

Ultrastructure of the spermatozoon of *Leiopelma hochstetteri* (Amphibia, Anura, Leiopelmatidae)

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ABSTRACT

The spermatozoon of the basal frog *Leiopelma hochstetteri* Fitzinger, 1861 is examined and compared with previously investigated lissamphibian spermatozoa. *L. hochstetteri* spermatozoa share with those of urodeles and *Ascaphus* the plesiomorphic (primitive) features of an elongate conical acrosome, and an elongate nucleus, with distinct nuclear shoulders, which tapers to a point (as the nuclear rostrum) in the subacrosomal space. The spermatozoon of *L. hochstetteri*, alone among the Anura, resembles those of urodeles in having a juxta-axonemal fibre at doublet 8. *L. hochstetteri* shares with *Ascaphus truei* (homoplastically) the reduction of the undulating membrane to a short, thick paraxonemal rod. *L. hochstetteri* spermatozoa have the plesiomorphic condition, also seen in dipnoans, of a cytoplasmic collar, containing mitochondria, around the axoneme. Although the sperm of *L. hochstetteri* lacks the endonuclear perforatorium seen in *Ascaphus*, Bombinidae, urodeles and caecilians, it has what may be remnants of an endonuclear perforatorium within the nuclear rostrum. No synapomorphic spermatozoal characters are present to support the placement of *Leiopelma* and *Ascaphus* in the same family. Spermatologically, *Leiopelma* is the plesiomorph sister taxon of all other anurans and is thus confirmed as the most basal (primitive) anuran. A further finding is that the Pelodytidae, sometimes considered basal archaeobatrachians, share the synapomorphy of a conical perforatorium divided into fibres with the Bufonoidea.

KEY WORDS

Amphibia,
Anura,
Leiopelmatidae,
spermatozoa,
ultrastructure,
phylogeny.

RÉSUMÉ

Ultrastructure du spermatozoïde de Leiopelma hochstetteri (Amphibia, Anura, Leiopelmatidae).

Le spermatozoïde de la grenouille primitive *Leiopelma hochstetteri* Fitzinger, 1861 est examiné et comparé aux spermatozoïdes de lissamphibiens précédemment étudiés. Le spermatozoïde de *L. hochstetteri* partage avec les Urodèles et *Ascaphus* les caractéristiques plésiomorphes (primitives) suivantes : un acrosome conique et allongé, un noyau allongé, avec des épaules nucléaires distinctes, et qui s'effile dans l'espace subacrosomal, formant un rostre nucléaire. Le spermatozoïde de *L. hochstetteri* est le seul parmi les Anoures à ressembler à ceux des Urodèles par une fibre juxta-axonémale associée au doublet 8. *L. hochstetteri* a en commun avec *Ascaphus truei* (de manière homoplasique) la réduction de la membrane ondulante en une baguette paraxonémale courte et épaisse. Les spermatozoïdes de *L. hochstetteri* ont l'état de caractère plésiomorphe, également rencontré chez les Dipneustes, d'un collier cytoplasmique contenant des mitochondries, autour de l'axonème. Bien que le spermatozoïde de *L. hochstetteri* ne possède pas le perforatorium endonucléaire trouvé chez *Ascaphus*, les Bombinidae, les Urodèles et les Caeciliens, il présente ce qui peut être les restes d'un perforatorium endonucléaire dans le rostre nucléaire. Aucune synapomorphie spermatique ne soutient l'inclusion de *Leiopelma* et d'*Ascaphus* dans la même famille. Du point de vue spermatologique, *Leiopelma* est le taxon-frère plésiomorphe de tous les autres Anoures, ce qui confirme sa position d'Anoure le plus basal. On remarque aussi que les Pelodytidae, parfois considérés comme des Archaeobatraciens basaux, partagent la synapomorphie d'un perforatorium conique divisé en fibres avec les Bufonoidea.

MOTS CLÉS

Amphibia,
Anura,
Leiopelmatidae,
spermatozoïdes,
ultrastructure,
phylogénie.

INTRODUCTION

Frogs of the genus *Leiopelma* Fitzinger, 1861 are endemic to New Zealand and are considered to be among the most primitive (basal) of all living anurans (Cannatella 1985; Green & Cannatella 1993; Duellman & Trueb 1994). *Leiopelma hochstetteri* Fitzinger, 1861 is a semi-aquatic, highly cryptic and sedentary frog which lives and breeds in scattered remnant populations in moist seepages and along the edges of forest streams on the North Island and Great Barrier Island of New Zealand (Bell 1978; Tessier *et al.* 1991). They grow to a maximum known snout-vent length of 46 mm (McLennan 1985; Slaven 1992) in close agreement with lengths of 50 mm in subfossil deposits (Worthy 1987). They are mostly dull brown frogs but dark green morphs also occur (Barwick 1961; Whitaker 1996).

As observed in captive *Leiopelma hochstetteri*, amplexus is inguinal and occurs on land, in shallow water, or at the waters edge between the months of October and May (Bell 1978). Breeding sites are generally under rocks and logs in and around water seepages and smaller streams, where clutches of 10 to 22 eggs are laid and from which tailed swimming larvae hatch (Bell 1982, 1985). Males may occur in association with egg clutches but do not actively incubate the eggs, unlike other species of this genus. Furthermore, the larvae differ from their congeners in that they do not metamorphose on the back of a resident male frog, but metamorphose post-hatching and disperse soon after (Bell 1985).

Among the extant Anura only *Ascaphus* Stejneger, 1899 and *Leiopelma* retain the plesiomorphic characters of nine presacral amphicoelous vertebrae and "tail-wagging" muscles. Although these are sym-

plesiomorphies, *Ascaphus* and *Leiopelma* have been united in a single family, the Leiopelmatidae, together with the extinct Patagonian genera *Notobatrachus* Reig, 1957 and *Vieraella* Reig, 1961 (Duellman & Trueb 1994). However, Green & Cannatella (1993) concluded that, as *Ascaphus* and *Leiopelma* share no synapomorphic characters, they should be placed in separate families, Ascaphidae and Leiopelmatidae respectively.

Other research into the phylogenetic relationships of these basal anuran families has also produced conflicting conclusions. Cannatella (1985) on the basis of morphological data considered *Ascaphus truei* Stejneger, 1899 to be the sister taxon of all other anurans (see also a reanalysis of Cannatella's data by Hillis 1991), whereas, from an analysis of isozyme loci, Green *et al.* (1989) considered *Leiopelma* to be the probable sister group of all other frogs. In contrast to the above views, an extensive study of amphibian 12S and 16S rRNA data by Hay *et al.* (1995) indicated that the two anuran suborders Archaeobatrachia and Neobatrachia are each monophyletic. That *Ascaphus* and *Leiopelma* are sister groups within the Archaeobatrachia, with the most basal lineage of the Archaeobatrachia being the Pelobatoidea (Pelodytidae + Pelobatidae). While Hay *et al.* (1995) found *Ascaphus* and *Leiopelma* to be sister groups, this needs not necessitate placing them in the same family, the Leiopelmatidae, as suggested by them.

In this study we attempt to clarify the confused phylogenetic relationships of *Leiopelma hochstetteri* to other families of frogs by comparing its spermatozoal ultrastructure with that of previously examined lissamphibian spermatozoa, in particular that of *Ascaphus truei*.

ABBREVIATIONS

svl snout-vent length;
TEM transmission electron microscopy.

MATERIALS AND METHODS

SPERMATOZOAL ULTRASTRUCTURE

Four specimens of *Leiopelma hochstetteri* collected from Whareorino forest, North Island, New

Zealand on June 29th 1998 were enclosed in 10 cm × 6 cm plastic containers lined with damp tissue paper. Each container was then placed within a 20 cm × 30 cm polystyrene container, maintained at 10 °C for transport to Massey University. Frogs were maintained on a diet of crickets and wax moth larvae for one week before transport to Victoria University. At Victoria University each frog was euthanased with Benzocaine. The testes from the only male frog collected, svl = 34.4 mm, (FT4071, C. Daugherty field collection number, Victoria University), were fixed for transmission electron microscopy (TEM) in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) at 4 °C for 2 h, being agitated for the first hour. Testis material was then posted to Brisbane, Australia, at ambient temperature for TEM processing. The testicular tissue was diced into 1-2 mm³ portions and then rinsed in 0.1M phosphate buffer, post-fixed for 80 min in similarly-buffered 1% osmium tetroxide, rinsed in buffer, dehydrated through an ascending ethanol series, and infiltrated and embedded in Spurr's epoxy resin. Sections were cut with diamond knives, on a LKB 2128 UM IV microtome. Thin sections, 50-80 nm in thickness, were collected on carbon stabilized, colloidal-coated, 200 µm mesh copper grids, rinsed in distilled water, stained for one minute in Reynold's lead citrate, rinsed in distilled water, then placed in 6% aqueous uranyl acetate for six minutes, rinsed again, and stained for a further three minutes in lead citrate before final rinsing. Electron micrographs were taken on an Hitachi 300 electron microscope at 75 kV.

Light microscopic observations of spermatozoa from glutaraldehyde-fixed tissue squashes, were made under Nomarski contrast using an Olympus BH2 microscope.

PARSIMONY ANALYSIS

A parsimony analysis using PAUP* version 4.0b2 was used to reconstruct the phylogenetic relationships of *Leiopelma* with other Anura based on sperm morphological characters. Species used, together with their sources, were:

Urodela

Plethodontidae *Eurycea quadridigitata* (Holbrook, 1842) (Jamieson 1999; pers. obs.).

Anura, Archaeobatrachia

Leiopelmatidae *Leiopelma hochstetteri* (this study). Ascaphidae *Ascaphus truei* (James 1970; Jamieson *et al.* 1993). Discoglossidae *Alytes obstetricans* (Laurenti, 1768) and *Discoglossus pictus* Otth, 1837 (Pugin-Rios 1980). Pipidae *Xenopus laevis* (Daudin, 1802) (Bernardini *et al.* 1986; Pugin-Rios 1980). Pelodytidae *Pelodytes punctatus* (Daudin, 1802) (Pugin-Rios 1980).

Anura, Neobatrachia

Myobatrachidae *Limnodynastes peronii* (Duméril & Bibron, 1841) (Lee & Jamieson 1992).

Data matrix

The characters and character coding used were as follows:

- (1) Endonuclear canal containing perforatorium: 0, present; 1, absent.
- (2) Juxta-axonemal fibre at doublet 8: 0, present; 1, absent.
- (3) Conical perforatorium divided into fibres: 0, absent; 1, present.
- (4) Distinct nuclear shoulders: 0, present; 1, absent.
- (5) Subacrosomal cone: 0, present; 1, absent.

Taxon	1	2	3	4	5
<i>Eurycea quadridigitata</i>	0	0	0	0	0
<i>Leiopelma hochstetteri</i>	1	0	0	0	0
<i>Ascaphus truei</i>	0	1	0	0	0
<i>Alytes obstetricans</i>	0	1	0	1	1
<i>Discoglossus pictus</i>	0	1	0	1	1
<i>Xenopus laevis</i>	1	1	0	1	1
<i>Pelodytes punctatus</i>	1	1	1	1	1
<i>Limnodynastes peronii</i>	1	1	1	1	1

Eurycea quadridigitata was used as the outgroup. Plesiomorphic character states were scored as zero. An exhaustive search was used, employing maximum parsimony as the optimality criterion. All character states were of type unordered and equally weighted. For PAUP, the initial "Max-

Trees" value was set to 100, branches were collapsed (creating polytomies) if maximum branch length was zero, the "MulTrees" option was in effect and topological constraints were not enforced. Character-state optimization was by accelerated transformation (ACCTRAN).

RESULTS

The ultrastructure of the spermatozoon of *Leiopelma hochstetteri* is illustrated diagrammatically in Figure 1. For ease of comparison the terminology of Jamieson *et al.* (1993) for *Ascaphus truei* is used throughout this account. Data are restricted to the numerous sperm from a single individual owing to the vulnerable status of *L. hochstetteri* and its full protection under New Zealand law. The use of a single individual is not of concern as Lissamphibia show very little variation in sperm morphology within a species and sperm abnormalities are too rare to be commonly sampled.

The testicular spermatozoa of *Leiopelma hochstetteri* are filiform and average 224 µm (n = 24, sd = 12) in total length (range 207 to 250 µm). The spermatozoon is composed of a curved cylindrical head region (acrosome and nucleus) 66.8 µm long (n = 32, sd = 4.6), a midpiece 2.3 µm long (n = 3, sd = 0.02), and a tail 154 µm long (n = 27, sd = 12) (Fig. 2A).

ACROSOME COMPLEX

The acrosome complex is composed of an elongate conical acrosome and an underlying subacrosomal cone (putative perforatorium) which caps the nuclear rostrum (Fig. 2C-G, Q). The acrosome is a membrane bound vesicle and is filled with moderately electron-dense material (Fig. 2C-O, Q). The subacrosomal cone lies free within the subacrosomal space. It consists of diffuse material basally and is not bounded by a membrane. Apically the material of the subacrosomal cone compacts to form a dense rod which is square-ended anteriorly (Fig. 2D, E, I, J). Basally the subacrosomal cone is closely adpressed to the nuclear rostrum (Fig. 2G, N, O, Q).

The subacrosomal cone extends posteriorly for a short distance beyond the base of the acrosome vesicle (Fig. 2G). In transverse section the base of the acrosome vesicle is peripherally heptagonal (Fig. 2N). Further apically it decreases in polygonality, becoming trilobed at the level of the apical tip of the subacrosomal cone (Fig. 2I, J) and circular at its apex (Fig. 2H).

NUCLEUS

The nucleus is elongate and cylindrical, and is composed of electron-dense material (Fig. 2G, P). Anteriorly, within the acrosomal complex, the nucleus tapers to a point (Fig. 2G, N-Q). This nuclear rostrum is 3.24 μm long ($n = 3$, $sd = 0.06$) and contains several axial lacunae which contain material which is possibly the homologue of a perforatorium (Fig. 2G, N, Q). Although no continuous endonuclear canal is present, what appear to be the vestige of a short, more continuous endonuclear canal has been observed in the very tip of the nuclear rostrum in a late spermatid (Fig. 2B). Distinct nuclear shoulders are present at the base of the nuclear rostrum (Fig. 2G). At their level the nucleus is 0.41 μm in diameter ($n = 8$, $sd = 0.03$); this increases throughout its length to a maximum diameter of 0.80 μm ($n = 9$, $sd = 0.06$) at the level of the basal nuclear fossa. The fossa is conical and 0.92 μm deep (Fig. 3A, E).

NECK/MIDPIECE

Two parallel centrioles, orientated in the long axis of the nucleus, lie at the base of the nuclear fossa (Fig. 3B). Each centriole is composed of nine, circularly arranged, triplets of short microtubules. The two centrioles lie adjacent to each other embedded in a common mass of electron-dense material, and do not extend into the nuclear fossa. One of the centrioles forms the basal body of the axoneme (Fig. 3E). The axial fibre of the flagellum extends through the neck region to the level of the base of the nucleus (Fig. 3B, E). A short cytoplasmic collar surrounding the flagellum contains a few, scattered mitochondria. A gap (termed the "cytoplasmic canal" for many vertebrate sperm) separates the cytoplasmic collar from the flagellum (Fig. 3B-E).

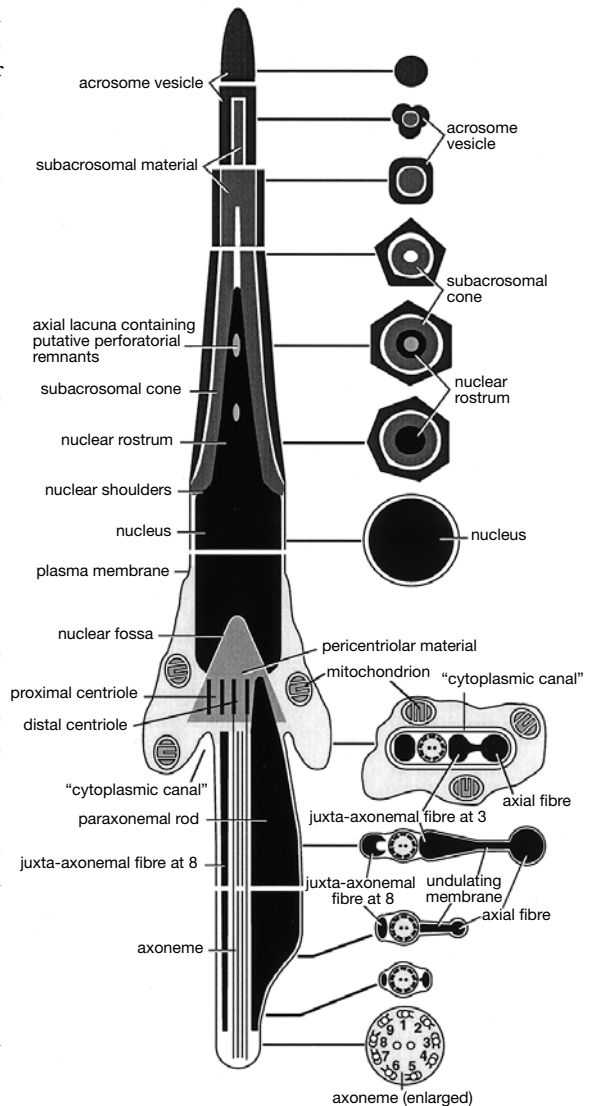


FIG. 1. — *Leiopelma hochstetteri*, highly diagrammatic representation of the ultrastructure of the spermatozoon.

TAIL COMPLEX

The tail complex (Fig. 3C-I) is composed of a 9 + 2 axoneme; an axial fibre, connected by a thickened undulating membrane to a juxta-axonemal fibre at doublet 3; and a further juxta-axonemal fibre at doublet 8. Anteriorly (in transverse section) the undulating membrane is shorter and with the juxta-axonemal fibre at 3

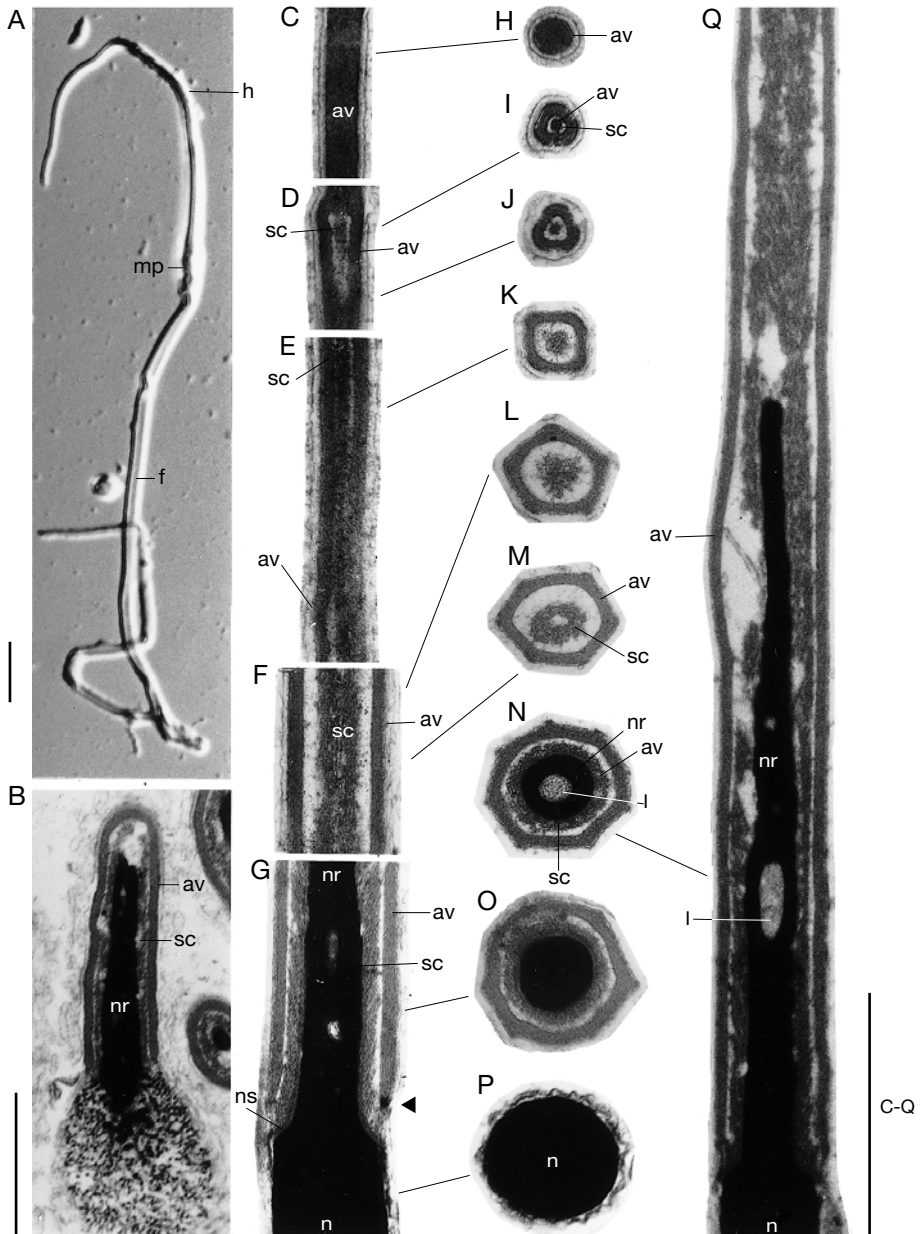


FIG. 2. — *Leiopelma hochstetteri*; **A**, Whole testicular spermatozoon shown by light microscopy; **B-Q**, transmission electron microscopy; **B**, longitudinal section (ls) of the nuclear rostrum region of a spermatid, showing what appears to be the remnant of a short endonuclear canal; **C**, ls of the apical region of the acrosome vesicles; **D**, **E**, ls's of the anterior acrosome, showing condensed "rod-like" subacrosomal material; **F**, ls of the mid region of the acrosome; **G**, ls of the base of the nuclear rostrum. Note the distinct nuclear shoulders (ns), the axially located lacunae (l) and that the subacrosomal material extend beyond the base of the acrosomal vesicle (arrow); **H-O**, successive transverse sections (ts's) through the acrosomal region indicated; **P**, ts through nucleus; **Q**, ls through the base of the acrosome surrounding the nuclear rostrum. Note the lacunae containing putative perforatorial material (see also N). Abbreviations: **av**, acrosome vesicle; **f**, flagellum; **h**, head (acrosome and nucleus); **l**, lacunae; **mp**, midpiece; **n**, nucleus; **nr**, nuclear rostrum; **ns**, nuclear shoulders; **sc**, subacrosomal cone. Scale bars: **A**, 10 μ m; **B-Q**, 1 μ m.

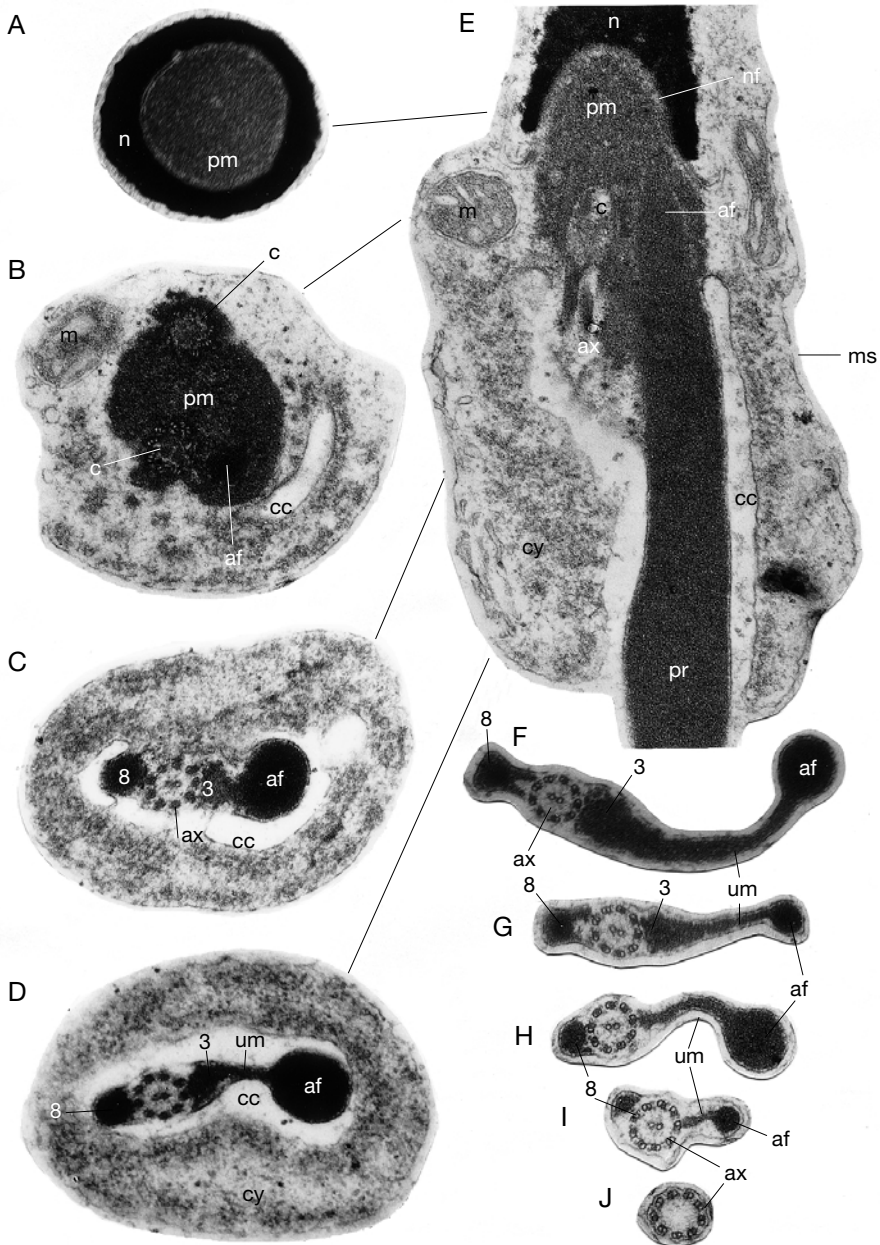


FIG. 3. — *Leiopelma hochstetteri*; spermatozoon by transmission electron microscopy; **A**, transverse section (ts) through the nuclear fossa; **B**, ts through neck region, showing the two centrioles lying adjacent and parallel to each other; **C**, **D**, ts's through the midpiece, showing the "cytoplasmic" canal. Note the juxta-axonemal fibres at doublets 3 and 8, the short thickened undulating membrane and the axial fibre; **E**, longitudinal section (ls) of the midpiece. Note that the centriole and axial fibre do not enter the nuclear fossa; **F-I**, successive ts's through the flagellum; **J**, ts through the distal end of tail (endpiece). All to the same scale as indicated. Abbreviations: **3**, juxta-axonemal fibre at 3; **8**, juxta-axonemal fibre at 8; **af**, axial fibre; **ax**, axoneme; **c**, centriole; **cc**, cytoplasmic canal; **cy**, cytoplasm; **m**, mitochondrion; **ms**, mitochondrial sheath (cytoplasmic collar); **n**, nucleus; **nf**, nuclear fossa; **pm**, pericentriolar material; **pr**, paraxonemal rod; **um**, undulating membrane. Scale bar: 1 μ m.

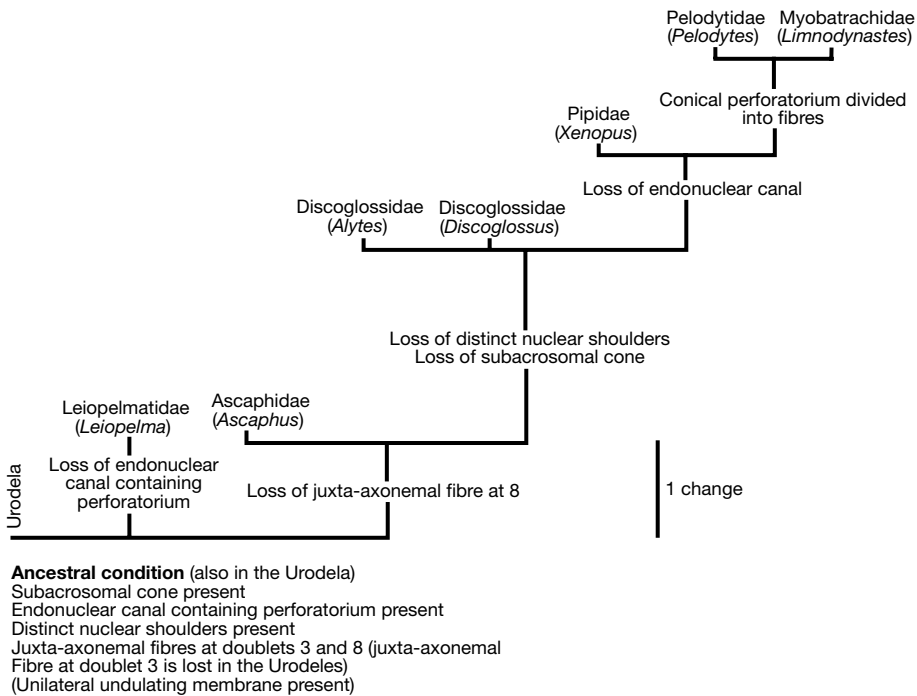


Fig. 4. — The single most parsimonious tree produced by an exhaustive search of anurans based on known spermatozoan ultrastructure. **6**; tree length; consistency index = 0.8333; homoplasy index = 0.1667; retention index = 0.8889; rescaled consistency index = 0.7407.

and the axial fibre, forms an electron-dense, hourglass shaped paraxonemal rod (Fig. 3C, D). Posteriorly, the juxta-axonemal fibre at 3 becomes less distinct from the undulating membrane (Fig. 3F-I). The juxta-axonemal fibre at doublet 8 extends along much of the length of the axoneme, decreasing in size posteriorly (Fig. 3C, D, F-I). For some of its length this fibre has two thin extensions, as seen in transverse section, attaching it to the axoneme (Fig. 3F). A short portion of the axoneme, the endpiece, extends beyond the juxta-axonemal and axial fibres (Fig. 3J). Within the endpiece the 9 + 2 structure of the axoneme becomes increasingly disrupted posteriorly.

PARSIMONY ANALYSIS

Analysis using PAUP evaluated a total of 10395 trees with only one shortest tree being found. This one best tree (Fig. 4) had a length of six steps. The worst tree found was 14 steps in length.

DISCUSSION

ACROSOME

An anterior acrosome, with or without a perforatorium, is present in all lissamphibian sperm. The acrosome of many urodelaes ends subapically in a distinct barb (Retzius 1906; Selmi *et al.* 1997; Wortham *et al.* 1982). Although the acrosomal vesicle in *Hynobius* spp. lacks a barb, in transverse section it is strongly trifoliate (Picheral 1967, 1979; Lee & Kwon 1996), a condition which is compatible with the presence of a barb or at least some unilateral modification of the tip of the vesicle (see review by Jamieson 1999). The barb has no equivalent in anurans. However, the acrosome vesicle of *Leiopelema* shows some similarities to that of *Hynobius* Tschudi, 1838 in that it is trilobed anteriorly. The acrosome vesicle of *Leiopelema* is similar to that of *Ascaphus* in being symmetrical and differentiated into regions of varying thickness and

with different contents. However, the polygonal structure of the acrosome vesicle, when seen in transverse section, in *Leiopelma* is unique (autapomorphic) within the Lissamphibia.

A subacrosomal cone covering the tip of the nucleus in *Leiopelma* is a synapomorphy of Lissamphibia and amniotes (Jamieson 1995, 1999) and therefore symplesiomorphic in *Leiopelma*. In *Leiopelma* the subacrosomal cone extends for a short distance beyond the posterior limit of the acrosome vesicle. A similar condition is seen in urodeles (Picheral 1979) and *Ascaphus* (James 1970; Jamieson *et al.* 1993). The acrosome complex of *Leiopelma* differs from that of *Ascaphus* in lacking a crenulated interior margin of the acrosome vesicle and in not being underlain by a perinuclear space.

At the base of the acrosomal region, the nucleus of basal amniotes (Healy & Jamieson 1992; Jamieson 1995), urodeles (Picheral 1979; Selmi *et al.* 1997), *Ascaphus* (Jamieson *et al.* 1993) and *Leiopelma* flares out abruptly to form distinct nuclear shoulders (tetrapod synapomorphy). Behind this, the nucleus forms a very elongate cylinder.

PERFORATORIUM

A rod-like perforatorium and endonuclear canal are characteristic of sperm of basal tetrapods. They occur in urodeles (Picheral 1967, 1979; Fawcett 1970; Selmi *et al.* 1997). They are seen in caecilians (Gymnophiona), though, there lodged posteriorly in a greatly shorter endonuclear canal (Van der Horst *et al.* 1991). They also characterize the more basal anurans, *Ascaphus* (Jamieson *et al.* 1993) and the bombinids *Bombina* Oken, 1816 and *Alytes* Wagler, 1830 (Sandoz 1970a, b; Furiere 1975a, b; Folliot 1979; Pugin-Rios 1980). Furthermore one or more are present in sarcopterygian fish (Dipnoi and *Latimeria chalumnae* Smith, 1939) (Mattei *et al.* 1988; see reviews in Jamieson 1991). It is thus reasonable to deduce that an endonuclear canal containing a long rod-like axial perforatorium is plesiomorphic for the Lissamphibia. This is endorsed by persistence of this condition in lower amniotes, the Chelonia, Sphenodontida and Crocodylia, and in palaeognath birds (Jamieson & Healy

1992; Jamieson 1995, 1999; Jamieson *et al.* 1997). However, although *Leiopelma* lacks a definite endonuclear canal, there are what appear to be remnants of an endonuclear canal and axial perforatorium in the form of axial lacunae, containing electron-pale material, within the nuclear rostrum. In the pelobatid *Scaphiopus couchi* Baird, 1854 an electron-lucent vesicle containing a "dense body" is present in the centre of the nucleus (Morrisett 1974). Nuclear vacuoles of variable size and location, and with granular contents, are also frequently seen in *Xenopus laevis* and *Bombina variegata* (Linnaeus, 1758) (Pugin-Rios 1980). A perforatorium traverses the endonuclear canal in the late spermatid of *Discoglossus pictus* (Sandoz 1970b) but is restricted to the prenuclear subacrosomal space in the mature spermatozoon, though the endonuclear canal persists (Pugin-Rios 1980).

NUCLEAR FOSSA

Within the Anura a basal nuclear fossa containing either one or both centrioles, which may constitute an implantation fossa, is poorly developed in species with a simple flagellum, well-developed in those with an undulating membrane and especially well-developed in *Discoglossus pictus* in which the tail is very long (1000 µm). No neck-piece of the type seen in salamandroid urodeles is seen in the Anura but the shape of the basal nuclear fossa of *Ascaphus* and *Leiopelma* appears to differ little from that of cryptobranch and hynobiid urodeles (pers. obs.). *Leiopelma* differs from *Ascaphus* and most other Anura in that no centriole is present within the nuclear fossa.

CENTRIOLES

The neck region contains the centrioles, the elements of the so-called centriolar annexe and the anterior part of the axial fibre if this is present. Within the Lissamphibia both centrioles are always present and have the classic structure of nine triplets of short microtubules; the proximal (anterior) centriole lies either parallel (*Leiopelma*, this study; *Scaphiopus* Holbrook, 1836, Morrisett 1974), oblique (*Ascaphus*, Jamieson *et al.* 1993; *Xenopus* Wagler, 1827, Pugin-Rios 1980; urode-

les, Picheral 1979) or perpendicular (*Bombina*, *Pelodytes* Wagler, 1830, Pugin-Rios 1980; most Neobatrachans, Lee & Jamieson 1992, 1993; Meyer *et al.* 1997) to the long axis of the spermatozoon. In sperm with a simple flagellum the distal (posterior) centriole is situated at the base of the axoneme, for which it forms the basal body, and is orientated in the long axis, but in sperm with an undulating membrane the distal centriole is often oblique. Where there are two flagella, as in *Telmatobufo australis* Formas, 1972, there is no differentiation into proximal and distal centrioles but each centriole forms the basal body of a flagellum (Pugin-Rios 1980). In *Leiopelma* both centrioles lie adjacent and parallel, and in the long axis of the spermatozoon, conditions which are unique to *Leiopelma* among lissamphibians with only one flagellum.

AXIAL FIBRE

In *Leiopelma*, as in most Anura, the base of the axial fibre is situated further posteriorly than, or adjacent to the distal centriole. However, in *Discoglossus pictus* the base of the axial fibre protrudes deeply into the implantation fossa and the centrioles occupy a lateral position at the caudal end of the fossa (Pugin-Rios 1980). This condition is also seen in the Myobatrachidae where it is considered a distinctive synapomorphy (Lee & Jamieson 1992) presumably homoplastic relative to *Discoglossus* Otth, 1837. In urodeles the axial fibre reaches the base of the fossa or extends only just into the fossa (Werner 1970; Picheral 1979; Selmi *et al.* 1997).

MIDPIECE

The number and disposition of the mitochondria in lissamphibian sperm are variable and they are often absent at maturity. In the primitive cryptobranch urodeles *Cryptobranchus alleganiensis bishopi* Grobman, 1943 (Baker 1963) and *Hynobius* spp. (Picheral 1979; Kuramoto 1995, 1997) the mitochondria are located in a protoplasmic bead around the nucleus and not around the tail, even in the mature sperm. In salamandrids, ambystomatids and plethodontids small, ovoid mitochondria are present in cytoplasm around a

long anterior region of the axial fibre where, in transverse section of the sperm, they form an arc (Baker 1966; Picheral 1967, 1979; Fawcett 1970; Wortham *et al.* 1977; Jamieson *et al.* 1993). The urodele type of midpiece has been reported to be absent in anurans (Pugin-Rios 1980) but in the myobatrachids *Limnodynastes peronii* and *Neobatrachus pelobatooides* (Werner, 1914) an incomplete ring of mitochondria surrounding the axial fibre occurs, much as in salamandrids (Lee & Jamieson 1992). A somewhat similar arrangement is also seen in *Bombina variegata* (Furieri 1975b; Folliot 1979; Pugin-Rios 1980). The mitochondria lie in a cytoplasmic mass in *Xenopus laevis*, *Mixophyes fasciolatus* Günther, 1864, *Caudiverbera caudiverbera* (Linnaeus, 1758), *Pleurodema thaul* (Lesson, 1826), *Nectophrynoides occidentalis* Angel, 1943, *Rana esculenta* (Linnaeus, 1758) and *Rana temporaria* (Linnaeus, 1758) (Pugin-Rios 1980; Lee & Jamieson 1992) but this location may be secondary in at least *Rana* and possibly in all of these species.

In *Xenopus laevis* a very short cytoplasmic canal is occasionally evident. In contrast, in bufonids, and most other eubufonoids including leptodactylids, the mitochondria are distributed around the base of the sperm tail in a collar, which may be lost at maturity in many of these species (Pugin-Rios 1980; Garrido *et al.* 1989; Lee & Jamieson 1992; Meyer *et al.* 1997). Mitochondria occur in one or more longitudinal series in a groove in the paraxonemal fibre in *Ascaphus truei*, a condition which does not appear to be plesiomorphic (arguments having been presented for a pedomorphic origin) despite the primitive (basal) status of this species (Jamieson *et al.* 1993). Large, elongate mitochondria are known only from the rhacophorids, *Rhacophorus arboreus* (Okada & Kawano, 1924), *Rhacophorus schlegelii* (Günther, 1859) and *Chiro-mantis xerampelina* Peters, 1854, which are highly aberrant, being "corkscrew-shaped" and biflagellate (Mizuhira *et al.* 1986; Wilson *et al.* 1991). In the caecilian *Typhlonectes natans* (Fischer, 1879), the collar forms a cylinder around the base of the axoneme and contains many mitochondria clustered in a spiral near its inner wall (Van der Horst *et al.* 1991).

The conditions seen in *Leiopelma* of slight development of a cytoplasmic collar and rudimentary cytoplasmic canal is similar to that seen in the lungfish *Neoceratodus forsteri* (Krefft, 1870) (Jamieson 1999; Jespersen 1971). Until now no extant species were known which presented an arrangement of mitochondria which could confidently be regarded as plesiomorphic for the Anura. It seems reasonable to deduce that the *Neoceratodus*-like condition seen in *Leiopelma* is plesiomorphic for anurans. The well-developed, but transient, mitochondrial collar in eubufonoids (Lee & Jamieson 1993), resemble a condition seen in many acanthopterygian fish (Jamieson 1991) and therefore on first analysis appear plesiomorphic, but may well be a reversal to a pre-lissamphibian condition.

TAIL COMPLEX

The structure of the sperm tail is highly distinctive of the Lissamphibia. The generalized lissamphibian sperm has three longitudinal fibres, one on each side of the axoneme adjacent to doublets 3 and 8 (juxta-axonemal fibres at 3 and 8), and a second fibre, the axial fibre, which is associated with doublet 3 but separated from it by an undulating membrane. The juxta-axonemal fibre at 8, characteristic of urodeles, is absent in caecilians and was previously thought to be absent in anurans. In urodele sperm, typically, there is no juxta-axonemal fibre at 3 (Baker 1963; Picheral 1979). However, *Plethodon albagula* Grobman, 1944 is exceptional, among urodeles studied, in having a dense structure on the adaxonemal end of the undulating membrane, although it is questionable if this is homologous with the anuran juxta-axonemal fibre at 3 (Jamieson *et al.* 1993; Lee & Jamieson 1993; Jamieson 1999).

The phylogenetic origins and, therefore, homologies of the lissamphibian flagellar complex have been discussed by Jamieson (1995, 1999) who suggested dipnoan affinities. Thus, in *Neoceratodus forsteri* where the anterior region of the sperm axoneme has a large fibre on each side, at doublets 3 and 8, and each fibre is continuous with a "fin" which terminates with a further, smaller lateral fibre (Jamieson 1999; Jespersen 1971).

Jamieson (1995, 1999) has tentatively recognized homology between each fin and an amphibian sperm undulating membrane, between the fibres at doublets 3 and 8 and the amphibian juxta-axonemal fibres, and between the terminal lateral fibres and the axial fibre. He considered that this, if valid, would suggest that lissamphibians have retained, from an ancestor shared with dipnoans (and with other sarcopterygian fish?), only one of two former, bilateral, undulating membranes, and only one of a former pair of axial fibres. He further argued that the two juxta-axonemal fibres of urodeles (and *Leiopelma*, this study) are a persistence of the paired ancestral condition, the fibre at doublet 8 normally being lost in the Anura. Thus the unilateral location of the undulating membrane and its axial fibre, rather than presence of undulating membranes *per se*, constitutes the synapomorphic condition for the Lissamphibia (Jamieson *et al.* 1993; Jamieson 1995, 1999). Sarcopterygian fish (for definition see Jamieson 1991), and particularly dipnoans, appear to be the nearest extant non-tetrapod relatives of amphibians. The plesiomorphic condition seen in *Leiopelma* of the presence of juxta-axonemal fibres at 3 and 8 is unique among the Anura. However, a temporary fibre is present in the spermatids of *Discoglossus pictus* (Sandoz 1975) and also in occasional *Bufo marinus* (Linnaeus, 1758) sperm (Swan *et al.* 1980).

With the discovery in *Leiopelma* sperm of a longitudinal fibre adjacent to axonemal doublet 8, the Anura cannot be defined spermatologically on the negative apomorphy of loss of this fibre. This loss was formerly the sole spermatozoal synapomorphy of the Anura. A juxta-axonemal fibre at doublet 8 (as at 3) is a feature of the anuran ground plan, as it is present in the basal frog *Leiopelma* and is very rarely retained in other anurans. A juxta-axonemal fibre at 3 is absent in urodeles (questionably present in *Plethodon*) and caecilians but cannot be considered an autapomorphy of the Anura as it appears to have a dipnoan precursor.

The plesiomorphic narrow unilateral undulating membrane with a fibre at 3 and an axial fibre (though in the absence of a fibre at 8) occurs in

the mature sperm of *Bufo*, *Litoria* and most lepodactylids (Pugin-Rios 1980; Lee & Jamieson 1992, 1993; Meyer *et al.* 1997). The flagellum may secondarily lack an undulating membrane and fibres and be single (*Xenopus laevis*, *Caudiverbera caudiverbera*, *Rana* spp., Pugin-Rios 1980) or double (*Telmatobufo australis*, Pugin-Rios 1980). Alternatively, the axoneme may be associated with a solitary parallel paraxonemal rod (*Ascaphus*, Jamieson *et al.* 1993; *Leiopelma*, this study; some *Cyclorana* spp., Meyer *et al.* 1997; and *Hyla meridionalis* Boettger, 1874; Pugin-Rios 1980); in *Ascaphus*, *Leiopelma* and *Cyclorana* Steindachner, 1867, at least, the rod represents axial fibre, lamina (equivalent to the undulating membrane), and juxta-axonemal fibre. The paraxonemal rod of *Ascaphus* differs from that of *Leiopelma* in that it carries a longitudinal groove which houses the mitochondria. Therefore, the paraxonemal rod is not considered here to be a shared apomorphy and is probably homoplastically derived in *Ascaphus* and *Leiopelma*. Presumed reduction of the undulating membrane has been considered a major advanced feature (apomorphy) of the *Ascaphus* spermatozoon (Jamieson *et al.* 1993).

LENGTHS OF SPERMATOZOEA

The sperm of the Anura are elongate, their length varying from 40 to 110 μm , though those of *Telmatobufo australis* and *Leiopelma hochstetteri* reach 240 μm and 250 μm , respectively, and *Discoglossus pictus*, attains the remarkable length of 2300 μm (Favard 1955; Sandoz 1975; Pugin-Rios 1980) to 2500 μm (Furieri 1975a). The length of *Ascaphus* sperm is unknown. The spermatozoa of urodeles are generally longer than those of anurans, with the exception of *Discoglossus pictus*. In urodeles, the sperm of the hynobiid *Hynobius takedai* Matsui & Miyazaki, 1984 is the shortest known, with an average length of 169 μm (Kuramoto 1997), while the longest known is that of the proteid *Necturus maculosus* (Rafinesque, 1818) which reaches a length of 920 μm (Baker 1963). The sperm of the lungfish *Neoceratodus forsteri* reaches an approximate length of 272 μm , with head, midpiece and tail

lengths of 70 μm , 2 μm and 200 μm respectively (Jespersen 1971), measurements very similar to those of *Leiopelma*.

CONCLUSION

Plesiomorphic features which the sperm of *Leiopelma hochstetteri* share with basal amniotes, urodeles and *Ascaphus truei* include, an elongate conical acrosome vesicle, a subacrosomal cone, distinct nuclear shoulders and an elongate nucleus which tapers to a point within the acrosome. However, *L. hochstetteri* does not exhibit the plesiomorphic feature present in *Ascaphus*, Bombinidae and urodeles of a well-defined axial perforatorium extending into an endonuclear canal (and albeit reduced, in caecilians), although axial lacunae present in the nuclear rostrum and containing putative perforatorial remnants are present in *L. hochstetteri*.

In contrast to all previously examined Anura, *Leiopelma hochstetteri* sperm has a well-developed juxta-axonemal fibre at doublet 8, a condition elsewhere seen only in urodeles within the Lissamphibia. Juxta-axonemal fibres at doublets 3 and 8 are deduced to be the plesiomorphic condition as they not only occur in one or both positions in anurans and urodeles but are also present, at both sites in the dipnoan *Neoceratodus forsteri*.

Our phylogram based only on sperm characters (Fig. 4) indicates that, spermatologically, *Ascaphus* and *Leiopelma* are paraphyletic. They share no synapomorphic sperm characters and there is accordingly no support here for their placement in the same family. Spermatologically, *Leiopelma* is the plesiomorphic sister-taxon of all other anurans. Our analysis also shows the spermatozoa of the Pelodytidae to be highly derived, sharing the apomorphic condition of a conical perforatorium divided into fibres with the Bufonoidea.

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