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Phylogenomics reveals habitat-associated body shape divergence in *Oryzias woworae* species group (Teleostei: Adrianichthyidae)

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ABSTRACT

The *Oryzias woworae* species group, composed of *O. asinua*, *O. wolasi*, and *O. woworae*, is widely distributed in southeastern Sulawesi, an island in the Indo-Australian Archipelago. Deep-elongated body shape divergence is evident among these three species to the extent that it is used as a species-diagnostic character. These fishes inhabit a variety of habitats, ranging from upper streams to ponds, suggesting that the body shape divergence among the three species may reflect adaptation to local environments. First, our geometric morphometrics among eight local populations of this species group revealed that the three species cannot be separated by body shape and that riverine populations had more elongated bodies and longer caudal parts than lacustrine populations. Second, their phylogenetic relationships did not support the presence of three species; phylogenies using mitochondrial DNA and genomic data obtained from RNA-Seq revealed that the eight populations could not be sorted into three different clades representing three described species. Third, phylogenetic corrections of body shape variations and ancestral state reconstruction of body shapes demonstrated that body shape divergence between riverine and lacustrine populations persisted even if the phylogenies were considered and that body shape evolved rapidly irrespective of phylogeny. Sexual dimorphism in body shape was also evident, but the degree of dimorphism did not significantly differ between riverine and lacustrine populations after phylogenetic corrections, suggesting that sexual selection may not substantially contribute to geographical variations in body shape. Overall, these results indicate that the deep-elongated body shape divergence of the *O. woworae* species group evolved locally in response to habitat environments, such as water currents, and that a thorough taxonomic reexamination of the *O. woworae* species group may be necessary.

1. Introduction

Body shapes of fishes vary greatly not only among species but also within species, and the variation is often related to habitat environments (Wootton, 1998; Langerhans and Reznick, 2010; Tytell et al., 2010). One striking pattern of body shape variation in fishes is the deep-elongated divergence, i.e., elongated vs. deep bodies (Claverie and Wainwright, 2014). Previous studies have demonstrated that these contrasting body shapes reflect adaptation of swimming performance to the physical properties of water, especially to water currents in the habitat. In general, an elongated body reduces drag and is hypothesized to be advantageous for cruising in open waters, such as in lakes and

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oceans (e.g. Walker, 1997; Svanbäck and Eklöv, 2004), or for swimming in rapid water currents (e.g., Hendry, 2001; Haas et al., 2010), whereas a deep body improves maneuverability and is favorable for unsteady swimming in spatially and temporally complex habitats (Blake, 1983; Webb, 1984; Wootton, 1998).

In addition to natural selection, fish body shapes are often under sexual selection. For example, deeper bodies are reported to increase male attractiveness (Head et al., 2013) and/or increase the social status (Quinn and Foote, 1994). Therefore, body shape dimorphism between males and females can evolve in fishes such that males have deeper body depths than females (e.g., Kitano et al., 2007; Hendry et al., 2006). Furthermore, the magnitudes of sexual dimorphism in body shape can differ between populations (e.g., Hendry et al., 2006; Spoljaric and Reimchen, 2008; Kitano et al., 2012). This is because natural selection in certain habitats constrains the evolution of exaggerated male body shape (e.g., Hendry et al., 2006; Langerhans and Makowicz, 2013) or because sexual selection pressures differ among habitats. Therefore, it is essential to investigate patterns of sex differences in body shape for a better understanding of the contribution of habitat environments to body shape variations among and within species.

One of the first and essential steps to test whether body shape divergence evolved as an adaptation to local environments is to find correlations between habitats and body shape. In such comparative studies of adaptation, however, phylogeny should be taken into account, because closely-related species/populations tend to share ancestral characteristics regardless of selection pressures to which they are exposed (Felsenstein, 1985; Harvey and Pagel, 1991; Garamszegi, 2014). Such a phylogenetic correction is necessary for making any strong inference that the deep-elongated body shape divergence is adaptive.

The family Adrianichthyidae, commonly referred to as either ricefishes or as medakas, is composed of two genera and 36 species (genus *Oryzias* having 32 species and genus *Adrianichthys* having four species), whose habitats range from freshwater to brackish-water environments (Parenti, 2008; Herder and Chapuis, 2010; Magtoon, 2010; Parenti and Hadiaty, 2010; Asai et al., 2011; Herder et al., 2012; Parenti et al., 2013; Mokodongan et al., 2014). This group is known to exhibit great variations in body shape even among closely-related species (Iwamatsu, 2006; Parenti, 2008). Especially, the deep-elongated body shape divergence is evident to the extent that it is used as a diagnostic character for some species. Three *Oryzias* species, *O. asinua*, *O. wolasi*, and *O. woworae*, which are called the *O. woworae* species group, have recently been described from southeastern Sulawesi, an island in the Indo-Australian Archipelago (Parenti and Hadiaty, 2010; Parenti et al., 2013). Parenti et al. (2013) reported that these three species can be distinguished from each other by body depth and caudal peduncle depth; *O. asinua* is relatively narrow-bodied compared with *O. wolasi* and *O. woworae*, whereas *O. wolasi* has a deeper caudal peduncle than the other two species. Because the *O. woworae* species group is monophyletic (Mokodongan and Yamahira, 2015) but found in a variety of habitats, ranging from fast-running upper streams to slow-running lower stream or ponds with still water (Yamahira et al., 2016), this species group provides a good model system to explore the evolutionary processes and mechanisms for adaptive changes in body shape in response to local habitat environments. Moreover, sexual dimorphism in body coloration and fin lengths is apparent in the three species (Parenti and Hadiaty, 2010; Parenti et al., 2013), suggesting that sexual selection is important in this species group, but sex difference in body shapes has never been investigated.

Several lines of evidence suggest that taxonomic reexamination of the *O. woworae* species group may be necessary. First, molecular phylogenetic analyses of Sulawesi adrianichthyids by Mokodongan and Yamahira (2015) revealed that two populations of *O. wolasi*, one from the type locality and another from the paratype locality (Parenti et al., 2013), are polyphyletic within the clade of the species group, suggesting that the current classification of this species group based on morphology may be incongruent with molecular phylogeny. Because the previous molecular phylogeny is based on a few loci (Mokodongan and Yamahira, 2015), construction of a robust phylogenetic tree using genomic data is essential. Second, we recently found many new localities of the *O. woworae* species group in the southeastern Sulawesi (Yamahira et al., 2016), suggesting that taxonomic status of this species group needs to be reconsidered after inclusion of these new populations.

In this study, we first used geometric morphometrics to examine body shape variations among eight populations of the *O. woworae* species group. Further, we reconstructed their phylogenies using mitochondrial and nuclear DNA sequences, where nuclear phylogenies were based on massively parallel RNA-Seq data. Using this phylogeny, we demonstrated that body shape of the eight populations examined are significantly associated with their habitat environments, i.e., riverine or lacustrine, even after controlling for phylogenetic effects. Based on these results, we discuss processes and mechanisms for the evolution of body shape divergence in this species group and discuss the necessity of taxonomic reexamination of the *O. woworae* species group.

2. Materials and methods

2.1. Materials

The *Oryzias woworae* species group, i.e., *O. asinua*, *O. wolasi*, and *O. woworae*, can be distinguished from its congeners by a bluish sheen on the body in live adult males and by orange to deep red dorsal and ventral margins on both the caudal fin and the ventral margin of the caudal peduncle in both sexes (Parenti and Hadiaty, 2010; Parenti et al., 2013). *O. asinua* is a relatively slender species compared to *O. wolasi* and *O. woworae*; body depth is 21–25% SL in *O. asinua* versus 23–32% and 22–30% SL in *O. wolasi* and *O. woworae*, respectively (Parenti and Hadiaty, 2010; Parenti et al., 2013). *O. wolasi* has a deeper caudal peduncle compared to *O. asinua* and *O. woworae*; caudal peduncle depth is 11–12% SL in *O. wolasi* versus 9–11% and 8–11% SL in *O. asinua* and *O. woworae*, respectively (Parenti and Hadiaty, 2010; Parenti et al., 2013).

The three species are also distinguished from each other by the number of procurrent caudal-fin rays; *O. asinua* has fewer procurrent caudal-fin rays in the lower lobe (4–5 versus 5–7 in *O. wolasi* and 5–6 in *O. woworae*) (Parenti et al., 2013).

2.2. Field collection

Wild *O. woworae* species group fish were collected from eight sites, i.e., five riverine and three lacustrine (Table 1), throughout the southeastern arm of the Sulawesi and Muna Islands (Fig. 1). Among the eight collection sites, Asinua River, Anduna River, and Fotuno Fountain are the exact type localities of *O. asinua*, *O. wolasi*, and *O. woworae*, respectively (Parenti and Hadiaty, 2010; Parenti et al., 2013). The Moramo Waterfall is also the same locality from which Parenti et al. (2013) collected *O. wolasi* paratypes. The other four collection sites,

Table 1
List of collection sites and studies specimens.

Locality	Habitat type	Collection date	Voucher number
Asinua River	Riverine	2014.11.27	MZB23359–23378
Anduna River	Riverine	2014.11.25	MZB23379–23398
Moramo River	Riverine	2014.12.14, 2015.11.9	MZB23399–23418
Moramo Waterfall	Riverine	2014.11.29	MZB23419–23438
Balano Fountain	Lacustrine	2014.12.6, 2014.12.9	MZB23439–23458
Laweau River	Riverine	2014.12.8	MZB23459–23478
Motobano Fountain	Lacustrine	2014.12.5, 2014.12.9	MZB23479–23498
Fotuno Fountain	Lacustrine	2014.12.7	MZB23499–23518

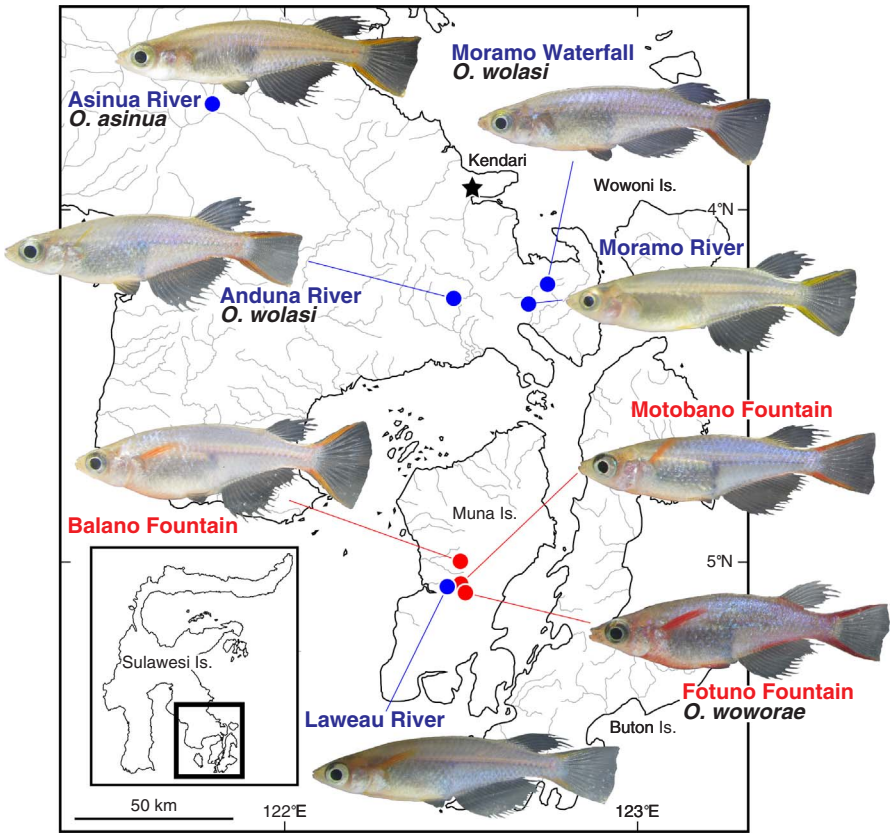

Fig. 1. Map showing the location of each collection site. Blue and red circles represent riverine and lacustrine populations, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Moramo River, Balano Fountain, Motobano Fountain, and Laweau River, were new localities discovered by Yamahira et al. (2016). The new four populations could not be diagnosed only from morphometric characters (Mokodongan, unpublished data). We collected ten adult males and ten adult females from each of the eight sites, 160 individuals in total, using a beach seine.

2.3. Body shape analysis

The collected individuals were brought back to our indoor facilities alive. Each individual was anaesthetized with MS-222 and photographed from the left side with a ruler, using a digital camera (Optio W90, Pentax, Tokyo, Japan). The digital pictures were later transferred to a personal computer, and 15 landmarks (Fig. 2) were placed on the images using image analyzing software (Illustrator CS6, Adobe Systems, San Jose, USA).

A Procrustes analysis was conducted on the landmark data to adjust the scale and placement of objects and to obtain “shape variables”, and subsequently a principal component analysis was performed on the shape variables, using MorphoJ 1.06d (Klingenberg, 2011). First, these geometric morphometric (GM) analyses were performed on the full data set of the 160 individuals (80 males and 80 females) to explore sexual dimorphism in body shapes. The mean of each population for the first and second principal components (PC1 and PC2, respectively) was obtained separately for males and females, and one-factor analysis of variance (ANOVA) with sex (i.e., male vs. female) as a fixed factor was performed on the means, using JMP 5.1.1 for the Macintosh (SAS Institute, Cary, USA). Moreover, to test if the degree of sexual dimorphism in body shapes differs between the riverine and lacustrine populations, one-factor ANOVA with habitat type (i.e., riverine vs. lacustrine) as a fixed factor was performed on the sex differences in the mean PC1 and PC2 values of each population.

Second, GM analyses were also performed separately for males and females to explore shape differentiation in each sex among the eight populations. To test if body shapes differ between the riverine and lacustrine populations, one-factor ANOVA with habitat type as a fixed factor was performed separately for males and females on the mean PC1 and PC2 values of each population.

2.4. Phylogenetic analysis using mitochondrial sequences

Either the pectoral or the caudal fin was collected from each

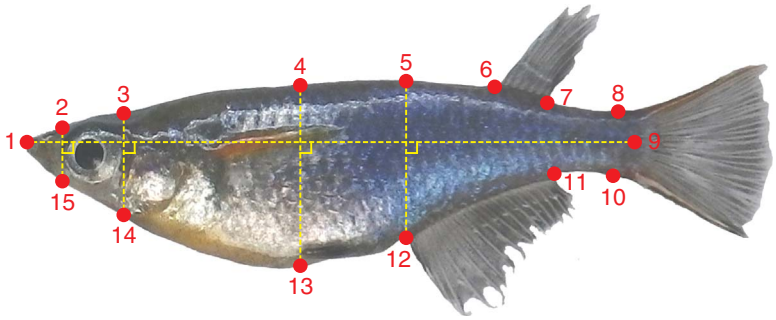

Fig. 2. Landmark configurations for the geometric morphometric (GM) analysis. 1: Anterior extent of maxilla. 2–5: Upper ends of perpendicular at anterior extent of orbit, at posterioventral extent of operculum, at anterior insertion of pelvic fin, and at anterior insertion of anal fin, respectively. 6 and 7: Anterior and posterior insertions of dorsal fin, respectively. 8: Insertion of the dorsalmost caudal-fin principal ray. 9: Posterior end of hypural. 10: Insertion of the ventral most caudal-fin principal ray. 11 Posterior insertion of anal fin, 12–13: Anterior insertions of anal and pelvic fins, respectively. 14: Posterioventral extent of operculum. 15: Lower end of perpendicular at anterior extent of orbit.

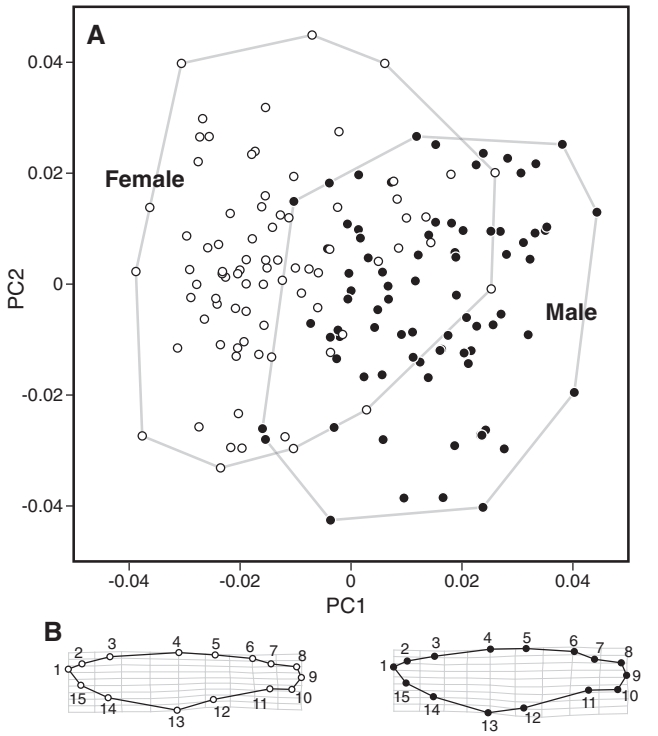

Fig. 3. Scatterplot of the first two axes of principal component analyses based on the geometric morphometric data set (A). Closed and open circles represent males and females, respectively. Deformation grids showing the maximum shape changes along the PC1 axis (B).

individual and preserved in 99% ethanol (other body parts were preserved in RNAlater for extraction of RNA; see below). In the laboratory, DNA was extracted from the fins using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol. We amplified and sequenced a mitochondrial region, including NADH dehydrogenase subunit 2 (ND2), using the methods and primers described in Mokodongan and Yamahira (2015).

All sequences for the 160 individuals from the eight populations were aligned using ClustalW version 1.4 (Thompson et al., 1994), and the ND2 gene region (1046 bp) was extracted. Forty unique haplotypes were detected among the 160 ND2 sequences using DnaSP version 5.10.01 (Librado and Rozas, 2009). The haplotype sequence data were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC316614–316653.

Phylogenies among these unique haplotypes were estimated using the maximum likelihood (ML) and Bayesian inference (BI) methods. Sequence data from two *O. celebensis* individuals from DDBJ (LC51719 and LC51720) were used as outgroups (*O. celebensis* is one of the species phylogenetically closest to the *O. woworae* species group; Mokodongan and Yamahira, 2015). ML analysis was performed in raxmlGUI version 1.31 (Silvestro and Michalak, 2012) using codon-specific GTR + G models, where a rapid bootstrap analysis of 10,000 bootstrap replicates was conducted. BI analysis was conducted in MrBayes version 3.2.4 (Ronquist et al., 2011). Appropriate substitution models were determined for codon positions, i.e., GTR + Γ, GTR, and GTR + Γ for the first, second, and third positions, respectively, in jModelTest version 2.1.7 (Darriba et al., 2012) based on the Akaike Information Criterion. The analyses were run using the following settings: ngen = 3,000,000, samplefreq = 100, and burnin = 750,000. Convergence of the MCMC (Markov chain Monte Carlo) chain to the stationary distribution and large ESS (effective sample size) (> 200) were confirmed for all parameters, using Tracer version 1.6 (Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2013. Tracer v1.6. URL: http://tree.bio.ed.ac.uk/software/tracer/).

2.5. Phylogenomic analysis using RNA-Seq data

Three individuals (one male and two females) collected from each of the eight wild populations, one lab-raised Fotuno Fountain individual (one female) maintained in the fish facility of the National Institute of Genetics, Japan, and two individuals (one male and one female) of *O. celebensis*, as an outgroup, which had been reared in the World Medaka Aquarium, Japan, i.e., in total 27 individuals were used in the following RNA-Seq. Total RNA was extracted from a single eye ball of each individual using the RNeasy Mini Kit (Qiagen). Libraries were constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, USA). Libraries with different barcodes were pooled and run on the Illumina MiSeq sequencer with the 2 × 300 bp paired-end mode at National Institute of Genetics, Mishima, Japan (average read number ± SD = 1,166,540 ± 829,064). All raw data were deposited in the DDBJ Sequence Read Archive under accession number DRA006053.

The quality of the MiSeq sequence reads was assessed by FaQCs.pl script (Lo and Chain, 2014), where (1) raw reads with average q-value < 20 were filtered out from the dataset, (2) raw reads were trimmed to remove low-quality bases (q-value < 20) mainly in the latter part, and (3) raw reads less than 50 bps were also excluded. A total of 27,730,260 paired-end trimmed reads from all of the 25 individuals of the *O. woworae* species group (i.e., 24 wild and one lab-reared individuals) were then assembled *de novo* using Trinity version trinityrnaseq_r20140413 (Grabherr et al., 2011). The quality of the initial transcriptome assembly was as follows: N50 (weighted average of contig sizes) = 1776 bp; total size = 136.0 Mbp; number of contigs = 160,907. To eliminate duplicate contigs, the initial transcriptome was clustered using CD-HIT-EST (Fu et al., 2012). In this step, contigs with > 80% nucleotide sequence identity were considered duplicate genes, splicing variants, and/or alleles, and only the longest isoform was kept. This "clustered" transcriptome was used as the transcriptome reference for mapping. The quality of the clustered transcriptome is as follows: N50 = 1263 bp; total size = 98.95 Mbp; number of contigs = 141,569.

Trimmed reads of each wild individual, including two *O. celebensis*, were then mapped to these 141,569 reference contigs using STAMPY v1.0.23 (Lunter and Goodson, 2011). Transcripts with average per-site coverage < 5× were excluded for each individual. Individual sites within a transcript were masked as N, if coverage at that site was < 5× or the variant quality score was < 40.

Nucleotide sequence alignments for each contig were generated by MAFFT v7.212 (Katoh and Standley, 2013). Only contigs shared among all 26 individuals (24 individuals of the *O. woworae* species group and two individuals of *O. celebensis*) were used for the phylogenomic analysis described below. As a result, 455 contigs of > 200 bp with a total alignment length of 0.87 Mbp remained. After excluding sites containing low quality bases (masked by "N") in one or more individuals and alignment gaps, the number of effective sites had a total alignment length of 0.34 Mbp.

After all contigs were concatenated, a ML tree of the 24 individuals of the *O. woworae* species group and two individuals of *O. celebensis* as outgroups was estimated using raxmlGUI version 1.31 (Silvestro and Michalak, 2012). In the ML analysis, each contig was set as a separate partition, the codon-specific GTR + I + Γ models was used, and the reliability of each tree node was assessed by a rapid bootstrap analysis of 100 replicates.

A coalescent-based population tree was reconstructed using SVDquartets (Chifman and Kubatko, 2014) implemented in PAUP* 4a (build 157) (Swofford, 2002). The analysis was run on the concatenated 455-contig sequence data set (three individuals per population except for two individuals for *O. celebensis*), where each contig was set as a separate partition using the command "charpartition". The reliability of each tree node was assessed by multilocus bootstrapping of 1000 replicates.

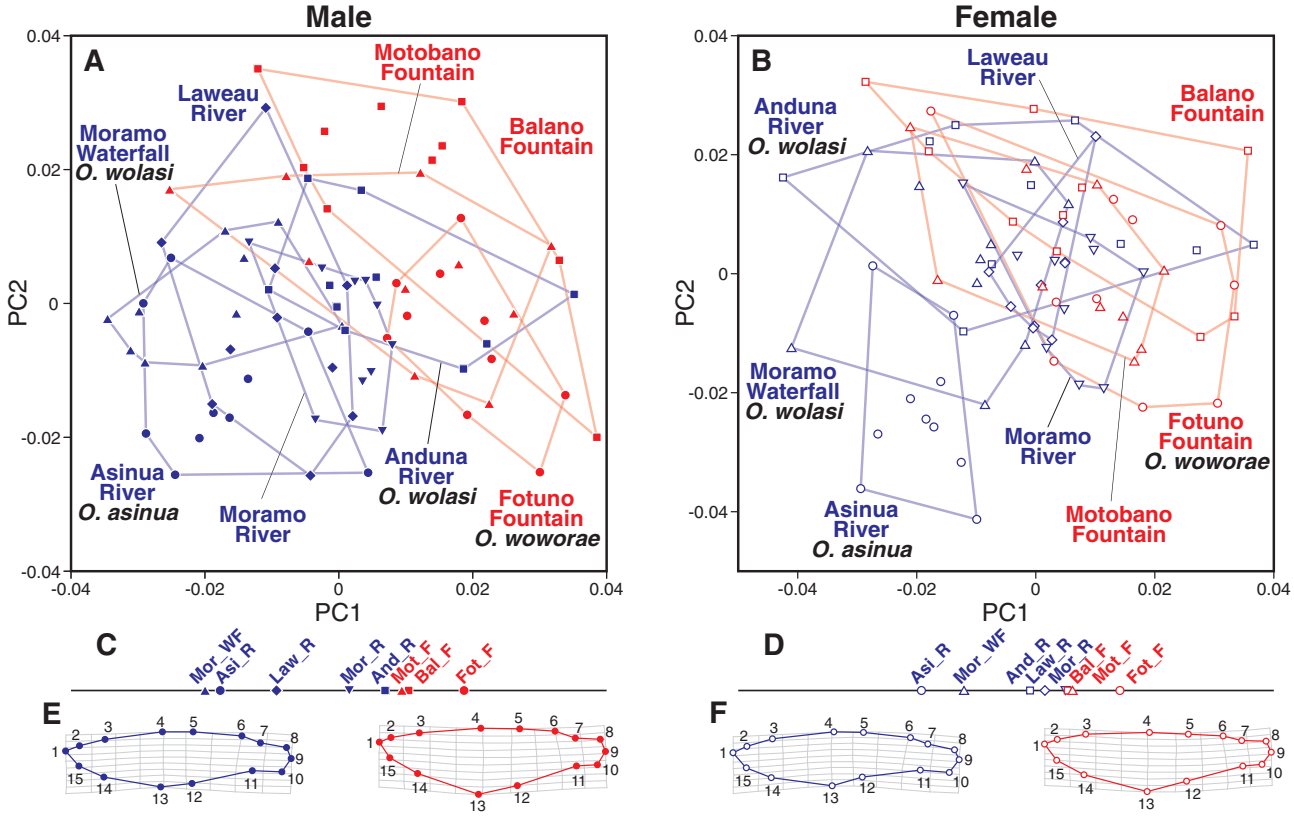

Fig. 4. Scatterplot of the first two axes of principal component analyses in males (A) and females (B) based on the geometric morphometric data sets. Blue and red symbols represent riverine and lacustrine populations, respectively. Mean PC1 score for each population is indicated separately for males (C) and females (D). Deformation grids showing the maximum shape changes along the PC1 axis in males (E) and females (F).

Population-averaged phylogeny was also constructed using the number of net nucleotide substitutions per site between two populations (d_A) (Nei and Kumar, 2000). We calculated a d_A value of all examined contigs for each pair among the eight populations, using the command-line version of Arlequin ver. 3.5 (Excoffier et al., 2005). A phylogenetic tree of the eight populations was then reconstructed using the matrix of d_A values with the neighbor-joining (NJ) method implemented in NEIGHBOR program for PHYLIP 3.6 software package (http://evolution.genetics.washington.edu/phylip.html).

2.6. Phylogenetic comparison and ancestral state reconstruction of body shape

We tested whether body shapes in each sex differ between the riverine and lacustrine populations when the phylogenetic relationships among populations were considered, using phylogenetic generalized least squares (PGLS) (Grafen, 1989; Martins and Hansen, 1997). The PGLS analysis was performed on the population mean PC1 and PC2 values obtained separately for males and females in the GM analyses above, using the "pgls" function in the R package "caper" (Orme et al., 2013). In the PGLS analysis, the population-averaged NJ phylogeny estimated by the d_A values among the eight populations (see above) was used as the tree data, and the branch lengths were optimized by ML estimations ("lambda" function = ML). Similarly, we tested whether the degree of sexual dimorphism in body shapes differs between the riverine and lacustrine populations by performing PGLS analysis on the sex differences in the population mean PC1 and PC2 values obtained in the GM analyses above without separating sexes, using the same population-averaged NJ phylogeny.

Ancestral values of PC1 at each node in the NJ phylogeny were also estimated separately for males and females, using the "fastAnc" function in the R package "phytools" (Revell, 2012), where the option "CI = TRUE" was set. The estimated state was mapped on the phylogeny using the "contMap" function in phytools.

3. Results

3.1. Body shape variation

GM analyses including both sexes revealed that 30.6% and 23.3% of the variance in body shape were explained by PC1 and PC2, respectively. A scatter plot of the PC1 and PC2 scores showed that males and females mainly differ along the PC1 axis (Fig. 3A); PC1 significantly differed between males and females (ANOVA, $F_{1,14} = 25.4387$, $P = 0.0002$). PC1 reflects overall body depth especially in the caudal parts (around landmarks 6, 11, and 12) and the positions of anterior insertions of anal (landmark 12) and pelvic fins (landmark 13), indicating that males tended to have deeper bodies with longer caudal parts than females (Fig. 3B). In contrast, PC2 did not significantly differ between males and females ($F_{1,14} = 1.5867$, $P = 0.2284$). The degree of sexual dimorphism (sex difference in the mean PC values) did not differ between the riverine and lacustrine populations for PC1 ($F_{1,6} = 0.6875$, $P = 0.4388$) or PC2 ($F_{1,6} = 0.7762$, $P = 0.4122$).

GM analyses performed separately for each sex revealed that in males 30.1% and 19.6% of the variance in body shape were explained by PC1 and PC2, respectively. A scatter plot of the PC1 and PC2 scores showed that the eight populations do not make distinct clusters separable from one another (Fig. 4A). The four populations from which type specimens of the three species were collected, i.e., Asinua River (*O. asinua*), Anduna River (*O. wolasi*), Moramo Waterfall (*O. wolasi*), and Fotuno Fountain (*O. woworae*), did not clustered into three groups; *O. wolasi* males from Anduna River and Morano Waterfall showed little overlap. Instead, fishes collected from different types of habitats (riverine vs. lacustrine) diverged along the PC1 axis; riverine populations

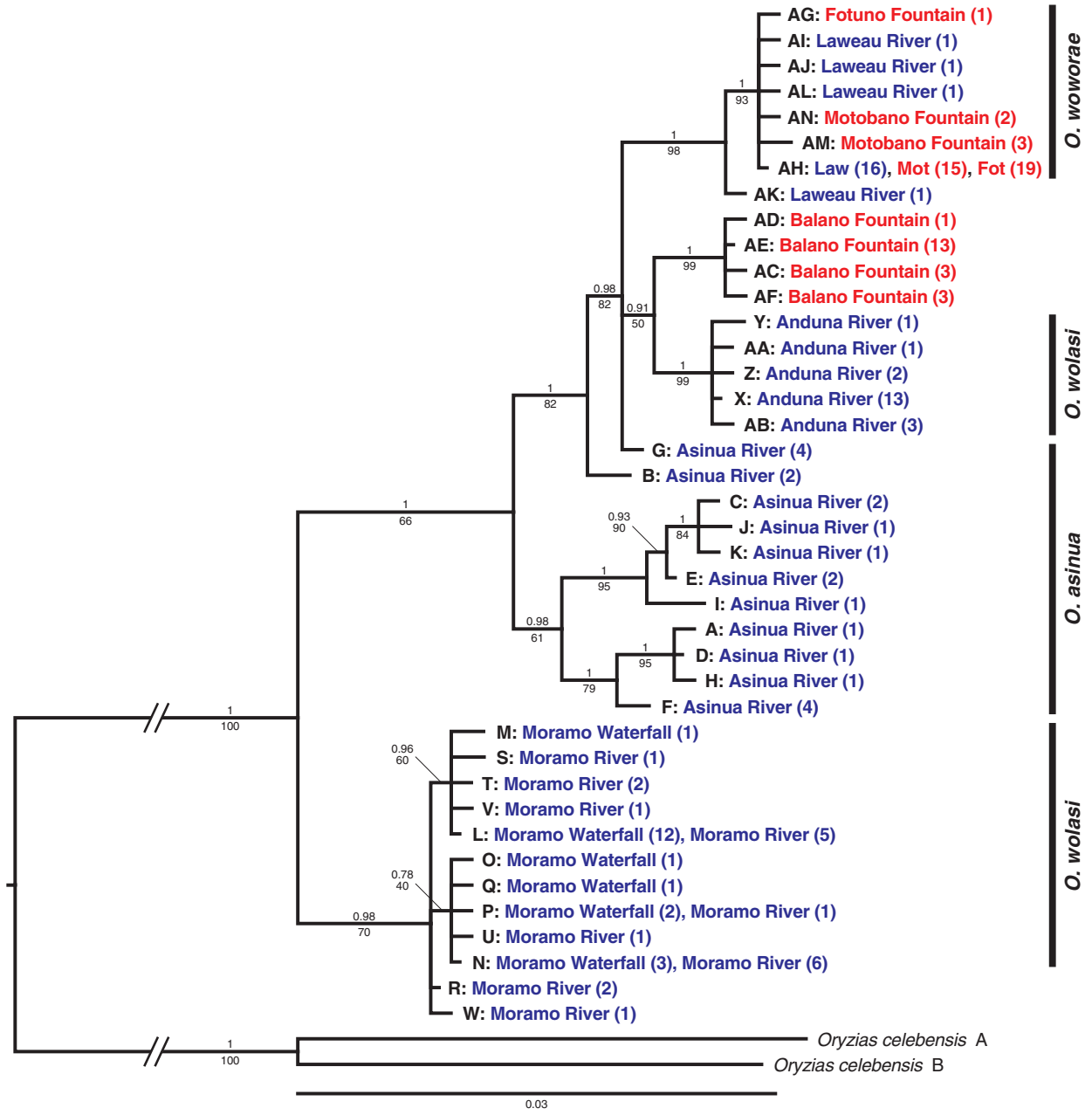

Fig. 5. Bayesian inference of phylogeny among haplotypes based on the mitochondrial ND2 sequences (104 bp). Blue and red characters represent riverine and lacustrine populations, respectively. Numbers on branches are Bayesian posterior probabilities (top) and maximum likelihood bootstrap values (bottom). Letters and numbers in parentheses after location names indicate haplotypes and number of individuals, respectively. Scale bar indicates the number of substitutions per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

generally had smaller PC1 values than the lacustrine populations (Fig. 4C). ANOVA revealed a significant effect of the habitat type (riverine vs. lacustrine) on PC1 ($F_{1,6}$ = 7.8690, P = 0.0310). In fish with larger PC1, landmarks 12 and 13 are located at more ventral sides (i.e., deeper body) and landmarks 5 and 12 are located at more posterior sides (i.e., shorter caudal parts) (Fig. 4E). In contrast, PC2 did not significantly differ between the habitat types ($F_{1,6}$ = 2.5335, P = 0.1626).

In females, PC1 and PC2 accounted for 29.4% and 24.9% of the variance in body shape, respectively. A scatter plot of the PC1 and PC2 scores in females showed patterns of shape variations qualitatively similar to those in males: riverine populations tended to have smaller PC1 values than lacustrine populations (Fig. 4B and D). PC1 was again related to overall body depth and length of caudal parts (Fig. 4F). The effect of habitat type on the differences in PC1 was close to significance ($F_{1,6}$ = 4.6085, P = 0.0754), while PC2 did not significantly differ between habitat types ($F_{1,6}$ = 0.6525, P = 0.4500).

3.2. Mitochondrial phylogeny

A phylogenetic tree based on the ND2 sequence revealed that haplotypes found in Moramo Waterfall, the paratype locality of *O. wolasi*, clustered with haplotypes from Moramo River at the most basal position (Fig. 5). However, haplotypes found in Anduna River, the type locality of *O. wolasi*, belonged to another clade, indicating that *O. wolasi* were polyphyletic. The haplotypes from Anduna River clustered with haplotypes from the four populations of Muna Island, i.e., Fotuno Fountain, Motobano Fountain, Laweau River, and Balano Fountain, at the most derived position. Most haplotypes from Asinua River were

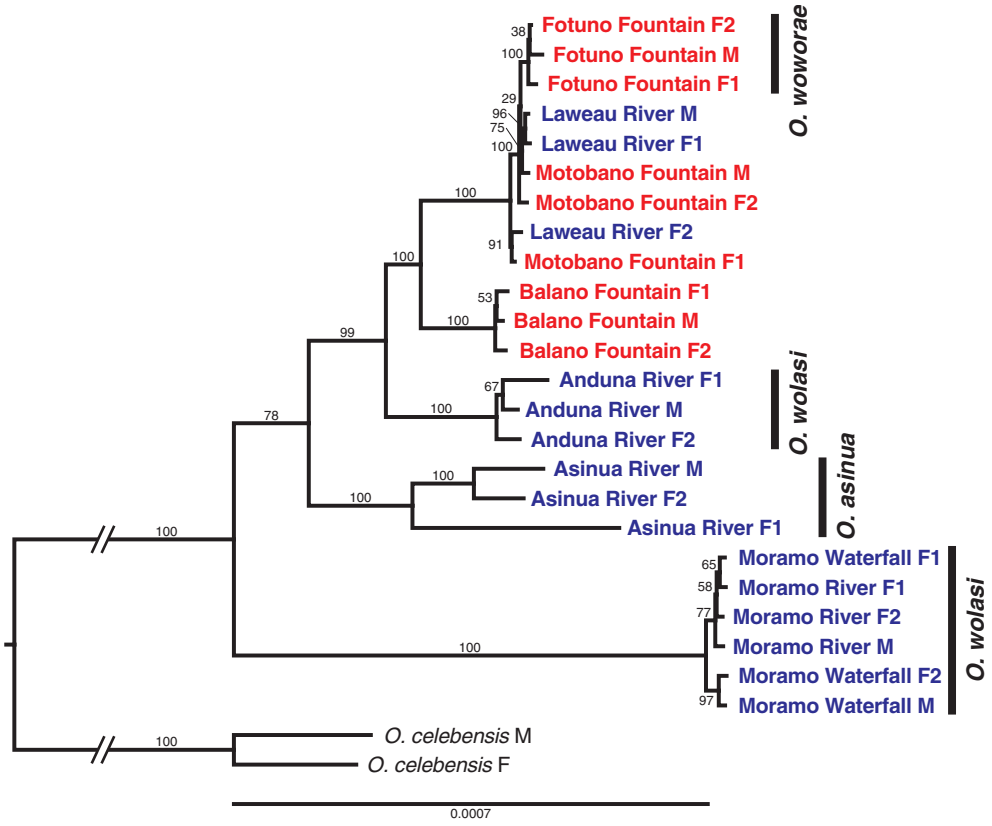

Fig. 6. Maximum likelihood (ML) nuclear phylogeny produced by concatenating 455 transcripts totaling 0.34 Mbp sites. Blue and red characters represent riverine and lacustrine populations, respectively. Numbers on branches show ML bootstrap values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

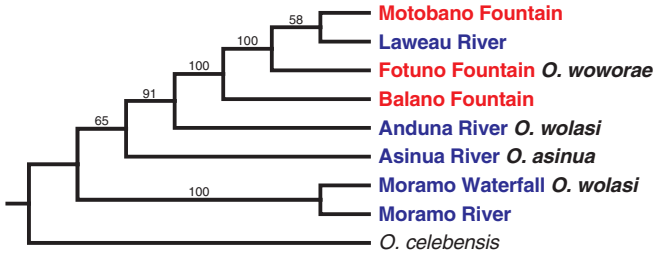

Fig. 7. The population tree using SVDquartets + PAUP* based on the three-individuals-per-population dataset (two individuals for *O. celebensis*). Each of the 455 transcripts was set as a separate partition. Numbers on branches show bootstrap values.

clustered with each other and sister to the Anduna-Muna clade, but some belonged to the Anduna-Muna clade.

3.3. Nuclear phylogeny

A ML phylogenetic tree based on the concatenated nuclear sequences obtained from RNA-Seq also revealed that *O. wolasi* is polypheletic such that the Anduna River population of *O. wolasi* is more closely related to the four populations of Muna Island than to the Moramo Waterfall population of *O. wolasi* (Fig. 6). As in the mitochondrial phylogeny, individuals from Moramo Waterfall clustered with individuals from Moramo River at the most basal position. Individuals from Anduna River were sister to individuals from Muna Island, i.e., Fotuno Fountain, Motobano Fountain, Laweau River, and Balano Fountain, at the most derived position. Individuals from Asinua River were sister to the Anduna-Muna clade.

The coalescent-based population tree yielded essentially the same topology as that of the individual-based ML phylogeny. The Moramo Waterfall and Moramo River populations formed a clade at the most basal position, but the Anduna River population was sister to the clade composed of the four populations in Muna Island at the most derived position (Fig. 7). The Asinua River population was sister to the Anduna-Muna clade. The NJ phylogenetic tree among the eight populations based on d_A values also yielded essentially the same topology as that of the coalescent-based population tree (Fig. 8).

3.4. Phylogenetic comparison and ancestral state reconstruction of body shape

PGLS analysis of GM data revealed that the effect of habitat type, i.e., riverine vs. lacustrine, on male body shape persisted, even if the phylogenetic relationships among populations were considered; the riverine populations had significantly smaller mean PC1 values than the lacustrine populations (estimate = −0.0202 ± 0.0076 SE, $t = -2.6643$, $P = 0.0373$). In females, the riverine populations also tended to have smaller mean PC1 values than the lacustrine populations even after phylogenetic corrections, but the effect of habitat type was not significant (estimate = −0.0137 ± 0.0065 SE, $t = -2.1131$, $P = 0.0790$). PC2 did not significantly differ between the riverine and lacustrine populations in males (estimate = −0.0105 ± 0.0063 SE, $t = -1.6740$, $P = 0.1452$) or females (estimate = −0.0038 ± 0.0071 SE, $t = -0.5307$, $P = 0.6147$) regardless of phylogenetic corrections. The degree of sexual dimorphism also did not differ between the riverine and lacustrine populations in PC1 (estimate = 0.0052 ± 0.0060 SE, $t = 0.8609$, $P = 0.4224$) or PC2 (estimate = 0.0029 ± 0.0040 SE, $t = 0.7269$, $P = 0.4947$) regardless of phylogenetic corrections.

The ancestral state reconstruction in males revealed that the ancestral body shape was intermediate between the elongated riverine form and the deeper-bodied lacustrine form (Fig. 7A). Bodies became more elongated in the Asinua River and Moramo Waterfall populations. The deep bodies with short caudal parts in the three lacustrine populations, i.e., Fotuno Fountain, Motobano Fountain, and Balano Fountain, evolved in their common ancestor. One riverine population on Muna Island, i.e., Laweau River, independently evolved an elongated

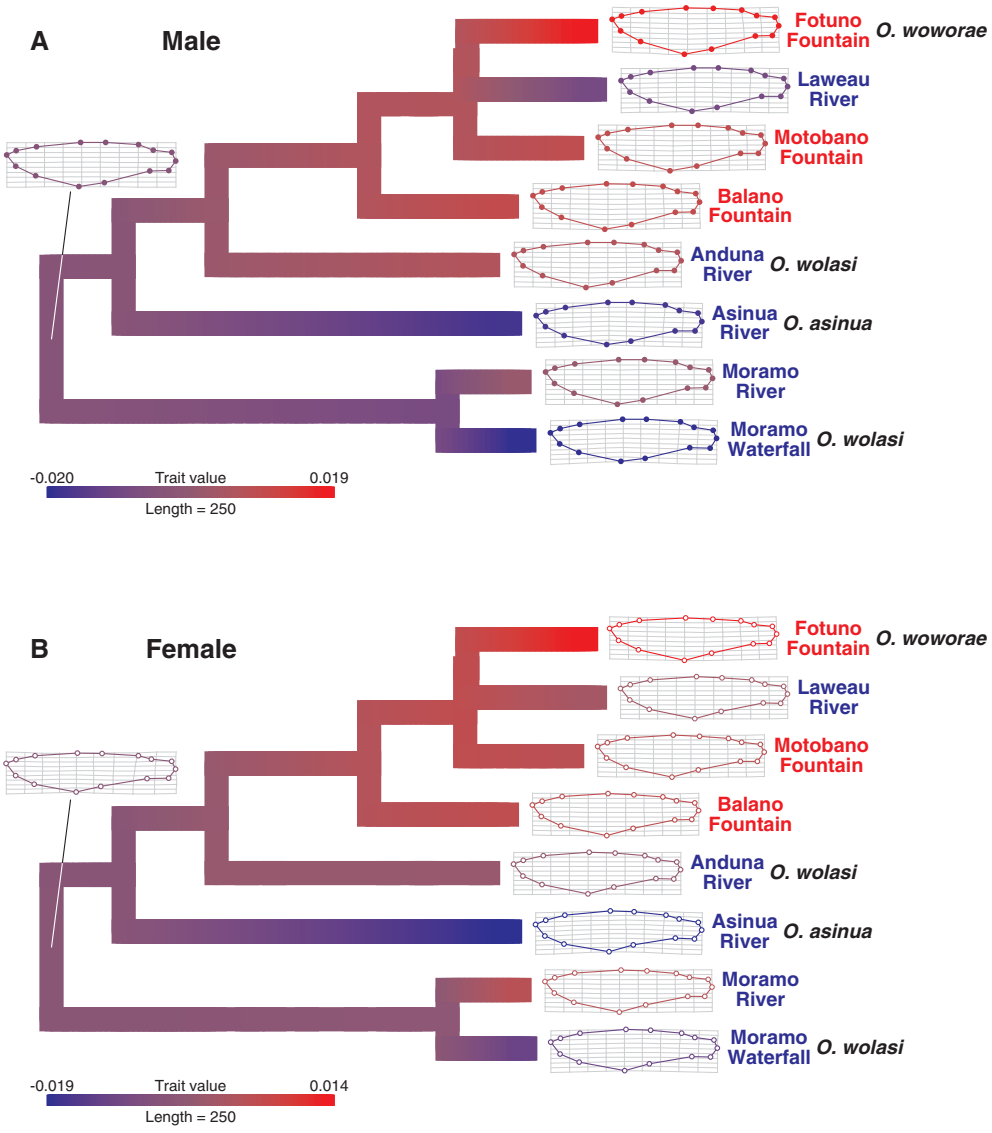

Fig. 8. Ancestral state reconstruction of body shape in males (A) and females (B). Estimated changes in PC1 values (see Fig. 4) on the phylogeny are represented by the branch color. Blue and red characters represent riverine and lacustrine populations, respectively. Scale bar indicates the number of net nucleotide substitutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

body from its deep-bodied ancestor. The ancestral state reconstruction in females showed essentially similar patterns with those in males (Fig. 7B).

4. Discussion

4.1. Processes and mechanisms of body shape divergence

Our GM analyses revealed that body shape of the *O. woworae* species group varied among local populations according to their habitat types, i.e., riverine vs. lacustrine. Individuals from the riverine populations had two common morphological features; riverine populations had more slender bodies and longer caudal parts both in males and females than lacustrine populations. In males, this body shape difference between habitat types persisted even after controlling for phylogenetic effects. The ancestral state reconstruction revealed that elongated bodies with longer caudal parts evolved several times in distinct branches, indicating that this body shape adaptation occurred independently in each local habitat. These results indicate that habitat-associated body shape divergence might result from local adaptation.

The slender body in riverine individuals probably reflects adaptation to riverine environments, such as rapid water currents. Similar body elongation in rapid streams is also known from many other fish taxa (e.g., Hendry, 2001; Brinsmead and Fox, 2002; Haas et al., 2010; see review by Langerhans, 2008). It has been reported that slender bodies hydrodynamically reduce drag and enhance swimming performance in fishes (Blake, 1983, 2004; Webb, 1992; Vogel, 1994; Kokita and Mizota, 2002). Longer caudal parts may also contribute to higher swimming performance in rapid water currents, because fish generate propulsion by beating their tails (Webber et al., 2001). In the Japanese medaka species complex (*O. latipes* species complex), it is known that individuals from lower latitudes swim faster than individuals from higher latitudes (Suzuki et al., 2010; Fujimoto et al., 2012), which is consistent with the patterns of the sizes of caudal parts with the former having longer caudal parts than the latter (Yamahira and Nishida, 2009).

In contrast, deep bodies are suitable for manoeuvrability and therefore may be favorable for unsteady swimming in spatio-temporally complex habitats, such as vegetated waters or environments with spatio-temporally variable water currents, (Blake, 1983; Webb, 1984; Wootton, 1998). The deep bodies in the lacustrine populations of the *O. woworae* species group may reflect adaptation to shallow vegetation areas of the ponds. In other taxa, lacustrine fishes are known to have a tendency to be elongated than riverine fishes (e.g., Webb, 1984; Berner et al., 2008; Kakioka et al., 2015), because open water areas of large lakes are less complex than river environments. However, it was not the case for the *O. woworae* species group; lacustrine fishes are more deep-bodied than riverine fishes.

Body shape is also often under sexual selection. In several fish taxa, deeper-body shapes increase reproductive success in males (Head et al., 2013; Quinn and Foote, 1994). The patterns of sexual dimorphism in body depth in the *O. woworae* species group with males having deeper bodies than females are concordant with the previous examples in other fish taxa, suggesting that body shape may be under sexual selection in this species group. Sexual dimorphism was also evident in the length of caudal parts with males having longer ones, suggesting that this trait may be also important for attracting females or male-male competition. The contribution of body shape to reproductive success in this species group should be investigated in more details in the future. Although the degree of sexual dimorphism often differs between habitats (Hendry et al., 2006; Spoljaric and Reimchen, 2008; Kitano et al., 2012), we found no differences in the degree of sexual dimorphism in body shapes between riverine and lacustrine populations of the *O. woworae* species group. Therefore, the observed body-shape divergence between habitat types was unlikely to be caused by the difference in sexual selection pressures.

Fish body shape is also often related to predation pressures; fish with deeper bodies and larger caudal parts tend to escape more successfully from piscivorous predatory fishes because of gape limitation (e.g., Hoyle and Keast, 1987; Hambright, 1991; Mittelbach and Persson, 1998) and faster escape swimming (e.g., Langerhans, 2009; Suzuki et al., 2010). Because our field collections and underwater observations showed that freshwater viviparous halfbeaks of the genus *Nomorhamphus*, the primary predator of *Oryzias*, are abundant in all of the eight habitats (Mokodongan et al., personal observation), it is unlikely that variations in predation pressures explain the majority of body shape variations in the *O. woworae* species group. However, it should be noted that further detailed quantitative field survey is necessary to make any conclusions.

4.2. Taxonomic status of the Oryzias woworae species group

Our molecular phylogenies did not identify any three distinct clusters representing three different species, contrary to the expectation from a phylogenetic species concept. Especially, it is worth mentioning that *O. wolasi* from Anduna River, the type locality, and Moramo Waterfall, the paratype locality, did not form a clade both in the mitochondrial and nuclear phylogenies, which agrees with Mokodongan and Yamahira (2015). Moreover, males from Anduna River and Moramo Waterfall differed in body shape to the extent that the PC1 and PC2 score plots in the GM analyses hardly overlapped with each other. These results indicate that *O. wolasi* may not be a valid species in terms of both phylogenetic and morphological species concepts; this species might be composed of two or more species or subspecies.

Alternatively, it may be more natural to consider that there are several ecotypes in the *O. woworae* species group rather than that clearly distinguishable valid species/subspecies exist. Given that the morphological variation was continuous and that the variation was correlated with water currents even after controlling for phylogenetic effects, the three species are probably just part of the continuum of adaptive variation. The presence of slender body fish in Laweau River, the only riverine populations in Muna Island, strongly suggests that body shape can evolve rapidly irrespective of phylogeny, and that convergent evolution of body shape can occur.

Our results indicate that examination of geographical variations in relation to habitats is essential for testing whether species are valid or not when species have wide distributions. It should be noted, however, that we did not examine any meristic characteristics, including the number of procurrent caudal-fin rays which was used as a diagnosis character among the three species of this group, although this number also overlaps with each other (Parenti et al., 2013). Overall, our data demonstrate that thorough taxonomic reexamination of the *O. woworae* species group should be done in the future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.10.005.

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