



SYMPOSIUM

Evolution and Functional Morphology of the Proboscis in Kalyptorhynchia (Platyhelminthes)

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Synopsis Predatory flatworms belonging to the taxon Kalyptorhynchia are characterized by an anterior muscular proboscis that they use to seize prey. In many cases, the proboscis is armed with hooks, derived either from the extracellular matrix that surrounds the muscles or from intracellular deposits in the epithelium covering the proboscis. Glands associated with the proboscis reportedly are venomous; however, there are few direct tests of this hypothesis. This article reviews the structure and current knowledge of the function of the proboscis in the Kalyptorhynchia, points to areas in which the current understanding of phylogenetic relationships within this taxon is incongruent with our hypothesis of how the proboscis evolved, and addresses areas in need of further research, especially as regards functional morphology and biomechanics.

Introduction and Background

The Kalyptorhynchia are predatory flatworms that use an anterior muscular proboscis to seize (and perhaps envenomate) their prey. The more than 550 described species are divided into two sub-taxa based on the structure of the proboscis. Members of the Eukalyptorhynchia possess a conorhynch—an anterior, cone-shaped bulb of muscle that is sometimes armed with hooks or teeth (Fig. 1A–D) (see De Vocht and Schockaert 1999, for terminology used here). Members of the Schizorhynchia possess a schizorhynch—an anterior pair of dorsoventrally opposed, finger-like muscular sheets or tongues that are, also, sometimes armed with hooks or teeth (Fig. 1E–H) (see Uyeno and Kier 2010, for terminology used here). In each case, the proboscis is located in a *proboscis-sheath*—an anterior invagination of the epidermal epithelium that opens through a sphincter at the tip of the body and extends to cover the end of the conorhynch or the surfaces of the tongues. The proboscis-sheath is underlain by a musculature

for at least part of its length, longitudinal in Schizorhynchia and circular and longitudinal in Eukalyptorhynchia (e.g., Karling 1961 and 1947, 1953, 1954, respectively). The musculature of the proboscis proper is separated from the underlying parenchyma by a layer of extracellular matrix; functionally, this structure is a muscular hydrostat. The armed proboscides can be regarded as a muscle articulation (Uyeno and Kier 2010; Uyeno and Clark 2015). In both groups, the proboscis is anchored by various muscles that enable its protrusion and retraction. In the Schizorhynchia, the musculature of the body-wall is modified to enable hydraulic protrusion of the proboscis, and, in some species, also modified in ways that allow bending of the body to take captured prey from the proboscis. In neither group is the proboscis connected to the gut; once prey is seized and subdued with the proboscis, it is either passed to the ventral mouth or removed from the proboscis by the protruded pharynx (Karling 1961).

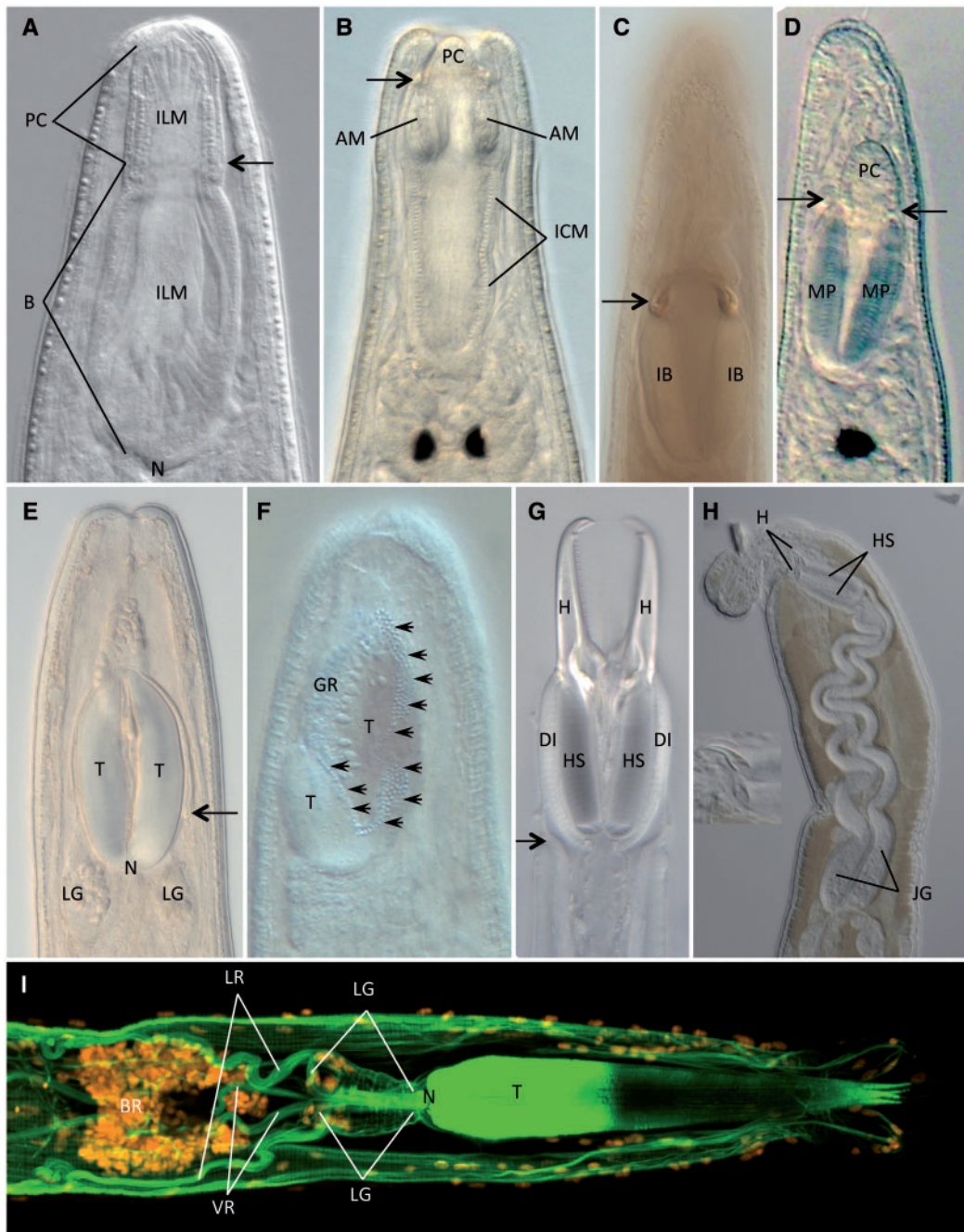


Fig. 1 Various proboscides in Eukalyptorhynchia (A–D) and Schizorhynchia (E–H), including some of the armed variants. (A) Typical conorhynch, with proboscis-cone protruding into its sheath (juncture at arrow) and bulb, showing internal longitudinal muscles; (B) Family Cicerinidae (*Cicerina debrae*) with glandular ampullae opening at juncture (arrow) and well-developed internal circular muscles; (C) Family Gnathorhynchidae (*Drepanorhynchides hastatus*) with dorsoventrally opposed hooks located at juncture (arrow) and muscular intrabulbs below hooks; (D) Family Placorhynchidae (*Placorhynchus* n. sp.), lateral view, with dorsoventrally opposed muscular plates in bulb and glands opening at juncture (arrow); (E) Typical schizorhynch proboscis (family Schizorhynchidae, n.gen. “inops”), lateral view, with paired, symmetrical tongues and lateral glands (juncture at arrow); (F) *Carcharodorhynchus* n.sp. with asymmetrical tongues, cone-shaped teeth on margins of tongues (arrowheads), and medial glandular ridge on larger tongue; (G) Family Cheliplanidae (n.gen. “gich”) with protruded proboscis, with hooks, hook-supports, and divaricators (juncture at arrow); (H) Family Karkinorhynchidae (*Karkinorhynchus* n.sp.) showing hook-supports and paired muscular tubes of the juncture glands (inset: hooks at higher magnification); (I) *Proschizorhynchus* n.sp. “spirale”, maximum-intensity projection of confocal stack in dorsal view (stained for nuclei and muscles), showing tongues, sac-like lateral glands, lateral retractor muscles and ventral retractor muscles; dorsal retractor muscles are also present in this species, but not included in the stack of optical sections. AM, ampullae; B, bulb; BR, brain; DI, divaricator; GR, glandular ridge; H, hook; HS, hook-support; IB, intrabulb; ICM, internal circular muscle; ILM, internal longitudinal muscle; JG, juncture glands; LG, lateral gland; LR, lateral retractors; MP, muscular plate; N, nodus; PC, proboscis-cone; T, tongue; VR, ventral retractors. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

Presently, there are approximately 425 described species of Eukalyptorhynchia and 153 described species of Schizorhynchia (Tyler et al. 2006–2015). It should be noted that this difference in species diversity between the two groups may be a consequence of the focused efforts of European taxonomists over the past 90+ years rather than an actual difference in diversity. Recent (2009–present) sampling at Bogue Banks, North Carolina has so far produced 31 species of schizorhynchs (only two of which are described) and 16 species of eukalyptorhynchs (only three of which are described). A similar proportion occurs at the island of Sylt in the North Sea, where intensive study over many years has uncovered 49 schizorhynchs and 25 eukalyptorhynchs (Noldt 1989a, 1989b).

For both groups, characters of the proboscis are used for taxonomic placement. However, the comprehensive review of the proboscis in kalyptorhynchs was published over 50 years ago (Karling 1961), and a modern review incorporating results from electron-microscopic studies is, by comparison, rather brief (Rieger et al. 1991). Furthermore, our current hypothesis of proboscis evolution in schizorhynchs is nearly a half-century old (Karling 1961; Schilke 1970a) and is not supported by molecular-phylogenetic results (Tessens et al. 2014).

The goals of the present review are to: (1) briefly summarize the anatomy of the proboscis in both groups, (2) synthesize what is known of the functional morphology of the proboscis in Schizorhynchia, (3) bring our hypotheses concerning evolution of the proboscis into line with recent results from molecular phylogenies, and (4) point to unanswered questions in the hope of stimulating further research on this interesting group of animals.

Anatomy of the Proboscis: Eukalyptorhynchia

The conorhynch (Figs. 1A, 2A) is a muscular bulb that lies in an epithelially lined proboscis-sheath. The proboscis is covered at its tip by a *cone-epithelium*, often divided into a distal sensory portion and a more proximal glandular portion. The cone-epithelium joins the epithelium of the proboscis-sheath at the *junction*. Proximally, the muscular *bulb* of the proboscis is separated from the surrounding parenchyma by a layer of extracellular matrix (*septum*). The septum encloses internal proboscis-muscles—usually an outer circular layer surrounding a bundle of inner longitudinal muscles (*cone-retractors*). The proboscis-sheath connects externally via a *proboscis-pore* that can be closed by a sphincter.

Sets of radially disposed muscles run from the proboscis to insert on the body-wall musculature, and include *protractors* (running anteriorly from the base of the bulb), one or two sets of *retractors* (running posteriorly from the posterior and, in some cases, anterior sides of the bulb out to the body-wall) and *fixators* (running radially from the proboscis to the body-wall). Additional musculature includes integumental retractors that shorten the forepart of the body and *sheath-dilators* (Fig. 2A). In most cases, *gland-necks* enter the conorhynch at the central part of the base of the bulb (*nodus*) and run anteriorly to open at the surface of the cone-epithelium. Additional glands (*junction-glands*) may open at the junction (Karling 1961).

Differentiation of the basic conorhynch has occurred in four families: Cicerinidae, in which there is a prominent ring of glandular ampullae at the junction in all but *Acrumena* (Fig. 1B); Gnathorhynchidae, in which there is a pair of dorsoventrally opposed hooks at the junction, supported by muscular cylinders called *intrabulbs*, except in species of *Uncinorhynchus* (Fig. 1C); Placorhynchidae, in which there is a pair of dorsoventrally opposed muscular plates differentiated within the bulb (Fig. 1D); Aculeorhynchidae, in which the bulb is greatly reduced, and the junction is equipped with a pair of dorso-ventrally opposed needles flanking the small terminal cone and supplied by a pair of two very large tube-like junction glands (not shown; see Karling 1983). In summary, within the Eukalyptorhynchia at least three dorsoventrally opposed specializations of the proboscis have evolved (see Conclusions section).

Electron-microscopic studies of the proboscis in various families of Eukalyptorhynchia reveal a few morphological trends of interest for future research. First, the epithelium at the junction in all proboscides that have been studied so far bears microvilli with electron-dense intracellular deposits (“stout microvilli”—see Rieger and Sterrer 1975; summary in De Vocht 1991; De Vocht and Schockaert 1999); once known, these can probably also be seen by light microscopy (e.g., Fig. 2B; Karling 1953, his Fig. 5). These reinforced microvilli are, presumably, applied to the surface of the prey during capture. Interestingly, the hooks in the single gnathorhynchid studied by electron microscopy are derived from electron-dense intracellular material deposited in these microvilli at the junction (Doe 1976). Furthermore, *Paragnathorhynchus subterraneus* has numerous small hooks on the cone-epithelium in addition to the usual large hooks mounted on intrabulbs (Schilke 1970a, his Fig. 17B–C; Karling 1983). Future research should examine other

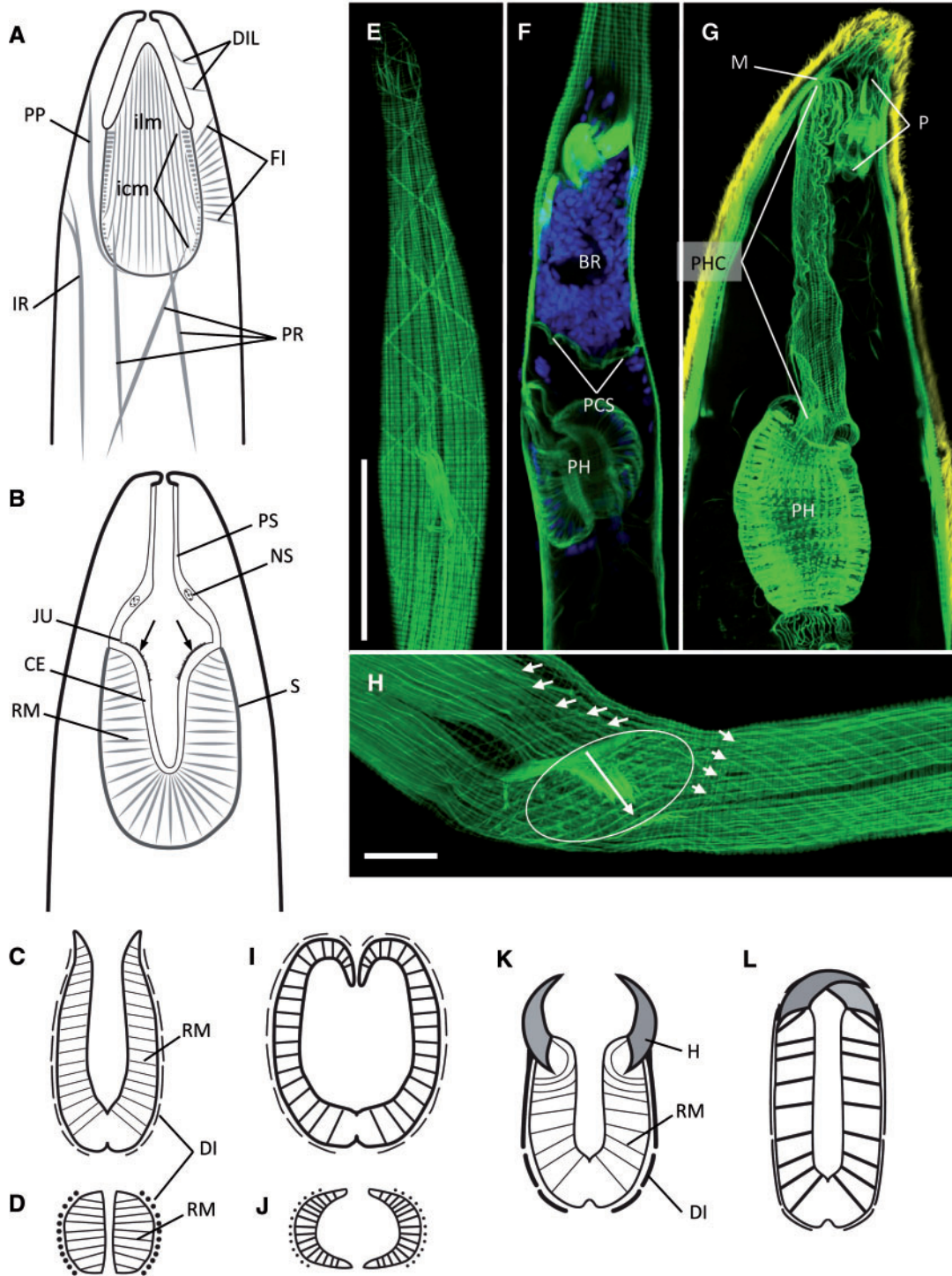


Fig. 2 Musculature involved in feeding movements. Unless otherwise noted, the anterior end is toward the top of the plate. (A) Relaxed conorhynch proboscis, showing internal and external musculature; fixators of the proboscis and dilators of the proboscis-sheath shown only on the right and proboscis-protractor and integumental retractor shown only on the left; (B) Contracted conorhynch proboscis, showing epithelium of sheath and terminal cone, contracted internal longitudinal muscles (internal circular muscles not shown), and “hardened epithelium” (arrows) of the terminal cone near the juncture; Diagram of schizorhynchid proboscis-tongues showing musculature in longitudinal section (C) and cross-section (D); (E) Anterior tip of the body in *Carolinorhynchus follybeachensis*, with confocal stack showing body-wall muscles (circular, longitudinal, and helical); (F) Region between the brain and the pharynx in *Proschizorhynchus* sp., lateral view from left, confocal stack stained for muscles and nuclei; (G) Anterior end of *Cheliplana* (n.sp. “blind october”), lateral view from left side, confocal stack stained for nuclei, muscles, and cilia; (H) *Proschizorhynchus* “shaunae”, body-wall musculature near pharynx, confocal stack stained for muscles, anterior to right, viewed from right, position of pharynx indicated by imposed oval, arrow indicates position of pharyngeal lumen and points toward the mouth; note the oblique muscles in the body-wall

(continued)

gnathorhynchids to see whether the teeth and hooks are always intracellular derivatives, and should also be directed at looking for less obvious “teeth” at the juncture-region of other proboscides.

Second, both the sheath-epithelium and the cone-epithelium have apparently been under selective pressure for sunken nuclei and for syncytiality (De Vocht and Schockaert 1999), ultimately leaving the nuclei of both epithelia connected by thin cytoplasmic extensions to the cytoplasm at the epithelial surface (or “epimyum”—Crezee 1975). Similar trends are seen in the epidermal epithelium, and reasons advanced for these changes include mechanical stress and placement of the muscles closer to the terminal web (Tyler 1984). The latter explanation is consistent with the fact that there are apodeme-like connections between the internal longitudinal muscles and the terminal web of the cone-epithelium in *Cicerina*, which has a relatively small cone (Fig. 1B; De Vocht and Schockaert 1988) and in *Mesorhynchus terminostylis* (De Vocht 1991). However, in most conorhynch proboscides, the internal longitudinal muscles insert on a basal lamina underneath the cone-epithelium, and a more likely reason could be the thinning of the epimyal layer, thereby more easily accommodating changes in epimyal volume during retraction of the cone (compare Fig. 2A and B). Future research should examine the cone-epithelium throughout the range of motion of the proboscis, between the relaxed position and the fully contracted condition, to test this hypothesis.

Finally, the location of the nucleated portions of the internal proboscis musculature needs to be identified. Muscle-cell nuclei (“Myoblastenkerne”) lying inside the septum were identified in the older light-microscopic literature (e.g., Karling 1954, his Fig. 1). However, such cases have, so far, turned out to be the sunken nuclei of the cone-epithelium (De Vocht 1990, 1991; De Vocht and Schockaert 1999). Although the lack of muscle-cell nuclei within the bulb has been mentioned (e.g., De Vocht 1989), we are unaware of any modern hypotheses concerning where, exactly, one might expect to find the nuclei belonging to the internal circular muscles or to the

longitudinal muscles. The internal longitudinal muscles of the proboscis (Fig. 2A) probably arose from anterior retractors—muscles originating on the body-wall and inserting at the anterior tip of the body (see summary by Rieger 1974, his Fig. 17). Muscle cells in flatworms have a sac-like nucleated portion appended near the mid-point of the elongated portion containing the myofilaments (Rieger et al. 1991). Given that the nuclei of the internal proboscis muscles must be located somewhere outside of the bulb, there appear to be two ways that this situation could have arisen—either the septum formed across the distal portion of the anterior retractor muscles, thus generating “internal longitudinal muscles” and leaving the nucleated portions of these muscles outside of the bulb, or the nucleated portions of the internal longitudinal muscles have sunken through the septum, like those of the cone-epithelium in some species (De Vocht 1990). In the former case, this would imply that the “internal longitudinal muscles” are actually extensions of muscles whose insertions lie outside of the bulb on the body-wall. This possibility is supported by Karling’s (1953) statement concerning the fixator muscles in *Cytocystis clitellatus*: “The histological connection of the inner and outer fiber systems of the proboscis musculature becomes clear, in that fine fibers in the posterior part of the bulb can pass through the septum unhindered, from the end-bulb retractors into the fixators (Fig. 5).” Karling’s observation from light-microscopy has been partially verified by transmission electron microscopy (TEM)—“... some fibers of the fixators seem to enter the bulb through the perforations and act as cone retractors” (De Vocht 1990). For the second alternative, one might expect to find these muscle-cell nuclei posterior to the nodus, as the necks of numerous glands and processes of nerves enter there, and there are no electron-microscopic studies of this region. Additionally, as noted below for schizorhynchs, the numerous internal proboscis-muscles need not each belong to a separate cell. It is clear that detailed studies of the conorhynch and surrounding musculature by TEM are sorely needed.

Fig. 2 Continued

(arrowheads); Diagram of contracted schizorhynchid proboscis-tongues in longitudinal section [I] and cross-section [J]; note that contraction of the radial muscle elongates and flattens the tongues; Diagram of relaxed [K] and contracted [L] proboscides in *Karkiorhynchus*; note elongation of the tongues due to contraction of radial muscles and that contraction of radial muscles inserting on medial portion of hook-bases could adduct the hooks (L). BR, brain; CE, cone-epithelium; DI, divaricators; DIL, dilator muscle; FI, fixator muscles; H, hook; ICM, internal circular muscles; ILM, internal longitudinal muscles; IR, integumental retractor; JU, juncture; M, mouth; NS, nucleus of proboscis-sheath; P, proboscis; PCS, post-cerebral septum; PH, pharynx; PHC, pharyngeal cavity; PP, proboscis-protractor; PR, proboscis-retractor; PS, proboscis-sheath; RM, radial muscle; S, septum. Scale bars: 2E, 100 μ m; 2H, 30 μ m. Figures 2A, B adapted from Meixner (1938); Figures 2C, D, I, J, K, L adapted from Karling (1961). (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

Anatomy of the Proboscis: Schizorhynchia

The schizorhynch proboscis (Fig. 1E) comprises paired, dorsoventrally opposed muscular *tongues* that are joined at their bases, taper distally, and lie within an inturned pocket of epithelium, the *proboscis-sheath*, which opens at the anterior tip of the body. The epithelium of the sheath is continuous with the epithelium covering the tongues, and as with the conorhynch, the point along the proboscis where the two epithelia join is called the *junction*. The *radial muscles* within the tongues are oriented primarily in the sagittal plane. Longitudinally oriented *divaricator muscles* run along the tongues on their outer (dorsal and ventral) surfaces (Fig. 2C, D). External musculature associated with the proboscis includes proboscis *retractors*, attached at or near the nodus and running to the body-wall (Fig. 1I); these usually occur in six pairs (dorso-lateral, lateral, and ventro-lateral); in some cases the dorsal pair is lacking. Finally, there are *sheath-retractor muscles* that run from the proboscis-sheath to the body-wall, and *epidermal retractors* that act to pull the tip of the body-wall posteriorly during protrusion of the proboscis (Karling 1961; his Figs. 63, 67). Glands associated with the proboscis include *lateral glands* (sac-like multicellular glands, often enclosed by muscle) which open into the proboscis cavity just distal to the nodus (Fig. 1I), *junction glands*, which open at the junction and send secretory ducts along the tongues, and, rarely, *nodal glands*, which open through the nodus at the base of the tongues (Schilke 1970b; Karling 1983).

The basic schizorhynch has been modified in a number of ways. In species of *Carcharodorhynchus* (Fig. 1F), the tongues are broad and flattened, hemi-oval in cross-section, and are armed with small teeth along their lateral margins. The tongues may be highly unequal in size; in this case, the dorsal tongue is usually the larger (Schilke 1970b). In species of *Thylacorhynchus*, the radial muscles are extremely short, the tongues are broad, greatly flattened, joined to each other laterally, and divaricators are lacking (Karling 1961). In the family Cheliplanidae (Fig. 1G), the tongues (here called *hook supports*) are greatly reduced in size and bear *hooks* at their tips. The divaricator muscles are well-developed. The junction extends all the way to the nodus, and hence the proboscis in cheliplanids can be protruded some distance from the body and are used to snare prey (Fig. 1G). The three genera constituting the family Karkinorhynchidae (Fig. 1H) also have hook supports bearing hooks and have

well-developed divaricator muscles. In some karkinorhynchids, the lateral glands are comparatively small, and very large tube-like juncture glands supply secretions to the hooks (Fig. 1H).

In the Schizorhynchia, there are specializations of the body-wall muscles that allow the proboscis to be protruded by increased hydraulic pressure in the anterior end of the body. This is thought to be mediated primarily by the action of the circular and longitudinal muscles in the anterior body-wall (Karling 1961). In addition, confocal microscopy reveals crossed-helical muscle fibers in the body-wall, generally located anterior to the posterior part of the brain (Fig. 2E). In three species of *Proschizorhynchus*, the average angle between these helical muscles and the longitudinal axis of the body ranged from 31° in relaxed specimens to 45° in contracted specimens (data not shown). Helical muscles with these fiber-angles could be expected to produce torsion, or, if both left- and right-handed helices contract simultaneously, shortening of the anterior tip of the body (Kier and Smith 1985); this action, if accompanied by simultaneous contraction of the circular muscles, could be used to assist hydraulic protrusion of the proboscis. In many schizorhynchs, there is a net-like muscular post-cerebral septum immediately behind the brain (or between the testes and the pharynx) that purportedly prevents increased hydrostatic pressure in the anterior end from being dissipated in the rest of the body (Fig. 2F) (Karling 1961; Whitson et al. 2011).

Following the capture of prey, there appear to be two general methods of transferring the prey to the mouth. In cheliplanids and *Lehardyia*, the pharynx lies at the end of a long pharyngeal cavity that is everted during feeding, thereby allowing the pharynx to be used to take the prey from the proboscis (Fig. 2G) (Karling 1961; Whitson et al. 2011). This method may also be used by other species with a long pharyngeal cavity leading to a mouth that opens shortly behind the proboscis, for example, in *Neoschizorhynchus longipharyngus* (Schilke 1970a).

A second method involves bending the anterior part of the body ventrally, bringing the proboscis with captured prey close to the mouth, as described for *Carcharodorhynchus* (Karling 1961). In this genus, there are single muscle-fibers that originate behind the proboscis on the ventral body-wall and run dorso-caudally, suggesting that they may facilitate this ventral bending of the anterior part of the body (Karling 1961). This movement is probably used also in other schizorhynchs in which the pharynx is located rather caudally (e.g., *Serratorhynchus stellatus*, *Schizorhynchoides* spp., and *Schizochilus*

spp.). However, most schizorhynchs have the pharynx and mouth located at or slightly anterior to the middle of the body. Karling (1961) attributes the similar ventral-ward bending movements in these species to “body-wall musculature that is especially powerful... and strong longitudinal muscles on the ventral side”. Although this may indeed be the case, confocal microscopy shows that there are specialized muscles placed near the pharynx that could facilitate this bending movement. Anterior to the pharynx, one set originates on the ventral body-wall and runs dorsocaudally just under the body-wall, and a second set originates dorsally behind the pharynx and runs ventrocaudally, again just under the body-wall (Fig. 2H).

Functional morphology of the proboscis in Schizorhynchia

Direct observations of feeding in kalyptorhynchs are relatively rare, and the functional morphology of the proboscides is, therefore, largely inferred from structure (Fig. 2C, D). Karling (1961) noted that the tongues in schizorhynchs often are elongated and curled inward following fixation; this position is accompanied by contracted radial muscles and tongues flattened in cross-section (Fig. 2I, J). This implies that extension of the tongues could be accomplished by contraction of the radial muscles and opening (abduction) of the tongues by the divaricator muscles (Fig. 2) (Karling 1961; Uyeno and Kier 2010). Analogous actions could explain the elongation of the hook-supports and abduction of the hooks in most schizorhynchs with armed proboscides (except in *Diascorhynchus*, in which the hook-supports are greatly reduced, and there appear to be separate sets of divaricator and occlusor muscles acting on the proboscis-hooks (Karling 1961, his Figs. 65–67). None of the above adequately explains closing (adduction) of the tongues (or hooks). Karling (1961) suggested that the distal-most radial muscles of the hook-supports in *Karkinorhynchus*, which appear to insert on the medial portion of the hook's base, could act to adduct the hooks (Fig. 2F, G). However, this observation does not explain how the tongues in the unarmed schizorhynch could curl inward. In a functional-morphological study of the armed proboscis in *Cheliplana*, Uyeno and Kier (2010) hypothesized that a medial structure that limits extension of the hook-supports could cause them to curl inward when the hook-supports were maximally extended (their Fig. 5).

Investigation of the unarmed proboscis in *Proschizorhynchella* n.sp (“2-belt”) reveals just such a

structure (Fig. 3A–D). The unarmed tongues are ~200 μm long when at rest. The proboscis possesses a V-shaped row of sheath-glands associated with the medial side of each tongue, extending from just above the nodus to the juncture (Fig. 3A). Each tongue possesses numerous radial muscles, which angle slightly from the outer margins of the tongue toward the midline as they run medially (Fig. 3B, C, C inset, D). The tongues are enclosed by a continuation of the sheath-epithelium, and the extracellular matrix (ECM) underlying the sheath-epithelium is continuous with the outer layer of ECM investing the tongues (data from longitudinal sections not shown; Fig. 3D, arrowheads). Strip-like expansions (marginal thickenings) of this layer run along the outer margins of the medial face of the tongues (Fig. 3C) and comprise mostly longitudinally oriented fibers (Fig. 3D, inset). We suggest that these strips act to limit extension of the medial face of the tongues, causing the tongues to curl inward when they are maximally extended. Structures that probably corresponds to these marginal thickenings are occasionally seen in the older literature based on light-microscopy (cf. Schilke 1970b, his Figs. 2B–D “me”). Finally, these marginal thickenings occupy the same position as the tooth-rows in *Carcharodorhynchus* and the toothed bands in *Serratorhynchus* (see next paragraph). As the teeth that arm the proboscis in *Carcharodorhynchus* appear as electron-dense condensations in the ECM surrounding the tongues (Rieger and Doe 1975), it is interesting that there are small, electron-dense inclusions in the longitudinal strips of ECM in *Proschizorhynchella* (Fig. 3D); these condensations may be homologous with the teeth in *Carcharodorhynchus*.

As a more detailed and general study of the proboscis in the Schizorhynchia is underway, we limit ourselves here to noting that the three-layered nature of the ECM surrounding the tongues and the structure of radial-muscle insertions on the inner layer of this ECM each appear to be the same as in *Carcharodorhynchus* (compare our Fig. 3D with Fig. 3B of Rieger and Doe 1975). Additional cases worthy of further investigation are *Serratorhynchus stellatus*, in which the proboscis tongues are long and finger-like, but armed with twin rows of teeth in the same relative position as those in the broader tongues found in *Carcharodorhynchus* (Noldt 1988) and in the single species of *Thylacorhynchus* for which light-microscopic studies have indicated the presence of hardened knobs or teeth (Rieger and Doe 1975, p. 30). Finally, as with the Eukalyptorhynchia, there appear to be no published speculations about the location of the nuclei belonging to the hundreds of radial muscles found in each

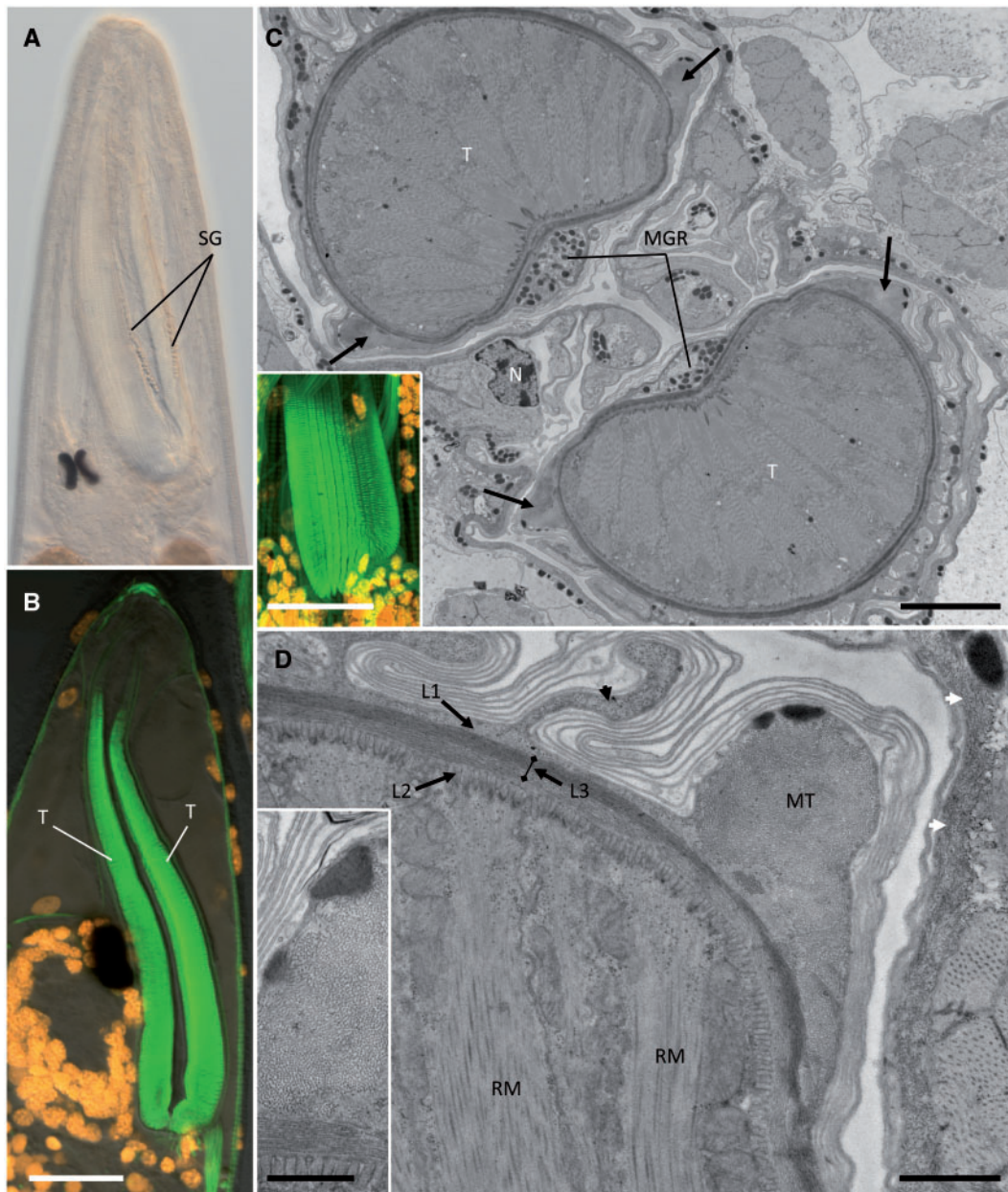


Fig. 3 Proboscis of *Proschizorhynchella* (?) n.sp. “2-belt”. (A) Oblique dorsal view of living specimen; note sheath glands along the margins of the tongues; (B) confocal stack, viewed from right side showing muscles of proboscis-tongues and nuclei of brain; (C) Electron-micrographic cross-section of the proboscis-tongues approximately half-way along their length; note marginal thickenings of ECM flanking medial face of tongues (arrows). Inset: Confocal stack stained for muscle and nuclei; oblique dorsal view showing radial muscles, here viewed end-on, aligned in longitudinal rows; (D) Higher magnification of the cross-section of one tongue from 3 C; note three-layered ECM around tongue: outer homogeneous layer (with wing-like extensions—short arrow), medial fibrous layer (bipartite), inner papillary layer (with electron-dense junctions to ends of muscle-units) and similarity between layer L1 of ECM surrounding tongue and ECM underlying sheath-epithelium (white arrows). Inset: Higher magnification of marginal thickening, showing cross-sectioned fibers and electron-dense deposits in the ECM. L1–L3, respectively the outer, inner papillary, and median fibrous layers of the ECM surrounding the tongues; MGR, median glandular ridge; MT, marginal thickening of ECM; N, nucleus of sheath-epithelium; RM, radial muscle; T, tongue. Scale bars: 3B, 30 μ m; 3 C, 5 μ m (inset, 40 μ m); 3D, 1 μ m (inset, 500 nm). (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

tongue. These muscles are far too numerous for each to belong to a single cell. As the radial muscles themselves are located in longitudinal rows, and in preparations for confocal microscopy, sometimes appear to occur in

three longitudinal groups (one medial, two lateral), we suggest that the lateral glands might contain the nuclei of the muscle-units in addition to those of the gland cells.

Phylogenetic considerations

As part of a larger molecular-phylogenetic study focused primarily on Eukalyptorhynchia, Tessens et al. (2014) provided the following broad results of interest here: (1) The genus *Toia* is the sister-group to all other Kalyptorhynchia; (2) The majority of the eukalyptorhynch family Cicerinidae lies outside of the Eukalyptorhynchia, and forms a trichotomy with Eukalyptorhynchia and Schizorhynchia; and (3) the subfamily Cheliplaninae, with its armed proboscis, is separate from other members of the Karkinorhynchidae and forms the sister-clade to all other Schizorhynchidae. We re-analyzed these relationships with new sequences of Placorhynchidae (2) Gnathorhynchidae (4), Koinocystididae (1), Cicerinidae (1), and Schizorhynchia (25), together with selected species from Tessens et al. (2014—see our Table S1). Our results (Fig. 4) reinforce the first and third results of Tessens et al. (2014), listed above, and add the following new insights concerning the evolution of the proboscis:

The gnathorhynchid genus *Uncinorhynchus* lies outside the remaining Gnathorhynchidae (Fig. 4, arrow). This is in accord with the morphological results, as *Uncinorhynchus* is unique among gnathorhynchids in that the proboscis-hooks are not supported by intrabulbs (Karling 1947). Furthermore, even within the core Gnathorhynchidae, there are different arrangements of muscle in the intrabulbs (Karling 1947, 1983). Clearly, a more detailed analysis of the clade containing the families Koinocystididae, Cystiplanidae, and Gnathorhynchidae (Tessens et al. 2014; their Fig. 2) might prove fruitful, especially in regard to evolution of the armed proboscis within the Gnathorhynchidae.

Within the Schizorhynchia, the Cheliplanidae *sensu* Tessens et al. (2014) remains the sister-group to all other Schizorhynchia (Fig. 4). Given the very long basal branch, long-branch attraction should not be excluded as the reason for this position, as the genera *Cheliplanana* and *Cheliplanilla* are arguably the two most derived of the four in the family Cheliplanidae. Unlike these two genera, *Baltoplana* and *Archipelagoplana* include species with paired ovaries (germaria). Paired germaria occur in the Cicerinidae and in the genus *Carcharodorhynchus*, taxa that bracket the Cheliplanidae in the current tree. Paired germaria, therefore, appears to be the plesiomorphic condition in the Schizorhynchia, as the remaining taxa in this group are characterized by a single germarium.

However, *Carcharodorhynchus*, with its armed proboscis, also lies at the base of the remaining

Schizorhynchia (Fig. 4), and hence, it would appear that the primitive condition of the proboscis in the Schizorhynchia is the armed one (contra Karling 1961; Schilke 1970b). Therefore, both morphological studies of the proboscis and molecular-phylogenetic analyses are sorely needed for additional species of Cheliplanidae.

The remaining well-supported clade (Fig. 4, clade 1) contains monophyletic genera (*Schizochilus*, *Thylacorhynchus*, *Lehardyia*, *Diascorhynchus*), but there is no support for a monophyletic Karkinorhynchidae, as *Lehardyia* and *Karkinorhynchus* do not group together. In addition, the Schizorhynchidae of Tessens et al. (2014) remains paraphyletic, as all of the remaining genera with armed proboscides (*Diascorhynchus*, *Lehardyia*, *Karkinorhynchus*) clearly lie within the Schizorhynchidae.

In summary, although the armed condition appears to be primitive within the Schizorhynchia, whether it has been lost repeatedly in schizorhynchs with unarmed proboscides, or lost once and re-appeared convergently in taxa such as *Karkinorhynchus*, *Lehardyia*, and *Diascorhynchus* remains to be seen. In either case, these phylogenetic results disprove the hypothesis (Schilke 1970b, his Fig. 30) that the proboscis in Schizorhynchia evolved from the unarmed to the armed condition, with, at most, two origins of the latter.

Finally, the cicerinids (*sensu* Tessens et al. 2014) are weakly connected at the base of the Schizorhynchia, and the placorhynchids are weakly connected at the base of the Eukalyptorhynchia in the present analysis.

Conclusions

Given the position of *Toia*, it is clear that the conorhynch proboscis is primitive in the Kalyptorhynchia. However, it must be borne in mind that dorsoventral specializations of this originally axially symmetrical proboscis have evolved at least three times within the Eukalyptorhynchia (Gnathorhynchidae, Aculeorhynchidae, and Placorhynchidae), and therefore, finding a fourth such dorsoventral specialization in the Schizorhynchia is unsurprising. However, there presently appear to be no clear hypotheses explaining the evolution of the dorsoventrally opposed proboscides in the Schizorhynchia. Karling's (1961) derivation of the schizorhynch proboscis from the contracted eukalyptorhynch proboscis seems unlikely, and the present molecular tree does not resolve this quandary. Accordingly, it can be assumed that further structural investigations and biomechanical studies of the proboscis in this

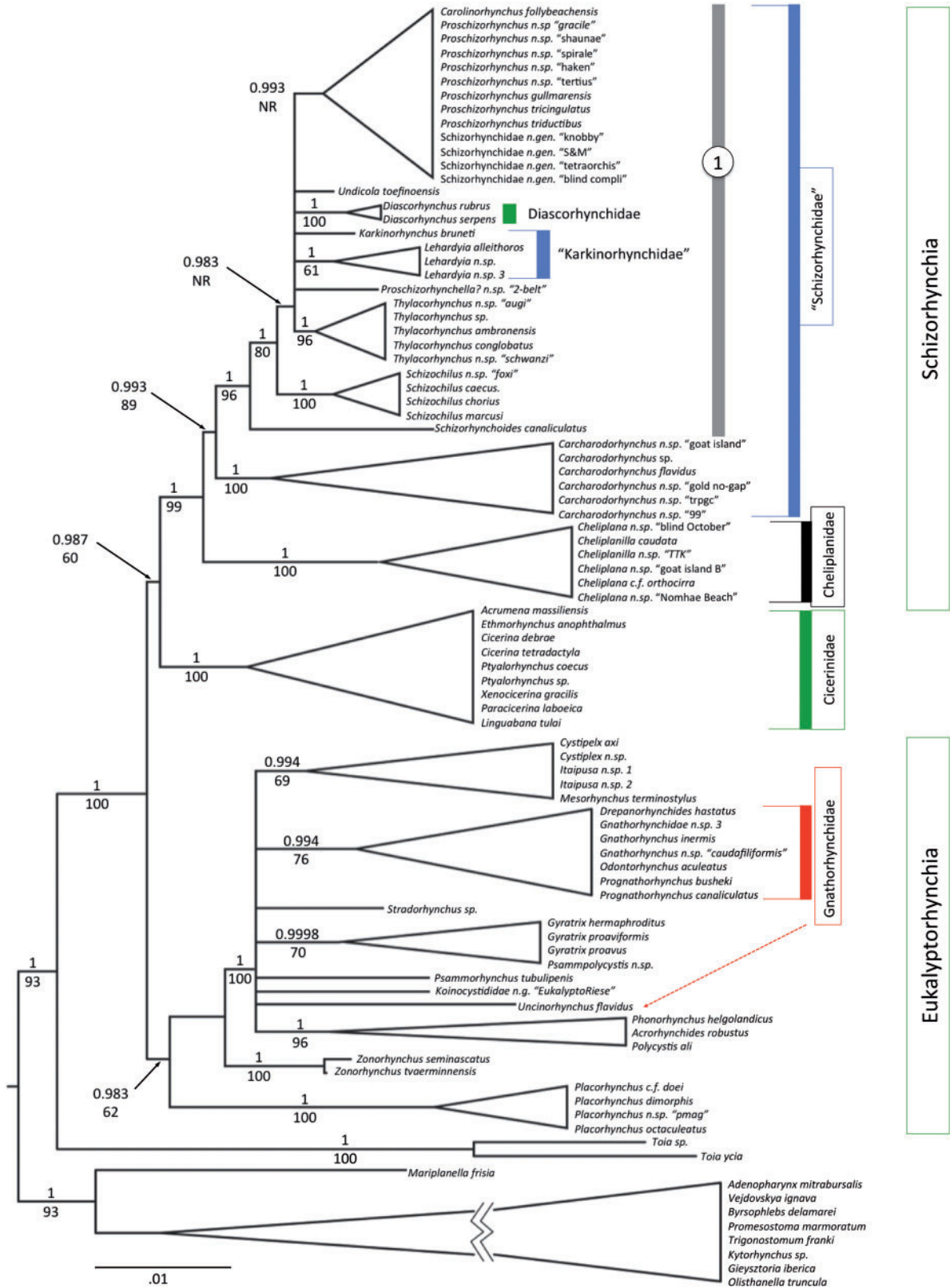


Fig. 4 Bayesian and maximum-likelihood analysis of concatenated 18S and 28S rDNA sequences for Schizorhynchida and selected Eukalyptorhynchida; selected Dalytyphloplanoida used as the outgroup. Posterior probability (bpp) shown above nodes and bootstrap values shown below. Selected genera and families cartooned; nodes with bpp < 0.95 are collapsed. Methods and sequences used are listed in Supporting Information (S1). NR, node not recovered in RAxML analysis. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

interesting group of animals, accompanied by appropriate molecular investigations of their phylogeny, would add much to our knowledge.

Author Contributions

J.S.III conducted the study of the proboscis tongues in *Proschizorhynchella* and drafted the manuscript. J.S.III and M.K.L. obtained the new sequences reported here. J.S.III conducted the Bayesian analysis and S.G. conducted the RAxML analysis. T.U. wrote the section on biomechanics and provided useful advice for the investigation of the proboscis in *Proschizorhynchella*. T.A. provided guidance on phylogenetic analysis and contributed to the section on the Eukalyptorhynch proboscis. All authors read and approved the final manuscript before submission.

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Supplementary data

Supplementary data available at *ICB* online.

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