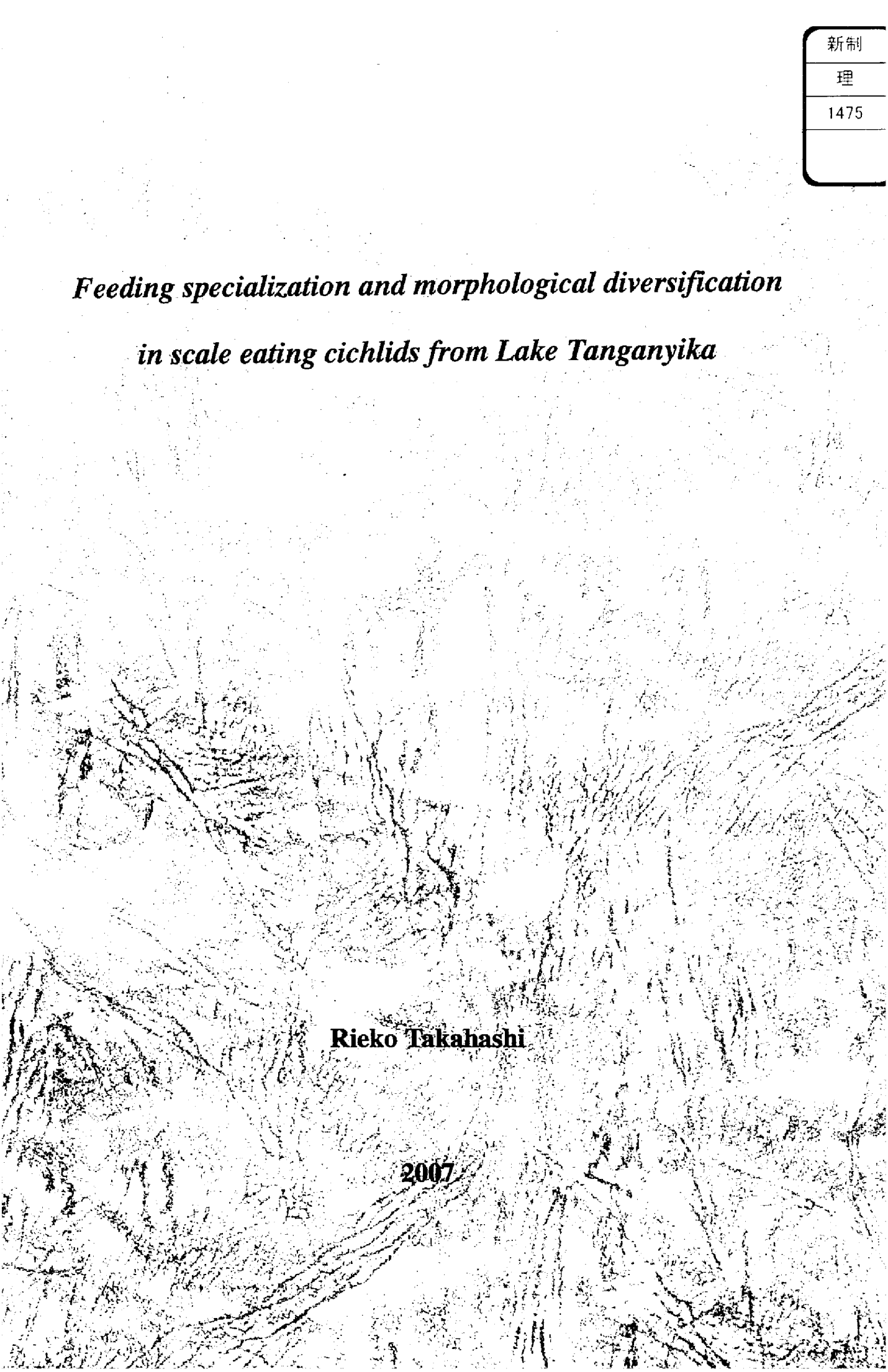
Feeding specialization and morphological diversification in scale eating cichlids from Lake Tanganyika



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Contents

Chapter 1. General Introduction

Adaptive radiation is referred as the evolution of ecological diversity within a rapidly multiplying lineage (Shulter 2000). It is a differentiation of a single ancestor into an array of species that inhabit a variety of environments or that differ in traits used to exploit those environments. Such process of adaptive radiation often involves the specialisation to fulfill the various niches. Simpson (1953) also identified specialisation as one of the dominant themes of adaptive radiation.

One of great examples for such specialisation is array of feeding evolution and specialisation found in the cichlid fishes of African Great Lakes (Fryer & Iles 1972). Within these lakes, Lake Tanganyika, the oldest of these lakes with an estimated age of 9–12 Myr (Cohen *et al.* 1993), contains the morphologically and ecologically most complex species flock of cichlid fishes (Greenwood 1984). Scale-eating cichlids of the tribe Perissodini are perhaps among the most specialised cichlids in the lake (Fryer & Iles 1972). The endemic tribe Perissodini comprises nine species (Poll 1986; Takahashi & Nakaya 1999). Liem & Stewart (Liem & Stewart 1976) classified them into two genera, *Perissodus*, which includes all scale eaters identified so far in the lake, and *Haplotaxodon*. We here follow their classification, although Poll (1986) further subdivided the former genus into three genera, *Perissodus* (*P. microlepis* and *P. eccentricus*), *Plecodus* (*P. multidentatus*, *P. paradoxus*, *P. elaviae*, *P. straeleni*) and *Xenochromis* (*P. hecqui*).

The success in diverse feeding habits is suggested to be coupled with the divergence in trophic morphology, particularly, oral jaw structure in cichlid fishes (Liem

1991). This appeared to be true for *Perissodus* species: these scale eaters are characterised by possessing unique oral jaw teeth that vary in shape among species, showing specialisation to scale eating (Liem & Stewart 1976). The leaf-like teeth pattern was found in five of *Perissodus* species (*P. hecqui*, *P. multidentatus*, *P. straeleni*, *P. elaviae*, and *P. paradoxus*), whereas the broad-based, truncated tooth type was found in *P. microlepis* and *P. eccentricus*. This unique tooth form was previously referred as "the utterly strange leaf-like teeth of *Plecodus* far transcend the limit of dental modification, not only among the family Cichlidae, but also of the order Percomorphi and of the entire class of bony fishes. Nothing recently like this exists" (Fryer & Iles 1972). The asymmetry in mouth opening found in scale eaters was also considered an adaptation for efficiently tearing off prey's scales and result of specialisation among the lineage (Hori 1993). The asymmetry is first described in *P. eccentricus* (Liem & Stewart, 1976). However, this asymmetry is also found in all of *Perissodus* species. Hori (1993) showed that the right-handed individuals only attack on the right side of the flanks, and vice versa.

Not only such feeding morphology are peculiarly unique, the field observations revealed the ecological importance of these morphological diversification among species or even among individuals. The asymmetry in mouth-morph in *P. microlepis* was maintained by frequency-dependent selection in a population in which minority morphs' advantage results in differential reproductive success (Hori 1993), or differential colour morphs in *P. straeleni* relate to the differential hunting strategies of each morphs (Nshombo 1994). The aggressive mimicry to prey species found in *P.*

microlepis is also considered to be related to diverse hunting strategies (Hori & Watanabe 2000). These behavioural and morphological diversifications are suggested to be important for the maintenance and stable coexistence of these scale eaters in the lake (Hori 1997).

Such specialisation in morphology, and unusually high degree of specialisation to scale eating (Fryer & Iles 1972, Hori 1987, Brichard 1989) indicates the importance of morphological differentiaions for the evolution and diversification process of this group, which in fact have large number of scale eaters compared with scale eating cichlids of Lake Malawi and Victoria (Fryer & Iles 1972). However, the origin of scale eating in *Perissodus* species has been only guessed from ecological observations, and the answer remains unclear. This is partly because the five of *Perissodus* species are deepwater inhabitants whose ecology is poorly known (Brichard 1989; Coulter 1991), and above all, a reliable phylogenetic framework, which is essential for evolutionary analyses, has not yet been obtained for *Perissodus* species. Clarifying the evolutionary history of *Perissodus* is important to understand the process of feeding specialisation and morphological diversification, and further understand the present diversity of *Perissodus* species in the lake.

Particularly, the oral jaw structure in *Perissodus* species itself has left interesting questions. The tooth morphs of *Perissodus* are previously suggested as adapted to scale eating (Liem & Stewart 1976). However, the role of differential tooth shapes among species, and its relation to their feeding behaviours have not clearly been understood. As another interesting question, the asymmetry in mouth morph is suggested to be inherited

in a mendelian one locus system from the observation of parents and young collected in field. To test whether this morph is inheritable trait, the laboratory breeding experiments is an asset to exclude the any environmental effects. In addition, this laterality in mouth morph was also suggested to be possessed by fishes other than scale eaters (Mboko *et al.* 1998; Seki *et al.* 2000; Hori *et al.* 2007). These results indicate that the asymmetry in mouth morph may not be unique trait to scale eaters. This needs to be tested with the comparisons of inheritance patterns among fishes.

The objective of this study is to examine the relation among feeding habits, morphology, and behaviours from evolutionary perspectives. I particularly focused on the morphological diversification of oral jaw structure from functional, evolutionary, and genetic perspectives. First, I examined the feeding behaviours of the two syntopic scale eating cichlids, *P. straeleni* and *P. microlepis*, with a high-speed video camera recording in laboratory in order to test the functional relation of feeding morphologies to their specialised feeding behaviours (Chapter 2). Second, I conducted the integrative analysis of feeding ecology and morphology based on the molecular phylogenetic tree for all nine known Perissodini species in order to reveal the evolutionary patterns and process of specialisation to scale eating (Chapter 3). Third, I focused on the inheritance pattern of asymmetry in mouth morphs to examine the inheritance pattern of the trait. In order to test whether this dimorphsm is inheritable, I conducted the breeding experiments using the model organism, *Oryzias latipes*, and the Tanganyikan sponge eating cichlid, *Julidochromis* cf. 'Gombi' (Chapter 4).

Chapter 2. The functional morphology of the oral jaw teeth in the scale eating behaviours of *Perissodus straeleni* and *Perissodus microlepis*

2.1 Introduction

The oral jaw tooth morphology of cichlids are as diverse as their feeding habits (Fryer & Iles 1972), and is considered to be a key component of the functional divergence (Yamaoka 1997). In Lake Tanganyika, *P. straeleni* and *P. microlepis* syntopically inhabit around shallow rocky areas (Hori *et al.* 1983; Hori 1997). These two species are considered to be specialised scale eaters; their primary diet consists of scales from other fishes, comprising up to 70% of the total stomach contents (Hori *et al.* 1983). These species are known for their specialised oral jaw tooth morphology, which differ markedly between the species (Fryer & Iles 1972): *P. straeleni* has laminar, recurved teeth, whereas *P. microlepis* has broad-based, truncated teeth (Liem & Stewart, 1976). Previously, the field study revealed their hunting behaviour, which slightly differ between the species: typically, P. straeleni approaches from close behind a target fish and moves quickly towards it, whereas P. microlepis approaches a target by rapidly advancing from far behind it. (Hori 1987). Hori (1987) suggested that the body conformation of each species, i.e. the deeper body of *P. straeleni* and the streamlined body of *P. microlepis*, is related to these differential hunting strategies.

The possible function of their tooth shapes for such specialised feeding behaviours, has also previously been suggested (Liem & Stewart 1976; Brichard 1989). However, it remains to be elucidated because their swift feeding motion has prevented detailed

observations of the foraging behaviour. Thus, for clarifying this, laboratory based observations of their feeding behaviour using high-speed video camera recording is necessary.

In this chapter, I aimed to clarify the function of the teeth in the feeding behaviour of the two scale eating cichlids, *P. straeleni* and *P. microlepis*. For that purpose, the observation of their feeding behaviour with a video camera recording was conducted in laboratory. Dental morphology was analyzed using scanning electron microscopy. The condition of teeth in wild-caught individuals was also examined. Finally, the functional relation of feeding morphology to the specialised scale-eating behaviour is discussed.

2.2 Materials & Methods

Dental morphology

Two specimens of each of *P. straeleni* and *P. microlepis* were collected in the field (Myako Point, Mahale District, Tanzania) and preserved in 10% formalin. The jaws were removed from the specimens, cleaned with water, dehydrated in 70% ethanol, and coated with gold. Teeth of the upper and lower jaws of the two species were then observed and photographed using a scanning electron microscope (SEM; JSM5800, JEOL, Tokyo, Japan).

Tooth condition

Tooth condition was evaluated in 44 individuals of *P. straeleni* (63.1—113.6 mm SL)

and 39 individuals of *P. microlepis* (70.5—111.6 mm SL). These individuals were collected at the same locality mentioned above. The dentition of each individual was stained with Alizarin red S solution, and each tooth was inspected under a stereomicroscope. The number of teeth on both upper and lower jaws was counted, and the position and condition of each tooth was recorded. The types of tooth conditions were classified as: missing, newly erupted, no damage, and wearing (partly broken, cracked, worn to make a rounded appearance). The ratio of wearing teeth in each individual was calculated, and analyzed between species using a Mann–Whitney *U* test.

Video recording of scale-eating behaviour

Two live *P. straeleni* and five *P. microlepis* were provided from a trader in Burundi, and a commercial vendor in Tokyo, Japan, respectively. Each individual was housed in a 57-L aquarium and maintained at 26°C on a 12:12 light: dark cycle. Fish were fed TetraMin ® twice a day.

To record scale-eating behaviour, live specimens including seven *Carassius auratus langsdorfii* and five *Cyprinus carpio* (both Cyprinidae) were used as prey. Though these fishes were not natural inhabitants of Lake Tanganyika, I had verified that the feeding behaviours of scale eaters to these prey fishes were mostly same as those of observed in the lake. The fishes were individually isolated in each aquarium, and maintained at room temperature around 23°C. They were fed commercially available dried food twice a day.

One prey fish was transferred to a 57-L aquarium in which one scale eater was

housed. Each recording trial started after the predator began approaching to the prey fish, and the recording conducted for 10–20 min. The foraging behaviours of scale eaters were filmed using a CCD camera (SONY CCD-V800 VHS-C) at 30.0 fields per second. The camera was positioned 2 m from the tank and recorded foraging events from a lateral view. A video camera light (Mitsubishi VLT-100) was positioned on both sides of the arena.

In these recording events, the evasive movements of the prey sometimes obscured the feeding behaviour of the predator, or the mouth movements of the predator were not visible in the lateral view. Therefore, only feeding events that were clearly visible from the camera were used in the analyses. The timing of feeding events was analysed field-by-field by visually locating the video field containing the event of interest, including the start of the forward dash, the onset of mouth opening, the start of a mouth strike on the body of the prey, mouth separation from the prey, and mouth closing. The time duration of forward dash, mouth opening time prior to strike, and time duration of strike on the body of the prey were measured and statistically compared between species using a mixed-model nested analysis of variance (ANOVA; Sokal & Rohlf, 1995). In this analysis, species was the fixed main effect, with the individual random effect nested within species. Variables were transformed using Box-Cox transformation. Data were tested for normal distributions using a Shapiro-Wilk test, and for homogeneity of variances using a Bartlett test.

2.3 Results

Dental morphology

In both *P. straeleni* and *P. microlepis*, teeth are arranged in a single row on both upper and lower jaws (Figure 2–1 A, D). The size of the teeth is dissimilar; in *P. straeleni*, the second and penultimate teeth are usually larger than the other teeth in the lower jaw, and the first teeth are usually smaller than the other teeth in the upper jaw. In *P. microlepis*, the second and penultimate teeth are usually larger than the other teeth in the lower jaw, and the first and last tooth are usually smaller than the other teeth in the upper jaw. The average number of teeth in wild samples was 18.3 ± 1.5 (mean ± S.D) in the upper jaw and 14.9 ± 0.9 in the lower jaw of *P. straeleni* (n = 44), and 24.1 ± 1.7 and 18.7 ± 1.0, respectively, in *P. microlepis* (n = 39). The teeth of *P. straeleni* tended to be closer to each other than those of *P. microlepis* in both jaws (Figure 2–1 B, E).

Tooth shape also differs between the two species. In *P. straeleni*, the overall shape of the tooth is leaf-like (Figure 2–1 C). Each tooth is strongly recurved, and the tip is mostly directed downward. The tooth becomes laminar toward the edges, forming sharp lines along the lateral sides. In *P. microlepis*, each tooth is thick and broad based (Figure 2–1 F). The tooth is strongly recurved, and the tip is dully pointed and directed backward. The corners of the upper side of each tooth project vertically, forming a pair of spine-like points.

Tooth condition

Of the total number of teeth in *P. straeleni*, 49.2 % exhibited chipping. Missing teeth,

and newly erupted teeth were 2.3 % and 1.1%, respectively. In *P. microlepis*, 10.3 % of teeth showed wearing on the upper parts of the teeth. *P. microlepis* individuals also possessed the teeth that were worn to make a rounded appearance (0.8%), or cracked (1.8 %). Missing teeth, and newly erupted teeth were 6.2 % and 2.5 %, respectively. The mean ± S.D ratio of wearing teeth, i.e., partly broken, cracked, and rounded, was significantly higher in *P. straeleni* than in *P. microlepis* (0.50 ± 0.17, $n = 44$ versus 0.12 ± 0.07, $n = 39$, respectively; $P < 0.001$).

Foraging behaviour of P. straeleni and P. microlepis

Approaching behaviour

The observed typical feeding behaviour was as follows. Before approaching prey, the predator often remained or hid near the corner of the arena, apparently watching for the prey. This hiding behaviour lasted several minutes, after which the predator began approaching the prey. After the predator reached a position near the prey, it oriented its head toward the flank of the prey and began rapidly advancing the prey (Figure 2–2 A, B, 2–3 A). Mouth opening began during this dash toward the prey, resulting in a strike of the maximally opened mouth against the flank of the prey (Figure 2-2 C, D, 2–3 A, B). The mean ± S.E time from the beginning of the dash until the mouth reached the flank of the prey was 135.7 ± 14.5 ms ($n = 15$) in *P. straeleni* and 144.9 ± 12.5 ms ($n = 23$) in *P. microlepis*, and the onset of mouth opening occurred 86.0 ± 12.0 ms ($n = 19$) and 68.8 ± 5.0 ms ($n = 48$) prior to the strike, respectively (Table 2–1). No significant difference was found between the two species in the timing of the dashing phase and

mouth opening prior to the strike ($P > 0.05$).

Scale-removing behaviour

In *P. straeleni*, following the strike of the mouth against the body of the prey (Figure 2–2 D), the predator continued attacking the body of the prey by holding its body nearly perpendicular to that of the prey (Figure 2–2 D, E). During this phase, the mouth of the predator was shifted backward along the body of the prey in response to the evasive forward movement of the prey, thereby scraping scales off of the prey (Figure 2–2 E, F). Subsequently, with the mouth widely opened, the head of the predator turned to the back end of the prey, and its mouth separated from the prey (Figure 2–2 G, H). The mean ± S.E time from the strike to the flank of the prey until separation was 91.7 ± 7.4 ms (n = 42). The predator then closed its mouth, which contained scales. Scales occasionally floated in the water and were collected by the predator.

In *P. microlepis*, following the strike to the body of the prey, the predator pressed its widely opened mouth against the flank of the prey (Figure 2–3 B). Although the prey attempted to escape, the predator continued to cling to the body of the prey, with its mouth pressed against the flank (Figure 2–3 C). During this strong attachment to the body of the prey, the predator rapidly rotated its own body several times in the attack position with its mouth widely opened, simultaneously wrenching scales off the body of the prey (Figure 2-3 D, E, F). The mouth of the predator then separated from the body of the prey (Figure 2–3 -G, H). The mean ± S.E time from the strike to the body of the prey until separation was 108.7 ± 8.0 ms (n = 54). The predator then closed its mouth,

which contained scales. Occasionally, the predator collected scales that were floating in the water. No significant difference was found between the two species in the time duration of strike on the prey's body ($P > 0.05$).

2.4 Discussion

The feeding behaviour of scale-eating fishes has been reported from various habitats (Fryer & Iles, 1972; Major, 1973; Sazima, 1977, 1983; Whitfield & Blaber, 1978; Roberts, 1970; Nshombo *et al.*, 1985; Hori, 1987, 1991; Yanagisawa *et al.*, 1990; Nshombo, 1994). For example, the neotropical characoid *Roeboides prognathus* strikes at the flanks of its prey with its mouth closed and uses its stout, external, forward-directed teeth (Sazima, 1983). The marine fish *Terapon jarbua* swims forward rapidly, opens its mouth widely before contacting the prey, and often rips the fins with its conical teeth (Whitfield & Blaber, 1978). In African scale-eating cichlids, feeding patterns have generally been categorised into two types, i.e., rasping the scales or the caudal fins using numerous small teeth [e.g., the Malawian cichlids, *Genyochromis mento* and *Corematodus* spp., and Victorian cichlids, *Haplochromis welcommei* ; Fryer & Iles, 1972], or biting with a single row of relatively large teeth (e.g., the Tanganyikan cichlids *Perissodus* spp.; Fryer & Iles, 1972; Liem & Stuart, 1976). These observations infer that scale-eating behaviour is often associated with specialised feeding morphology, particularly of the teeth.

The results of this study confirmed that the feeding behaviour of *P. straeleni* and *P. microlepis* include more than biting; rather, these fishes exhibit species-specific scale-

eating behaviour. In *P. straeleni*, following the strike of the mouth against the flank of the prey, the mouth position is shifted to the back of the prey body. During this shifting motion, the teeth of *P. straeleni* is considered to scrape scales from the flank of the prey by pressing and shifting the sharp edges of the teeth laterally along the body of the prey. Attacks by *P. straeleni* leave denuded, lateral spots on the flanks of the prey (unpublished). Thus, the sharp edges of the teeth appear to function as blades for scraping. In contrast, *P. microlepis* quickly rotates its body, with the teeth of both jaws pressed tightly against the flank of the prey. During this rotational movement, the teeth also appear to rotate on the flank of the prey and effectively catch scales with their spine-like points, resulting in the simultaneous wrenching off of scales. A ringed spot is often observed on the prey after an attack by *P. microlepis* (unpublished). The points of the teeth thus function as hooks for wrenching scales off prey fishes. These observations demonstrate that the specific feeding behaviour of each scale eater is closely correlated with the functional diversification of their teeth, i.e., the scraping teeth of *P. straeleni* and the wrenching teeth of *P. microlepis*.

Webb (1984) showed that laterally compressed, taller body shapes are advantageous for slow swimming and manoeuvring while searching for food, stalking, or feeding, whereas a streamlined body is more suitable for sprint swimming. Recently, two species of sympatric three-spined sticklebacks, *Gasterosteus* spp., were found to differ in morphology and swimming performance; the streamlined limnetic species showed greater swimming endurance and lower drag coefficients than the deeper-bodied benthic species (Blake *et al.*, 2005). These findings are in accord with the

feeding behaviour of *P. straeleni* and *P. microlepis*: the deeper-bodied *P. straeleni* may be better at approaching prey from a nearby location (Hori, 1987) and maintaining a stable body position against the prey during scale-eating, whereas the streamlined body of *P. microlepis* is more effective for greater locomotion, including approaching rapidly from a distance (Hori, 1987) and quickly rotating the body. These results suggest that body conformation *per se* may be an important specialisation in the feeding behaviour of these two species.

The position and wear of teeth allow some predictions about the mode of attack and scale removal (Roberts, 1970; Sazima, 1983). In *Roeboides* spp., the foremost, external teeth of both jaws which point straight ahead were invariably worn and sometimes missing, probably by striking at prey with the mouth closed (Sazima, 1983). These tooth wear of scale eaters sometimes affect their feeding capacity of scale removal. Sazima (1983) mentioned one individual of characoid scale eater, *Probolodus heterostomus* with most of teeth worn out only takes food items such as insects or plant material. In our analyses, the ratio of wearing teeth was significantly higher in *P. straeleni* than in *P. microlepis*. These higher wearing on the teeth of *P. straeleni* may affect their dependence on scales. In fact, *P. straeleni* often takes food other than scales such as spawned eggs of cichlids or fish skins from scale-less fishes such as catfish or Mastacembelid spiny eel whereas *P. microlepis* mostly feed on scales (Nshombo *et al.*, 1985; Nshombo, 1994). The difference in tooth wear between the two species also indicates that the tooth shape of *P. microlepis* may be more damage resistant to strong collision against the flank of the prey than those of *P. straeleni*. Such damage resistant

structure would be important for sucessful hunting techniques. This functional significance is suggested to be an important factor that promotes the differentiation of tooth shapes in these species.

The tooth wear, however, is also influenced by tooth replacement patterns of each species: if the rate of tooth replacement is slower, the species may contain higher number of older, worn out teeth. This must be taken into consideration when comparison is made between the two species. Further study on tooth formation and replacement patterns of each species may clarify this issue in more detail.

From the present study, it is showed that the two scale eaters exhibit differential scale-eating behaviour based on the specialised function of their teeth. These specialisations seem to be important to effectively remove scales and overcome the low hunting success previously reported by Hori (1987). Furthermore, it is suggested that species using similar food resources by specialising in different hunting techniques often increase the hunting success of individuals by making prey less cautious of any one predator as a result of diverse hunting techniques (Hori, 1987, 1997; Matsuda *et al.*, 1993, 1994, 1996). This situation, termed 'exploitative mutualism' (Matsuda *et al.*, 1993) could be an important mechanism that promotes the differentiation in morphology and behaviour between *P. straeleni* and *P. microlepis*.

Chapter 3. Evolutionary patterns and process to scale eating: integrative analyses based on a molecular phylogeny

3.1 Introduction

Integrative study of phylogeny, ecology, and morphology for *Perissodus* species would give us important insights about the mode of the specialisation to scale eating and differentiation in divergent trophic morphology. Particularly, understanding the phylogenetic relationships of *Perissodus* species from molecular approach is important to know the evolutionary pattern of their specialised oral jaw shape, which has only been discussed from morphological phylogeny (Liem & Stewart 1976).

Previous molecular phylogenetic studies on the Tanganyikan cichlids have placed the tribe Perissodini within the "H-lineage" (Nishida 1991; Salzburger *et al.* 2002). The phylogenetic relationships within Perissodini have not been clarified since a molecular phylogenetic approach using mitochondrial DNA (mtDNA) data has potential problems, sometimes resulting in patterns incongruent with the species phylogeny based on morphology or nuclear DNA, when hybridisation or incomplete lineage sorting is involved (Parker & Kornfield 1997; Schelly *et al.* 2006). Mitochondrial DNA and any single nuclear locus would be susceptible to this problem (Chow & Kishino 1995; Sota & Vogler 2001; Shaw 2002). In recent years, the amplified fragment length polymorphism (AFLP) method, in which large numbers of restriction fragments from whole genome digests can be examined, has provided powerful phylogenetic markers to overcome the above problems and is especially useful for analyses of closely related

cichlid species (Albertson *et al.* 1999; Allender *et al.* 2003; Kidd *et al.* 2006).

In this study, I aimed to clarify the evolutionary process of scale eating habits in Tanganyikan scale eaters, *Perissodus* species. For that purpose, molecular phylogenetic analysis based on AFLP data was conducted. I also examined the stomach contents, oral jaw tooth morphology, and habitat depths for all known Perissodini species. Subsequently, I compared these characteristics based on the obtained phylogenetic framework. Finally, discussion is made on the evolutionary process of specialisation to the scale eating habits, and associated feeding morphology and feeding behaviour of *Perissodus* species.

3.2 Materials & Methods

Specimens

Specimens of all nine described species from the tribe Perissodini (two *Haplotaxodon* and seven *Perissodus*), and representatives of lineages nested close to the Perissodini in the analysis of Salzburger *et al.* (2002) (two cyprichromine species, two benthochromines, and three lamprologines), were collected at Kasenga, Zambia, and from several other sites in Lake Tanganyika (Figure 3–1). Specimens were collected using gill nets, anaesthetised by storing in an icebox, and preserved in 99% ethanol. Specimens for the analysis of stomach contents were injected with 10% formaline into the stomach and were subsequently preserved in 10% formaldehyde solution. Specimens for the observation of jaw morphology were also preserved in 10% formaldehyde solution. These collection procedures were approved under the guidelines

for animal experiments enacted by the Japan Ministry of Education, Culture, Sports, Science and Technology (MEXT).

The habitat depths of all Perissodini species were estimated from collection records conducted at various water depths near Kasenga, Zambia. These samples were collected using gill nets (see Table 3–1).

Amplified fragment length polymorphism (AFLP) analysis

The AFLP analysis followed a protocol modified from Vos *et al.* (1995). The AFLP Plant Mapping Kit protocol (Applied Biosystems, Foster City, CA, USA) was used. DNA digestion was performed using *Eco*RI (20 units; New England Biolaboratories, Beverly, MA, USA), and *Mse*I (5 units) at 37°C for 5 h in a thermal cycler. At the end of 5 h, a ligation reaction was performed with restriction mixture containing each *Eco*RI and *Mse*I adaptor at 16°C overnight. Pre-selective amplification with one selective base on each primer (*Eco*RI–A and *Mse*I–C) and 11 different selective amplifications were performed using following combinations of primers with two additional bases (CT–TT, CG–TT, CA–TT, CG–TG, GG–TC, CT–TA, CA–TA, GG–AC, CT–AC, CA–AC, AG–AC). PCR was performed on a PC808 thermal cycler (ASTEC, Fukuoka, Japan). The DNA concentration was checked prior to restriction reactions. Fragments were electrophoresed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with internal size standards (GS 500 ROX; Applied Biosystems). Signal detection was carried out using GeneScan ver. 3.1 (Applied Biosystems). The fluorescence threshold was set to 50 r.f.u. And the correct fit of size standards was checked for all

electropherograms. Scoring to presence/absence was conducted between 50 and 499 bases using Genotyper ver. 2.5 (Applied Biosystems). Peaks with values <0.4 were considered the same.

Nei and Li (1979) genetic distances was calculated with the site (nucleotide) length set at 16 using the program Restdist in PHYLIP ver. 3.65 (Felsenstein 2005). Trees were constructed using the neighbour-joining (NJ) algorithm (Saitou & Nei 1987) implemented in Neighbour in PHYLIP. Alternative restriction site length parameters of 10 and 26 for the distance program were also used to examine the robustness of the distance model. To assess the robustness of the NJ tree, 1000 bootstrap replications were conducted with each site length parameter using Seqboot and Neighbour in PHYLIP.

Stomach content analysis

Stomach fullness was assessed under a light microscope according to Hynes' (1950) point method with minor modification, i.e., 4 points for 1/4 fullness, 8 points for 1/2 fullness, 16 points for complete (1x) fullness, and 32 points for twice (2x) fullness. After each food item in the stomach was identified, its volume relative to the fullness points was judged. Points were allotted to each food item according to the relative volume. The percent contribution of each food item in each species was calculated by pooling the total points for each food item and dividing by the sum of stomach fullness points.

Observation of oral jaw tooth morphology

The oral jaws were removed from one specimen for each *Perissodus* and *Haplotaxodon* species, cleaned with water, dehydrated in 70% ethanol. The oral jaw teeth were then observed and photographed either using a scanning electron microscope (SEM; JSM5800, JEOL, Tokyo, Japan) for *Perissodus* species, and a digital microscope (VHX–100, Keyence, Osaka, Japan) for *Haplotaxodon* species. These teeth were coated with gold prior to SEM photographing. The numbers of teeth on both jaws were also enumerated under a binocular microscope. The individuals of full adults with relatively a few numbers of lacked teeth were used to minimize the effects of natural replacement or wearing out of the teeth.

Ancestral state reconstruction

Ancestral reconstruction of feeding habits, oral tooth morphology and habitat depths were undertaken using the Neighbor-Joining tree from AFLP dataset. For reconstruction of feeding habits, oral jaw tooth morphs, and habitat depths, maximum parsimony ancestral reconstruction was performed using Mesquite (Maddison & Maddison 2006). Additionally, maximum–likelihood ancestral reconstruction was performed for reconstruction of ancestral states of feeding habits, using Mesquite, based on Markov k-state one-parameter model (Maddison & Maddison 2006).

3.3 Results

AFLP phylogeny

In total, 1582 fragments including 996 informative characters were scored for 72 individuals in the AFLP analysis. The neighbour-joining (NJ) analysis produced a tree (Figure 3–2) in which the monophyly of the tribe Perissodini, *Haplotaxodon*, and *Perissodus* was supported (BP = 100%, 96–99%, and 90–91%, respectively). The monophyly of each species was also strongly supported in this AFLP tree (BP = 89–100%). Among the *Perissodus* species, *P. hecqui* was placed as most basal, followed by *P. multidentatus* (BP = 61–63%). The remaining five *Perissodus* species constituted a monophyletic group (BP = 98%) in which *P. straeleni* and *P. microlepis* were clustered together (BP = 100%), with *P. paradoxus* as the likely sister taxon (BP = 61–64%).

Stomach content analysis

Scales, primarily those of cichlids, were a major component in the diet of five *Perissodus* species (*P. microlepis*, *P. straeleni*, *P. paradoxus*, *P. eccentricus*, and *P. elaviae*). Except in *P. straeleni*, scales accounted for about 90% of the diets (Figure 3–3). Fry of the clupeids *Limnothrissa miodon* and *Stolothrissa tanganicae*, which are abundant in pelagic open waters (Brichard 1978), or those of littoral cichlids were occasionally found in the stomach contents of these species. Fish skin (epidermal and dermal tissues) was observed in the stomach contents of *P. straeleni* (16.0%).

In contrast, the proportion of scales in the diets was lower among the basal lineages of *Perissodus* species than in the above five species. Fish skin (47.7%) and

scales (46.8%; mainly of clupeids) were major diet components of *P. multidentatus*. The scales were not found alone, but always with skin. *Perissodus hecqui* appeared to be a zooplankton feeder, with calanoid copepods as its main prey (54.5%), although it also consumed clupeid fish fry (16.5%). The stomach contents of this species also contained detrital materials, including plant tissue, sponges, a few scales, pieces of fish fin, and sand grains (11.5%).

Haplotaxodon species fed on several prey items, with the pelagic shrimp *Mysis* sp. as the main prey (46.4% and 39.5%, respectively, in *H. microlepis* and *H. trifasciatus*), followed by aggregates of small organic particles, which included a variety of planktonic remains such as crustacean moults, plant cells, and phytoplankton. Planktonic algae, mostly blue-green algae (mainly *Microcystis* and *Anabaena*), green algae (e.g., *Gloeocystis, Coelastrum*), and diatoms, were also found in the stomach contents. The clupeid fry was also consumed by *H. trifasciatus* (10.2%).

Habitat depths

Perissodus microlepis and *P. straeleni* were collected mainly from shallow rocky regions less than 70 m in depth (Table 3–1). *Perissodus paradoxus* was found at depths of 2 to 162 m, ranging from shallow rocky regions to deep areas. The remaining four *Perissodus* species were collected only at depths greater than 40 m. *Haplotaxodon* species were found in shallow waters.

Morphology of the oral jaw teeth

Teeth were arranged in a single row on both the upper and lower jaws in all perissodine species. Five of *Perissodus* species, *P. microlepis*, *P. eccentricus*, *P. straeleni*, *P. paradoxus*, and *P. elaviae*, had fewer but larger teeth (see Figure 3–4B and Figure 3–5) than did the other *Perissodus* and *Haplotaxodon* species, and these teeth were strongly recurved backward. In *P. microlepis*, the corners of the upper side of each tooth projected vertically, forming a pair of spine-like points. In *P. eccentricus*, one side of each tooth was sharply edged with a blunt point on the tip, forming a fist-like projection. In *P. straeleni*, *P. paradoxus*, and *P. elaviae*, each tooth had a laterally widened leaf-shaped crown, forming sharp edges laterally. *P. hecqui* and *P. multidentatus* had a large number of small teeth, which were also recurved backward. The teeth of *P. multidentatus* had slim, elongated stems with right-angled crowns, whereas those of *P. hecqui* had short stems with leaf-shaped crowns. The teeth of *Haplotaxodon* species were generalised, small, and conical in shape, and slightly recurved backward.

Ancestral reconstruction of feeding habits and morphology

The ancestral reconstruction of feeding habits based on MP and ML methods both yielded similar results: the general carnivorous / plankton feeding found in *Haplotaxodon* species and *P. hecqui* to be most ancestral in Perissodini (Figure 3–4A). The skin eating habit has most likely evolved once in *P. multidentatus*. The specialised scale eating habit appeared to be monophyletic in the clade containing *P. elaviae*, *P. eccentricus*, *P. paradoxus*, *P. straeleni*, and *P. microlepis*. The feeding habit prior to

specialised scale eating was undetermined: ML analysis gave the probability of each character state at the ancestral node of *P. multidentatus* and the five specialised scale eaters as 0.45, 0.10, 0.45, for general carnivorous / plankton feeding, skin eating, and scale eating respectively.

Reconstruction of the tooth shapes indicated that the ancestral state of tooth shape was recurved pattern in Perissodini (see Figure 3–4B). Sharply pointed tooth patterns occurred twice independently in *P. microlepis* and *P. eccentricus*. Parsimony reconstruction of habitat depths unambiguously indicated that the deepwater habitat type is the most ancestral (Figure 3–4B): the transitional shift from deepwater to shallow rocky region occurred in *Perissodus*.

3.4 Discussion

Phylogenetic relationships of Perissodini species

The present molecular phylogenetic study based on the AFLP method demonstrates the highly supported monophyly of each species, and suggests important implications for the phylogenetic relationships in Perissodini (Figure 3–2). In the Perissodini, it was not rejected that the genus *Haplotaxodon* is the sister group to the genus *Perissodus* as is suggested by Poll (Poll 1986). In the genus *Perissodus*, *P. hecqui* and *P. multidentatus* appeared as basal to other *Perissodus* species, as with the morphological phylogeny of Liem & Stewart (Liem & Stewart 1976). The phylogenetic relationships of the remaining five species revealed the sister relationship between *P. microlepis* and *P. straeleni*, with *P. paradoxus* likely being the sister taxon. This does not agree with a

traditional classification, which divides these into two groups: "*Plecodus*" group (including *P. paradoxus*, *P. elaviae*, and *P. straeleni*), and "*Perissodus*" group (including *P. microlepis* and *P. eccentricus*), mainly based on the oral tooth morphology (see Figure 3–4B) (Liem & Stewart 1976; Poll 1986). Tooth shape is an adaptive morphological trait, which could potentially be susceptible to homoplasy through natural selection (Hufford 1996; Rüber *et al.* 1999). Thus, the traditional morphological classification of this fish group cannot be concluded to reflect their evolutionary relationships. Rather, the recurrent evolution of similar feeding morphology was suggested to have occurred with specialisation to scale eating, which will be discussed more in the later section.

The present AFLP phylogenetic tree partly agrees with the AFLP tree suggested by Koblmüller *et al.* (Koblmüller *et al.* 2007), particularly in the sister relationship of *P. microlepis* and *P. straeleni*, and the placement of *Haplotaxodon* species as sister to *Perissodus* species. The differences are also observed in resolution and phylogenetic relationships between the two studies, most probably due to the limited number of AFLP characters and lack of *P. eccentricus* in their AFLP tree (Koblmüller *et al.* 2007). Our AFLP tree gained better resolution with over 1000 multilocus AFLP characters, though some of basal nodes in *Perissodus* species were still not well resolved. This low resolution at some nodes with short branch lengths can be partly explained by the rapid cladogenesis events that may have occurred at the onset of the diversification of those scale-eating cichlids as also previously suggested from mtDNA study for Perissodini and other Tanganyikan tribes (Koblmüller *et al.* 2004; Duftner *et al.* 2005; Koblmüller *et al.* 2007).

The mtDNA phylogeny for Perissodini was recently reported to show the strong incongruence from AFLP phylogenetic trees (Koblmüller *et al.* 2007). Our phylogenetic analyses based on mitochondrial cytochrome *b* sequences support their result (see supplemenrary figure 1). The ancestral polymorphisms due to rapid cladogenesis events were most likely explanation for these discrepancy, as suggested for several Tanganyikan cichlids (Takahashi *et al.* 2001; Koblmüller *et al.* 2007). Furthermore, the effect of hybridisation should also be considered as factors for such incongruence. In our cytochrome *b* genealogy, *P. microlepis* and *P. straeleni* from the northern and southern regions, clustered together, and *P. elaviae* and *P. paradoxus* shared similar haplotypes. These results suggest the past and / or recurrent hybridization events may have occurred between these pairs of species.

Evolution of scale eating

The ancestral reconstruction analyses of feeding habits based on our new phylogenetic framework suggest the evolution of feeding in the Perissodini from general carnivorous feeding to highly specialised scale eating (Figure 3–4A). *Haplotaxodon* species appear to be general carnivorous feeder that mainly collect mysid shrimp by effectively using their upwardly pointed mouth. At the basal lineage of *Perissodus*, *P. hecqui* appears to be a zooplankton feeder. The result agrees with the observation that *P. hecqui* has twice as many gill rakers as other *Perissodus* species (Poll 1956). However, the specialised recurved oral teeth of *P. hecqui*, similar to those of the other *Perissodus* species, suggest some biting function. This implies that the ancestral feeding habit of *Perissodus* species

involved some carnivorous habit. Another basal taxon, *P. multidentatus*, appeared to feed on both fish skin and scales of fish at high rates. The fish skin was always found with scales, implying that this species probably bites the flanks of fish, rather than simply tearing off scales. Such skin-eating habit may reflect some original mode of scale eating in this group.

The highly specialised scale-eating habit appears in the five remaining species, which form a monophyletic group, suggesting a single origin of the specialisation in this lineage (Figure 3–4A). Among these species, *P. straeleni* also uses other resources such as fish skins and fish fry (Figure 3–3). This may be an alternative feeding strategy of older, large *P. straeleni*, which may show a decreased ability to feed on scales, probably because of worn teeth (Chapter 2). In fact, scale-less non-cichlid fishes such as catfish *Chrysichthys* spp. and *Synodontis* spp. are frequent targets of skin eating by older *P. straeleni* (Yanagisawa *et al.* 1990). Therefore, the most likely explanation for the variation in food habits is secondary divergence from scale eating as a consequence of supplementary feeding.

Evolution of oral jaw tooth

Our study further indicates that the oral tooth shapes have divergently differentiated, particularly among the specialised scale eaters: the tooth tended to become large in size, and show remarkable differentiations in shape and structure among species. Within them, the tooth shape of *P. microlepis* and *P. eccentricus* is noteworthy. Their teeth are broad based and thicker than those of other *Perissodus* species, that have laminar

recurved teeth pattern. Particularly, the spine-like projection is the unique trait that is only found in *P. microlepis* and *P. eccentricus*. Interestingly, parsimonious reconstruction of jaw tooth shape suggests that this tooth shape pattern has evolved independently in each lineage of *P. microlepis* and *P. eccentricus* (Figure 3–4). *P. microlepis* uses its jaw teeth for wrenching scales in a screw like manner from the prey, showing close associations of feeding morphologies and feeding behaviours (Chpter 2). Furthermore, the teeth pattern of *P. microlepis* appeared to have much damage-resistant structure than that of *P. straeleni* (Chapter 2). Such functional significance may also have promoted the convergent oral tooth structure in *P. microlepis* and *P. eccentricus*. However, note that whereas the teeth of these two species have similar shapes for wrenching feeding action, the sharp edge of the teeth of *P. eccentricus* imply a somewhat different function from that of *P. microlepis*; a scraping feeding action such as that observed in *P. straeleni* may also be additionally involved in the feeding behaviour of *P. eccentricus*.

Mutual exploitation

Our study also revealed a sister relationship of the coexisting specialised scale eaters *P. microlepis* and *P. straeleni*, both of which are very common in the shallow waters of Lake Tanganyika, and exploit similar food resource (Hori *et al.* 1983). These species exhibit differential feeding morphologies and hunting behaviours (, chapter2Hori 1987). Notably, these two species increase their hunting success by diverting the caution of the prey through diverse hunting techniques (Matsuda *et al.* 1993; Matsuda *et al.* 1994, 1996). This situation, termed 'exploitative mutualism' (Matsuda *et al.* 1993), would play

an important role in the stable coexistence of *P. microlepis* and *P. straeleni*, and may have promoted further morphological and behavioural divergence of these two species under sympatric conditions. Although nothing is known about the behaviour of deepwater scale eaters, such a relationship might also be found between the deepwater scale eaters, *P. elaviae* and *P. eccentricus*. The examination of feeding relationships in deepwater habitats may be of interest to further consider the inter-specific relationships among the scale eaters in deepwater habitat.

The deepwater origin of scale eating

Our present study suggests the deepwater origin of the scale eating. In the shallow rocky habitat, scale eaters share prey species with some specialised pursuit piscivores such as *Lepidiolamprologus* spp. (Hori *et al.* 1993). On the other hand, in the deepwater habitat, such benthic pursuit hunters are absent, though some pelagic piscivorous cichlids such as *Bathybates* spp. and general carnivorous cichlids such as *Telotrematocara macrostoma* are common (Coulter 1991). Thus, the feeding niche, which is dominated by the specialised pursuit hunters in the shallow habitat, seems to be vacant in the deepwater habitat. Thus, it can be speculated that such niche may have been explored by the ancestor of scale eating cichlids in the deepwater habitat. Their large recurved oral jaw tooth shape, which differs from the conical tooth shape of true piscivorous species, may also have been suited for biting off the small portion of the prey's flank. Such morphological constraint might also have led this group to further specialisation for the full-dress scale eating habit.

Conclusions

Although scale-eating cichlids also inhabit other younger African lakes, i.e., Lake Victoria and Lake Malawi, the number of species and the degree of specialisation for scale eating are greatest in *Perissodus* species of Lake Tanganyika (Fryer & Iles 1972). The present study revealed the phylogenetic relationships of the Perissodini, and based on the resultant tree, proposed the comprehensive evolutionary sequence of the specialisation of scale eating habits in this lineage for the first time. The mtDNA and AFLP phylogenetic trees both suggested the rapid speciation at the onset of diversification of Peerissodini. The mtDNA phylogenetic tree also suggests that the diversification of *Perissodus* species occurred roughly in the late Neogene (1.7–7 Mya), implying that these species may have experienced the dramatic geological events of the lake, including lake level changes that occurred 2.5 – 3 Mya (DeMenocal 1995). The remarkable diversity of *Perissodus* would be attributed to this complex geological history of Lake Tanganyika and complex interspecific relationships among fishes in the lake.

Chapter 4. Inheritance pattern of lateral dimorphism in fishes

4.1 Introduction

The lateral dimorphism in mouth morph was first described in Tanganyikan scale eating cichlids, *Perissodus* (Liem & Stewart 1976; Hori 1991, 1993). One type has its mouth opening to the right, causing the left-side of its head to face the front (termed " lefty"; Nakajima *et al.* 2004), while the other type has its mouth to the left, causing the right side of the head to face frontward (termed "righty"). This asymmetry in mouth opening was caused by laterally asymmetrical joint of mandible to suspensorium (Liem & Stewart 1976). It is a typical example of anti-symmetry, which has a bimodally distributed frequecny of asymmetric character in populations. Note that Nakajima *et al.* (2004) changed the definitions of lefty and righy to those described previously; previous papers (Hori 1991, 1993; Mboko *et al.* 1998; Seki *et al.* 2000) called individuals with its mouth-opening to the right as "right-handed" or "dextral" whereas those with mouth-opening to the left as "left-handed" or "sinistral".

Hori (1993) also showed that this lateral dimorphism is inheritable in *Perissodus microlepis* based on the samples of parents and their offspring from the natural habitat, and suggested a simple Mendelian one locus-two alleles system. However, non-related young may have been mingled in those broods due to frequent occurrence of intraspecific brood-mixing (Yanagisawa 1985). Therefore, the inheritance pattern was re-analyzed by collecting the parents and its offspring soon after the spawning, and raised in laboratory (Hori *et al.* 2007). This study has indicated that the laterality is

inherited in a mendelian one locus-two-alleles manner, in which lefty gene is dominant over righty gene with the dominant gene acting as lethal when in a homozygote, and thus, the lefty phenotypes should be heterozygous. Cross incompatibility is also suggested as alternative cause for the absence of dominant homozygote (Hori *et al.* 2007).

Interestingly, such morphological laterality has been reported in several other fishes: Tanganyikan cichlids (*Telmatochromis temporalis*: Mboko *et al.* 1998; *Neolamprologus moorii*: Hori *et al.* 2007), a scale eating tricanthodid (*Macrorhamphosodes uradoi*: Nakae & Sasaki 2001), and even a Japanese riverine goby, *Rhinogobius flumineus* (Seki *et al.* 2000). The inheritance of such asymmetry in mouth morph of these fishes was also examined in *R. flumineus* (Seki *et al.* 2000), and the Tanganyikan algae-eating cichlid, *N. moorii* (Hori *et al.* 2007). These studies have suggested that the laterality is inherited in a similar manner to *P. microlepis*. However, high mortality of eggs observed in *R. flumineus* might affect the observed segregation ratios (Seki *et al.* 2000). Or any environmental effects cannot be fully eliminated in sampling of parents and offspring collected in field. Thus, laboratory-based breeding experiments including F1 and F2 generation analyses have been desired to confirm the inheritance pattern of this trait.

P. microlepis seems to be the best material for such breeding experiment because of many studies having been accumulated on the laterality, and the easiness in judging of the mouth asymmetry. However, the fish is sensitive to breeding conditions, and our efforts for breeding their broods did not result in success. Instead, the Tanganyikan

algae eating cichlid, *Julidochromis* cf. 'Gombi' and Japanese Medaka, *Oryzias latipes* (Adrianichthyidae) are relatively easy to breed, and large number of progeny are available in experimental conditions. *Julidochdomis* species are substrate-brooding cichlid fish endemic to Lake Tanganyika (Konings 1998). The breeding ecology of this group has been studied both in field (Awata *et al.* 2005) and laboratory (Awata, prepared). *Julidochromis* cf. 'Gombi' is a commercially popular race, and their reproductive biology is basically same to wild *Julidochromis* species. *O. latipes* is an egg-laying freshwater fish native to Japan, Korea and China, and has been used as a model organism for vertebrate developmental study and genetics.

In this chapter, I have conducted the breeding experiments using *Julidochromis* cf. 'Gombi' (hereafter *J.* 'Gombi') and *O. latipes* in laboratory for the purpose of examining the inheritance pattern of the laterality in these fishes. I here demonstrate the segregation pattern of laterality in F1 progeny of these fishes is consistent with the ratios previously found in the scale eating cichlid, *P. microlepis* and other two fishes (*N. moorii* and *R. flumineus*), and suggest mendelian one-locus system from the result of F1 and F2 segregation ratios.

4.2 Materials & Methods

The fishes

The young individuals of *J.* 'Gombi' were obtained from a commercial vendor in Osaka. The fishes were maintained in aquaria until they became mature, and used as parents for

breeding experiments. The adult individuals of *O. latipes* were collected from Kamigamo Experimental Forest Station of Kyoto University, Kyoto, Japan in May 2004.

Pairing and Breeding

The single-pair cross experiments were performed for the two species. For *J.* 'Gombi', pairing was conducted in 9L tank maintained in 14/10 dark/light at 26°C. An artificial nest, which was made of two slate tiles (10 x 10 x 0.5 cm) with the entrance widths of around 30 mm, was situated in each breeding tank. The female parents successfully laid their eggs on these tiles. The tiles were carefully transferred with eggs to the 3L aquarium. Dead eggs were counted and removed from the tiles with tweezers every day until hatching. They were fed with alive brine shrimp during juveniles, and thereafter with commercially available flake food (SeraSan®).

In *O. latipes*, paring cross was conducted in 1L tank maintained in 14/10 dark/light at 23 ± 1°C. The eggs were mainly collected in the morning soon after the light was on. These eggs were removed from the belly of females gently with a small hand net, preserved in a dish with egg water (0.65% NaCl, 0.04% KCl, 0.1% NaHCO3), and maintained in an incubator at 26°C until hatching. The development of *O. latipes* eggs was checked by eye or under binocular microscope, and dead eggs were removed from a dish each day. The number of dead eggs was counted every day until hatching. Hatched larva of each brood was transferred to 1L tank. They were fed with commercially available powder food (Ranchuu-kizoku®) during juveniles, and

thereafter with alive brine shrimp.

Cross scheme and judgment of laterality

The pairing was conducted as follows for F1 crosses; lefty x lefty parents, lefty (♂) x righty (♀) parents, righty (♂) x lefty (♀) parents, and righty x righty parents. More than two pairing crosses were conducted for each breeding scheme. Total of 12 and 14 pairs laid eggs in *J.* 'Gombi' and *O. latipes*, respectively. These F1 individuals were raised until sexually maturing, thereafter being anesthetized with approximately 5% phenoxy-methanol, and preserved in either David solution or 10% formaline except for individual used for F2 testcrosses. Some individuals were dead before maturity. These fishes were also included for the analyses. The standard length of F1 individuals used for segregation analyses were in the range of 1.8–9.5, and 1.2–3.0 cm in *J.* 'Gombi', and *O. latipes*, respectively. P0 parents were preserved as the same procedure for F1 individuals.

F2 testcrosses were conducted under the hypothesis that the lefty is dominant over righty, and the righty is recessive homozygotes. Thus, test-crosses were conducted under following two pairing schemes: in cross 1 scheme, paring cross was conducted between lefty morph individual of F1 bred from lefty and lefty parents, and righty morph individuals of F1 from righty and righty parents. In cross 2 scheme, pairing was conducted between righty morph individuals of F1 from righty and righty parents. F2 individuals obtained from these parings were bred for more than 2 months with same conditions as described above. Total of 7, and 5 pairs with the standard length larger

than 1.8 cm and 1.5 cm, respectively were obtained for testcross of *J.* 'Gombi' and *O. latipes*, respectively. They were preserved with same procedures mentioned above.

The laterality of F1 and F2 individuals was checked under binocular microscope with the same method applied in the previous studies (Seki *et al.* 2000, Hori *et al.* 2007, Nakajima *et al.* in press). In the righty morph, the right joint of the mandible to suspensorium is positioned toward the front, ventrally, and outside, compared to the left joint, whereas in the lefty morph, the left joint has these characteristics. When the mouth opened rightward, the individuals were judged as lefty, whereas when the mouth opened leftward, the individuals were considered as righty. The segregation ratio was tested using a chi-squared test.

4.3 Results

The egg hatchability

In *J.* 'Gombi', the hatching normally occurred in 2 to 3 day after oviposition. The egg hatchability of *J.* 'Gombi' was lower than those of *O. latipes* (Table 4–1). Most of egg deaths were detected on 1 to 2 day after oviposition. The observation of developing embryos was impossible because of the untransparent eggs being coated with green-opaque membrane. No difference was observed among pairing schemes of *J.* 'Gombi' (ANOVA; $P > 0.05$).

In *O. latipes*, the hatching normally occurred in 10–12 day after oviposition. As shown in Table 4–1, the hatchablity of *O. latipes* was high. Most of unhatched eggs appeared to be unfertilized, because in these cases, no fertilization membrane was

observed. Occasionally, the development of embryos appeared to stop in cleavage stages, and hatching did not occur. The large decline in hatchability in some pairing schemes, which would be expected under some lethal effect during developmental stages, was not observed. No difference was detected in egg hatchability among paring schemes (ANOVA; $P > 0.05$).

Inheritance patterns in F1 and F2 progenies

In the F1 progeny of *J.* 'Gombi', the observed segregation ratios were similar to those of *O. latipes* (Table 4–2a). The ratios of the phenotypes in F1 progeny of both lefty parents closely fitted to the ratio of 2:1. The ratios of lefty to righty from lefty and righty parents were closely akin to 1:1. The progenies from both righty parents were all righty.

The segregation ratios of F2 progenies showed that individuals from cross 1 scheme showed the ratios of lefty to righty were close to 1:1. The individuals from cross 2 showed all righty phenotypes. These segregation ratios were consistent with those of *O. latipes.*

In the F1 progeny of *O. latipes*, those from both righty parents exhibited all righty phenotypes (Table 4–2b). On the other hand, those from the righty and the lefty parents, or those from both lefty parents exhibited the lefty and the righty phenotypes in every case. The ratios of lefty and righty phenotypes in F1 progenies were close to 1:1 from lefty and righty parents, whereas close to 2:1 from lefty parents.

The testcross was conducted for examining the genotypes of the lefty phenotypes

under the hypothesis of righty phenotypes as recessive homozygotes (Table 4–2b). The segregation ratios of F2 progenies showed that individuals from cross 1 schemes showed the ratios of lefty to righty close to 1:1. Individuals from cross 2 schemes showed all righty phenotypes. These results suggest that the segregation ratios of laterality appear to be genetically determined. Lefties appear to be dominant over righties.

4.4 Discussion

The inheritance of laterality has previously been inferred in several fish species (Hori 1993; Seki *et al.* 2000; Hori *et al.* 2007). In this study, I have conducted the laboratory based breeding experiments using the two distantly related species, Tanganyikan cichlid, *J.* 'Gombi' and Japanese Medaka, *O. latipes*. The present study gives us important implications regarding the inheritance pattern of laterality.

The observed difference in segregation ratios in F1 and F2 generation proved that the phenotype ratios of progeny were related to the phenotypes of parents, indicating that the strong genetic factors were involved. The segregation ratios in F1 generations of the two species are in agreement with previous results that progenies from lefty pairs had 2:1 segregation ratios of lefty: righty, progeny from lefty and righty pairs had 1:1 ratios, and progenies from righty pairs had all righties (Seki *et al.* 2000; Hori *et al.* 2007). The test cross experiments in F2 generation further confirmed the hypothesis that lefty is dominant heterozygote over recessive righty.

The observed segregation ratios in progenies from lefty parents did not fit to the

1:0 or 3:1 segregation ratios, which are expected under the hypothesis of normal mendelian inheritance of one-locus two-alleles system. Those exhibited ratios from lefty parents have been explained with the hypothesis that the dominant homozygotes may be absent, acting as lethal gene. However, in spite that genes responsible for morphogenesis are mostly expressed in embryonic stages in zebrafish or medaka (Wittbrodt 2002), the large decline in viability in embryonic stage was not found. Thus, lethal gene seems not to be involved in the development of the laterality. As an alternative hypothesis, cross incompatibility was suggested as possible cause for the absence of dominant homozygotes (Hori *et al.* 2007). The cross incompatibility is widely obsereved in domesticated plants such as maize and rice (e.g. Rashid & Peterson 1992; Matsubara *et al.* 2003). However, this mechanism does not also fully explain the observed segregation ratio in the present study. Further investigation is required to make our genetic model more conclusive.

The much similar inheritance pattern in *J.* 'Gombi' and *O. latipes*, and other three fishes investigated so far suggest that the laterality in fishes may be regulated by the same genetic mechanism. An unpublished result of F1 breeding experiment for zebrafish, *Danio rerio*, also showed a similar inheritance pattern (see Supplementary table 1). These results infer that the laterality may be prevalent in fishes, and one of important traits contributing to the craniofacial development of fishes in general. Recent molecular studies on left-right patterning of body plan have been focused on the craniofacial development of fishes, and lack of some genes important for left-right patterning have shown asymmetrical development of jaws and organs in left and right

sides in mutant zebrafish (Albertson & Yelick 2005; Kawakami *et al.* 2005). Though actual relation of such genetic mechanism, and the laterality of the present study have not been investigated, the present finding in inheritance patterns in *O. latipes*, in which numerous developmental studies were conducted with molecular methods, may provide us some way to analyse the trait from developmental and molecular perspectives.

Laterality in foraging behavior is clearly assorted with mouth-opening dimorphism in scale-eaters in Lake Tanganyika (Hori 1991, 1993). Righties of *P. microlepis* at present definition only attack on the right side of prey, and vice versa (Hori 1991, 1993). Such specialised feeding behaviours may relate to the evolution of the enhanced asymmetry in *Perissodus* species. Furthermore, in the herbivorous cichlid, *T. temporalis*, the lefties tend to use the right side jaw more frequently, and the righty the left side jaw (Mboko *et al.* 1998). In *R. flumineus*, Seki *et al.* (2000) also showed that in the stationary state, righties had rightward curved patterns more frequently than leftward patterns, and vice versa. Nakajima *et al.* (2004, 2005) suggested the mechanism of " cross predation" as factors for maintaining dimorphism in populations. In their model, the predators tend to prey on preys of opposite laterality; lefties and righties prey on righties and lefties, respectively. Such mechanism would be related to the prevelance of laterality in fishes of various trophic levels. Future study for the laterality may be more interesting if the prelevance and degree of the laterality in various groups of fishes are evaluated.

Chapter 5. General Conclusion

In this study, I have investigated the relation of feeding specialisation and morphological diversification in Tanganyikan scale eating cichlids from multiple viewpoints. In chapter 2, it was shown that the morphological difference in oral jaw tooth have functionally close associations with the scale eating behaviours of the two species. The leaf-shaped jaw tooth of *P. straeleni* has scraping function while shifting its mouth laterally along the body of the prey whereas the broad-based tooth with spine-like projections of *P. microlepis* exhibits the wrenching functions while quickly rotating its body. This comparative study suggests the diversification in oral jaw tooth plays a significant role to perform the differential feeding behaviours in each species.

In chapter 3, I have examined the evolutionary process of feeding specialisation and morphological diversification in *Perissodus* species. The scale eating habit appeared to have evolved once in the deepwater habitat from the general, carnivorous feeding. With the specialisation to scale eating, *Perissodus* species broadened their habitats from deepwater to shallow water habitat, and concomitant divergence in jaw teeth shapes occurred among specialised scale eaters. These results indicate that the evolution in feeding habits, and feeding morphologies were closely associated to each other. In particular, from the sister relationship of *P. straeleni* and *P. microlepis*, the mechanisms of 'mutual exploitation' is noted as an important species relationship that may promote the morphological and behavioural diversification under sympatric conditions. The study suggests the importance of the inter-specific relationship both for the maintenance of the present diversity of these scale eaters, and for further evolution

and diversification of *Perissodus* species.

In chapter 4, the genetic analyses of the laterality confirmed that the laterality first found in scale eating cichlids is also inherited in *O. latipes* and *J.* 'Gombi'. The result also suggests that the segregation pattern of F1 and F2 generations are similar to those of *P. microlepis* and other speies infered from field observations, suggesting that the same genetic mechanisms control the asymmetry in mouth morphs found in these fishes. From these results, it is speculated that the asymmetry in mouth morph may be inherited in various fish groups, and can be considered as one of important traits contributing to the craniofacial development of fishes in general. The present study gives us the basic knowledge regarding the laterality in fishes, and opens the way to develop the study from genetic perspectives (Chapter 4).

From the present results, it is suggested that the trophic specialisation to scale eating triggered the diversification of *Perissodus* species. Particularly, the differentiaion in oral jaw morphology closely relate to the evolution and divergence in their feeding ecology and behaviours among specialised scale eaters. These close relations of morphology, ecology, and behaviour are key to maintain the present diversity of *Perissodus* species, and promotes further evolution and diversification of each other. For future perspectives, it is necessary to examine the feeding relationships of deepwater scale eaters. This study will help to understand the inter-specific relationships in deepwater scale eaters, and consider what kind of feeding niches was initially explored by the ancestor of this scale-eating group. This knowledge would further lead us to consider any ecological factors for the diverse feeding morphology of deepwater

scale eaters. Furthermore, the analyses of laterality from morphological, developmental, and genetic aspects would be of importance to know the actual genetic factors responsible for the trait, and how enhanced aysmmetry is genetically maintained in *Perissodus* species.

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Table 2.1. The mean time duration (ms) of each phases of feeding behaviours for *P. straeleni* (two individuals) and *P. microlepis* (four individuals), and statistical comparisions between the species.

	P. straeleni		*P. microlepis*		
Variables	Number of observations	Mean ± S.E (ms)	Number of observations	Mean ± S.E (ms)	*P*-value
Forward dash	15	135.7 ± 14.5	23	144.9 ± 12.5	NS*
Mouth opening prior to strike	19	86.0 ± 12.0	48	68.8 ± 5.0	NS
Strike duration	42	91.7 ± 7.4	54	108.7 ± 8.0	NS

*ANOVA test, NS: $P > 0.05$

Table 3–1. Number of times and depths of sampling for Perissodini species

Species	Number of individuals caught within each depth range (m)*				
	1–19	20–39	40–69	70–99	≥100
Perissodus microlepis	60 (12)	–	6(1)		
Perissodus straeleni	25 (9)	–	2 (1)		
Perissodus paradoxus	10 (4)	–	8 (4)	6 (3)	2 (1)
Perissodus eccentricus		–	8 (1)	4 (4)	31 (20)
Perissodus elaviae		–	7 (3)	1 (1)	14 (11)
Perissodus multidentatus		–	3 (1)	6 (3)	26 (17)
Perissodus hecqui		–	14 (4)	9 (6)	13 (9)
Haplotaxodon microlepis	20 (4)	–			
Haplotaxodon trifasciatus	25 (3)	–			

* The numbers of samplings conducted at each depth range are given in parentheses. In waters deeper than 40 m, gill nets were deployed for about 1 h. In shallow rocky areas less than 20 m in depth, gill nets were set using SCUBA. Sampling was not conducted at depths of 20–39 m. The anoxic region begins at depths around 120–140 m in this region.

Table 4–1. The percentages of egg hatchability in each cross scheme of *O. latipes* and *J.* 'Gombi'.

			O. latipes		*J.* 'Gombi'
P0 laterality		*N*	F1	*N*	F1
♂	♀		Egg hatchability (%, mean ±SD)		Egg hatchability (%, mean ± SD)
Lefty	Lefty	2	94.8 ± 0.2	2	82.5 ± 13.2
Lefty	Righty	2	93.1 ± 2.9	1	77.3
Righty	Lefty	2	93.8 ± 4.3	1	74.5
Righty	Righty	2	87.5 ± 3.9	2	82.4 ± 12.3
F1 laterality			F2		F2
♂	♀		Egg hatchability		Egg hatchability
Lefty	Righty	2	80.5 ± 13.2	2	68.5± 23.3
Righty	Lefty	2	75.2 ± 3.1	1	78.5
Righty	Righty	3	70.0 ± 10.4	2	65.5 ± 12.5

**N* indicates the number of pairs used for observation of egg hatchability.

Table 4–2a. Segregation ratios of the F1 and F2 generations of single-pair crosses in *J.*'Gombi'.

J. 'Gombi'							
Cross (♂x♀)	Pair	*n*	L	R	Observed ratio	Expected ratio	Fit to the expected ratio*
F1							
L x L	J1	54	36	18	2.00:1	2:1	1.000
	J2	47	32	15	2.13:1	2:1	0.836
	J3	12	7	5	1.40:1	2:1	0.540
	total	113	75	38	1.97:1	2:1	0.947
L x R	J4	113	60	53	1.13:1	1:1	0.510
	J5	60	30	30	1.00:1	1:1	1.000
	total	173	90	83	1.08:1	1:1	0.595
R x L	J6	44	23	21	1.13:1	1:1	0.763
	J7	90	43	47	1.10:1	1:1	0.553
	J8	54	22	32	0.68:1	1:1	0.174
	J9	57	26	31	0.84:1	1:1	0.508
	J10	27	15	12	1.25:1	1:1	0.550
	J11	87	44	43	1.02:1	1:1	0.914
	total	359	173	186	0.93:1	1:1	0.493
R x R	J12	57	0	57	0:1	0:1	——
	J13	58	0	58	0:1	0:1	——
	J14	96	0	96	0:1	0:1	——
	total	211	0	211	0:1	0:1	——
F2							
L x R	F2-J1	45	22	23	0.96:1	1:1	0.881
	F2-J2	23	11	12	0.92:1	1:1	0.835
	total	68	33	35	0.94:1	1:1	0.808
R x L	F2-J3	28	15	13	1.15:1	1:1	0.705
R x R	F2-J4	29	0	29	0:1	0:1	——
	F2-J5	27	0	27	0:1	0:1	——
	total	58	0	58	0:1	0:1	——

*Chi-square test

Table 4–2b. Segregation ratios of the F1 and F2 generations of single-pair crosses in *O. latipes*.

O. latipes							
Cross (♂x♀)	Pair	*n*	L	R	Observed ratio	Expected ratio	Fit to the expected ratio*
F1							
L x L	O1	90	58	32	1.81:1	2:1	0.250
	O2	32	21	11	1.91:1	2:1	0.900
	O3	17	11	6	1.83:1	2:1	0.863
	O4	59	38	21	1.81:1	2:1	0.713
	O5	34	20	14	1.43:1	2:1	0.332
	O6	60	41	19	2.16:1	2:1	0.784
	Total	292	189	103	1.83:1	2:1	0.481
L x R	O7	157	72	85	0. 85:1	1:1	0. 284
	O8	42	23	19	1.21:1	1:1	0.537
	Total	199	95	104	0.91:1	1:1	0.523
R x L	O9	27	15	12	1.3:1	1:1	0.550
	O10	11	4	7	0.57:1	1:1	0.366
	Total	38	19	19	1:1	1:1	1.0
R x R	O11	54	0	54	0:1	0:1	——
	O12	35	0	35	0:1	0:1	——
	Total	89	0	89	0:1	0:1	——
F2							
L x R	F2-O1	24	11	13	0.85:1	1:1	0.683
	F2-O2	21	12	9	1.3:1	1:1	0.512
	Total	45	23	22	1.04:1	1:1	0.881
R x L	F2-O3	18	8	10	0.8:1	1:1	0.637
	F2-O4	20	10	10	1:1	1:1	0.179
	Total	38	18	20	0.90:1	1:1	0.746
R x R	F2-O5	13	0	13	0:1	0:1	——
	F2-O6	11	0	11	0:1	0:1	——
	F2-O7	15	0	15	0:1	0:1	——
	Total	39	0	39	0:1	0:1	——

* Chi-square test.

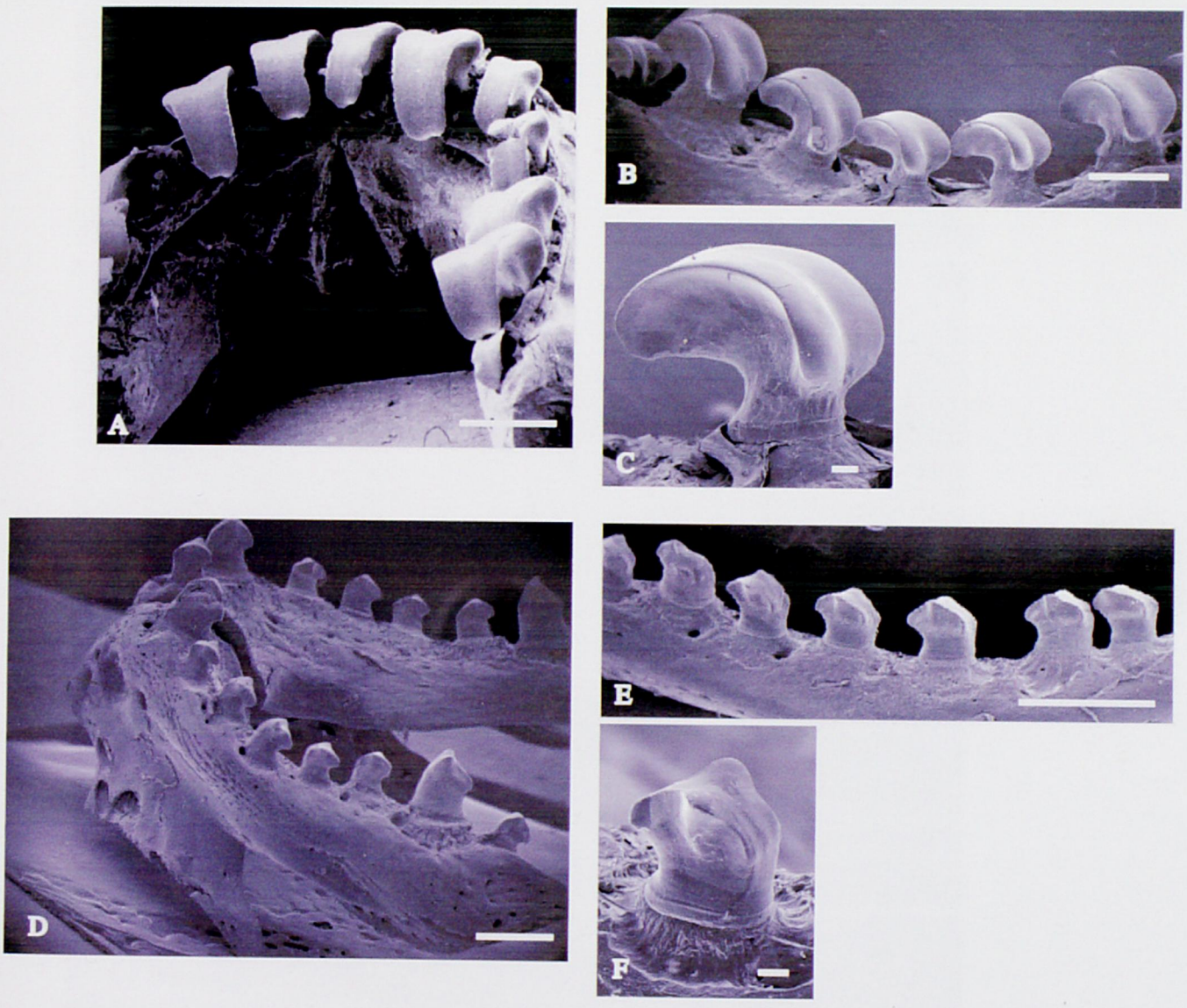


Figure 2–1. Scanning electron micrograph photographs of the teeth of *P. straeleni* and *P. microlepis*.

A: Teeth of the lower jaw in *P. straeleni*. **B**: Lateral view of (A).

C: One tooth of the upper jaw in *P. straeleni*.

D: Teeth of the lower jaw in *P. microlepis*; **E**: Lateral view of the teeth of the upper jaw in *P. microlepis*.

F: One tooth of the lower jaw in *P. microlepis*.

Bar in A, B, D, E, incates 1mm. Bar in C, F indicates 0.1mm.

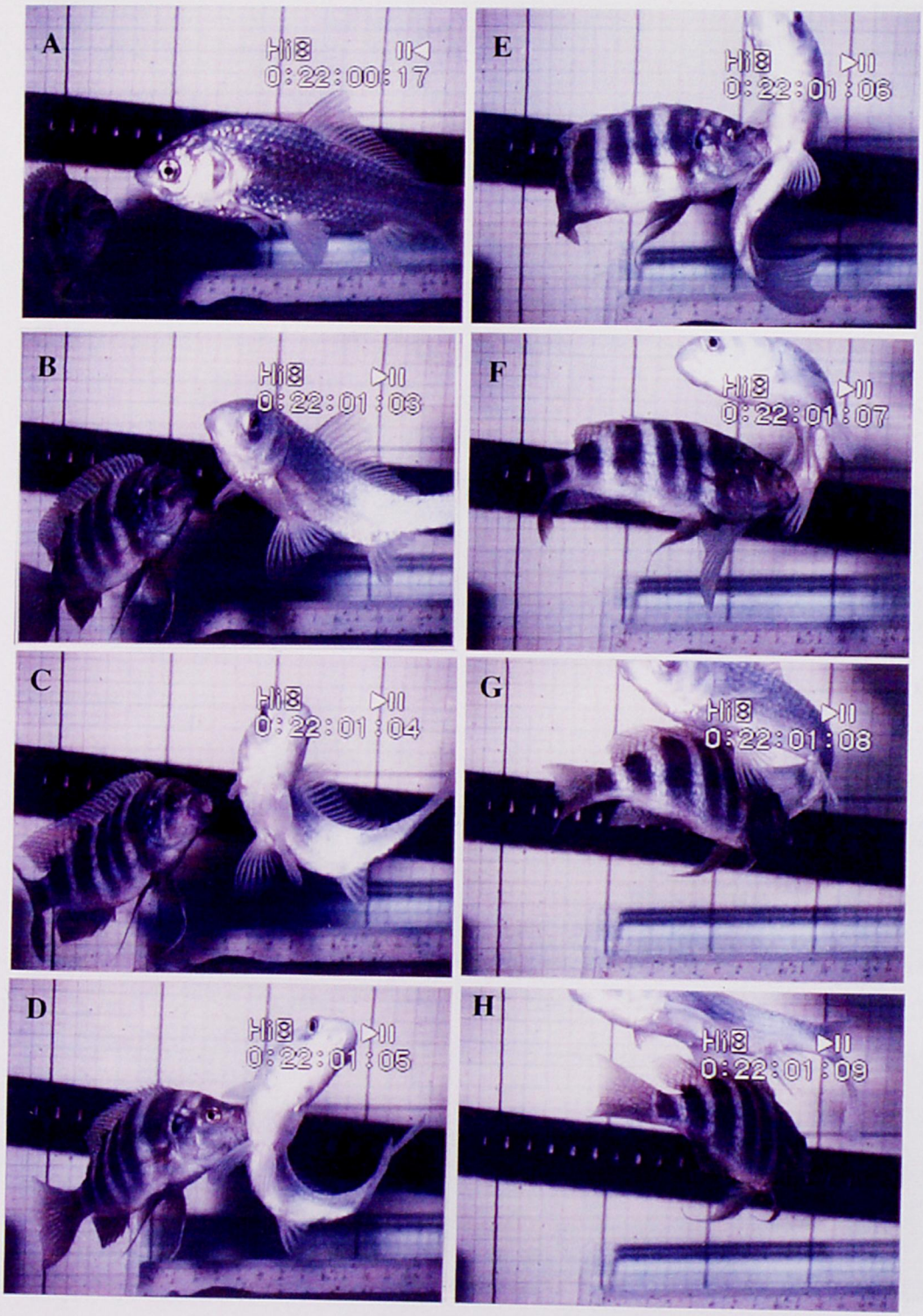


Figure 2.2. Attack sequence of *P. straeleni* from approach to separation from the prey. The time code in the upper right is in units of 1/30 s.

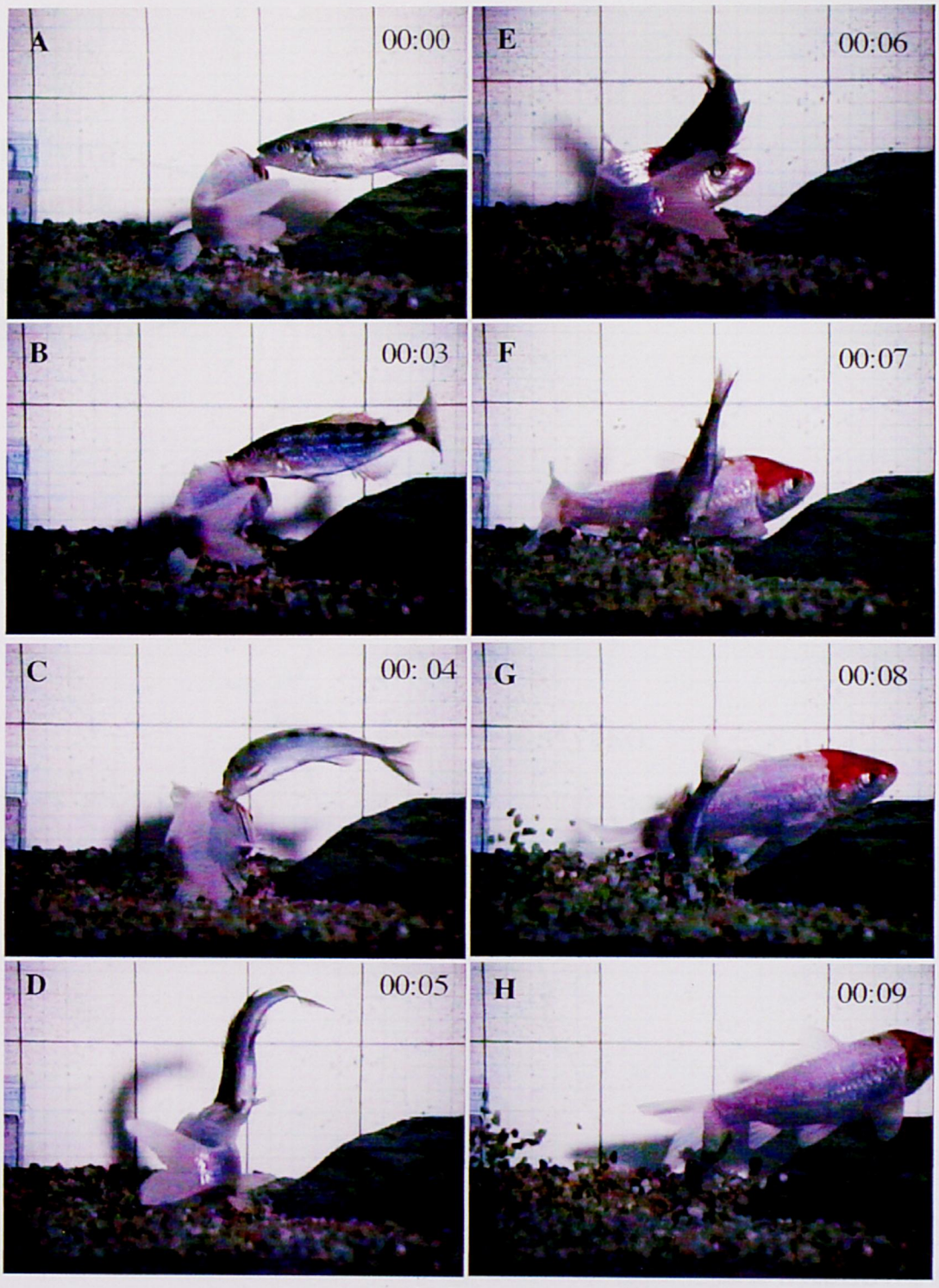


Figure 2–3. Attack sequence of *P. microlepis* from approach to separation from the prey. The time code in the upper right is in units of 1/30 s.

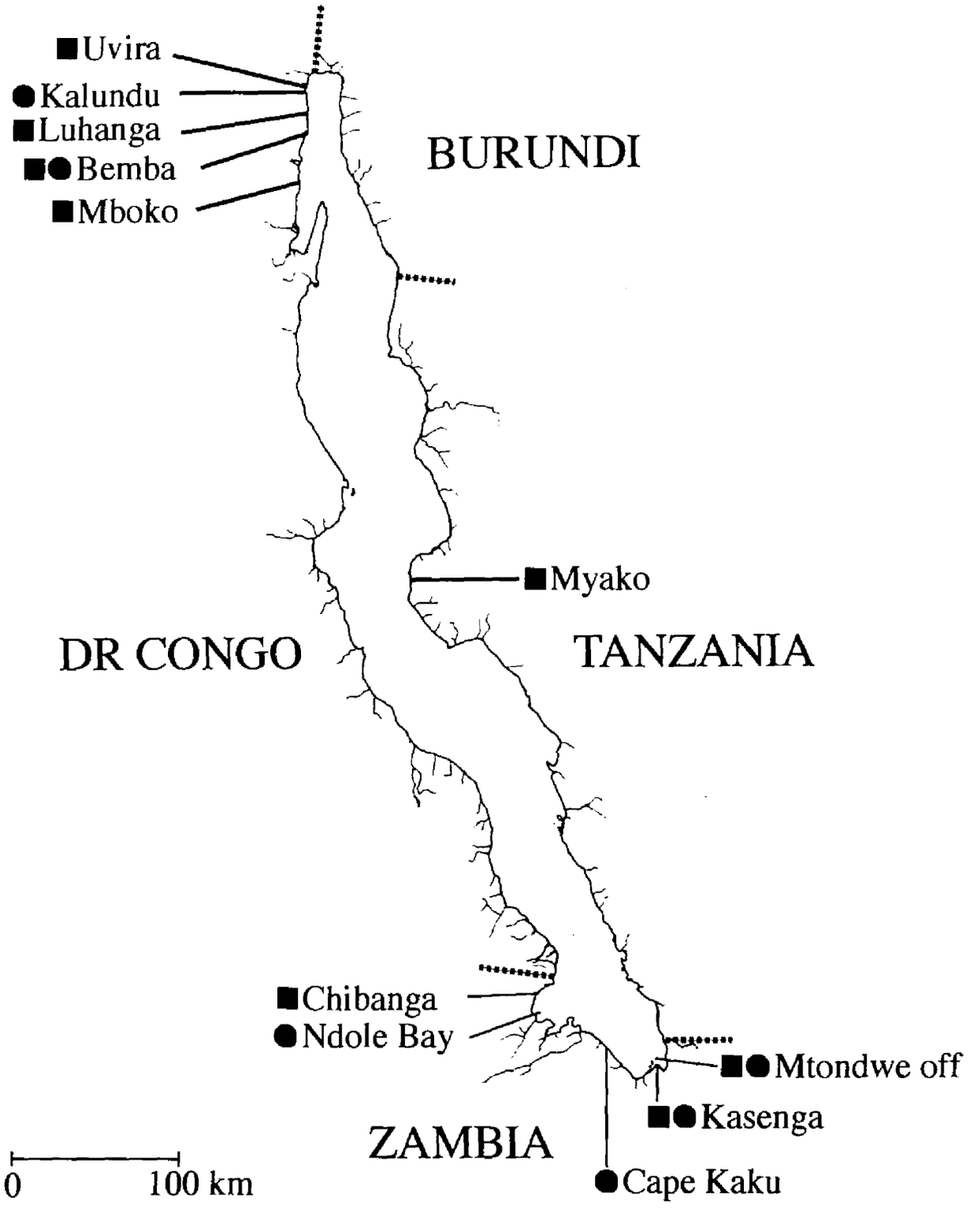


Figure 3–1. Map of Lake Tanganyika
Sampling sites of specimens used in phylogenetic analyses (circles) and stomach content analyses (squares).

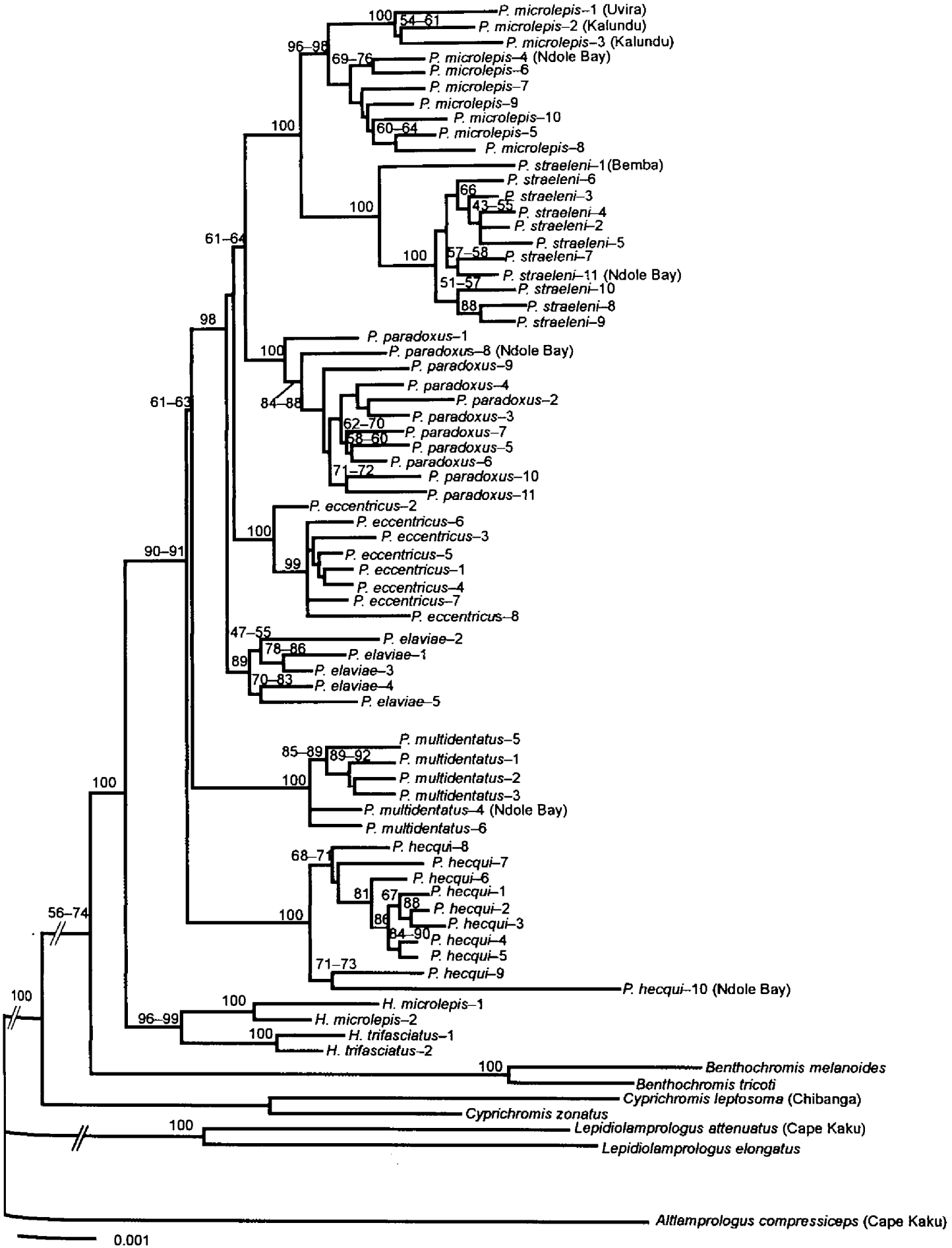


Figure 3–2. Neighbor-Joining tree obtained from 1582 AFLP characters.
Numbers at nodes indicate the ranges of bootstrap values using three different parameter sets for recognition site length (for details, see Materials & Methods) at 10, 16, and 26 bp (values <50% not shown). Sampling localities other than the main sampling site, Kasenga, Zambia (see Figure 3.1) are provided in parentheses after the species names.

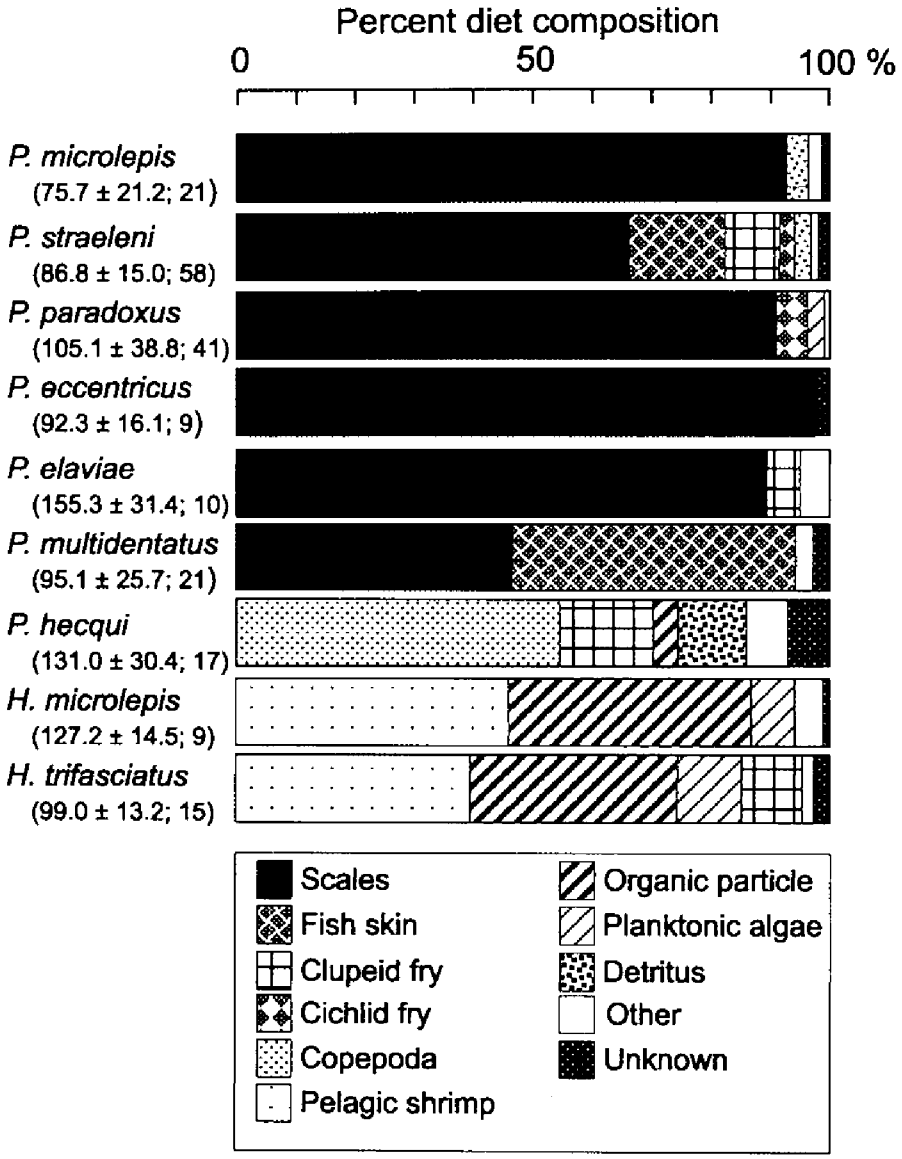


Figure 3–3. Diet composition of Perissodini species.
The mean standard length ± standard deviation (mm) and the number of specimens examined for each species are given in parentheses. Items that comprised <2% of the diet were included in "Other".

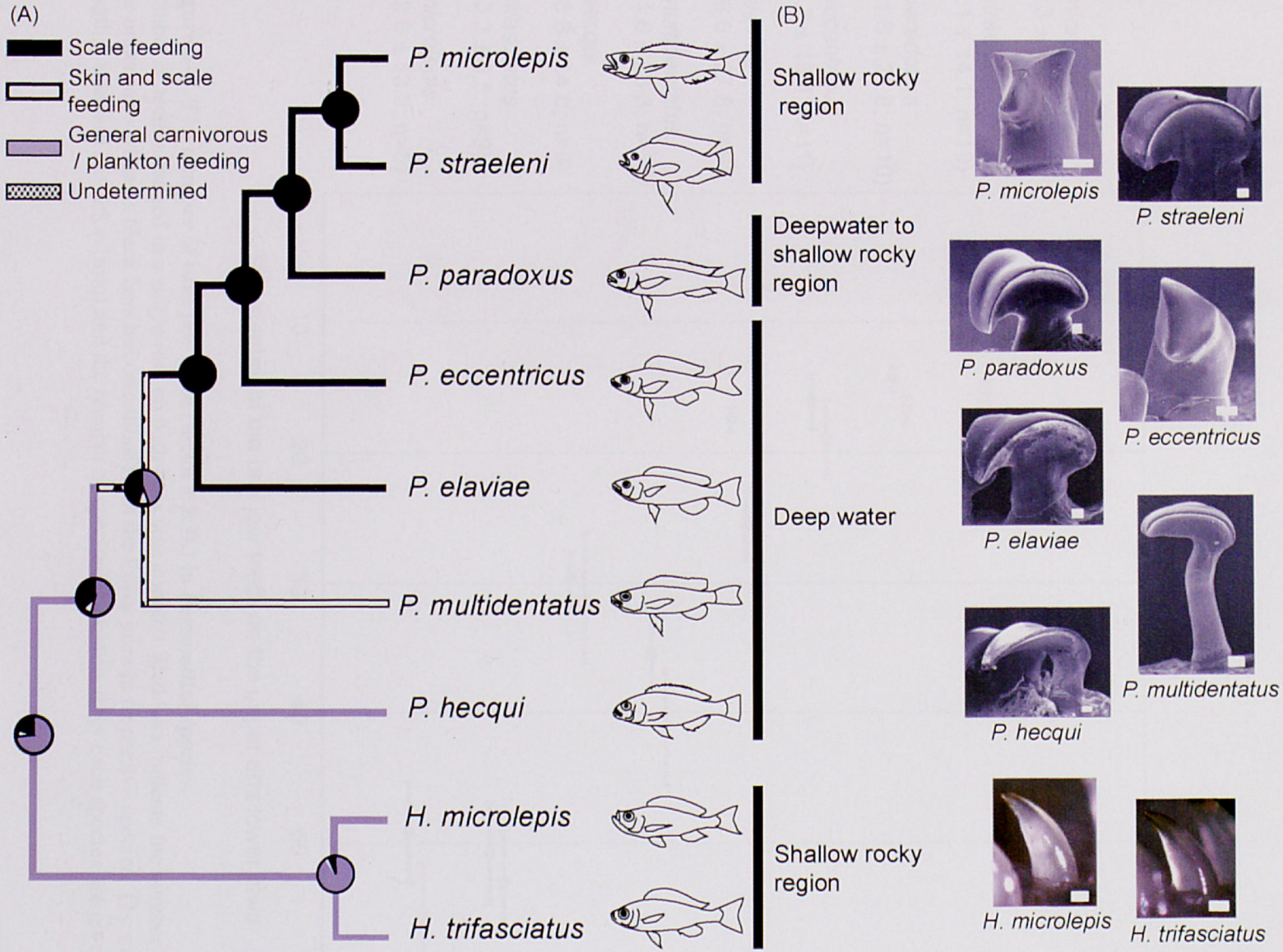


Figure 3–4. Ancestral state reconstruction for the feeding habits (A) and the habitat depth and oral tooth shape (B) of Perissodini.
A) Ancestral state of the feeding habits estimated using maximum parsimony method was indicated on the tree. The colored portion in each pie diagram corresponds to the calculated probability of the reconstruction of the respective feeding habit using maximum likelihood method. Tree used for ancestral state estimation is that inferred from AFLP data. Branch lengths were not scaled in this diagram. B) The habitat depth and oral tooth shape of each species are shown to the right of the species names.Bars in the photographs indicate 0.1 mm.

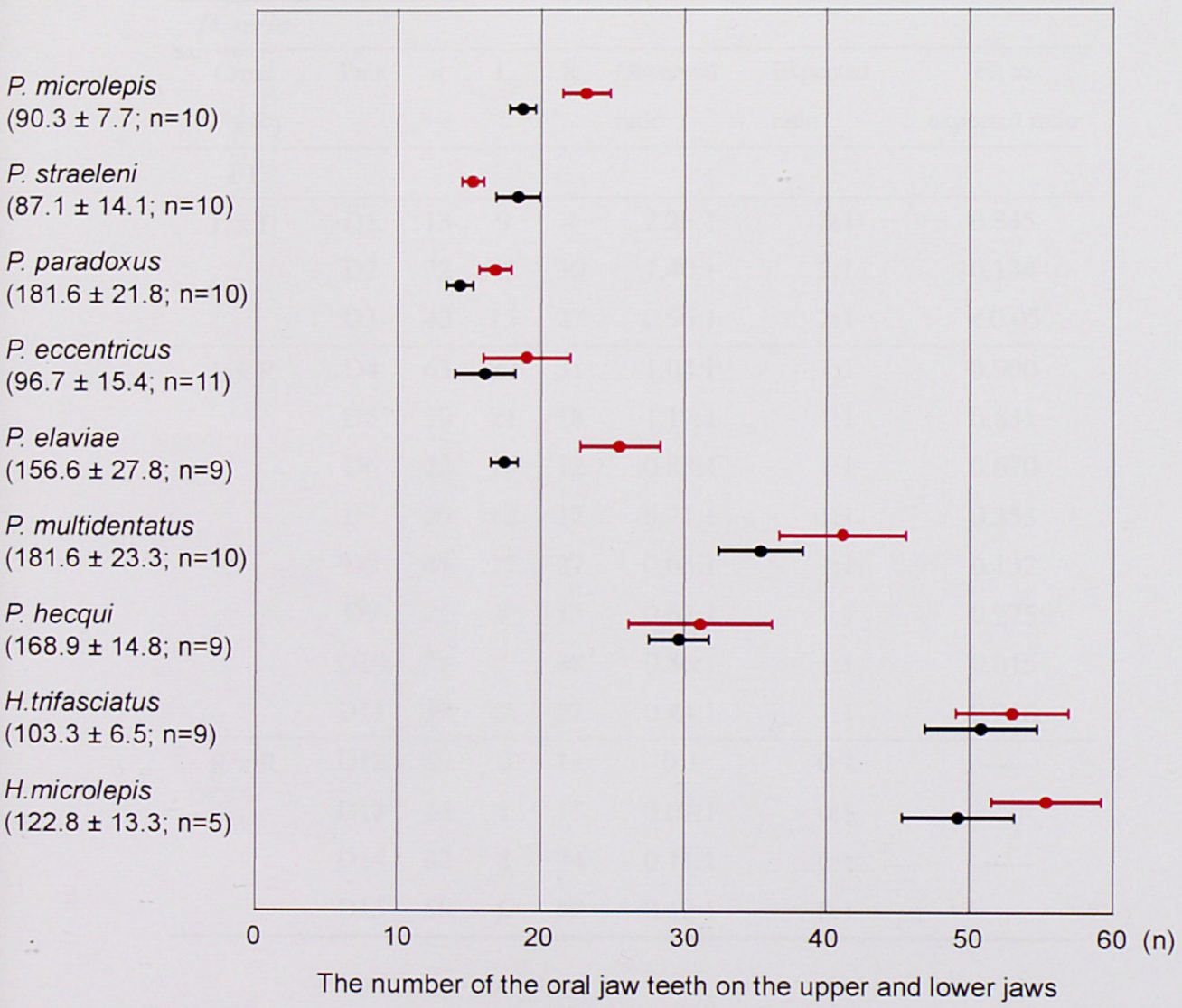


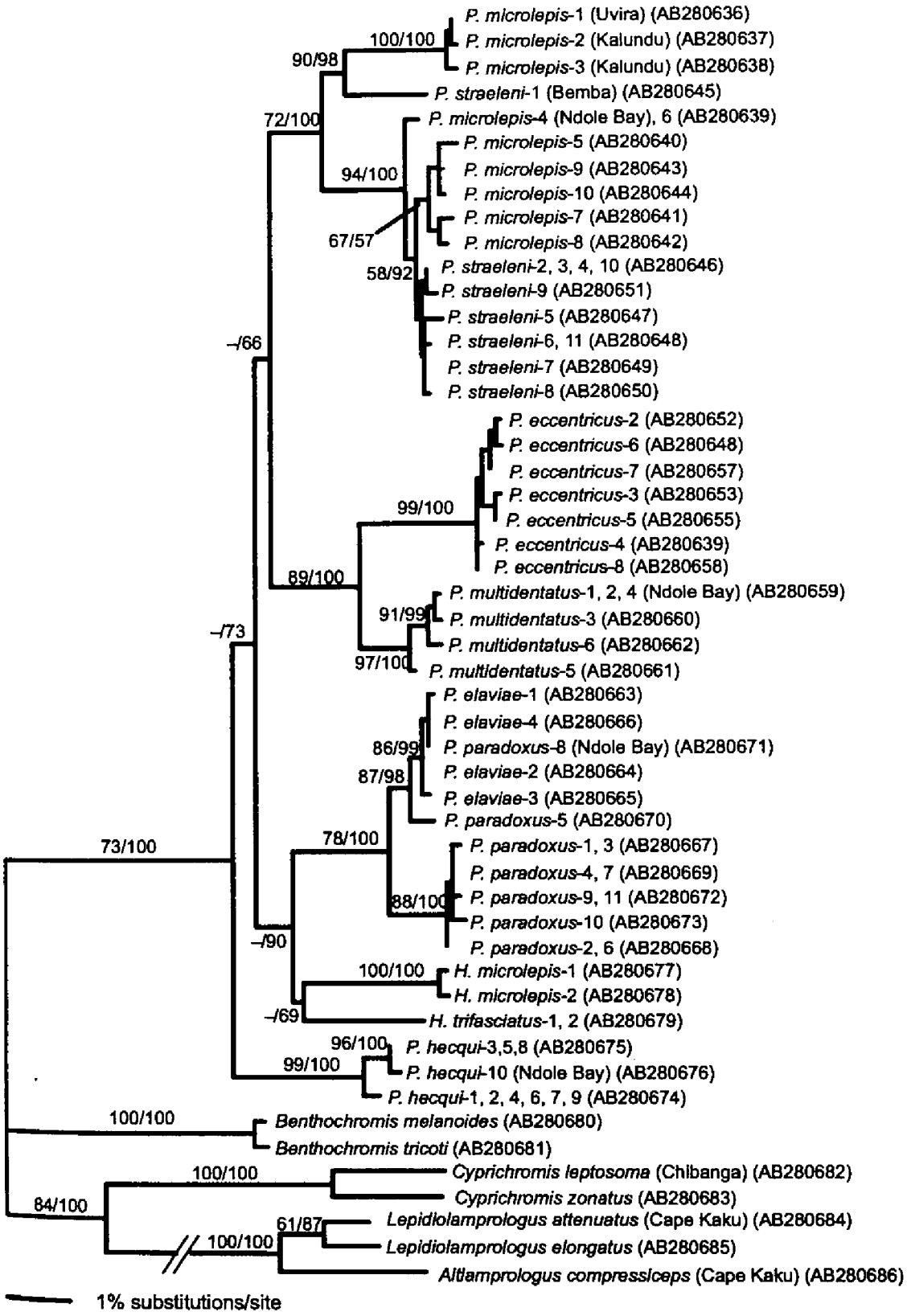
Figure 3–5. The number of oral jaw teeth (mean ± S.D.) in Perissodini species.
Number of teeth arranged in a single row on both jaws was counted. Red bars indicate the number of teeth from the upper jaws whereas black bars indicate those from the lower jaws in respective species. The mean standard length ± standard deviation (mm) and the number of specimens examined for each species are given in parentheses.

Supplementary table–1.

Segregation data* of lefty (L) and righty (R) individuals in F1 progeny of zebrafish, *Danio rerio*.

D. rerio							
Cross (♂x♀)	Pair	*n*	L	R	Observed ratio	Expected ratio	Fit to expected ratio
F1							
L x L	D1	13	9	4	2.25:1	2:1	0.845
	D2	72	42	30	1.40:1	2:1	0.134
	D3	42	15	27	0.56:1	2:1	< 0.05
L x R	D4	63	32	31	1.03:1	1:1	0.900
	D5	39	21	18	1.17:1	1:1	0.631
	D6	22	10	12	0.83:1	1:1	0.670
	D7	29	12	17	0.71:1	1:1	0.353
	D8	44	17	27	0.63:1	1:1	0.132
	D9	21	8	13	0.62:1	1:1	0.275
	D10	75	27	48	0.56:1	1:1	0.015
	D11	39	12	27	0.44:1	1:1	0.016
R x R	D12	13	0	13	0:1	0:1	——
	D13	16	1	15	0.07:1	0:1	——
	D14	82	8	74	0.11:1	0:1	——
	D15	56	6	50	0.12:1	0:1	——

*The data is cited from the dissertation thesis for bachelor's degree by Asada, H., Lab. of Animal Sociology, Dept. of Biosciences, Osaka City University.



Supplementary figure 1. Maximum likelihood tree of Perissodini based on mitochondrial cytochrome b gene (1133bp) sequences. Numbers at nodes correspond to bootstrap probabilities (values ≤50% not shown) on the left and Bayesian posterior probabilities on the right. The 63 specimens used for this study correspond to those for AFLP analyses though two *Perissodus* specimens (*P. eccentricus*-1 and *P. elaviae*-5) were lacked in the mtDNA tree because of failure in mtDNA PCR reactions. Sampling localities other than the main sampling site, Kasenga, Zambia (see Figure 3.1), and the accession numbers are provided in parentheses.