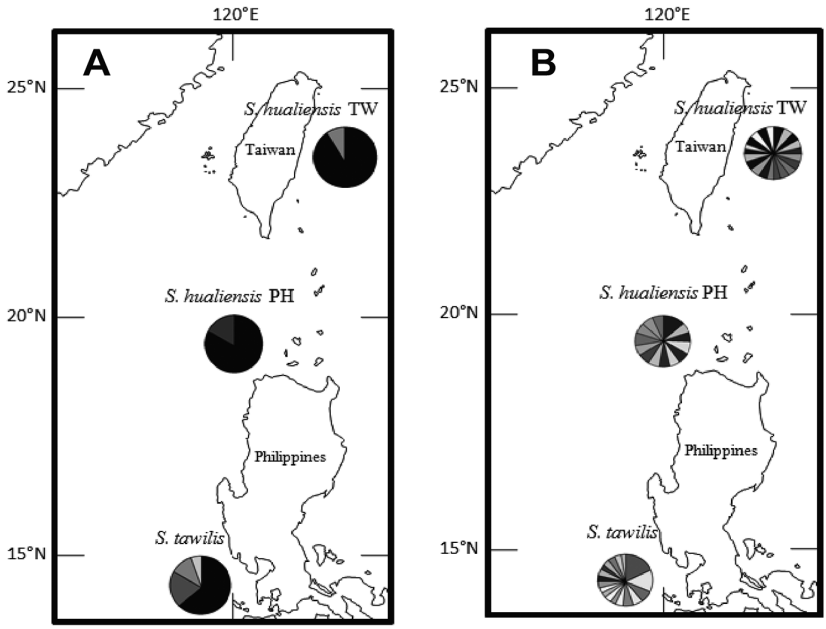

Figure 2. Haplotype frequency diagrams for specimens from three sampling locations, *Sardinella hualiensis* Taiwan (TW), *S. hualiensis* Philippines (PH), and *Sardinella tawilis* Taal Lake for (a) 16S gene region, and (b) control region sequences. Cyt *b* and S7 results are not shown, but were similar to 16S results. Each color represents a distinct haplotype. Shared haplotypes are the same color between sites.

Table 2. Sample size (*n*), number of unique haplotypes, haplotype diversity (*h*), nucleotide diversity (π%), and the Tajima's *D* and Fu and Li's *D** neutrality tests for rRNA16S, mtDNA Cytochrome *b*, nDNA S7, and mtDNA control region for *Sardinella hualiensis* sample from Taiwan (TW) and the Philippines (PH), and *Sardinella tawilis*. *Sardinella tawilis* was subdivided into two inferred clusters from the control region haplotype network. Statistically significant neutrality test values ($P < 0.05$) are in bold.

Gene/species	*n*	No. of haplotypes	No. of unique haplotypes	*h*	π%	Tajima's *D*	Fu and Li's *D**
16S							
S. hualiensis TW	10	2	1	0.20	0.04	−1.401	−1.587
S. hualiensis PH	6	2	1	0.33	0.06	−0.933	−0.950
S. tawilis	16	4	3	0.59	0.16	−0.280	−0.039
Cyt *b*							
S. hualiensis TW	13	4	3	0.64	0.19	**−1.863**	**−2.323**
S. hualiensis PH	7	3	2	0.67	0.26	0.206	−0.059
S. tawilis	14	5	4	0.50	0.13	−1.278	−1.037
S7							
S. hualiensis TW	6	4	3	0.80	0.19	−1.295	−1.325
S. hualiensis PH	7	5	3	0.91	0.47	−0.197	−0.076
S. tawilis	7	5	3	0.91	0.24	0.239	−0.069
Control region							
S. hualiensis TW	21	21	21	1.00	4.33	−0.578	−0.843
S. hualiensis PH	15	14	14	0.99	3.81	−0.321	−0.438
S. tawilis	42	24	24	0.93	3.32	0.609	−0.212
S. tawilis (cluster 1 only)	29	15	12	0.88	1.04	**−1.690**	**−2.86**
S. tawilis (cluster 2 only)	13	9	7	0.91	1.23	0.476	0.068

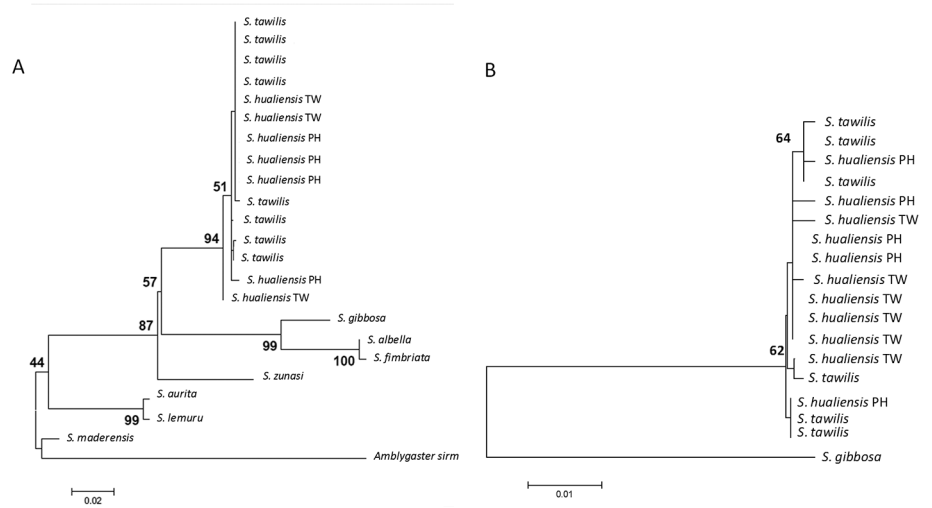

Figure 3. Maximum likelihood tree inferring phylogenetic relationship of *Sardinella tawilis,* Sardinella hualiensis, and other Sardinella species using (A) 16S sequence data and (B) S7 sequence data. Bootstrap probability (%, 1000 replicates) shown for values >40.

MtDNA control region sequences were obtained from 15 *S. hualiensis* individuals from the Philippines (PH), 21 *S. hualiensis* individuals from Taiwan (TW), and 42 *S. tawilis* from Taal Lake. Fifty-nine haplotypes were identified with no shared haplotypes between sites (Fig. 2), with high haplotype diversity (0.93–1.00) and high nucleotide diversity (3.32%–4.33%) at each sampling location (Table 2). The phylogenetic reconstruction inferred five clades (two clades each for *S. tawilis* and *S. hualiensis* TW, one for the *S. hualiensis* PH individuals) supported by low bootstrap probability values (<50%) from one another and indicative of a polytomy (data not shown). The five clades were, however, clearly distinct from the *S. lemuru* outgroup with a genetic distance of 36%–38% (SE 5.5%). The median-joining parsimony haplotype network showed two *S. tawilis* clusters, two *S. hualiensis* TW clusters, and one *S. hualiensis* PH cluster (Fig. 4), groupings similar to the clades described from the maximum likelihood analysis (data not shown). Low-frequency haplotypes were common at all sites with no clear dominant haplotypes. This may suggest a period of separation between the lineages, as the five haplotype clusters were separated by 10 or more single nucleotide mutations, and haplotypes within clusters were often separated by several mutational steps (Fig. 4). Evidence for recent population expansion, population bottlenecks or selective sweeps would be inferred from the presence of star-like polytomies in the haplotype network (Grant and Bowen 1998); however, such patterns were not observed. Both Tajima's *D* and Fu and Li's *D** neutrality tests were negative for nearly all species and genes, suggesting recent population expansion (Grant and Bowen 1998; Table 2). However, none of these values were significant, except for the *S. hualiensis* Taiwan Cyt *b* results. Finally, mismatch distribution results for control region sequence data for all three sampling sites were bi- or multi-modal (see Online Appendix), patterns reflecting constant population size (Schneider and Excoffier 1999).

MtDNA intraspecific genetic distance from control region data was lower [*S. tawilis* 3.5% (SE 0.5%), *S. hualiensis* 5.5% (SE 0.7%) than interspecific distance (*S.*

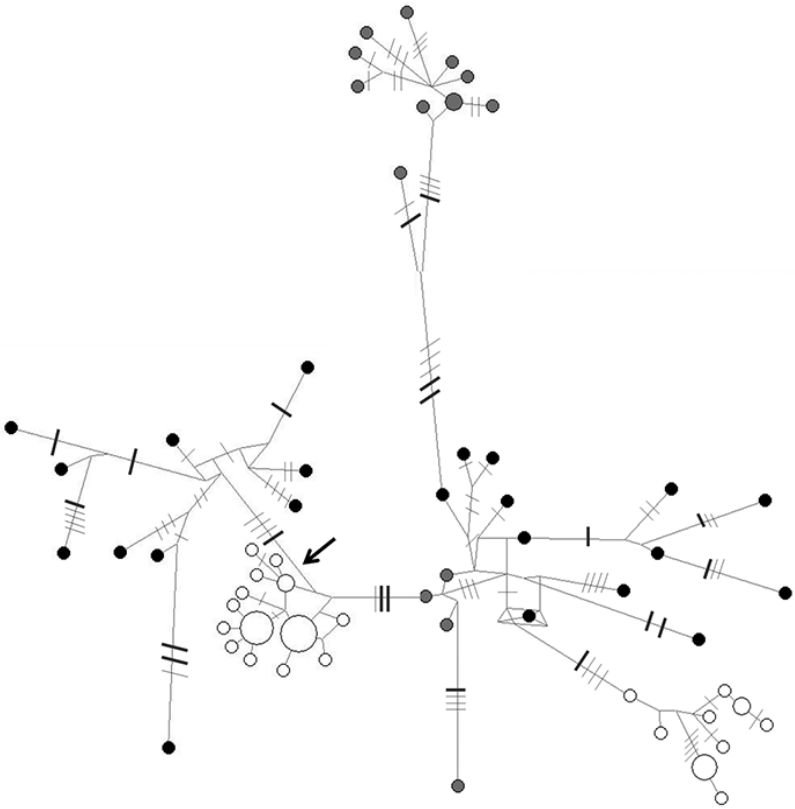

Figure 4. Median-joining parsimony network based on 435 bp of mitochondrial control region (n = 78) from *Sardinella tawilis* (white circles), *Sardinella hualiensis* Taiwan population (black circles), and *S. hualiensis* Philippine population (grey circles) samples. Each circle represents a haplotype with size of the circle proportional to frequency of a given haplotype. Branch lengths signify one mutational step with one additional step indicated by thin bar, five additional steps by thick bar. Black arrow just left of center in the network indicates the approximate location of where *Sardinella lemuru* (outgroup) would join the network based on a rooted median-joining parsimony network.

hualiensis – *S. tawilis* = 5.9% (SE 0.7%)]. MtDNA control region interspecific distance between *S. hualiensis* and *S. tawilis* and the out-group species was 28.8% or greater. The mean sequence diversity within each location was 0.035 for *S. tawilis*, 0.045 for *S. hualiensis* TW, and 0.040 for *S. hualiensis* PH. In examining population structure between the two *S. hualiensis* sites, significant genetic structure was found (Φ_{ST} 0.123, $P < 0.05$) suggesting a barrier to gene flow between the two *S. hualiensis* populations. Structure was also observed when *S. tawilis* sequences were included in the analysis (Global Φ_{ST} 0.149, $P < 0.05$).

The species tree models all indicate two population divergence times for *S. hualiensis* and *S. tawilis*. For the Cyt *b* mutation rate of 2%/MY, population divergence occurred first between the ancestral *S. hualiensis* TW lineage and the Philippine populations approximately 59,950 years ago (lower boundary of HPD interval 50,400 yrs ago), followed by a divergence between *S. hualiensis* PH and *S. tawilis* approximately 41,050 yrs ago (lower boundary of HPD interval – 30,200) (Fig. 5). For the

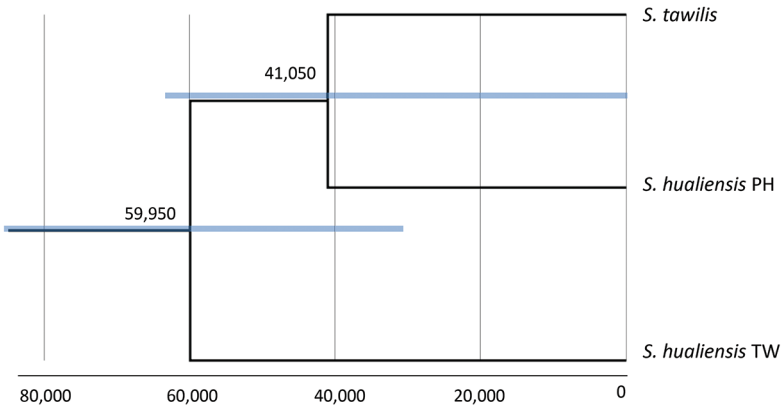

Figure 5. Species trees inferred from mtDNA 16S, Cyt *b*, and control region and nDNA S7 sequence data representing population divergence times among *Sardinella tawilis*, *Sardinella hualiensis* PH, and *S. hualiensis* TW as estimated by *BEAST. Species tree applies a 2%/MY mutation rate (divergence time above node) and 10% rate (divergence time below node) for the Cyt *b* gene region; the rate for other gene regions are estimated by *BEAST (species trees for other mutation rates not shown). Error bars (grey horizontal bars) represent 95% HPD intervals on age estimates. Scale bar is in years before present, ranging from 75,000 yrs ago to present day (0) for the 2%/MY rate (top axis), and 15,000 yrs ago to present day for the 10%MY rate (bottom axis).

rates of 1%/MY, 3%/MY, 5%/MY, and 10%/MY the first population divergence between the lineages occurred approximately 112,350, 41,350, 26,300, and 11,990 yrs ago, respectively; and the second population divergence, that between the marine *S. hualiensis* PH and freshwater *S. tawilis*, occurred approximately 76,500, 28,300, 18,000, and 8210 yrs ago, respectively. For the 10% mutation rate, the lower boundaries of HPD intervals for first and second divergence times were 10,080 and 6040 yrs ago (Fig. 5). All divergence times, with the exception of the fastest rate of 10%/MY, arise within the late Pleistocene period.

Bray-Curtis Similarity test on morphological and meristic data produced groups consistent with sampling location, with *S. hualiensis* samples from Taiwan and the Philippines most similar to one another, and sister to *S. tawilis* samples (Fig. 6). A single *S. tawilis* outlier grouped with the *S. hualiensis* PH specimens and is attributed to this aberrant individual's very high gillraker count and longer snout/head length proportions. The principal component analysis of all specimens revealed 30% of variation attributed to snout length proportion of fish and 17% of the variance attributed to gillraker counts. These two features are diagnostic features used to distinguish many *Sardinella* species (Whitehead 1985), although plasticity in gillraker counts has been attributed to natal origin (Kinsey et al. 1994).

Discussion

Our phylogenetic analyses indicate the closest extant relative for the freshwater *S. tawilis* is the marine *S. hualiensis*. This is further supported by small genetic distances and a large proportion of shared haplotypes between the species in the S7, Cyt *b*, and 16S gene regions. Our incomplete phylogeny of *Sardinella* based on the 16S gene

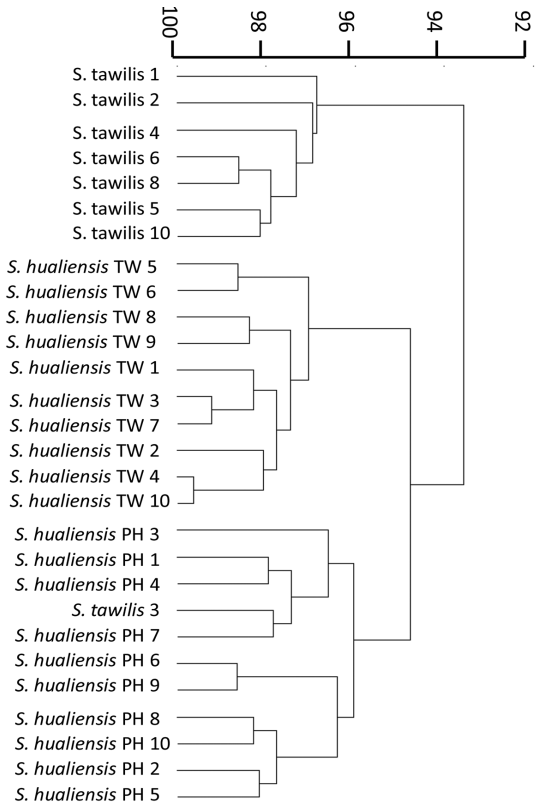

Figure 6. Tree based on similarity of morphological and meristic features between specimens of *Sardinella tawilis*, and *Sardinella hualiensis* from Taiwan (TW) and the Philippines (PH) obtained from a Bray-Curtis Similarity analysis. Percentage similarity between individuals and species is noted on the horizontal axis, individual specimens are listed along the vertical axis. One *S. tawilis* grouped with *S. hualiensis* PH, an outcome attributed to the individual having a very high gillraker count and longer snout/HL proportion which are similar to the *S. hualiensis* PH average. These features account for nearly half of the observed variance between groups in the PCA (not shown).

and the more complete phylogeny of the genus by Quilang et al. (2011) rule out most other potential sister species to *S. tawilis* and *S. hualiensis*. The only other *Sardinella* in the region not covered by these two phylogenies are *S. brachysoma*, *S. fijiense*, and *S. richardsoni,* which are all morphologically different from the morphologically similar *S. tawilis* and *S. hualiensis* (Whitehead 1985). This supports the sister species relationship of *S. tawilis* and *S. hualiensis* and what remains is an interpretation of their observed phylogenetic and phylogeographic patterns.

Despite unresolved gene trees (Figs. 3, 4), widely accepted species concepts (Hausdorf 2011) would define *S. tawilis* and *S. hualiensis* as separate species. For example, from the perspective of the evolutionary and biological species concepts, the physiological and geographic barriers between *S. tawilis* and *S. hualiensis* result in complete allopatry and reproductive isolation with no plausible path for natural interbreeding or for evolutionary reticulation. Although more similar to one another than any other *Sardinella* species, morphological differences were clearly diagnosable between *S. tawilis* and *S. hualiensis* (except one aberrant specimen, Fig. 6) therefore

meeting the criterion of a phylogenetic species (Hausdorf 2011). Furthermore, the species have also diverged physiologically, as *S. tawilis* has evolved the osmoregulatory mechanism necessary to adapt to a strictly freshwater environment. Marine to freshwater species transitions are relatively rare in fishes because of the stringent contrasting physiological requirements in these two habitats (Bloom and Lovejoy 2012). The loss of demographic connectivity (Mayr 1963, Lowe and Allendorf 2010), the relatively high proportion of unique haplotypes in the mitochondrial and nuclear genes (Craig et al. 2009) and the statistically significant Φ_{ST} values in the mtDNA control region data, support recognition of *S. tawilis* as a distinct freshwater species in Taal Lake. For sister species that exist allopatrically in both marine and freshwater habitats, however, species distinction can be contentious because of different interpretations of species concepts, and taxonomy is often left unresolved, defaulting to a common Latin binomial (Taylor 1999). We follow the current taxonomy (Whitehead 1985, Munroe et al. 1999) and accept the evidence for recognition of both *S. tawilis* and *S. hualiensis* as separate species as consistent with our interpretation of a species.

Unraveling the history of evolutionary divergence is difficult in species like sardines that go through sequential population expansions and contractions and whose ranges may have changed multiple times in the past (Bowen and Grant 1997, Quenouille et al. 2011) in response to fluctuations in environmental or ecological conditions (Chavez et al. 2003, Takasuka et al. 2007). The polyphyletic Control Region, S7, Cyt *b,* and 16 S gene trees (Fig. 3) could be explained by incomplete lineage sorting, or contemporary migration or hybridization between *S. tawilis* and *S. hualiensis*. Contemporary migration or hybridization seem unlikely due to the species' widely disjunct ranges, oceanographic currents that would prevent southward migration, osmoregulatory physiological differences, and no reports of either species occurring outside their described ranges (Fig. 1; Bognot and Mutia, Philippine National Fisheries Research and Development Institute, pers comm). Instead, the data indicate incomplete lineage sorting and suggest recent divergence between *S. tawilis* and *S. hualiensis*. A late Pleistocene (McMillian and Palumbi 1997) divergence is consistent with the species tree results at 1%/MY, 2%/MY, 3%/MY, and 5%/MY (Fig. 5), diversity indices, and neutrality test results. The very rapid 10%/MY rate suggests a more recent divergence, but still prior to the putative 18th century formation of Taal Lake. The S7, Cyt *b,* and 16S genes demonstrate high-to-low haplotype diversities with low nucleotide diversities, suggesting the populations had either experienced a bottleneck or were historically founded by a few lineages (Grant and Bowen 1998). Further, the faster-evolving control region sequences demonstrated high nucleotide and haplotype diversities and bi-modal mismatch distributions (see Online Appendix), implying large, stable populations (Table 2; Rogers and Harpending 1992). This is also consistent with non-significant neutrality tests across the four genes for all but one population and one gene (Table 2). Therefore, *S. tawilis* and *S. hualiensis* apparently have had enough time to establish stable populations after having undergone a population bottleneck and divergence in the late Pleistocene, or just after in the case of the 10%/MY rate. In general, the genetic results do not support a divergence of *S. tawilis* as recent as the 18th century when a Taal Volcano eruption reconfigured the hydrography of Taal Lake and the Pansipit River to its present day condition (Wolfe and Self 1983, Hargrove 1991).

The mtDNA control region data indicated two discrete *S. tawilis* haplotype clusters separated by approximately 20 mutational steps (Fig. 4). Several potential scenarios

may explain this divergence within *S. tawilis*. First, there may have been two independent freshwater invasions into Taal Lake by ancestors of the *S. tawilis* lineage. Although marine-freshwater transitions are rare in fishes, they are not uncommon within Clupeiformes (Wilson et al. 2008, DeFaveri et al. 2011, Bloom and Lovejoy 2012). Two invasions would be supported by separate divergence times for the two *S. tawilis* haplotype clusters; however, this is not consistent with the mean species divergence data (Fig. 5) that indicate a single divergence point for *S. tawilis*, and a divergence prior to the putative 18th century formation of the freshwater lake. Further, if the inferred population divergence did not co-occur with the very recent isolation of Taal Lake, then it may have not been caused by a freshwater invasion event. Second, Taal Lake may have historically been partitioned into separate lakes with allopatric populations of *S. tawilis* that diverged and subsequently coalesced into one. The bathymetry of Taal Lake shows a north and south basin partitioned at Volcano Island east to west by a ridge only 30 m or shallower from the water surface (Ramos 2002). Volcanic activity or fluctuations in lake water levels may have fully separated these basins, and then more recently united the basins and fish populations. This scenario also predicts different divergence times of the two haplotypes, but this is unfounded. Lastly, different haplotype clusters may have existed in the marine environment prior to establishment within Taal Lake, lineages that may have subsequently given rise to the present day Taal Lake *S. tawilis*. This third scenario is most supported by the species tree results that are indicative of a single event that caused the isolation of *S. tawilis* and the Philippine population of *S. hualiensis* (Fig. 5). This argues that the two different lineages existed in the marine environment before *S. tawilis* became isolated in Taal Lake. Neutrality tests suggest a stable population size for cluster 2, but significant negative Tajima's *D* and Fu and Li's *D** values indicate deviation from neutrality in cluster 1, a result suggesting either recent population expansion or purifying selection (Table 2).

Sardinella tawilis is both a valuable natural resource and a unique evolutionary lineage that requires conservation effort because of overfishing, introduction of nonnative species, and potential impacts from aquaculture (Mutia et al 2001, Cagauan 2007, Aquilino et al. 2011, Papa and Mamaril 2011). In addition to cultural and economic value, the study of its physiological adaptation to a freshwater environment may provide insights into evolutionary processes, particularly in view of recent advances in genomics (Czesny et al. 2012, Jones et al. 2012). The evolution of *S. tawilis* and identification of its subtropical sister species populations will provide ample opportunity for evolutionary investigation if efforts to conserve this precarious species are successful.

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