Morphometric differentiation among haplochromine cichlid fish species of a satellite lake of Lake Victoria

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Abstract

Lake Victoria holds a young but species-rich assemblage of cichlid fishes, which form a monophyletic assemblage with additional species from surrounding water bodies, termed the Lake Victoria super flock. Lake Victoria is surrounded by smaller lakes that are somewhat disconnected from the main lake. Lake Kanyaboli is such a small lake, having markedly reduced species diversity, in part comprised of Lake Victoria species and endemics. Here, we studied the modern haplochromine component of the cichlid fauna, represented by Lipochromis maxillaris, Astatotilapia nubila, Xystichromis phytophagus and Astatotilapia sp. ‘Bigeye’, as well as a number of unidentified modern haplochromine specimens. We used landmark-based geometric morphometrics to study the degree of morphological divergence among those young entities. Twenty landmarks and 14 interlandmark distances were used for shape analysis. Multivariate analysis revealed significant differences between all four species, but principal component analysis and canonical variate analysis did not clearly discriminate between the entities. To test their reproductive distinctness and to demonstrate potential hybridization, nuclear genetic data are needed.

Key words: Lake Victoria – Lake Kanyaboli – landmark analysis – interlandmark distances – Eastern Africa

Introduction

With an estimated geological age of 250 000–750 000 years (Temple 1969; Johnson et al. 2000), Lake Victoria is not only the largest and shallowest, but is also the youngest of the great lakes of Eastern Africa. The cichlids of this lake are renowned for their adaptive radiation and explosive speciation (Fryer and Iles 1972; Kaufman et al. 1997; Kocher 2004). Various causes have been discussed to be the driving forces behind this rapid speciation, e.g. sensory drive in cichlid fish (Seehausen et al. 2008), sympatric speciation by disruptive sexual selection (Seehausen and van Alphen 1999), heritability and heterochrony of polychromatism (Maan et al. 2005), selection on coloration and male–male competition (Carleton et al. 2005; Dijkstra et al. 2006, 2008; Maan et al. 2006 and Maan et al. 2008, Seehausen and Schluter 2004) or sex reversal (Lande et al. 2001). Concerning the time scale of this adaptive radiation, the remarkably diverse species flock was suggested to have evolved in situ in less than 200 000 years (Meyer et al. 1990; Verheyen et al. 2003; Genner et al. 2007). Considering the findings of Johnson et al. (1996) who revealed that the lake dried out (almost) completely during the late pleistocene about 12,400 14C-years ago, the cichlid fauna in Lake Victoria might have even evolved and radiated within this remarkably short period of time (Seehausen 2002). While the actual age of the flock is currently disputed (Fryer 1997, 2001, 2004; Nagl et al. 2000; Seehausen et al. 2003) and Elmer et al. 2009), it is clear that irrespective whether some endemic cichlid species survived the drought inside or outside the lake basin, cichlid speciation in Lake Victoria must have been truly explosive (Seehausen 2000).

Whereas the cichlids of older lakes like Lake Tanganyika are morphologically, genetically and ecologically more distinct, the haplochromine cichlids of Lake Victoria are highly similar morphologically (Greenwood 1979, 1980; Meyer et al. 1990; Verheyen et al. 2003) and offer the most complex taxonomic puzzle (Van Oijen 1982, 1991; Seehausen 1996; Van Oijen and Witte 1996; Seehausen et al. 2003a,b), both at the generic and at the species level (Barel et al. 1991; Booton et al. 1999). Greenwood already highlighted the narrow clustering around the modal form, the presence of all intergrades within morphoclines of ‘species’ (Greenwood 1974, 1981), which was later confirmed by biochemical and molecular phylogenetic studies (Meyer et al. 1990; Nagl et al. 2000), altogether supporting that the Lake Victoria haplochromines are at an early stage of radiation. Additional human-induced problems need to be considered: the dramatic decline of the haplochromine cichlids in the lake in the 1980s as a result of the introduction of the Nile perch and frequent blooms of blue-green algae because of deforestation and overfertilization (Witte et al. 1992, 2005; Seehausen et al. 2003a,b). Natural hybridization because of turbid water conditions (Seehausen et al. 2003a,b) complicate taxonomic classification (Witte et al. 2007).

Haplochromine cichlids exhibit sexual dimorphism, with females usually exhibiting a totally different color from that of males, because they are usually camouflaged as a consequence of their breeding mode as mouthbrooders. So while live body coloration, especially that of adult males, is an important diagnostic character (at least for preliminary field identification), it can mostly be applied only on live male specimens, which may also lose their colour in response to stress, bright light, emotional state of the fish (Conte 2004) and after...
preservation. DNA sequences as tools for bar coding proved inefficient because of the stage of incomplete lineage sorting, because several species share haplotypes or alleles (Nagl et al. 1998, 2000). All these factors led to the quest for an objective, reliable and repeatable procedure for quantifying morphological differences and discriminating between different species.

The classic works solely applied traditional morphometrics (TM) in their morphological analyses, mainly involving linear measurements (trusses) or angles from different points on the body (Barel et al. 1977; Snoeks 1994), and proved to be only partially able to characterize and distinguish species. To overcome these problems, geometric morphometrics (GM) was applied in more recent works (Kassam et al. 2002, 2003; Costa et al. 2006) and turned out to be a powerful tool for addressing shape differences, even in preserved specimens. Further, GM remains compatible with and complementary to modern statistical analyses (Adams et al. 2004) and provides a unique opportunity to visualize the distinctive body regions using thin plate splines and deformation grids – a fact which greatly enhances interpretation of results with respect to the ecological significance of the observed differences (Bookstein 1991).

While many of the Lake Victoria haplochromine species have been classified based mainly on coloration of mature males, little effort has been put on establishing the shape differences between the species, so that quantifiable differences in shape are not known to date. This study is one of the first to apply GM on Lake Victoria cichlids (but see, Fermon and Cibert 1998). We selected the relatively simple species community of a satellite of Lake Victoria – Lake Kanyaboli. The lake has a surface area of approximately 11 km², a mean depth of 3 m and is separated from the main lake by a papyrus swamp (Fig. 1). Because of its dramatic effects on the haplochromine fauna in Lake Victoria, it is important to note that the Nile perch Lates niloticus Linnaeus, 1758; has not yet been reported from Lake Kanyaboli. Lake Kanyaboli still harbours cichlids and haplochromines that are currently believed to be extinct in the main Lake Victoria. The lake harbours six species of haplochromines: Astatoreochromis alluaudi Pellegrin, 1904; Pseudocrenilabrus multicorl victoriae Seegers, 1990; Astatotilapia nubila Boulenger, 1906; Xystichromis phytophagus Greenwood, 1966; Lipochromis maxillaris Trewavas, 1928, a rare and undescribed species – Astatotilapia sp. ’Bigeve' and possibly more undescribed entities. Of these, Astatoreochromis alluaudi and Pseudocrenilabrus multicorl victoriae represent ancient splits within the haplochromines. The remaining species are part of the 'modern' haplochromine species superlock (Salzburger et al. 2005), which are part of the c-lineage sensu Clabaut et al. (2005).

Using landmark analysis in combination with interlandmark distances (ILD) generated from the landmarks, the study attempts to test the degree of morphological distinction among the four recognized species of modern haplochromines in Lake Kanyaboli and to analyse the assignment of about 90 unidentified specimens which could not be assigned to either of the recognized entities. The fit to nuclear genetic markers is a long-term goal of this approach and will be addressed in a forthcoming work.

**Material and Methods**

**Sample collection and data acquisition**

Between May 2007 and April 2008, samples were collected from 33 localities covering the entire lake (approximately 11 km²) using gill nets, traps and baited hooks. In total, 145 (139 males, six females) individuals were sampled of Astatotilapia nubila, 174 (136 males, 38 females) of Lipochromis maxillaris, 191 (131 males, 60 females) of Xystichromis phytophagus and three (one male, two females) individuals of the rare and yet undescribed species Astatotilapia sp. ’Bigeve’. Our sample also comprises 90 (47 males, 43 females) specimens that we could not assign to any of the known species and we named them ‘unidentified’ specimens. We further included 10 male specimens of Astatotilapia burtoni (Günther, 1894) in our analysis, sampled by Walter Salzburger in Kalambo River (a tributary of Lake Tanganyika) to better assess the degree of morphological distinction of the Kanyaboli species. To retain live colour and shape, all specimens used in this study were taken live and then anesthetized using clove oil (two drops per litre). The mouth of each specimen was pinned shut to minimize errors that could be associated with gape articulation. Each specimen, together with a corresponding label and a measuring scale,
was then scanned using a modified flatbed scanner (Herler et al. 2007). All specimens are deposited in the Zoology Department of the National Museums of Kenya in Nairobi, Kenya.

The images obtained were then converted into TPS file format using the tpsUtil software (Rohlf 2008). To minimize bias potentially associated with working in a systematic order, the images were randomly picked for landmark placement. Twenty homologous landmarks were defined to cover the overall shape of the fish (Fig. 2a).

These were digitized using the tpsDig2 software (Rohlf 2006). The landmarks included the following: (1), tip of the snout, excluding the lips; (2) and (3), anterior and posterior insertion of the dorsal fin; (4) and (6), upper and lower insertion of caudal fin; (5), mid-point of the caudal fin base; (7) and (8), posterior and anterior insertion of the anal fin; (9), anterior insertion of the pelvic fin; (10), the most ventral border between the interoperculum and the suboperculum; (11), the point where preoperculum, suboperculum and interoperculum meet; (12), upper insertion of the pectoral fin; (13), dorsal origin of the operculum; (14), dorsal end of the preopercular groove; (15) and (16), extreme points on the anterior and posterior parts of the orbit – covering its width; (17), centre of the orbit; (18) and (19), anterior and posterior origin of the maxilla; (20), most posterior point of the lips. The landmark at the centre of the orbit was placed by first zooming out the landmark to circle the entire orbit so that reducing the size of the landmark left it at the centre. Fourteen ILD were also generated from the landmarks using the Morphometric and Distance Computation Software for evolutionary studies (MODICOS; Carvajal-Rodrıgüez and Rodrıgüez 2005) (Fig. 2b). This was performed by first converting the data set from the TPS format to NTS format using the tpsUtil software (Rohlf 2008) and then to MODICOS using the Conver-Thor0.2 utility software (Carvajal-Rodrıgüez 2005). All ILD were expressed as ratios of standard length before analysis. Abbreviations and definitions of ILD are given in Table 1.

Geometric morphometric analysis

The raw coordinates were aligned using the partial procrustes superimposition (Rohlf 1999; Slice 2001 in CoordGen6f (IMP programs, Sheets 2003). To assess the degree of morphological distinction of Kanyaboli species and the outgroup, we applied canonical variate analysis (Mardia et al. 1979), a method of finding a set of axes that allows for the greatest possible ability to discriminate between two or more groups. The program CVAgen (IMP programs, Sheets 2003) was used to compute partial warp scores to a common reference, on which the consecutive CVA was based. In this way, we determined the number of distinct CVA axes in the data at a p-value of 0.05 and computed the canonical variates scores of all the specimens entered. For investigation of unidentified specimens, we performed an assignment test using a CVA-distance-based method, in which the specimens entered. For investigation of unidentified specimens, we performed an assignment test using a CVA-distance-based method, which determines the probability that one specimen is closer to the mean of the group to which it was assigned a priori than to the mean of another group (Zelditch et al. 2004; Nolte and Sheets 2003). The assignment test was also carried out in the CVAgen software.

Relative warp analysis (RWA) (which is a principal component analysis (PCA) of shape variables) was performed using the software TPSRelw (version 1.42, Rohlf 2002). The relative warps were computed to summarize the variation among the specimens (with respect to their partial warp scores) in as few dimensions as possible. For \( \alpha = 0 \), this is a principal components analysis of the covariance matrix of the partial warp scores. A graphic visualization of the shape differences in the form of a deformation grid was generated, which pinpoints the deformation in shape from the reference.

For the degree of morphological distinction of Kanyaboli species to *Astatotilapia burtoni* representing a basal split within the modern haplochromines and the Lake Victoria superflock. This CVA showed the clear morphological differentiation of the four Kanyaboli study species (Fig. 3). Moreover, we found evidence for sexual dimorphism in *L. maxillaris* and *X. phytophagus*. This was predominantly expressed in head size and eye diameter, as shown in Supplementary Fig. S1.

Relative warp analysis analysing the four known Kanyaboli haplochromines and the group of unidentified individuals highlighted morphological differentiation between three of the four study species and arranged the unidentified specimens on the positive side of RW 1 close to the *A. nubila* and *X. phytophagus* specimens. The first two relative warps explained 20% and 12.5% of the total variation among species. *L. maxillaris* and *A. sp. ‘Bigeye’* could be distinguished from the other three groups of specimens. The associated shape change was visualized as splines relative to the extreme values of the relative warp axis. Scatter plot and corresponding deformation grids are shown in Fig. 4. We could see that most specimens of *A. nubila*, *X. phytophagus* and the unidentified group were located on the positive side of relative warp 1 and could be more associated with the grid at the positive extreme of RW 1. That means they had a slightly deeper body and a much smaller mouth than *L. maxillaris*. *Astatotilapia sp. ‘Bigeye’* was separated along RW 2, which indicates that *A. sp. ‘Bigeye’* had a more elongated body with smaller maxilla and – faithfully to its nickname – larger eyes than the other species (Fig. 4).

To corroborate and concretize those findings, we carried out a PCA on 14 ILD (Fig. 5a). PC axis one explained 30.8% and PC axis two 26.9% of total variance in our ILD data set. *Lipochromis maxillaris* could be separated through PC axis 2 based on remarkable differences in the length of maxilla. *Astatotilapia sp. ‘Bigeye’* is far separated from the other group means. PC 1, as shown in bar graphs of loadings in Fig. 5b, is...
mostly defined by body depth, dorsal fin length and eye diameter. There was also a separation trend between *A. nubila* and *X. phytophagus* along PC 1 axis even if there were no discrete character differences differentiating those two species. Concerning measurements on the unidentified specimens, PCA on ILD yielded morphological similarity to *X. phytophagus*. To get more information about classification of the unidentified group, we carried out a RWA and CVA on *A. nubila*, *X. phytophagus* and the unidentified specimens. As shown in Fig. 6a, RWA could not discriminate those three groups. Most unidentified specimens were arranged on the negative side of RW 1 (18.2%) but again seemed to be more like *X. phytophagus* specimens concerning overall shape. CVA revealed two distinct axes (CV1: eigenvalue = 1; CV2: eigenvalue = 0.32). This analysis did separate unidentified individuals along CV 1 from *A. nubila* and *X. phytophagus*. As shown in the deformation grid according to CV axes 1 (Fig. 3c), there were differences in the position of posterior insertion of the dorsal fin and some differences concerning eye size and head region in general. The CVA-based assignment test revealed that 70% of the three morphologically overlapping entities were assigned to the three clusters (71.9% of *A. nubila*, 67.9 of *X. phytophagus* and 74.5% of the unidentified specimens; see Table 2).

**Discussion**

Geometric morphometrics has been shown to be a highly selective method for discriminating between closely related species of fish (Loy et al. 2000; Albertson and Kocher 2001; Kassam et al. 2002; Costa et al. 2006) and even between populations (Maderbacher et al. 2008). Furthermore, it has been successfully used to discriminate between wild and captivity-bred populations (Hard et al. 2000), and it was shown to be informative for distinguishing osteological structures (Postl et al. 2008). In this study, GM was applied to a set of four closely related species of haplochromines occurring in a small satellite lake of Lake Victoria, to test for their degree of morphological distinction. This lake is fed by the Yala River and separated from the main lake by a large papyrus swamp. Three of the four species also occur or at least occurred in Lake Victoria; *X. phytophagus* might have gone extinct because of the introduction of the Nile perch. In addition to seven non-cichlid species, the lake also harbours four tilapiine cichlids and at least six haplochromines. The tilapiine species are *Oreochromis esculentus*, *Oreochromis leucostictus* and the two introduced species *Oreochromis niloticus* and *Tilapia rendalli*. Apart from the four study species, two other haplochromines, *Astatotilapia alhaudi* and *Pseudocrenilabrus multicolor victoriae*, occur in the lake, but they are considered to belong to ancient lineages and to be clearly distinct from the other modern haplochromines both morphologically and genetically (Kobmüller et al. 2008). We concentrated on three described species of modern haplochromines, *Lipochromis maxillaris*, *Astatotilapia nubila* and *Xystichromis phytophagus* and one undescribed species, *Astatoilapia* sp. ‘Bigeye’, which seems to be the only true endemic of Lake Kanyaboli. However, we also included all sampled specimens of modern haplochromines in this analysis, which could not be classified with any of the four known entities, to gain more insights into their status.

**Table 1. Abbreviations and definitions for interlandmark distances**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Traditional measurement</th>
<th>Distance (mm) between landmarks</th>
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<tbody>
<tr>
<td>PDD</td>
<td>Predorsal distance</td>
<td>1–2</td>
</tr>
<tr>
<td>PPED</td>
<td>Prepelvic distance</td>
<td>1–9</td>
</tr>
<tr>
<td>PPD</td>
<td>Prepectoral distance</td>
<td>1–12</td>
</tr>
<tr>
<td>HL</td>
<td>Head length</td>
<td>1–13</td>
</tr>
<tr>
<td>POD</td>
<td>Preorbital distance</td>
<td>1–15</td>
</tr>
<tr>
<td>ML</td>
<td>Mouth length</td>
<td>1–20</td>
</tr>
<tr>
<td>DFB</td>
<td>Dorsal fin base</td>
<td>2–3</td>
</tr>
<tr>
<td>BD</td>
<td>Body depth</td>
<td>2–9</td>
</tr>
<tr>
<td>CPD</td>
<td>Caudal peduncle depth</td>
<td>4–6</td>
</tr>
<tr>
<td>CPL</td>
<td>Caudal peduncle length</td>
<td>5–7</td>
</tr>
<tr>
<td>AFB</td>
<td>Anal fin base length</td>
<td>7–8</td>
</tr>
<tr>
<td>DPP</td>
<td>Distance between pelvic and pectoral fin insertion</td>
<td>9–12</td>
</tr>
<tr>
<td>ED</td>
<td>Eye diameter</td>
<td>15–16</td>
</tr>
<tr>
<td>LMX</td>
<td>Length of maxilla</td>
<td>18–19</td>
</tr>
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</table>

**Fig. 3. CVA scatter plot including males and females of four species of modern haplochromine cichlids (**Lipochromis maxillaris** □, *Astatotilapia nubila* ◆, *Xystichromis phytophagus* ○, *Astatoilapia* sp. ‘Bigeye’ △) in Lake Kanyaboli and *Astatotilapia burtoni* ■ as outgroup**

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In terms of overall morphology, all four species could be consistently distinguished, albeit to different degrees. Moreover, we observed clear sexual dimorphism in particular body proportions, in agreement with several previous studies (see e.g. Oliveira and Almada 1995; Barnett and Bellwood 2005; Herler et al. 2010). This was qualitatively demonstrated for all four species and quantified for *X. phytophagus* and *L. maxillaris* only, for which sufficient specimens of both sexes were available. Consequently, we used male individuals only for further analyses.

*Astatotilapia* sp. ‘Bigeye’, clearly the most distinct entity, is an algae scraper (Abila et al. 2008), so that its distinctness can also be aligned with the ecological background (Bouton et al. 2002). Clear differences were also found between *L. maxillaris* and the other species. More specifically, the differences between *L. maxillaris* and the other species were mainly concentrated in the head region, in that *L. maxillaris* consistently had a larger head, mouth and maxilla. This observation could also be explained by the trophic ecology of *L. maxillaris*, which is a paedophage feeding on the eggs and fry of other mouthbrooding haplochromines, displaying a specific predatory behaviour, ending with the suction of eggs or fry from the brooding female. The observed clear distinctions conform to the classic argument that cichlid fishes diversify through trophic specializations and as such, the structures of the trophic apparatus differ most among species (Fryer and Iles 1972). Such differences were shown to primarily concern the head shape (Bouton et al. 2002), in addition to the underlying anatomical structures with trophic function (Bouton et al. 1999). *Astatotilapia nubila* and *X. phytophagus*, however, were highly similar in their overall body shape, even if they are clearly separated trophically. The two species were classified as ‘insectivore’ and ‘plant eater’ by Kaufman and Ochumba (1993) and were found to have distinct diets conforming to the classification after analysis of their gut content, albeit showing much dietary overlap (Abila et al. 2008). Their close overall morphological similarity is therefore surprising but plausible. Interestingly, both species were found in Lake Victoria before the Nile perch was introduced, but *X. phytophagus* became extinct in the main lake. Their phylogenetic relationships need to be analysed by means of nuclear genetic markers in a larger phylogenetic framework including a representative set of Lake Victoria species.

Analyses of ILD (see Fig. 5) mirroring traditional morphometric measurements not only supported the results of the landmark analysis but added valuable information: *L. maxillaris* was most distinguished by its much longer maxilla, head length, prepectoral- and predorsal distance, while *X. phytophagus* and the unidentified specimens were characterized by...
greater body depth and dorsal fin length. *A. sp. ‘Bigeye’* was characterized by its larger eye diameter. However, it must be noted that unlike those traditional morphological measurements carried out with digital callipers, the third dimension could not be taken into account. Moreover, information in the spaces, curves or surfaces between the landmarks were not captured.

Taking together the outcomes of alternative approaches (Figs 4 and 5), it became clear that the majority of the unidentified specimens were clearly neither *L. maxillaris* nor *A. sp. ‘Bigeye’*. Our secondary analysis excluding *L. maxillaris* and *A. sp. ‘Bigeye’* (Fig. 6) casts the unidentified specimens to be a rather different entity from *A. nubila* and *X. phytophagus*, with a similar proportion of morphologically overlapping individuals (Table 2). This observation casts doubts on a hybrid origin of these specimens, as they were not resolved as morphological intermediates of *A. nubila* and *X. phytophagus*. Clearly, further analyses based on genetic data such as microsatellite markers are needed to clarify the species assignment of these unidentified specimens. Such molecular genetic analyses are underway and will be presented in a forthcoming paper.

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### Zusammenfassung

Morphologische Differenzierung von haplochrominen Buntbarschen im Kanyaboliisee, einem Satellitensee des Victoriasees


**References**


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Relative Warp analysis on single species data (X. phytophagus and L. maxillaris) to assess shape differences between sexes.

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