The Phylogenetic Position of the Tuatara, Sphenodon (Sphenodontida, Amniota), as Indicated by Cladistic Analysis of the Ultrastructure of Spermatozoa

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The phylogenetic position of the tuatara, *Sphenodon* (Sphenodontida, Amniota), as indicated by cladistic analysis of the ultrastructure of spermatozoa

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SUMMARY

Sphenodon has traditionally been regarded as a little changed survivor of the Permo-Triassic thecodont or eosuchian 'stem reptiles' but has alternatively been placed in the Lepidosauria as the plesiomorphic or even apomorphic sister-taxon of the squamates. A cladistic analysis of 16 characters from spermatozoal ultrastructure of *Sphenodon* and other amniotes unequivocally confirms its exceedingly primitive status. The analysis suggests that monotremes are the sister-group of birds; squamates form the sister-group of a bird + monotreme clade while the three sister-groups successively below the bird + monotreme + squamate assemblage are the caiman, the tuatara and the outgroup (turtles). The monotreme + bird couplet, supports the concept of the Haemothermia, but can only be regarded heuristically. The usual concept of mammals as a synapsid-derived outgroup of all other extant amniotes is not substantiated spermatologically. All cladistic analyses made, and a separate consideration of apomorphies, indicate that *Sphenodon* is spermatologically the most primitive amniote, excepting the Chelonia. It is advanced (apomorphic) for the amniotes in only two of the 16 spermatozoal characters considered. A close, sister-group relationship of *Sphenodon* with squamates is not endorsed.

1. INTRODUCTION

Sphenodon has traditionally been regarded as a little changed survivor of Permo-Triassic 'stem reptiles' of the Thecodontia (von Wettstein 1931; Crook 1975) or Eosuchia (Carroll 1969) since erection of the order Rhynchocephalia by Günther in 1867 to receive it. Although endorsing its primitive status, several investigators have placed *Sphenodon* with the squamates in the Lepidosauria (Rieppel 1978; Schwenk 1986; Peterson 1984; Laurin 1991) or Lepidosauromorpha (Gauthier *et al.* 1988). Alternatively, an advanced status for *Sphenodon* relative to lizards, within the Lepidosauria, has been argued on the basis of the head skeleton (Whiteside 1986).

The effectiveness of phylogenetic analysis of the structure of spermatozoa (spermiocladistics, Jamieson 1987) in resolving hitherto intractable taxonomic and phylogenetic problems or in arbitrating between conflicting views of relationship has been demonstrated by many workers (see, for example, Franzén (1970); Jamieson (1978, 1984*a*, 1987, 1991); Afzelius (1979); Ferraguti (1983); Wirth (1984); Justine *et al.* (1985); Healy (1988); Alberti (1990)). In an attempt to elucidate the relationships of the tuatara, Healy & Jamieson (1992) described the ultrastructure of the spermatozoon of *Sphenodon punctatus punctatus*, the nominate subspecies of one of at least three species (one extinct) of the genus which are currently recognized (Daugherty *et al.* 1991). They concluded that the spermatozoa of the tuatara strongly resemble those of turtles and crocodiles and unequivocally indicate that the living tuatara (and by association other sphenodontids) is truly primitive and is a basal amniote with no special relationship to the Squamata (lizards, skinks, gekkos and snakes). This conclusion was based on an intuitive consideration of comparative sperm ultrastructure in *Sphenodon* and other amniotes but also on a computerized cladistic analysis which sought to find the shortest phylogenetic tree from a set of 16 spermatozoal characters. The cladistic analysis is the subject of the present paper.

2. MATERIAL AND METHODS

(*a*) *Program and taxa used*

Reconstruction of the phylogenetic relationships of *Sphenodon* with other amniotes ('reptiles', birds and monotremes) from spermatozoal ultrastructure was attempted using the Phylogenetic Analysis Using Parsimony Program (PAUP) of Swofford (1990), version 3.0L.

Species included in the analysis, in the order presented in the data matrix, together with sources, are as follows. Groups for which the data from the different

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species were pooled because they were invariable for the characters used are indicated by asterisks:

Turtles* (Emydidae): *Emys orbicularis*, *Clemmys guttata*, *C. insculpta*, *Terrapene carolina*, *Chrysemys picta picta*, *C. picta belli* (Furieri 1970). *Emydura kreffti*, *Em. macquarii*, *Em. signata*, *Elseya latisternum*, *El. dentata*, *Chelodina expansa*, (B. G. M. Jamieson & A. Georges, unpublished results). (Testudinidae): *Testudo hermanni* (Furieri 1970).

Crocodile: *Caiman crocodylus* (Saita *et al.* 1987).

Tinamou (Tinamidae): *Eudromia elegans elegans*, (Asa *et al.* 1986).

Rhea: (Rheidae) *Rhea americanus albisceus* (Phillips & Asa, 1989).

Rooster (Phasianidae): *Gallus domesticus* (Xia *et al.* 1986; Thurston & Hess, 1987).

Guinea fowl (Numididae): *Numida meleagris* (Thurston & Hess, 1987).

Tuatara: *Sphenodon punctatus punctatus* (present study).

Lizards* (Lacertidae): *Algyroides alleni*, *Lacerta sicula campestris*, *L. lepida lepida*, *L. laevis*, *L. viridis* (Furieri 1970). *Podarcis taurica* (Butler & Gabri 1984).

Skinks* (Scincidae): *Chalcides ocellatus tiligugu* (Furieri 1970; Carcupino *et al.* 1989).

Gekkos* (Gekkonidae): *Hemidactylus frenatus*, *H. mabouia*, *Lygodactylus picturatus*, *Tarentola mauritanica mauritanica* (Furieri 1970).

Snakes* (Colubridae): *Coluber viridiflavus viridiflavus*, *Natrix tessellata tessellata*, *N. natrix*, *Coronella austriaca* (Furieri 1970). (Viperidae): *Vipera aspis* (Furieri, 1970).

Monotremes*: *Ornithorhynchus anatinus*, *Tachyglossus aculeatus*, *Zaglossus brujini* (Carrick & Hughes 1982).

(*b*) *A review of amniote spermatozoa in relation to selection of characters for cladistic analysis*

This section is directed towards defining spermatozoal characters that vary between taxa and which can therefore be used in phylogenetic analysis. Although outgroup rooting obviates the need to determine the polarity of characters, a further aim is to recognize the ground plan, that is the primitive organization, of the spermatozoon for each major group of the amniotes. Little attention will therefore be focused on the ultrastructure of sperm of advanced groups where sperm structure is clearly highly derived. Consideration of passerines, in the Aves, and Metatheria and Eutheria in the Mammalia will therefore be minimal. This section also constitutes a brief review, the first to our knowledge, of the sperm of the amniotes as a whole. To that end, some anticipation of the results of the cladistic analysis is included. For references to the literature on the various taxa, the reader is referred to the list of species investigated cladistically, in addition to references cited in the text. Those descriptions of ultrastructure given here which are derived from the literature are drawn from text and perusal of micrographs and the terminology employed and homologies recognized are not necessarily those of the authors of the accounts. Further discussion of the characters may be found in Healy & Jamieson (1992).

The characters, excepting character 10 which (like 13) was uninformative, are diagrammatically illustrated for *Sphenodon* in figure 1, together with their distribution in other included amniotes.

The acrosome, endonuclear canals and perforatoria (characters 1–3)

In all amniote classes the acrosome plesiomorphically forms an elongate, narrow cone symmetrically located on the tip of the nucleus which it overlaps and constricts. The acrosome vesicle, with this form, encloses a similarly shaped subacrosomal cone. The pointed form of the acrosome, presence of the subacrosomal cone, and constriction of the nuclear tip are seen in the Chelonia, Crocodilia, *Sphenodon*, Squamata, non-passerine birds and monotremes. They are deduced to be plesiomorphies because of their presence in less modified members of the respective amniote groups and because they are also seen in the Lissamphibia, including the primitive frog *Ascaphus* (B. G. M. Jamieson, M. S. Y. Lee & K. Long, unpublished results); they presumably characterized the common ancestor of Amphibia and Amniota. The subacrosomal cone is lost in ratites.

In passerines the acrosome vesicle becomes an elongate single-keeled helix, again with no evident subacrosomal cone, in, for instance, finches (Kondo *et al.* 1988) whereas in *Grus vipio*, the white-naped crane, the vesicle is dome-shaped, also with no recognizable subacrosomal cone, although retaining a rod-like perforatorium which extends posteriorly into an endonuclear canal (Phillips *et al.* 1987).

Monotremes retain the elongate conical bipartite structure of the acrosome but this is greatly modified in Metatheria and Eutheria.

In the Squamata, Furieri (1970) recognizes a paracrystalline substructure of the subacrosomal cone. This paracrystalline organization is confirmed by Butler & Gabri (1984) for the lizard *Podarcis taurica* and by Carcupino *et al.* (1989) for the skink *Chalcides ocellatus tiligugu*. It is found in the analysis to constitute a synapomorphy of the Squamata.

All classes of amniotes possess one or more endonuclear canals which perforate the anterior end of the nucleus to varying depths. This condition is seen in basal lissamphibians: urodeles (Picheral 1967) and the primitive anurans *Ascaphus* (B. G. M. Jamieson, M. S. Y. Lee & K. Long, unpublished results) and *Discoglossus* (Sandoz 1969, 1970). Absence, as in squamates, is therefore clearly apomorphic. The number of endonuclear canals and of enclosed perforatoria is one in basal Lissamphibia, in the caiman, tinamou, rhea and non-passerines but in the Chelonia there are two or three canals and there are two in *Sphenodon*. The plesiomorphic number of canals may well be one for the Lissamphibia–Amniota and it is possible that that multiple number in chelonians and *Sphenodon* is secondary and that in this respect chelonians are misleading as the outgroup. However, there are three endonuclear canals in sturgeon and two in the dipnoan *Neoceratodus*. Although there appears to be only a single canal in the coelacanth, *Latimeria*, this contains two or three perforatoria (see Jamieson 1991). It is

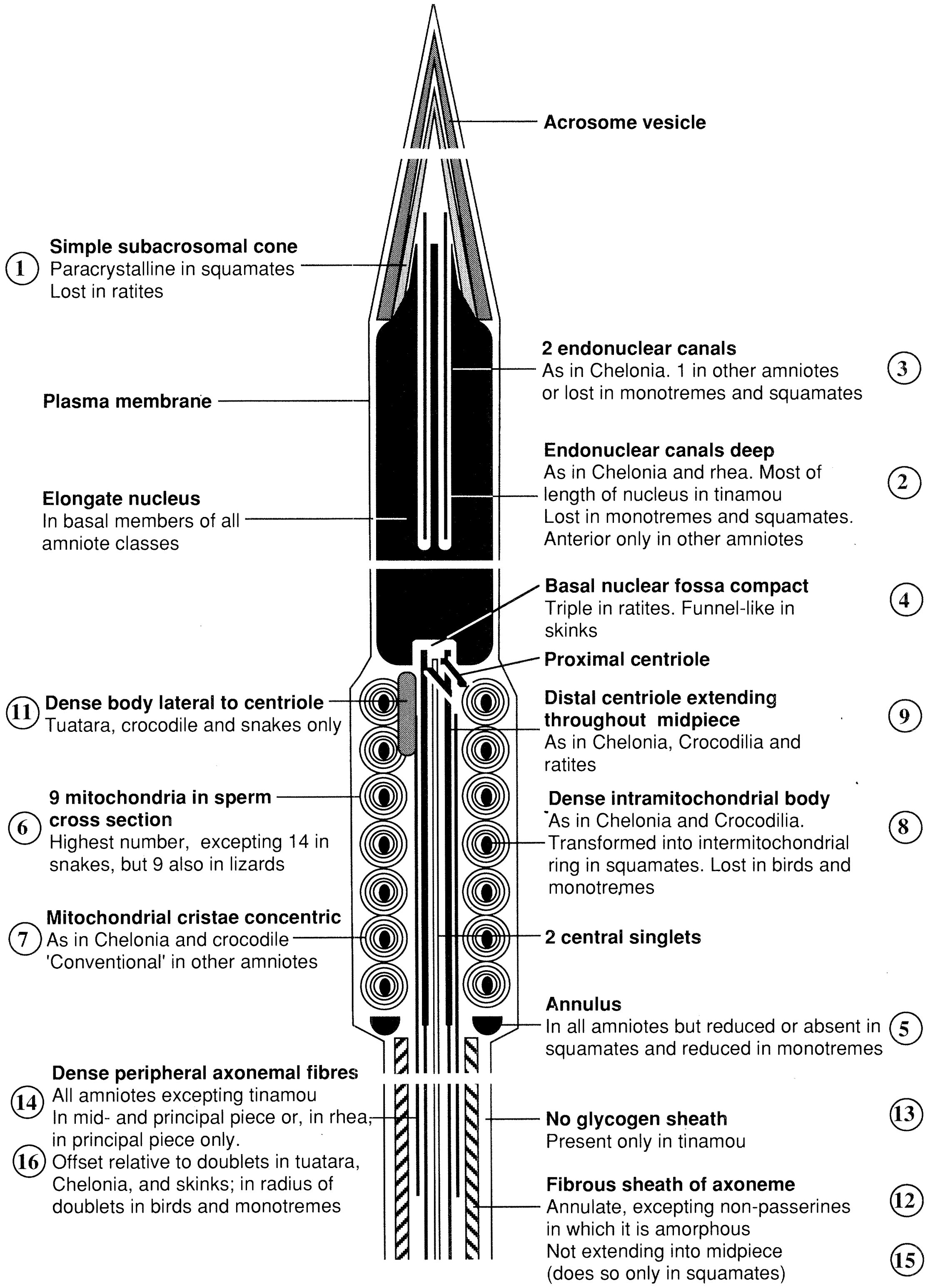


Figure 1. Diagram of the spermatozoon of *Sphenodon punctatus* reconstructed from transmission electron micrographs. Major characters are labelled and numbered and their occurrence in the other amniotes studied, if variable, is indicated. The invariable characters labelled were not used in the analysis.

therefore possible that the presumed common ancestor of Amphibia and amniotes possessed more than one endonuclear canal, although an apomorphic increase to two or more in chelonians cannot be ruled out.

In the cladistic analysis for character 3 the plesiomorphic number of canals for the ingroup emerges as two, as in the outgroup turtles and in *Sphenodon*. Possession of only a single canal computes as basic to all amniotes above turtles and *Sphenodon*. Absence is seen as a synapomorphy of the squamates and a homoplasy of these with non-passerines and monotremes.

Character 2, including depth of penetration of the endonuclear canal is, again, refractory to intuitive determination of polarity relative to most amniotes. In the analysis, the outgroup condition of a deep endonuclear canal is retained in *Sphenodon* and retraction to an anterior location is seen as a basal apomorphy of the remaining amniotes. Deep penetration appears to have been re-evolved in ratites, with secondary extension to the base of the nucleus in tinamou. The canal is lost in monotremes and homoplasically in squamates. This series of transformations, although reasonable, can only be viewed heuristically.

The nucleus and nuclear fossa (character 4)

The nucleus is an elongate cylinder narrowly constricted within the base of the acrosome in all amniotes here investigated. This form, also seen in *Ascaphus* and urodeles (B. G. M. Jamieson, unpublished results), is clearly plesiomorphic for amniotes. As it is universal for the basal members of each class it is not used in the cladistic analyses. The condition of the endonuclear canal has been discussed above.

Representation of the basal nuclear fossa (character 4) is variable. It is poorly developed in the sperm of the caiman, and is small and compact in turtles, tuatara, rooster, guinea fowl, and squamates excepting the skinks. In skinks it is narrowly funnel-shaped. In the ratites it has a triple profile. In the analysis the compact form is plesiomorphic as it characterizes the outgroup, and it also computes as plesiomorphic for the in-group.

The annulus (character 5)

A dense ring, the annulus (character 5), at the posterior end of the midpiece is a feature of many metazoan sperm and is clearly plesiomorphic for amniotes. In the taxa analysed here it is absent (or at least negligibly developed) only in the squamates (as shown for lizards and skinks, it has not been described for gekkos and lizards but there absence requires confirmation). Absence of the annulus in squamates possibly relates to their unique synapomorphy (autapomorphy) of extension of the fibrous sheath anteriorly into the midpiece. The annulus is weakly developed in monotremes.

The number of mitochondria (character 6)

The number of mitochondria seen in transverse section of the midpiece, where possible near its anterior end, is very variable (see character 6 in data matrix) between the taxa investigated and the extent of intra-taxon variation is little known. The outgroup (chelonian) number is six but because phylogenetic trends are not as evident by inspection as for most other characters, some anticipation of transformations in the cladistic results seems warranted here.

With a consistency index of 0.833 (unordered) or 0.500 (Dollo up), the character was less 'noisy' than might have been expected. A trend towards reduction from nine computed as plesiomorphic for the in-group to four in birds and monotremes is apparent. It is four in ratites and although not known for the two non-passerines is (Thurston and Hess, 1987) also four in the turkey. The tuatara, with nine, does not differ significantly from the highest ingroup number (eight) recorded for the caiman and skinks. The crocodile was scored as eight (given as eight or nine by Saita *et al.* 1987) but in their line-drawing is illustrated as six, as in turtles. In squamates the number remains plesiomorphically high in lizards or, in snakes, shows apomorphic increase to as many as 14 whereas a reduction to two in gekkos is occasioned by intrusion of intermitochondrial material of supposed mitochondrial origin into the transverse section of the midpiece.

Structure of the mitochondria (characters 7 and 8)

The form of the cristae of the mitochondria (character 7) is a most striking character. In turtles, caiman and tuatara, the mitochondria have a form known only in the sperm of one other amniote, the Woolly opossum, *Caluromys philander* (see Phillips 1970; Fawcett 1970). The mitochondrial cristae in these three taxa are concentric (character 7) and usually surround a large central dense body (character 8). In all other amniotes included, the cristae have a 'conventional' appearance, being linear or curved, as in Lissamphibia, but never concentric, and do not surround a dense body.

Linear cristae in spermatozoal, as in somatic mitochondria, must be accepted as a plesiomorphic condition for tetrapods as it is normal for metazoan sperm, including fish. The concentric arrangement with dense body is here considered to be an apomorphy acquired early in amniote evolution, as evidenced by its occurrence in Chelonia. The question then arises as to whether it is a synapomorphy of its possessors, turtles, caiman and tuatara, or, although acquired as an apomorphy, is a simultaneous plesiomorphy for the entire extant amniote assemblage which has been superseded by reversion to the linear condition in all but these three taxa.

To anticipate, the cladistic analysis favours the view that concentric cristae are a basic amniote feature (figure 2). However, if turtles, caiman and tuatara are constrained as a monophyletic group (figure 3) the tree retains the same length and consistency index. Figure 3 has the advantage of regarding the linear cristae of other amniotes as a retained plesiomorphy. Exclusive monophyly of the tuatara–crocodile–turtle assemblage seems improbable and, it must be remembered, is obtained only by applying a constraint on the tree. Presence of concentric cristae and the intramitochondrial body in the woolly opossum also militate against such monophyly. Furthermore, in spermatids of the caiman (Saita *et al.* 1987) and, we have observed, in at least some mitochondria of spermatids of tuatara, the cristae have the linear appearance usual for metazoan sperm and the concentric arrangement is a late development. Phylogenetic 'reversion' of mitochondrial cristae to the linear condition would need only suppression of this final transformation.

The intermitochondrial rings which are limited to squamates are here regarded as derivations of the dense bodies and as such a 'reminiscence' of the occurrence of concentric cristae in the ancestry of squamates (character 8). Carcupino *et al.* (1989) independently concluded that the rings were mitochondrial derivatives. Rescoring the characters in such a way that the rings are not regarded as mitochondrial derivatives does not alter the topology of the tree.

Characters 7 (condition of the cristae) is the only single character for which deletion changes the topology of the tree. If it is deleted a trichotomous tree is produced, the three clades of which are (i) turtles, (ii) a tuatara–crocodile–squamate clade, and (iii) the bird–monotreme clade. This is an implausible phylogeny but again indicates tuatara as the most plesiomorphic taxon other than turtles.

The centrioles (characters 9 and 10)

The distal centriole, forming the basal body of the axoneme, is plesiomorphically short in vertebrates, including the Lissamphibia, as in most Metazoa. In contrast, in the tuatara the distal centriole extends the entire length of the long midpiece (character 9). We have re-interpreted the condition in turtles and the caiman as being similar to that in the tuatara (Healy & Jamieson 1992). This condition is retained in ratites. The shorter, although still elongate distal centriole in the rooster and the somewhat shorter centriole in guinea fowl, the short centriole in squamates, and the vestigial, possibly absent, centriole in monotremes are deduced to represent secondary reduction in length of the centriole. This interpretation of the evolution of the centriole in amniotes is endorsed by the cladistic analysis. Great elongation of the distal centriole is thus seen as a basal synapomorphy (and simultaneously a plesiomorphy) of amniotes, contrasting with the plesiomorphically short organelle in lissamphibians.

Phillips & Asa (1989) attempt a functional explanation for selection for the long centriole in ratites. In these birds the annulus forms at the distal end of the centriole as in mammalian spermatids but it never moves, unlike mammals, and the length of the distal centriole becomes the length of the midpiece. Thus, they consider, the extra-long centriole of ratite spermatozoa allows the spermatozoa to form a midpiece without moving the annulus.

The distal centriole is embedded in a ring of dense material in all of the amniotes in the present study for which this has been investigated (character 10) and the character therefore computed as constant and uninformative. Data on this character are not available for squamates and monotremes.

The axoneme and appurtenances (characters 11–16)

Lateral to the centriole in the tuatara there is a large dense body which appears to be an enlargement of one of the peripheral dense fibres (character 11). A similar body is seen in the caiman although its direct homology with that of the tuatara is uncertain. In the caiman the dense body shows in longitudinal section a transverse periodicity which suggests that the body may be homologous with the striated columns of eutherian sperm. We have found evidence of this periodicity in late spermatids of the tuatara and consider it likely that it survives in the spermatozoon (Healy & Jamieson 1992). Descriptions of other amniotes included in this study are insufficient to indicate further taxonomic distribution of the dense body. It appears to be absent from rooster and guinea fowl sperm. Occurrence in ratites has not been reported; it is probably absent but has been scored as unknown ('?'). It is absent from monotremes and the squamates included excepting snakes. In snakes, dense material is illustrated on each side of the centriole by Furieri (1970). Homology of this material in snake sperm with that observed in the tuatara and the caiman is doubtful but it is seen in the analysis as a retained plesiomorphy. Deletion of this imperfectly known character does not affect the topology of the tree.

An annulated, helical, dense fibrous sheath (character 12) must, clearly, have developed in the earliest amniotes as it is present in all amniote classes. Homology of this sheath across the different classes is reinforced by the presence of two spurlike inwardly directed triangular processes, seen in cross section of the sperm, in the vicinity of doublets 3 and 8, in mammals (although at most weakly developed in monotremes) and in the two ratites. Only in the two non-passerines of taxa here studied, is the fibrous sheath transformed into an amorphous sheath.

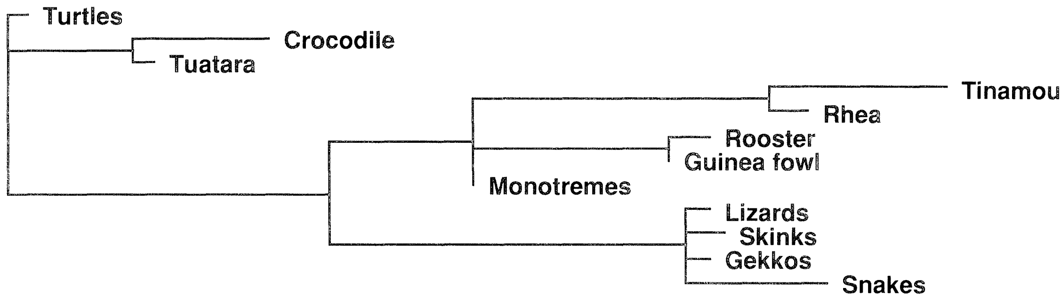
The isolated peripheral fibres 3 and 8 in amniotes may well be homologous with columns at this point in other sarcopterygians, the coelacanth, *Latimeria*, and Dipnoi. However, such modifications at doublets 3 and 8, which are approximately in the plane of the two central singlets, could be independent acquisitions as they presumably are in chondrichthyan sperm (see references in Jamieson (1991)).

Modification of the axonemal complex in lissamphibians has been so profound, including the development of the undulating membrane, that most crossopterygian affinities of the lissamphibian axoneme would have been obscured. A fibrous sheath is absent from amphibian sperm. A long dense body at the head of the axoneme in lissamphibians is probably, however, homologous with the retronuclear body of *Latimeria* and of Dipnoi (see Jamieson 1991).

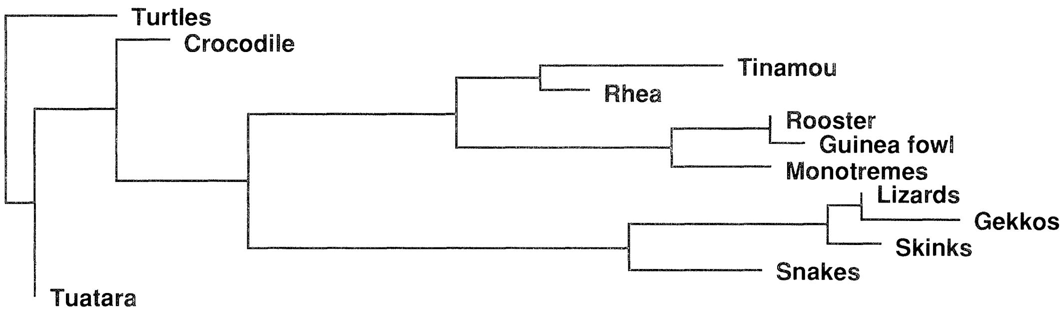
An external longitudinal protuberance (rib) on each side of the fibrous sheath is seen in many amniotes, including eutherians, and is particularly well developed in the tinamou. It has not been separately coded in the analysis.

A sheath of putative glycogen external to the fibrous sheath was found to be limited to the tinamou and was therefore uninformative but is included in the analysis for its descriptive value (character 13). Phillips & Asa (1989) have suggested that a function of the glycogen may be to nourish the spermatozoa during storage. The absence of a glycogen sheath in other birds which store sperm (Hatch 1983; Shugart 1988) would then need further explanation.

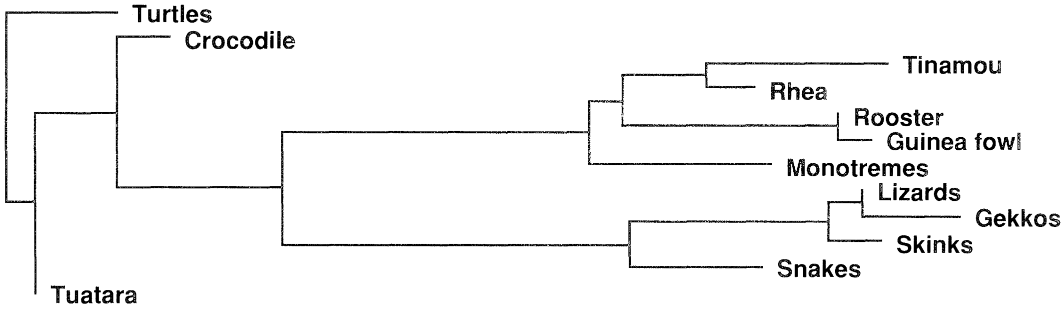
Nine longitudinal dense fibres peripheral to the nine axonemal doublets are a fundamental feature of amniote sperm, being found in all classes. They are an autapomorphy and simultaneous symplesiomorphy of the amniotes, although they appear homoplasically in other groups, such as heterobranch and cephalopod molluscs (Healy 1988, 1989). The dense fibres are small in most amniotes investigated: turtles, the caiman, the tuatara, squamates and monotremes. The



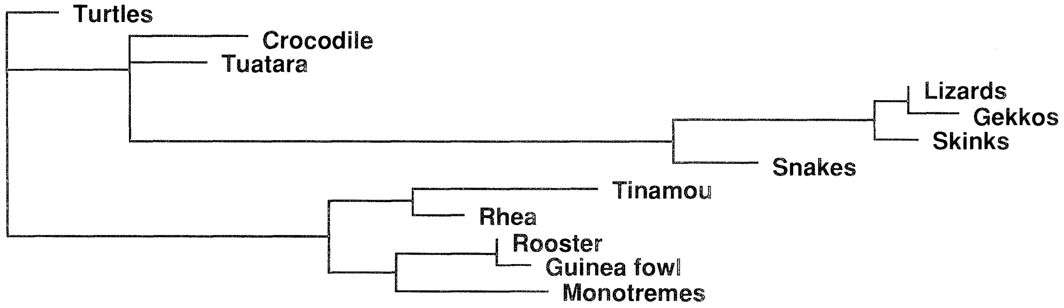
2 Unconstrained consensus tree. All characters unordered.
Length 17096. Consistency Index 0.828. Rescaled CI 0.744



3 Single unconstrained tree. Character 1 unordered, 2 ordered, others Dollo up.
Length 21194. Consistency Index 0.693. Rescaled CI 0.639

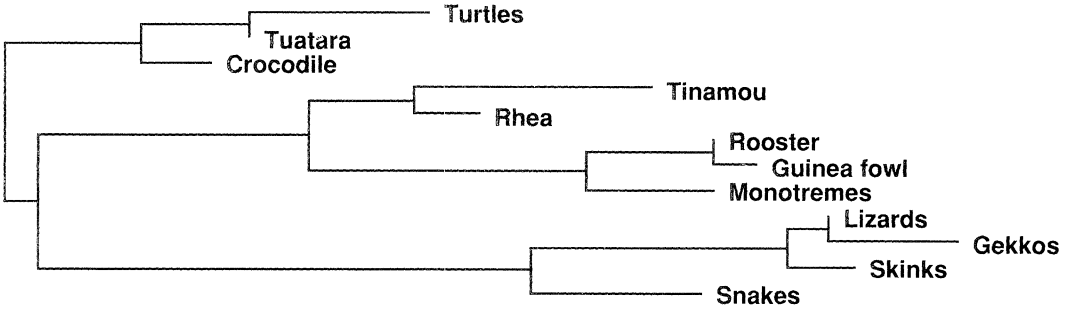


4 Single tree constrained to monophyletic birds. Character 1 unordered, 2 ordered, others Dollo up.
Length 21860. Consistency Index 0.671. Rescaled CI 0.613



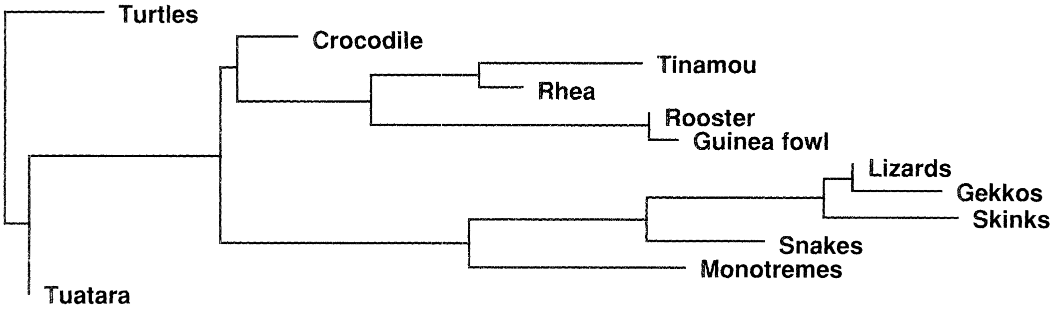
5 Single tree constrained to monophyletic squamates+tuatara.
Character 1 unordered, 2 ordered, others Dollo up.
Length 22893. Consistency Index 0.639. Rescaled CI 0.575

Figures 2–9. Most parsimonious trees of amniote relationships suggested by the present analysis of spermatozoal ultrastructure with or without the constraints indicated in the captions. Note the very primitive status of *Sphenodon*, the tuatara, in all trees. Options employed: branch-and-bound; search setting initial upper bound unknown (computed via stepwise); addition sequence simple; branches having maximum length zero collapsed to yield polytomies; character-state optimization accelerated transformation (ACCTRAN); characters all scaled; rooting outgroup. Characters in figure 1 were run unordered but in figures 2–8, character 1 is unordered, character 2 ordered and the remaining characters under the option Dollo up. See summary in table 1.



6

Single tree constrained to monophyletic turtles+tuatara+crocodile (as outgroup).
Character 1 unordered, 2 ordered, others Dollo up.
Length 21194. Consistency Index 0.693. Rescaled CI 0.639



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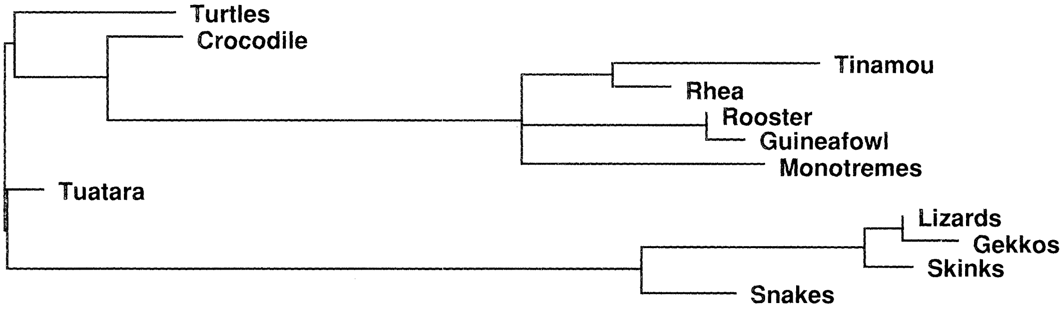
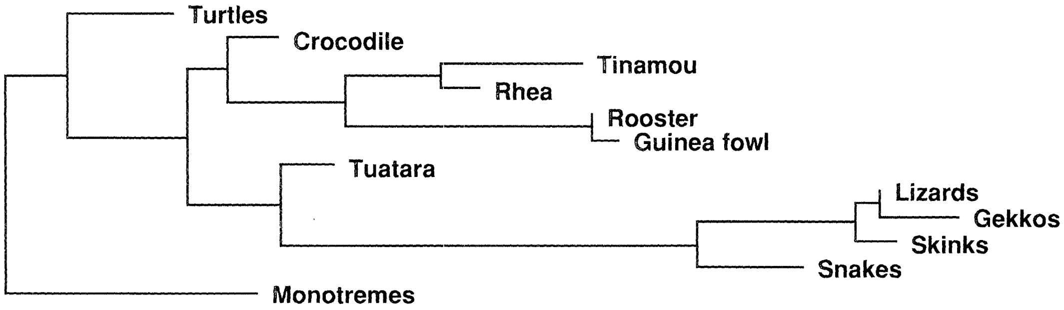
Single tree constrained to monophyletic archosaurs.
Character 1 unordered, 2 ordered, others Dollo up.
Length 24061. Consistency Index 0.607. Rescaled CI 0.535



8

Tree constrained to phylogeny of Gardiner (1982). Lepidosauria (Tuatara + squamates) as outgroup
Character 1 unordered, 2 ordered, others Dollo up.
Length 24159. Consistency Index 0.604. Rescaled CI 0.532



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Tree constrained to phylogeny of Gauthier et al. (1988). Monotremes as outgroup.
Character 1 unordered, 2 ordered, others Dollo up.
Length 26926. Consistency Index 0.540. Rescaled CI 0.454

Table 1. *Relative lengths and consistency index (CI) of alternative amniote trees tested*

Constraints	Character type	Number of trees	length	CI	rescaled CI
unconstrained	all unordered	45	17096	0.828	0.744
unconstrained	Dollo, 1 unord. 1 ord.	1	21194	0.693	0.613
monophyletic turtles + tuatara + crocodile	Dollo, 1 unord. 1 ord.	1	21194	0.693	0.639
monophyletic birds	Dollo, 1 unord. 1 ord.	1	21860	0.671	0.613
monophyletic tuatara + squamates	Dollo, 1 unord. 1 ord.	1	22893	0.639	0.575
monophyletic archosaurs	Dollo, 1 unord. 1 ord.	1	24061	0.607	0.535
Gardiner topology	Dollo, 1 unord. 1 ord.	1	24159	0.604	0.532
Gauthier *et al.* topology	Dollo, 1 unord. 1 ord.	1	26926	0.540	0.454

fibres are described as 'tiny' for the rhea and are absent from the tinamou; their presence in guinea fowl requires confirmation; they are present in suboscine and the more apomorphic oscine passerines, being larger in the latter. They are large and diverse in shape in marsupials above the didelphids, and in eutherian mammals. There thus appear to be trends to enlargement of the peripheral fibres in passerines and non-monotreme mammals, with diversification in the latter, and reduction in ratites. They are usually situated in the midpiece, in addition to the principal piece (character 14) as in turtles, the caiman, non-passerines, tuatara, squamates and monotremes (present survey) but are restricted to the principal piece in the rhea. They extend through most of the length of the sperm cell in oscine passerines (Phillips & Asa, 1989).

In most of the amniotes investigated in the present study the fibrous sheath commences immediately behind the midpiece (character 15). This condition is seen in turtles, the caiman, the ratites, non-passerines and monotremes but in squamates the fibrous sheath extends anteriorly well into the midpiece, a clear squamate autapomorphy, as emerges in the analysis.

The dense fibres lie in the same radius as the doublets in the rhea (absent in the tinamou), at least some non-passerines (turkey) and monotremes (character 16). In turtles, the tuatara and skinks the fibres are partly displaced into the gaps between adjacent doublets. Their exact radial position is unknown in the crocodile and in squamates other than the skink *Chalcides*. In the matrix the rooster has been scored as for the turkey which is the only non-passerine for which location is known.

Characters, with states indicated, which were employed in the cladistic analysis are listed in §2*c* below. Character states are presented for each character in what is deduced to be an increasing order of apomorphy; this ordering is necessary, despite outgroup rooting, so as to permit use of the Dollo option.

For greatest objectivity, all characters were initially treated as unordered. This manifestly produced unacceptable evolutionary pathways for some characters. For example, the endonuclear canal was represented as having been lost and redeveloped in the line leading to the ratites. Although the authors accept, and, indeed, have proposed (Jamieson, 1984*b*), that character states may be switched on or off in this way, *ad hoc* postulation of this phenomenon is to be avoided and it is closer to Hennigian principles to adopt a procedure involving fewer assumptions. The 'Dollo up' option has accordingly been applied to most characters. This option prohibits redevelopment of a state if a more apomorphic state has been acquired and is a reflection of 'Dollo's law', that a character, once lost, cannot be reacquired.

(*c*) *The characters and states used*

1. Subacrosomal cone: simple (1); paracrystalline (2); absent (3).
2. Endonuclear canal(s): extending to base of nucleus (1); deep into nucleus (2); anterior only in nucleus (3); absent (4).
3. Number of endonuclear canals: 2, 1, 0.
4. Nuclear fossa: absent or very weakly developed (0); compact (1); triple (2); funnel-like (3).
5. Annulus: present (1); absent (2).
6. Number of mitochondria in TS midpiece: 2, 4, 6, 8, 9, E(= 14).
7. Cristae: linear or curved (1); concentric (2).
8. Dense body in mitochondria: absent (0); present (1); replaced with intermitochondrial rings (2).
9. Distal centriole: short (1); slightly elongate (2); elongate (3); extending through entire midpiece (4).
10. Distal centriole, embedding in dense material: absent (0); present (1). The state of this character is unknown for several taxa and is constant for those for which it is known. It did not, therefore, contribute to the analysis.
11. Dense body or rod lateral to centriole: absent (0); present (1).
12. Fibrous sheath of axoneme: absent (0); annulated (1); amorphous (2).
13. Glycogen sheath external to fibrous sheath: absent (0); present (1). This character was found to be limited to the Tinamou and was therefore uninformative but is included for its descriptive value.
14. Axonemal dense fibres: in principal piece only (1); also in midpiece (2); absent (3).
15. Fibrous sheath, extension into midpiece: absent; (0) present (1).
16. Axonemal dense fibres offset relative to doublets (1), or in the same radius as these (2).

(*d*) *The data matrix*

character										1	1	1	1	1	1	1
number	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6
turtles	1	2	2	1	1	6	2	1	4	1	0	1	0	2	0	1
crocodile	1	3	1	0	1	8	2	1	4	1	1	1	0	2	0	?
tinamou	3	1	1	2	1	4	1	0	4	1	?	1	1	3	0	?
rhea	3	2	1	2	1	4	1	0	4	1	?	1	0	1	0	2
rooster	1	3	1	1	1	?	1	0	3	1	0	2	0	2	0	2
guineafowl	1	3	1	1	1	?	1	0	2	1	0	2	0	?	0	?
tuatara	1	2	2	1	1	9	2	1	4	?	1	1	0	2	0	1
lizards	2	4	0	1	2	9	1	2	1	?	0	1	0	2	1	?
skinks	2	4	0	3	2	8	1	2	1	?	0	1	0	2	1	1
gekkos	2	4	0	1	?	2	1	2	1	?	0	1	0	2	1	?
snakes	2	4	0	1	?	E	1	2	1	?	1	1	0	2	1	?
monotremes	1	4	0	1	1	4	1	0	1	?	0	1	0	2	0	2

(*e*) *Constraints optionally applied*

Monophyletic birds = ((tinamou, rhea, rooster, guinea fowl)).

Monophyletic squamates + *Sphenodon* = ((tuatara, lizards, skinks, gekkos, snakes)).

Monophyletic basal triplet = ((turtles, crocodile, tuatara)).

Monophyletic archosaurs = ((crocodile, tinamou, rhea, rooster, guinea fowl)).

Phylogeny of Gardiner (1982) = (((tuatara) (lizards, skinks, gekkos, snakes)) (turtles (crocodile ((tinamou, rhea, rooster, guinea fowl) (Monotremes))))).

Phylogeny of Gauthier *et al.* (1988) = (monotremes (turtles ((lizards, skinks, gekkos, snakes, tuatara) (tinamou, rhea, rooster, guinea fowl, crocodile)))).

(*f*) *Options employed*

Branch-and-bound search settings: initial upper bound unknown (computed via stepwise). Addition sequence: simple. Branches having maximum length zero collapsed to yield polytomies. Character-state optimization: accelerated transformation (ACCTRAN). Trees are unrooted. Rooting outgroup. Characters all scaled so that each scores unity (expressed as 1000) irrespective of number of states. Outgroup defined as turtles except where indicated.

3. RESULTS

(*a*) *Tree topologies*

The topologies of trees obtained in the various analyses are shown in figures 2–9. The lengths and consistency indices of the trees are summarized in table 1.

Unconstrained trees using unordered characters

To avoid *a priori* assumptions, the first parsimony analysis was done with all characters unordered. It gave 45 most parsimonious trees (length 17 096, consistency index (CI) excluding uninformative characters = 0.828, rescaled CI 0.744). The resultant consensus tree (figure 2) was, however, of questionable acceptability in view of the fact that the sequence of changes in some characters was of doubtful evolutionarily plausibility. For instance, loss and redevelopment of the endonuclear canals in the ancestry of ratites. The consensus tree nevertheless, like all subsequent trees, indicated a very plesiomorphic position for *Sphenodon*. Sister-group relationship of *Sphenodon* with the caiman in the tree is less acceptable than its isolated, basal position in further trees to be discussed below. The sister-group relationship of monotremes and birds in this tree is of the greatest interest in view of the evidence presented by Gardiner (1981) for this relationship of mammals and birds which he unifies in the group Haemothermia. We stress, however, that analyses in the present paper, although strongly supporting a plesiomorphic status for *Sphenodon*, allow only tentative support for recognition of the Haemothermia.

Unconstrained tree under the Dollo principle

To avoid unacceptable reversals the Dollo up option was applied. The evolutionary pathway for the first two characters was however more acceptable when the first was run unordered and the second ordered, with the remaining characters Dollo up, although the tree was unchanged. If character 1 was treated as Dollo up, a paracrystalline subacrosome cone was found to be basal to squamates, birds and monotremes with subsequent loss in ratites, and reversion to a simple form in non-passerines and monotremes. Fewer assumptions were necessary if it was run unordered as it merely became paracrystalline in the squamates and was lost in the ratites. If character 2 was run Dollo up or unordered, the endonuclear canals were lost at the base of the bird–monotreme–squamate clade and regained in birds, an unsatisfactory scenario. When run ordered, the endonuclear canals were lost only at the base of the squamates, without re-evolution. All further analyses were run with this set of character types.

The single tree (length 21 194, CI excluding uninformative characters 0.693, rescaled CI 0.639) (figure 3) resembled the unordered tree with the exception that the tuatara appeared more basal than the caiman and, of doubtful acceptability, the ratites formed the sister-group of the monotremes plus non-passerines. The latter relationship nevertheless underlined the spermatological proximity of monotremes and birds. Henceforth this topology will be referred to as the standard tree. The character-state transformations occurring in this tree are shown in figure 10.

Tree constrained to monophyletic birds.

The single tree constrained to give a monophyletic Aves (figure 4) agreed with the standard topology except in necessarily representing the monotremes as the sister group of all included birds. It was slightly less parsimonious and had a marginally lower consistency index (length 21 860, CI excluding uninformative characters 0.671, rescaled CI 0.613) than the standard tree. This tree is here regarded as the most acceptable tree in terms of a combination of parsimony, rational evolutionary pathways of individual characters, and in avoiding the improbable associ-

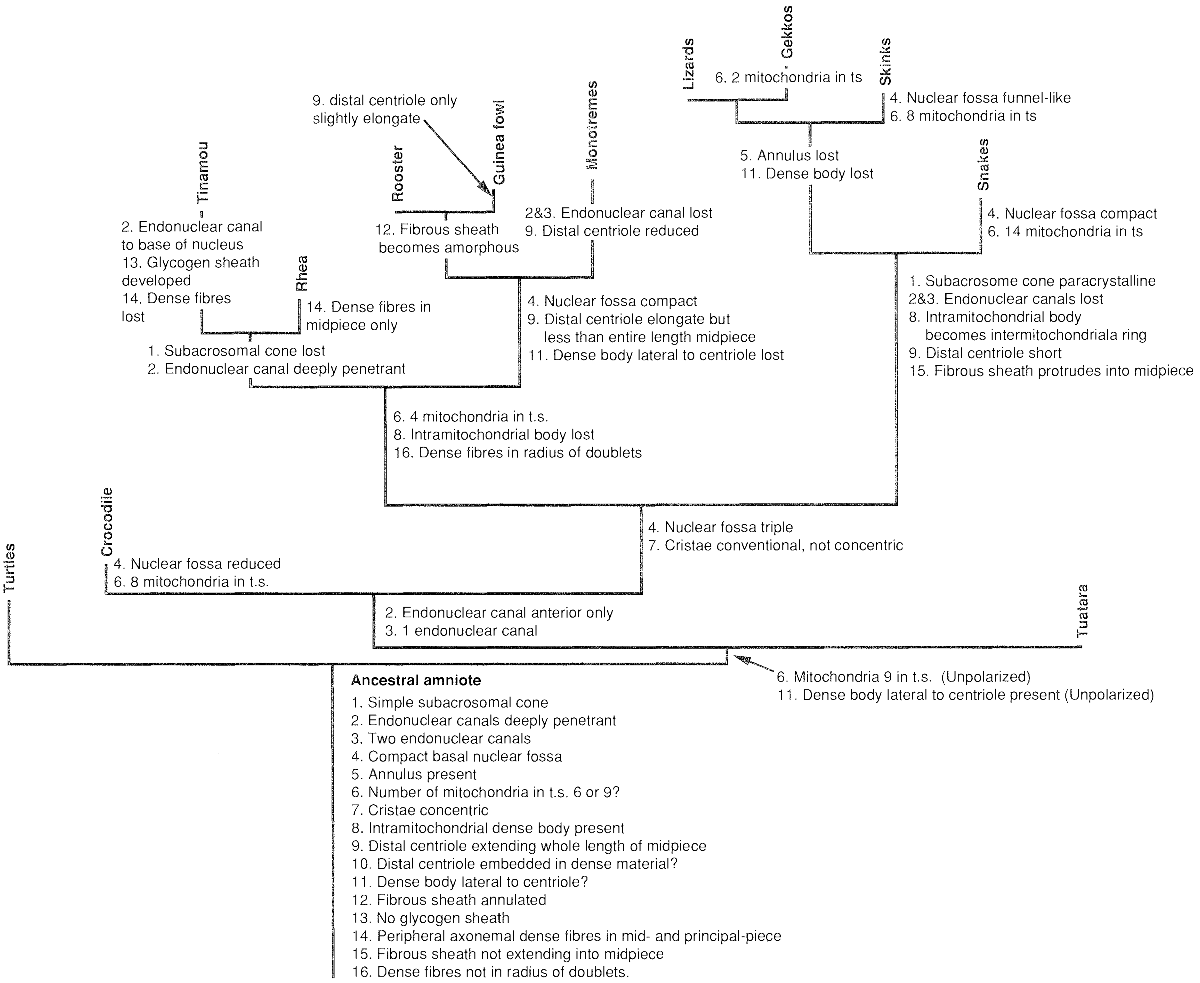

Figure 10. Most parsimonious tree, corresponding with figure 2, with no phylogenetic constraints and with the mixed character settings of figures 2–8, showing the apomorphic changes at each node.

ation of monotremes with non-passerines with its consequent (although heuristic) paraphyly of the birds.

Tree constrained to monophyletic tuatara + squamates

Constraining the tree so that the tuatara is the sister-taxon of the squamates, the relationship commonly accepted from comparative somatic morphology, again gives a single tree (length 22 893, CI excluding uninformative characters 0.639, rescaled CI 0.575) (figure 5). This is a little less than two steps longer than the standard tree but it cannot be considered to confirm a tuatara–squamate relationship because, despite constraint to this effect, the tuatara is exceedingly basal relative to the squamates and differs from the crocodile only in a questionably significant increase in the number of mitochondria in transverse section from eight to nine. Monotremes preserve the relationship with non-passerines birds seen in the standard tree.

Tree constrained to monophyletic turtles + tuatara + crocodile

In constraining the tree (figure 6) to give a monophyletic grouping turtles + tuatara + crocodile it is necessary to change the outgroup from turtles to this triad. Except for placing the three taxa in a single, basal clade, the resultant tree has the same length and CI as the standard tree (length 21 194, CI excluding uninformative characters 0.693, rescaled CI 0.639). This tree indicates that it would be equally parsimonious to conclude that the concentric cristae and dense bodies of the mitochondria of the midpieces of the three taxa were apomorphic, rather than plesiomorphic conditions as the hypothetical taxon at the base of the in-group has 'conventional', not concentric, cristae. This has the attraction of suggesting that the 'conventional' condition of these structures in the remaining amniotes was not a retention of a developmental condition but was a plesiomorphy of mature sperm. However, a special relationship of turtles, crocodiles and the tuatara seems less acceptable than the standard topology which places the crocodile as the sister-taxon of birds + monotremes + squamates. The latter configuration, although giving a paraphyletic 'Archosauria' does at least place the crocodile in a closer proximity to the remaining extant 'archosaurs', the birds. Furthermore, we have seen in the review of characters that concentric cristae and the intramitochondrial body also occur in the sperm of a didelphid mammal, suggesting that these features were plesiomorphic for amniotes and have been lost in those amniotes which lack them.

Tree constrained to monophyletic archosaurs

Forcing the crocodile to group with the birds gives a single tree (figure 7) (length 24061, CI excluding uninformative characters 0.607, rescaled CI 0.535) which is substantially longer than the standard tree. The tuatara retains its very primitive position, but an unacceptable change, particularly in view of the increased length, is grouping of monotremes with squamates. The present analysis does not, therefore, support recognition of the Archosauria.

Tree constrained to the phylogeny of Gardiner (1982)

In the phylogeny proposed by Gardiner (1982), the Lepidosauria (squamates + *Sphenodon*) were seen as the sister-group of all other amniotes. Mammals and birds were regarded as sister-groups (the Haemothermia). The sister-group of the Haemothermia was the Crocodilia and the sister-group of Haemothermia + Crocodilia was the Chelonia. It was acknowledged that recognition of the Haemothermia, though radical, owed much to Ray, writing in the 17th century. In the present analysis Gardiner's proposals are better supported than the conventional view of amniote interrelationships (see below) for which Gauthier *et al.* (1988) have, albeit ably, argued. Nevertheless, in the tree constrained to Gardiner's phylogeny, relationship between the tuatara and squamates is only very marginally closer than that between it and a crocodile + haemotherm clade or between tuatara and turtles (figure 8). Although the tuatara is constrained to be the sister-taxon of the squamates, it is extremely basal relative to the latter. Squamates are highly evolved in terms of newly acquired apomorphies relative to the tuatara. The tree is nearly three steps longer than the standard tree, with reduced CI (length 24159, CI excluding uninformative characters 0.604, rescaled CI 0.532). It cannot, therefore, be said that Gardiner's view that the Lepidosauria are the sister-group of all other amniotes is supported (this view is also rebutted if tuatara is not included in the Lepidosauria) but we have seen that other analyses, including that giving the standard tree, give some support to Gardiner's postulation of the group Haemothermia. The present tree, unequivocally supports the very plesiomorphic and isolated status of *Sphenodon*.

Tree constrained to the phylogeny of Gauthier et al. *(1988)*

When constrained, for taxa included in this study, to conform with the phylogeny proposed by Gauthier *et al.* (1988), a single tree (figure 9) is obtained (length 26926, CI excluding uninformative characters 0.540, rescaled CI 0.454) which is substantially longer (by nearly six steps) than the standard tree. The tree of Gauthier *et al.* differs very substantially from the topology which is produced from spermatozoal ultrastructure when no constraints are imposed, as in the standard or the unordered tree, notably in showing a special relationship between tuatara and squamates, in severing the relationship between mammals (here monotremes) and birds and in making the crocodile the sister-group of birds, all widely accepted relationships but here enforced by constraints. Although bootstrapping gives only two groupings a high significance (that of the two ratites and that of the squamates) the substantially greater length and low consistency of the Gauthier *et al.* topology in the present analysis leads us to conclude that this topology is not supported by sperm ultrastructure.

(*b*) ***Plesiomorphy of*** **Sphenodon**

All of unconstrained analyses and even the constrained analyses show the very plesiomorphic nature of *Sphenodon*. This plesiomorphy can also be expressed by examining the number of spermatozoal apomorphies of the tuatara and other amniotes relative to the outgroup. Table 2 shows the number of apomorphies, out of the maximum of 16 characters, for each member of the in-group relative to Chelonia (turtles). The Chelonia are scored as having no apomorphies, as they form the outgroup, but they presumably have some distinctive spermatozoal apomorphies. The only case in the analysis where the condition of a character in the ancestor at the base of the ingroup differed from that in the chelonian outgroup concerned, however, the number of mitochondria (six, against nine basal for the in-group).

Thus the table and all cladistic analyses indicate that *Sphenodon* is spermatologically the most plesiomorphic amniote, excepting the Chelonia. *Sphenodon* is apomorphic for the in-group in only two of the 16 known characters, that is, only 12.5 per cent of the characters computed. Squamates are approximately four to five times more apomorphic than the tuatara, with an apomorphy index relative to turtles of 57–63% compared with 12.5% in the tuatara. Further evidence which, like spermatology, lies outside conventional morphology and which separates squamates from *Sphenodon* is the demonstration that it possesses haemoglobin D which is also present in turtles and some birds but is absent from squamates (Abbasi *et al.* 1988).

The great apomorphy of the caiman indicates that the Crocodilia are more plesiomorphic and ancient

Table 2. *Apomorphy index for computed amniotes from spermatozoal data in increasing order of apomorphy relative to turtles which formed the outgroup*

taxon	number of apomorphies	total characters known	percent apomorphies (apomorphy index)
turtles	0	16	0
tuatara	2	16	12.5
crocodile	5	15	33.3
monotremes	7	16	43.8
guinea fowl	6	13	46.2
rooster	7	15	46.7
geckos	8	14	57.1
snakes	9	15	60.0
lizards	9	15	60.0
rhea	9	15	60.0
skinks	10	16	62.5
tinamou	9	14	64.3

than is generally believed. This is reflected in representation of the caiman as the sister-taxon of the bird–monotreme–squamate assemblage in the unconstrained, standard tree (figure 3). If the caiman is constrained in a monophyletic archosaur assemblage (figure 7) the tree is considerably less parsimonious and an improbable sister-group relationship of monotremes and squamates is produced. Saita *et al.* (1987) have previously recognized (in ignorance of the sperm of the tuatara) that 'the sperm of the alligator is more similar to the turtle spermatozoa than to the sperm of other reptiles'.

The tinamou has the most apomorphic sperm of all taxa included in the analysis. Nevertheless, non-passerines, included by Sibley *et al.* (1988) with ratites in the Eoaves, Passerines (Neoaves *sensu* Sibley *et al.*), not included in the analysis, have many unique spermatozoal apomorphies and their spermatozoa must be considered more highly derived than those of ratites.

It is thus demonstrated that spermatologically the tuatara has the very primitive status which is often accorded it on the grounds of somatic morphology and absence of an intromittent organ. Where no constraints are placed on its relationships in cladistic analysis *Sphenodon* is found to be the plesiomorphic sister-taxon of the caiman + birds + squamates. Even if a slightly longer tree which constrains *Sphenodon* with the squamates were accepted, and there is no strong reason why they should be, the primitive status of *Sphenodon* is strongly supported spermatologically in contradiction to recent claims (e.g. Whiteside, 1986) of the 'modernizing of a living fossil'.

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