A SYSTEMATIC REVIEW OF *AMIETIA VERTEBRALIS* (HEWITT, 1927) AND *STRONGYLOPUS HYMENOPUS* (BOULENGER, 1920) (ANURA: PYXICEPHALIDAE)

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Walk away quietly in any direction
and taste the freedom of the mountaineer...
Climb the mountains and get their good tidings.
Nature's peace will flow into you
as sunshine flows into trees.
The winds will blow their own freshness
into you, and the storms their energy,
while cares drop off like autumn leaves.

John Muir (1838 – 1914)

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DISSERTATION SUMMARY

For decades the taxonomic position of the Aquatic River Frog, *Amietia vertebralis*, and the Berg Stream Frog, *Strongylopus hymenopus*, has been a point of contention. A review of the literature, observations in the field and examination of preserved specimens, have led to speculation regarding the current classification of both species. *Amietia vertebralis* and *S. hymenopus* are highly aquatic anurans and both are endemic to the Drakensberg and Lesotho Highlands. There are a number of reasons for the inadequate information on both species, including that their initial descriptions were largely incomplete resulting in a complicated taxonomic history, and that the distribution area has been relatively poorly surveyed in terms of herpetofauna.

The aim of this dissertation is to provide clarification on the taxonomic confusion associated with these two species and to determine whether possible additional related species exist (as has been suggested). To ensure that the systematic review be as comprehensive as possible, both morphological and molecular techniques were employed. External morphological characters of most available specimens of both species from institutions within South Africa, as well as type specimens from museums abroad, were examined with the aim of determining clear diagnostic characters and to distinguish any clear trends that may indicate separate species. Because of the high level of morphological homoplasy among anurans, molecular techniques have proven invaluable in distinguishing between so-called cryptic species. Molecular analyses using both mitochondrial DNA (16S and ND2 fragments) and nuclear DNA (RAG1 and RAG2 fragments) was conducted to determine the extent of intraspecific variation within each species, as well as their phylogenetic position in relation to each other and the African clade of pyxicephalids in which they are currently placed.

Morphological assessment of museum specimens revealed a number of interesting discrepancies, especially with regard to the type and paratype specimens of both *A. vertebralis* and *S. hymenopus*. The type series of *A. vertebralis* appear to in fact be specimens of *S. hymenopus*, while the holotype of *S. hymenopus* (from the Natural History Museum, London) also does not match the species with which the name is

currently associated. In addition, the history and label information pertaining to this specimen revealed numerous inconsistencies and conflicts with what has been recorded in the literature. In its features this specimen most closely resembles a form of *Amietia fuscigula* from the Western Cape and it is suggested here that the name *Strongylopus hymenopus* be made *incertae sedis*. Statistical analysis of the morphological data confirmed the suspected differences between this holotype specimen and specimens currently identified as *S. hymenopus*, as well as mis-identifications a number of other specimens, and corrections for these have been suggested. Furthermore, morphometric analysis confirmed that *A. vertebralis* and *S. hymenopus* are very similar in terms of their body proportions, explaining, to some extent, why these species have sometimes been confused with one another.

Similarly, the molecular analysis produced some unexpected findings. Very little genetic variation was found to occur in *A. vertebralis* throughout its distribution, thus dispelling the hypothesis that additional species exist. Importantly, *S. hymenopus* was found not to be monophyletic with the *Strongylopus* genus, but rather to be a sister species to *A. vertebralis*. Together, *A. vertebralis* and *S. hymenopus* form a clade with *Amietia* sensu Frost (2006). In conclusion, name changes are suggested for both species, so that *Amietia vertebralis* is referred to as *Amietia umbraculata* and *Strongylopus hymenopus* becomes *Amietia vertebralis*. The current study adopts the nomenclature proposed by Frost *et al.*, 2006. Please note that, for ease of discussion, throughout this study both of the taxa under review are referred to by the names by which they are currently known, i.e. *Amietia vertebralis* and *Strongylopus hymenopus*. The concluding chapter discusses the nomenclatural changes that are necessary to correct the current taxonomy and the justification for these.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Systematics encompasses two broad domains, namely phylogenetics and taxonomy, which are used to explain the patterns and evolutionary history of biological diversity. Phylogenetics involves generating hypotheses regarding the evolutionary relationships between biological entities, while taxonomy uses these phylogenies to provide classifications of the taxa concerned (Hillis *et al.,* 1996; Kelly, 2005). This knowledge is essential to understanding the origins and constituents of biodiversity. With the aid of molecular and morphometric analysis, this study aims to elucidate the systematic status of two anuran species, the Aquatic River Frog (*Amietia vertebralis*), (Hewitt, 1927) and the Berg Stream Frog (*Strongylopus hymenopus*) (Boulenger, 1920), and thereby provide answers to questions pertaining to the taxonomy of these species that have been raised since their first description.

The necessity for comprehensive systematic records is illuminated by the fact that despite continuous new discoveries and significant advances in the technologies used to assess biological diversity, it is estimated that as many as 90% of living species remain undocumented (Hanken, 1999). This is particularly worrying at a time when environmental disturbance due to burgeoning human populations and the concomitant unsustainable utilisation of resources is likely to result in the reduction and probable extinction of untold numbers of species before they are described (Frost, 2006; Mayr, 2001; Meffe & Carroll, 1997).

This problem is especially relevant with regard to amphibians, which despite having one of the highest rates of descriptions of new species of all vertebrate groups, (Hanken, 1999; Köhler *et al.*, 2005) have experienced massive worldwide declines in recent decades (e.g. Houlahan *et al.,* 2000, Kiesecker *et al.,* 2001; Mendelsohn *et al.,* 2006). Two reasons for the significant increase in the number of described amphibian species are firstly, the recent use of molecular analysis to reveal cryptic species and

secondly, the ongoing debate over what constitutes a species (Hanken, 1999). Commonly known as "the species problem", the emergence of numerous definitions (or concepts) of species and their application can radically alter the number of species in question (Mayr, 2001). The correct differentiation of species is crucial to biology, since species form the basic units upon which diversity is measured as well as providing the study entities for evolutionary, biogeographical and ecological analysis (Kelly, 2005). Resolution of these problems will largely determine future estimates of future amphibian diversity and part of the solution lies in ongoing biological research together with a concerted effort to conserve the remaining biodiversity (Frost, 2006; Hanken, 1999; Kelly, 2005). Systematic reviews are integral to this process, and the most recent comprehensive review of amphibian systematics by Frost *et al.* (2006) has provided a good starting point for more in-depth testing of phylogenetic hypotheses among smaller groups.

While the systematics of southern African amphibians has received increased attention in recent years, especially in terms of molecular analysis (e.g. Cunningham & Cherry, 2004; Scott, 2005; van der Meijden *et al.,* 2005) there remain large gaps in the knowledge of the phylogeny of many genera. This is especially true for those species that occur in the relatively remote, and therefore poorly surveyed, Drakensberg and Lesotho highlands. Both *A. vertebralis* and *S. hymenopus* are endemic to this mountainous region and have not received as extensive systematic attention as those species in more accessible regions of southern Africa.

The classification of *A. vertebralis* has undergone several changes since the species' initial description by Hewitt (1927) and this, together with specific studies on certain morphological aspects, have led to the suggestion that additional species of this taxon may exist (Lambiris, 1991; Van Dijk, 1966). Similarly, observations in the field and conflicting evidence from museum records have led to doubt concerning the taxonomy of *S. hymenopus*. In addition this species differs significantly from all other species in the genus *Strongylopus*. Furthermore, similarities between *A. vertebralis* and *S. hymenopus* (both in lifestyle and morphology) have contributed to the confusion surrounding the classification of these species. These observations together with a long and contentious taxonomic history have led to the need for a complete revision of the current classification of both species.

1.2 A review of the literature

1.2.2 Taxonomic history of the Aquatic River Frog, *Amietia vertebralis*

Until recently, *Amietia vertebralis* was a monotypic species belonging to the Family Ranidae. Following Frost *et al.'s* (2006) publication of 'The Amphibian Tree of Life', it has been placed in the family Pyxicephalidae, along with a number of genera that comprise the African clade described by van der Meijden *et al.* (2005). In addition, all species previously in the genus *Afrana* have been placed together with *A. vertebralis* in the genus *Amietia* (Dubois, 1987). The name is derived from the genus *Amietia* (named after the herpetologist J.L. Amiet) and the specific name, *vertebralis,* which refers to the markings found along the back of the frog (Bates, 2004; Channing, 2001).

Hewitt first described the species as *Rana vertebralis* in 1927 based on a single immature specimen (with a snout-vent length [SVL] of 38 mm) and five additional juvenile specimens collected by Robert Essex in 1926 from the summit of Mont-aux-Sources, at the source of the Thukela River in the Northern Drakensberg (Hewitt, 1927). A separate female (SVL 101 mm) found at Rebaneng Pass was also considered by Hewitt to be of the same species (Bates, 2002), but is not included in the type series. Some of the characteristics noted by Hewitt include the broad and depressed head, small tympanum, slender toes with weak subarticular tubercles, dark cross bars on legs and the pale yellow underside to the femur.

Hewitt also referred to a number of tadpoles found in the vicinity of Mont-aux-Sources and "Thaba Putsua" noting in particular the characteristics of the oral disc (Bates, 2002). Part of the reason for the current confusion over the taxonomy of the species is due to this initial incomplete description. Hewitt (1927: 405) himself remarked that, "The exact status of this form is a little doubtful". In 1948 FitzSimons described 32 topotypic specimens (although he only gave diagnostic measurements for one specimen) as well as tadpoles found in the type locality, which were similar to those described by Hewitt (FitzSimons, 1948). In this description FitzSimons gives a comparison of the dimensions of *Rana vertebralis, Rana angolensis* and *Rana fuscigula.*

A separate species, *Rana umbraculata*, was described by Bush in 1952, based on a female specimen (assigned the Museum number U.N. 401) collected by Robert Crass (personal communication) in the Mzimkulu River, Drakensberg Gardens, KwaZulu-Natal and on eight additional specimens, which served as co- and paratypes, collected at the same time and location (Bush, 1952). *R. umbraculata* was so named for its possession of a large umbraculum – a process of the mid-dorsal edge of the iris, which overhangs the pupil of the eye (Bush, 1952; Ewer, 1952) and was distinguished from *R. vertebralis* on the basis of its larger size (SVL up to 140 mm), relatively broad head and differences in sternal apparatus:

1. The head of *R. umbraculata* is broader relative to the body-length than in *R. vertebralis.*
2. Specimens of *R. umbraculata* were much larger, with males being 1 ¾ and females up to 2 ¾ times the length of the largest known specimen of *R. vertebralis.*
3. The sternal apparatus differs in that the presternum in *R. umbraculata* terminates anteriorly in an expanded cartilaginous plate, which is absent in *R. vertebralis*; the ossified omosternum is more slender in *R. umbraculata*; the ossified metasternum is longer and more slender and lacks the double indentation at the posterior end as seen in *R. vertebralis*; and the xiphisternum of *R. umbraculata* is broader and more deeply notched than in *R. vertebralis* (Bush, 1952).

However, studies by Poynton in 1964 found that head width of sub-adults was intermediate between *R. vertebralis* and *R. umbraculata*, suggesting that *R. umbraculata* represented the adult form of *R. vertebralis* (Poynton, 1964; Bates, 2004). He also found the differences in sternal apparatus to be insignificant and consequently *R. umbraculata* has been considered to be synonymous with *R. vertebralis* (Poynton, 1964).

This synonymy, however, did not put an end to the debate and a number of authors have continued to question this classification. In his work with metamorphosing tadpoles, Van Dijk (1966) concluded that *R. vertebralis* and *R. umbraculata* were two

sympatric species that could be differentiated on the basis of keratodont, spiracular opening and neuromast organ characters. Later, Lambiris (1991) suggested that as many as three possible species exist – *R. vertebralis, R. umbraculata* and a third undescribed taxon, *Rana* "sp. A" based on differences in laryngeal morphology. In this study Lambiris suggested that the third potential species (*Amietia* "sp A") may also be distinguished by differences in distribution, vocalisation and morphological features and keratodont formula (Table 1.1).

The advertisement calls of males are essential for recognising amphibian species (Lambiris, 1991; 1994). Lambiris based his study of laryngeal and buccopharyngeal internal morphology on the assumption that these attributes are directly related to calls and therefore may be taxonomically useful in differentiating between species (Lambiris, 1991). Lambiris's suggestion of three separate species was based largely on tadpole buccopharyngeal morphology. Adults are indistinguishable in terms of external morphology, but differ in laryngeal morphology. However adult male larynxes were not available from *Rana vertebralis*, while only three adult specimens of both *Rana umbraculata* and *Rana sp. A* were examined. Furthermore, no topotypic larval material was available for assessment of *R. umbraculata.*

TABLE 1.1: Three species of *Amietia vertebralis* according to Lambiris (1991).

	Distribution	**Altitude**	**Tadpole length (Stage 38-40)**	**Labial tooth-row formula**
Rana vertebralis Hewitt 1927	Northern Drakensberg range (Mont-aux-Sources) and Lesotho	Above 2800m	50mm	5(3-5)/4 to 7(3-7)/4
Rana umbraculata Bush 1952	Central Drakensberg range	Below 2500m	Not given	8(3-8)/4 to 10(3-10)/4
Rana sp. A	Lesotho plateau above central and southern KZN/Lesotho Drakensberg range	Above 2900m	75 – 80mm	5(2-4)/3(1)

Thus a number of conflicting views have arisen regarding the taxonomic position of *A. vertebralis* since Hewitt first described the species in 1927. The multiple name changes and the establishment of the genus *Amietia* (Dubois, 1987) have further contributed to the confusion surrounding the species. This confusion is also reflected in well-respected field guides. For example, the description of *Rana vertebralis* in, "South African Frogs: A complete guide" (Passmore and Carruthers, 1995) shows photographs of two distinct species, one recorded from Sani Pass (which looks like *A. vertebralis*) and the other from Mont-aux-Sources (which looks like *S. hymenopus*). Additional inconsistencies are evident from habitat descriptions and locality information.

In order to resolve whether more than one taxa can be assigned to *Amietia vertebralis* further assessment of both morphological features and genetic relationships by molecular methods is required, as well as an in-depth study of vocalisation over the species' range (Bates, 2002).

1.2.3 Taxonomic history of the Berg Stream Frog, *Strongylopus hymenopus*

The description of "*Rana hymenopus*" by Boulenger (1920) is based on a single female specimen with SVL 57 mm. This specimen, apparently collected by Sir Andrew Smith, is presumed to have come from "South Africa". The collection date is not known, but, a re-registration date of 1933 is recorded, and a second acquisition date by the British Museum is recorded as 1947 (following the Second World War when specimens where removed from the museum in London and hidden in caves in the country). Sir Andrew Smith passed away in 1872, while Boulenger described the specimen in 1920. Furthermore, Sir Andrew Smith is not thought to have surveyed the north-eastern regions of the Drakensberg and Lesotho during his collections in southern Africa. This immediately raises questions as to the validity of this holotype specimen and its associated collection information. A more extensive discussion of this holotype specimen is given in Chapter 3.

Some of the characteristics Boulenger describes with regard to this specimen are the broad head, rounded snout, distinct tympanum, smooth skin and slender fingers. He describes the subarticular tubercles as being large and prominent and the hind feet as being half-webbed, with three phalanges of the fourth, and two of the third and fifth toe free. The colouring is described as greyish olive with dark and irregular spots above and white with a spotted throat below. The hind limbs are dark brown with regular dark "cross-bars". In reference to this specimen, Boulenger (1920: 106) comments "In its half-webbed toes this frog constitutes an interesting link between the typical *Rana* and the group *Strongylopus* of Tschudi (1838)." As in the case of *A. vertebralis*, the incompleteness of this initial description (based on a single specimen, lacking detailed locality information and without diagnostic comparison to other specimens) has resulted in much subsequent confusion regarding the species.

Lambiris (1987) suggested the possibility of two species, namely *Strongylopus hymenopus* and a cryptic and undescribed species whose tadpoles he described. The *S. hymenopus* tadpoles are described as having an umbraculum, a large spiracle, 4 rows of keratodonts, a flattened body and tail and fins originating well behind the base of the tail The undescribed taxon lacks an umbraculum, has a small spiracle and only 3

rows of keratodonts (Lambiris, 1987). In a subsequent study of buccopharyngeal characteristics (Lambiris, 1991), only one larval specimen was examined, since most of the material available was in too poor a condition for accurate assessment. Furthermore, no accurate locality information for the examined specimens was available; suggesting that further description of this taxon is necessary.

In terms of morphology *S. hymenopus* differs substantially from other *Strongylopus* species. *Strongylopus* species are characterised by having slender, streamlined bodies, pointed snouts and long legs. The toes are very long and usually have very restricted webbing (only up to the first phalange). Most species occur in grassland and forest and lay their eggs on moist earth out of water (Channing, 2004). In characters such as the stocky build, relatively short toes, extensive webbing, rough skin, presence of umbraculum, rounded snout, position of nostrils and habitat, *S. hymenopus* differs from the other species in the genus, but resembles *A. vertebralis*. In addition, the distribution of *S. hymenopus* is similar in some areas to that of *A. vertebralis*, and both species are adapted to an exclusively aquatic lifestyle.

1.3 Research aims and objectives

Observations in the field and conflicting evidence from museum specimens have led to the need for a taxonomic revision of both *Amietia vertebralis* and *Strongylopus hymenopus*. The broad aims of this study therefore, through the examination of museum specimens, more extensive fieldwork and molecular analysis, are to test various hypotheses regarding the phylogeny of both species, in particular, the possible existence of additional species of *A. vertebralis* (as has been suggested) and to apply the findings to questions regarding the taxonomy of the species and provide suggestions for nomenclatural changes. This dissertation is structured as follows:

- Chapter 2 gives notes on the biology and distribution of the study species and a description of the study area. It provides a brief overview of materials and methods used for the different aspects of the study.

- Chapters 3 and 4 are the experimental chapters of this dissertation. As such they are each set out in the form of a scientific paper and include their own abstract, introduction, methods, results and discussion. Chapter 3 discusses the use of morphological assessment for determining clear diagnostic characteristics for both *A. vertebralis* and *S. hymenopus*.

- Chapter 4 provides a phylogenetic overview of *A. vertebralis* and *S. hymenopus* and its main aim, through the use of DNA sequences, was to elucidate the evolutionary relationships of and these species in relation to each other and within the African family Pyxicephalidae.

- Chapter 5 gives a brief re-description of *S. hymenopus* based on recently collected specimens from Mont-aux-Sources.

- Chapter 6 provides a summary of the conclusions of the previous chapters and discusses potential future work as well as the conservation implications for the two species concerned.

CHAPTER 2

GENERAL MATERIALS & METHODS

2.1 Study area

2.1.1 Lesotho and the Drakensberg Mountains

The study area included the regions of Lesotho and the Drakensberg mountain range (Fig. 2.1). The Kingdom of Lesotho is a small, landlocked country, completely surrounded by South Africa. It lies between latitudes 28°34' and 30°31S and longitudes 27°00' and 29°28'E and has an area of roughly 30 355 km^2. The Caledon River demarcates its north-western boundary to the Free State, while the Drakensberg-Maluti escarpment forms its eastern boundary with Kwa-Zulu Natal. Political borders from Wepener to Mohale's Hoek form the southwest boundary. There are currently 23 species of frog that are recorded from the country (Bates & Haacke, 2003).

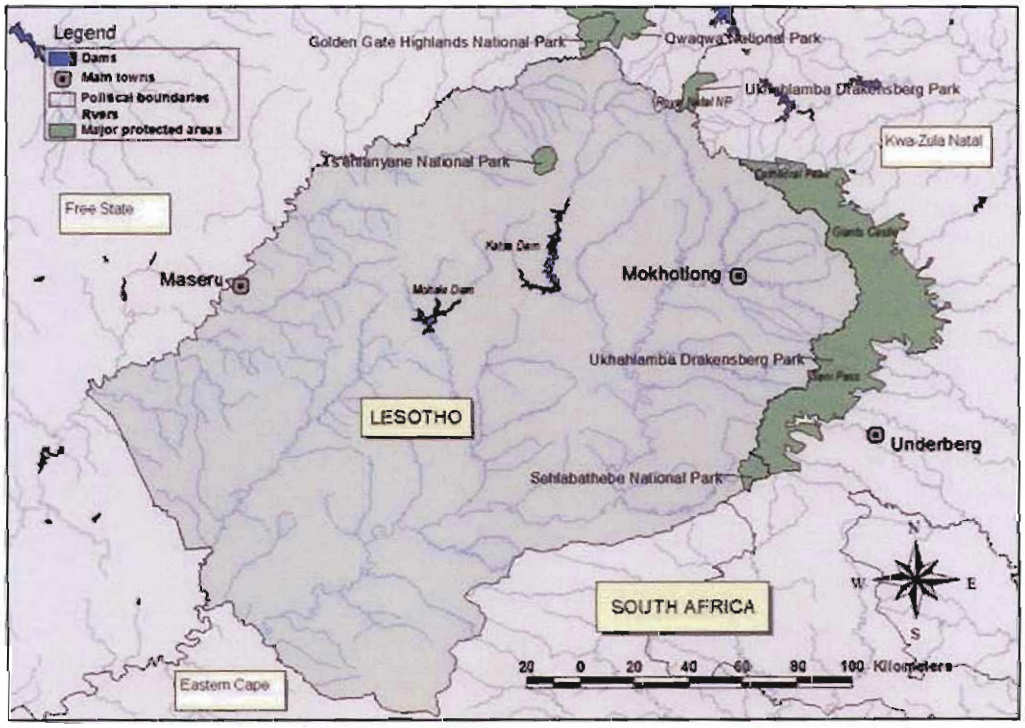

FIGURE 2.1: Map of Lesotho and the Drakensberg.

The Drakensberg forms the eastern boundary of Lesotho with Kwa-Zulu Natal, the Eastern Cape and the Free State and its diverse array of habitats host as many as a quarter of all frog species that occur in South Africa (Lambiris, 1988). Many of these species occur throughout southern Africa, but some are unique to the high altitudes of the Drakensberg Mountains.

Geology and Topography

Also, known as "the Mountain Kingdom", Lesotho is the only independent state that lies entirely above 1000m in elevation (http://en.wikipedia.org/wiki/Lesotho). Also of interest is that it has the "highest low point" of any country in the world (at 1380 m in the south-west at the junction of Kornetspruit and the Senqu river) (Suchet, 2006). It's highest point, Thabana Ntlenyana at 3482 m, is the highest point in southern Africa south of Mt Kilimanjaro. Ironically the name means "The Little Mountain that is a Little Bit Nice" (Suchet, 2006). Most of the country is a mountain plateau, carved out by river valleys, most of which drain into the Senqu basin. The corridors formed by the rivers traversing the mountains create steep gradations in relief, altitude and habitat within relatively short distances (Bates & Haacke, 2003).

The mountain ranges within Lesotho are collectively known as the Maluti Mountains. These ranges run from the northeast to the southwest of the country, with the area between the Caledon River and the first mountain ranges forming what is known as the Lowlands (somewhat of a misnomer since this area makes up the elevated plateau of inland southern Africa and averages 1600 m above sea level). Here the eroded sandstone forms many interesting rock formations. The mountain peaks and ridges of the Malutis are formed from volcanic basalt, which has resisted extensive erosion (Suchet, 2006). The Drakensberg forms the eastern border of Lesotho and extends from Metjhatjhaneng Peak (~3100 m) in the Free State, where the north-facing Drakensberg meets the main ridge of the Maluti Range from the south. From this northern-most point the escarpment curves to the east, along the border of Kwa-Zulu Natal and Lesotho with crest elevations ranging between 2900 m and 3200 m. Landmark features of the Berg include the Amphitheatre, The Saddle, Cathedral Peak, Champagne Castle and Giant's Castle. From Giant's Castle, the escarpment turns abruptly to the south-west, continuing past Sani Pass and Drakensberg Gardens to

Bushman's Nek near the border between Lesotho and Kwa-Zulu Natal, again rarely dropping below 2800 m in elevation. From here the basaltic highland recedes into Lesotho, as a peninsula flanked by river valleys, with the sandstone Little Berg forming the border around Sethlabathebe National Park in Lesotho, extending to the Kwa-Zulu Natal – Eastern Cape Provincial border. From this point the escarpment turns west-south-west and runs along the international border parallel to Senqu valley of Lesotho. The average elevation here is 2300 m, dropping below 2200 m at Qacha's Nek, forming an altitudinal and climatic barrier between the highland plateau to the north and south. The escarpment turns South again near Ongeluksnek, reaching the southernmost point of Lesotho near Naudésnek Pass, and then continuing for about 80 km in the direction of Indwe in the Eastern Cape (Bristow, 2003; Michael Cunningham, personal communication).

The Drakensberg is comprised of the rocks from the Stromberg Group, which is made up of a number of layers. The Molteno Beds form the lowest level and consist of the sandstones that comprise the base of the Little Berg (or foothills of the Drakensberg). The second layer, the Red Beds, is largely mudstones and shales and makes up the steep slopes of the mid-Little Berg. Fossils of the mammal-like reptiles from the Karoo dynasty are common in this layer. The top layer, known as the Cave Sandstone layer, is comprised mostly of aeolian deposits and as such is very soft and prone to erosion and makes up the cliffs and caves that characterise the Little Berg. The main peaks of the Drakensberg escarpment are a result of lava flows from the time of the Gondwanaland break-up and are comprised largely of basalt, which is gradually being weathered away (Bristow, 2003).

Rivers and wetlands

The Senqu River in Lesotho gives rise to the largest river system in South Africa (the Orange River) (Swartz, 2005). Together with its tributaries, especially the Senqunyane and Malibamatšo rivers, the Senqu absorbs most of the drainage in Lesotho. Other major rivers include the Makhaleng, Mohokare (Caledon), Khubedu, Mokhotlong, Sehonghong and Maletsunyane. Pans and marshes in the western regions are also an important seasonal source of water (Bates & Haacke, 2003). Shallow pools on sandstone beds known as tarns are particularly common the southern sandstone plateau, such as at Sehlabathebe National Park. Sponges and fens

are common throughout the highlands, especially near river sources. These wetland areas provide important breeding habitat for montane frogs and are unique due to the association of peat accumulation and gravel beds in alluvial fans (Bates & Haacke, 2003). Large areas of open water do not occur naturally in Lesotho, but has increased due to dam building in recent years. The Katse dam in the central mountainous region is part of the Lesotho Highlands Water Project and covers 36 km^2 and is now the primary source of the country's GDP through the export of water and electricity to supply Gauteng in South Africa (http://en.wikipedia.org/wiki/Lesotho). The Mohale Dam and Matsoku Weir are similarly large, and construction of additional dams has recently been approved as a continuation of this scheme.

Climate

The weather in this region varies considerably between seasons, with cold, dry winters and hot, humid summers, and can also fluctuate rapidly on any given day (Suchet, 2006). In general, due to its altitude, it is cooler throughout the year than other regions at the same latitude (http://en.wikipedia.org/wiki/Lesotho). The mean annual temperature in the elevated regions in the east is 12°C and 14 - 16°C in the lower west and Senqu valley in the southwest. Mean daily temperature in January is 18°C in the eastern and central parts of the country and 20°C in the west, while mean daily temperature in July is 6°C in the east, increasing to 8°C in the west (Bates & Haacke, 2003). Extreme variations occur in some areas, for example at Letseng-la-Terae, temperatures of 31°C in summer and –20.4°C in winter have been recorded, while the average annual temperature for the area is just 5.7°C (Bates & Haacke, 2003).

Lesotho experiences clear skies for the majority of the year, with an annual mean of 8.8 hours a day of sunshine. During the winter months 82% of all possible sunshine is experienced and even in the cloudiest months 67% of possible sunshine occurs. The total annual solar radiation for the country is approximately 5700 – 7700 MJ/m^2 (Bates & Haacke, 2003).

The mean annual precipitation for Lesotho is 725 mm, with the majority of this occurring during spring and summer (October to April). During summer thundershowers and hail storms with strong winds are common. Lesotho experiences

one of the highest incidences of lightning in the world, with approximately 5 – 12 strikes per km^2 per year, resulting in a high occurrence of lightning-induced fires. Mean January precipitation ranges between 50 – 150 mm across most parts of the country, but can reach as much as 200 mm in the Drakensberg area in the east. Average rainfall can fluctuate considerably from year to year, for example Sani Pass summit had annual rainfall of 1441.6 mm in 1938/39, but only 439.3 mm in 1944/45 (Bates & Haacke, 2003). Winter precipitation occurs in the form of snow, especially in the eastern highlands, where in the highest parts it has been recorded to fall at any time of the year. Mean July precipitation for most of the country is 10 – 50 mm, with the exception of a small area in central and eastern Lesotho where it is 0 – 10 mm.

The eastern escarpment experiences a high incidence of mist, with up to 403 mm being contributed to the annual amount of precipitation in the Drakensberg foothills. The highlands experience frost during the winter months, and this too varies considerably between areas and from year to year. Evaporation in winter has a monthly mean of 60 – 70 mm, increasing to 175 – 225 mm in summer. This rate of evaporation usually exceeds the annual rainfall, especially in summer, for most of the country (Bates & Haacke, 2003).

Vegetation

Most of Lesotho's vegetation can be classified as belonging to the Grassland Biome, with the eastern alpine region falling under the Nama-Karoo Biome and a very small section being represented by the Forest Biome (Killick, 1978; Low & Rabelo, 1996). The lack of trees is one of the most striking aspects of Lesotho (Suchet, 2006). The Drakensberg grasslands are dominated by C_3 cool-temperate species, many of which are endemic to the area. Of the five Centres of Plant Endemism (CE) that have been identified within the Grassland Biome, the Drakensberg Alpine CE has the highest endemism (13%), which is thought to be linked to the speciation events brought about as a result of the climatic changes during the Plio-Pleistocene (circa 5mya) (Mucina & Rutherford, 2006).

There are six vegetation types that comprise the Grassland Biome vegetation, with Mountain Grassland types being dominant (76.5%). These types consist of Afro Mountain Grassland, Alti Mountain Grassland and Moist Upland Grassland. The

remainder are made up of Highveld Grassland types, namely Moist Cold Highveld Grassland, Moist Cool Highveld Grassland and Wet Cold Highveld Grassland. The Afro Mountain Grassland covers 52.4% of the country and is found on the moist, steep slopes of the Kwa-Zulu Natal mountains. Alti Mountain Grassland (24.1%) occurs on the steep, treeless Upper Mountain region, while Moist Cold Highveld Grassland (22.6%) covers most of western Lesotho (Bates & Haacke, 2003).

The flora of the Drakensberg foothills (Little Berg) is mostly Afro-montane, while that of the summit is Afro-alpine. These broad categories can be further split into three zones: the montane zone (made up of the grassland and temperate forest of the lower slope); the sub-alpine zone (which consists mostly of grassland up to the base of the Escarpment cliffs); and the alpine zone (heath and scrub at the summits). These divisions in vegetation are largely due to variation in altitude (Bristow, 2003).

Conservation areas

Much of the country has been modified to meet the pastoral needs of what is largely a farming population. Because of the lack of trees, the woody shrubs have been exploited to provide firewood. Most large wildlife has been hunted to extinction, although a number of smaller mammals, such as the grey rhebok and jackals are common and bird life, especially birds of prey, is plentiful (Suchet, 2006).

Protected areas include the Ts'ehlanyane National Park, Sehlabathebe National Park and the Bokong Nature Reserve. South Africa's fifth transfrontier park, the Maloti Drakensberg Transfrontier Park between Sehlabathebe and the uKhahlamba Drakensberg Park World Heritage Site was proclaimed on 4 September 2007.

Much of the Drakensberg escarpment is protected within the uKhahlamba-Drakensberg Park, which encompasses 243 000 hectares and is one of the world's 24 World Heritage Sites. The area is rich in indigenous flora and fauna, including a 2500-strong herd of eland as well as numerous other antelope and other small mammals. The Park is also home to over 300 bird species, including the rare but well-known Bearded Vulture (or Lammergeyer) (Bristow, 2003).

2.2 Species Description

A description is essential to the process of systematic revision so that the taxon involved can be subsequently recognised and distinguished from others (Mayr & Ashlock, 1991). General descriptions of *A. vertebralis* and *S. hymenopus* are given here, detailing morphological characteristics and other broad information about the two species as we currently know them. The following chapter on morphological assessment provides more in depth diagnosis to provide specific details particular to each taxa, which can be used to compare them with other species. *A. vertebralis* and *S. hymenopus* both belong to the Anuran family Pyxicephalidae and occur in the high-altitude streams and rivers of Lesotho and the adjacent Drakensberg Mountains of Kwa-Zulu Natal, the Free State and the Eastern Cape. Both species are endemic to this area. In addition to both having a complicated taxonomic history, the species are similar to each other in a number of ways (both morphologically and in life history) and for this reason it is imperative that clear diagnostic characters be assigned to each species to enable easy identification.

2.2.1 Description of the Aquatic River Frog, *Amietia vertebralis*

Adults

Figs 2.2 – 2.4 show examples of *A. vertebralis*. The primary, and most noticeable, characteristic of the adults of *A. vertebralis* is that they can grow to be very large, reaching snout – vent lengths (SVL) of 145 – 160 mm. With the legs extended the frogs can reach up to 350 mm from snout to toes (personal observation). They are the second biggest species in southern Africa, after the Giant Bullfrog, *Pyxicephalus adspersus* (Bates, 1991). The head is notably very wide and flattened, and up to half the body length (Channing, 2001; Wager, 1991). Bates (2001) found significant variation in head width, with adults having wider heads relative to snout-urostyle and tibia length. The body has a strong, compact build with muscular legs enabling powerful swimming (Bush, 1952). The colouring of the back is usually grey-brown with rings of black spots and often with three to four light grey vertebral spots located along the middle of the back (Bates, 2002; Wager, 1991). The hind legs are banded, with the dark bands being equal in width to the paler bands.

This dull colouring with lack of bright markings or bold patterning suggests the habit of living on stony or muddy substrates, with infrequent periods spent at the surface or on land (Bush, 1952). The venter is creamy white with dark vermiculations (Carruthers, 2001). The extent of these vermiculations can vary considerably from being quite pale and only on the throat to being thick and dark and extending throughout the ventral area. One of the best diagnostic features is the extensive webbing (Fig. 2.4), which extends beyond the last subarticular tubercle of the longest toe, again indicative of the fully aquatic lifestyle (Passmore & Carruthers, 1995). The tips of the fingers and toes are squared-off. The eye has a protective outgrowth above the pupil called an umbraculum, which is thought to block UV radiation and is found in a number of high-altitude animals (Channing, 2001).

FIGURE 2.2: Dorso-lateral view of *Amietia vertebralis* in its natural habitat.

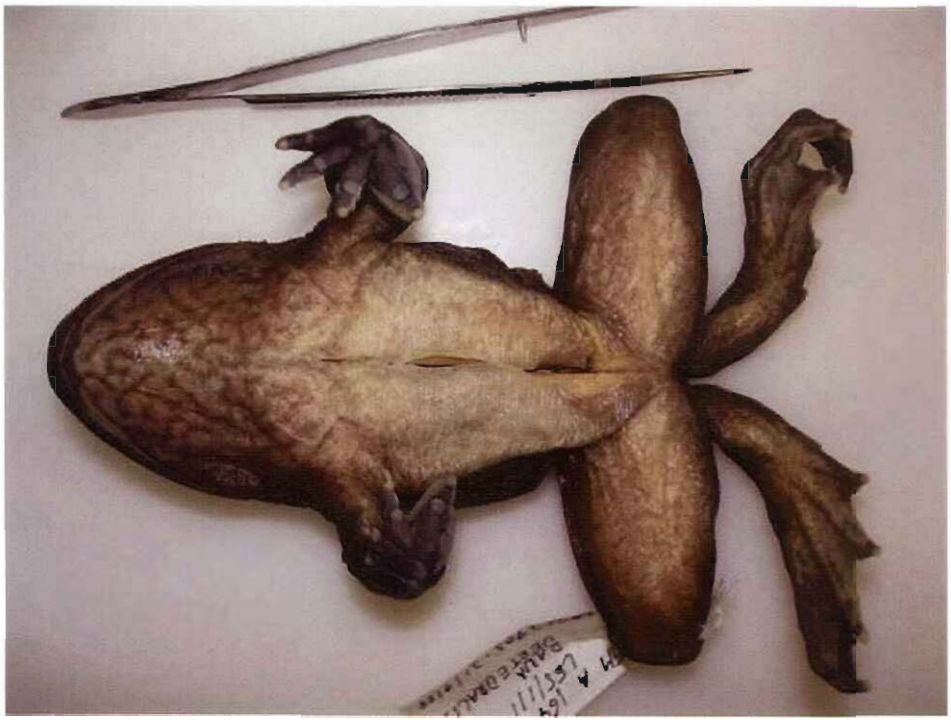

FIGURE 2.3: Ventral view of a preserved specimen of *Amietia vertebralis* (PEMA 1646 Sehonghong River, at bridge on Sani Rd).

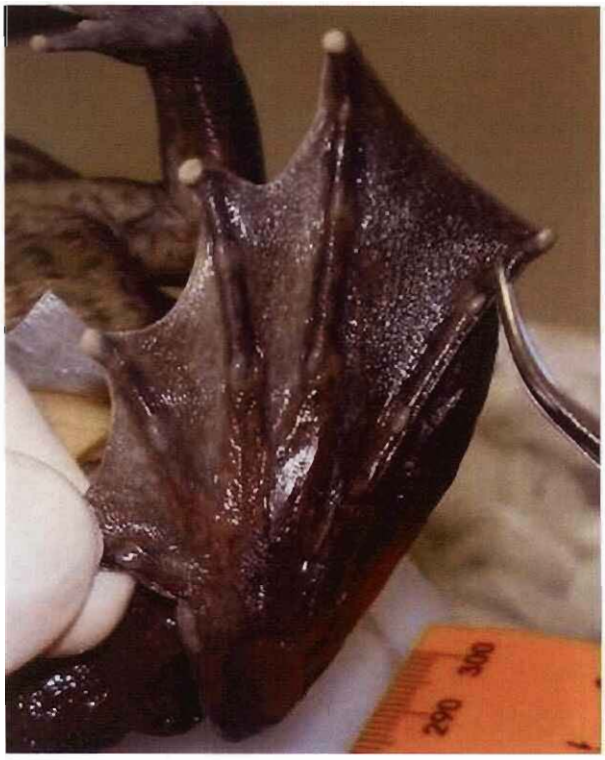

FIGURE 2.4: Detail of the extensive webbing on the hind foot of *Amietia vertebralis* (PEMA1688, Sehonghong River, at bridge on Sani Rd).

Tadpole description

Tadpoles reach 50 mm in length at Gosner stage 36. The colouring is mottled brown on the dorsum, pale on the ventrum and cloudy on the tail (Fig. 2.5). The body is dorso-ventrally flattened and the dorsal fin rises sharply, well beyond the base of the tail and is bluntly rounded (Lambiris, 1988). Tadpoles also have a relatively large, wide mouth containing many rows of keratodonts (Channing, 2001). The labial tooth row formula according to Lambiris (1988) is 5(3-5)/4 to 10(3-10)/4.

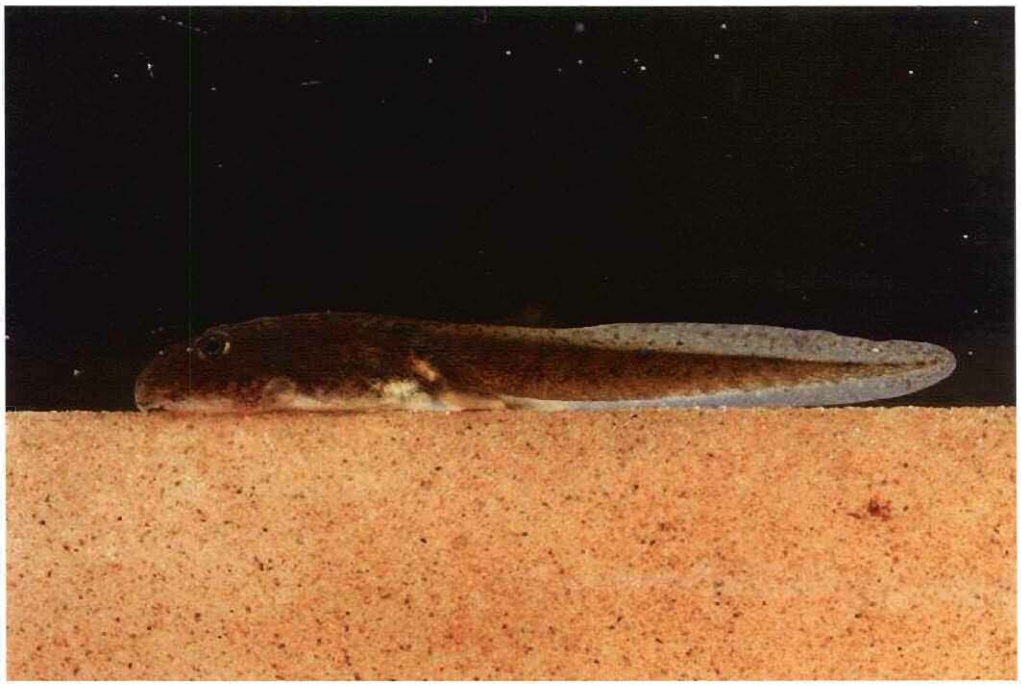

FIGURE 2.5: Tadpole of *Amietia vertebralis*.

Van Dijk (1966) found that tadpole mouthparts vary considerably within species, even at the level of local populations. However, the variability found in *A. vertebralis* seems to exceed that of most other tadpoles of South African species (Bates, 2002). Apparently, this variability among tadpoles is quite common among high altitude species (Miguel Vences, personal communication). Tadpole development is slow, and has been recorded as taking up to two years in captivity (Bates, 2004). The sucker-like oral disc enables the tadpoles to be well adapted to life in fast-flowing water (Bates, 2004).

Habitat and life history

The adults are good swimmers and are largely aquatic, preferring cold, clear mountain streams with rocky substrates (Lambiris, 1991). They can sometimes be found on or under rocks or amongst vegetation at the water's edge or in swampy areas (Bates, 2004). The adults, because of their large skin surface area are able to remain completely submerged for long periods, and have been observed to spend up to 30 hours under water (Channing 2001; Wager, 1991), while tadpoles and juveniles spend more time closer to the surface and in shallower pools (Bates, 2004). The literature reports that both adults and tadpoles are intolerant of high temperatures (above 8°C) (Lambiris, 1991) However during the course of this study specimens were found in water with temperatures up to 16°C during the summer months (both in captivity and in the wild). During the winter months both adults and tadpoles have been observed swimming under the ice that frequently forms a layer on the rivers in the highlands.

Although they are found most commonly where conditions are classified as pristine, during this study large numbers of adults were observed in the Sani River near the Lesotho border post, the water of which is quite polluted from the laundry activities and debris from the nearby village. It is thought that their abundant occurrence here may be a result of the overall increased productivity in the area.

Adults have been observed to remain in the same position, with only the head and shoulders protruding from the water, for long periods during daylight hours (Bush, 1952). If disturbed, they will quickly retreat below water, and re-emerge to resume their crouching position after about 10 minutes. They have been observed to repeat this behaviour for periods of up to three weeks, and this, as well as the extensive webbing on the hind feet and smooth skin, suggests that the species is almost completely aquatic (Bush, 1952; Channing, 2001). The tadpoles also favour cold (often ice-covered and generally below 8°C) flowing, rocky-bottomed streams and have flattened bodies adapted to such conditions (Channing, 2001).

Breeding occurs during the warmer months (September- February), with males calling from under the water or with just the head protruding (Bates, 2004). Eggs are laid in

large, sticky clutches in slow-flowing water and become attached to sunken vegetation (Bates, 2004).

Diet

Adults eat a range of arthropods, gastropods, crustaceans (especially crabs), and smaller species of frogs, which are consumed under water (Channing, 2001). Specimens in captivity have even been observed to eat mice (Bates, 2004) and one specimen caught by the SAIAB electro-fishing expedition of 2001 was observed to eat the trout with which it was captured (Ernst Swartz, personal communication). The diet of tadpoles consists largely of unicellular and small multi-cellular algae (Lambiris, 1991). As they grow they also begin to scavenge on detritus (Bates, 2004).

Advertisement call

There is a need for good quality recordings of vocalisation for this species. According to Channing 2001, " The call consists of a very long series of low knocks, followed by a short croak. The knocks are uttered at a rate of 6.2 – 8.4/s, for a duration of 3 – 11 s. The croak lasts 0.03 – 0.57 s, and exceptionally up to 3 s." The species is diurnal and therefore most calls can be heard during the day (Michael Cunningham, personal communication, 2006).

2.2.2 Description of the Berg Stream Frog, *Strongylopus hymenopus*

Adults

The adults of *S. hymenopus* are comparatively small, reaching SVL of 45 – 60 mm. This species too is fully aquatic and has a compact body shape, relatively broad head and rounded snout. The colouring is mostly slate grey to brown on top with darker grey markings in a V or X shape (Carruthers, 2001), while the underside is yellow-white with dark stippling on the edge of the throat and legs. The skin of the dorsum is covered in wart-like projections (Fig. 2.6). The hind legs are banded, with the width of dark and light bands being roughly equal. The toes are thin and tapering, with weakly pronounced tubercles and extensive webbing (Lambiris, 1987). The body is muscular and the forearms, especially in males, are robust. The webbing extends to the tips of the toes, but is more deeply incised than that of *A. vertebralis* (Fig. 2.8). The tympanum is relatively small and the nostrils are located centrally on top of the snout. This species also possesses the umbraculum in the eye.

FIGURE 2.6: Dorso-lateral view of *Strongylopus hymenopus* in its natural habitat.

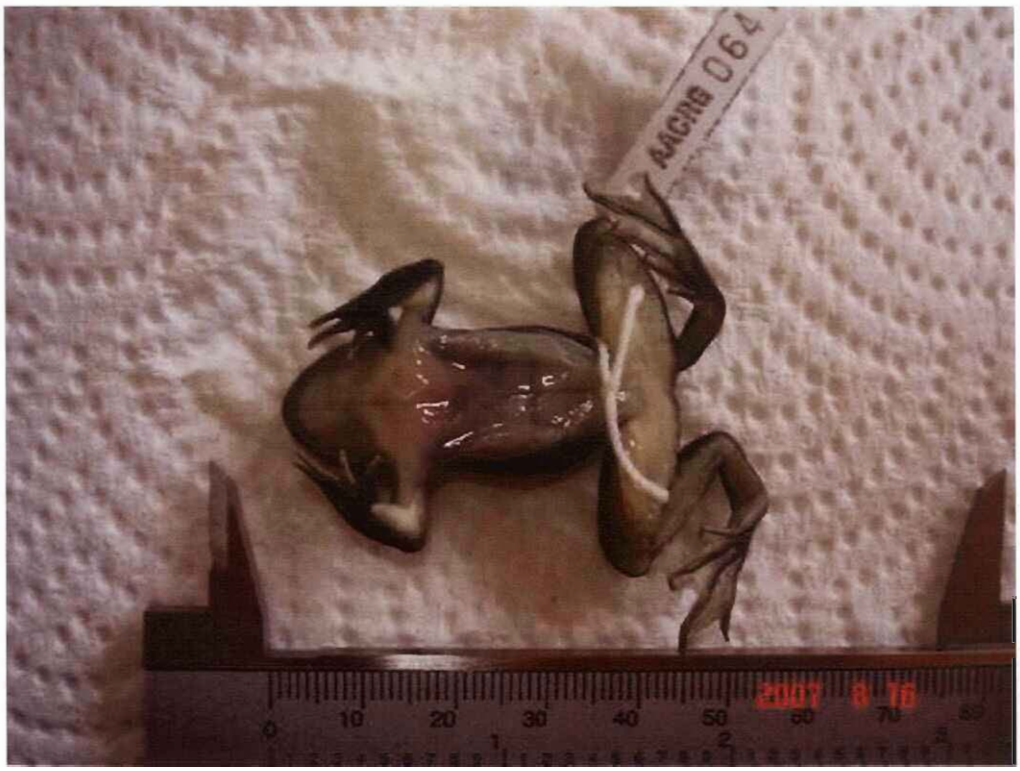

FIGURE 2.7: Ventral view of a preserved specimen of *Strongylopus hymenopus* (specimen AACRG 0647, Mont-Aux-Sources).

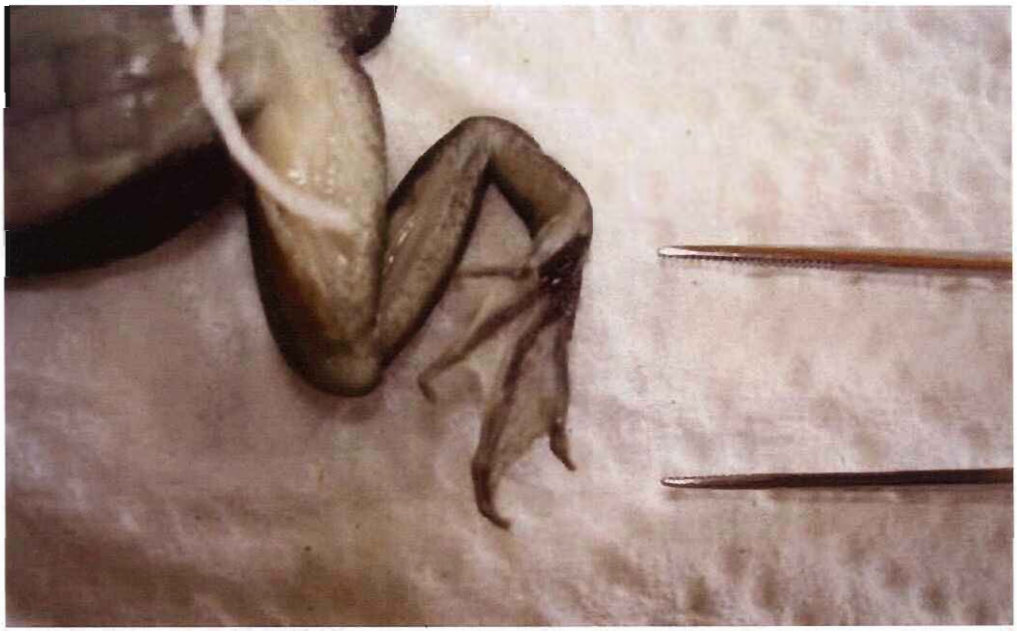

FIGURE 2.8: Detail of the webbing of *Strongylopus hymenopus* AACRG 0647, Mont-Aux-Sources).

Tadpole description

Tadpole SVL reaches 40 mm at Gosner stage 38. The colouring of the dorsum is brownish-grey with dark spots and the underside has a pale golden hue (Fig. 2.9). The tail fins are stippled. The body itself is plump and rounded and the tail is narrow, broadening at the base of the body and blunt at the end. The spiracle is large and rounded and visible from above. The oral disc has a single row of papillae and is not visible from above. The labial tooth row formula is 3 (2 - 3) / 3 or 3 (2 - 3) / 3(1-2) (Lambiris, 1987; 1988).

FIGURE 2.9: Tadpole of *Strongylopus hymenopus*.

Habitat and life history

These frogs are also highly aquatic and occur in high-altitude streams surrounded by Alti Mountain Grassland (Lambiris, 1991; Channing, 2004). Adults forage in vegetation on stream banks and on gentle slopes of the plateau (Channing, 2004). They breed in slow-flowing, sandy-bottomed streams and near the edges of pools, often preferring marsh-like conditions as breeding grounds. Advertisement calls have been heard after the first spring rains in September though to March, but amplecting pairs have been seen as early as July, indicating that the species are opportunistic

breeders. Amplexus can last a number of days, resulting in welts developing under the female's arms from the male's grip (Michael Cunningham, personal communication).

Clutches of 200 – 500 eggs are laid in the water (unlike other *Strongylopus* members) under banks and attached to rock in fast flowing streams or deposited in shallow pools in marshy areas. The tadpoles are found in shallow, cold (often ice-covered during the winter months) streams with a sand/pebble substrate (Lambiris, 1987). The tadpoles have been reported to show signs of hypothermic distress at temperatures above 8°C (Lambiris, 1987), although from personal observation (tadpoles where kept from October – December 2006) this is not the case. Tadpole development is thought to take up to two years.

Diet

Adults feed mainly on insects. The diet of tadpoles consists of small filamentous algae (Lambiris, 1991). In captivity tadpoles can subsist on a diet of pre-frozen lettuce and fish flakes.

Advertisement call

The distinctive call is issued under water (Michael Cunningham, personal communication) or at the water's edge and is described as a burst of rattling followed by long silences (Carruthers, 2001; Lambiris, 1991). Two notes are issued approximately 1 second apart, over a low continuous tone (Lambiris, 1991). The call is very dissimilar to other species in the *Strongylopus* genus.

2.3 Species Distribution

2.3.1 Distribution of *Amietia vertebralis*

A. vertebralis is a high-altitude montane anuran (usually occurring between 1600 – 3400 m above sea level) and is endemic to the study area. It occurs mainly in the Afro Mountain Grassland and Alti Mountain Grassland areas of Lesotho and is found in most of the major rivers and their tributaries, as well as in the upper reaches of the Thukela and Mzimkulu rivers in Kwa-Zulu Natal, the Elands River in the Free State, and the Bell River in the Eastern Cape (Fig. 2.10) (Bates, 2002).

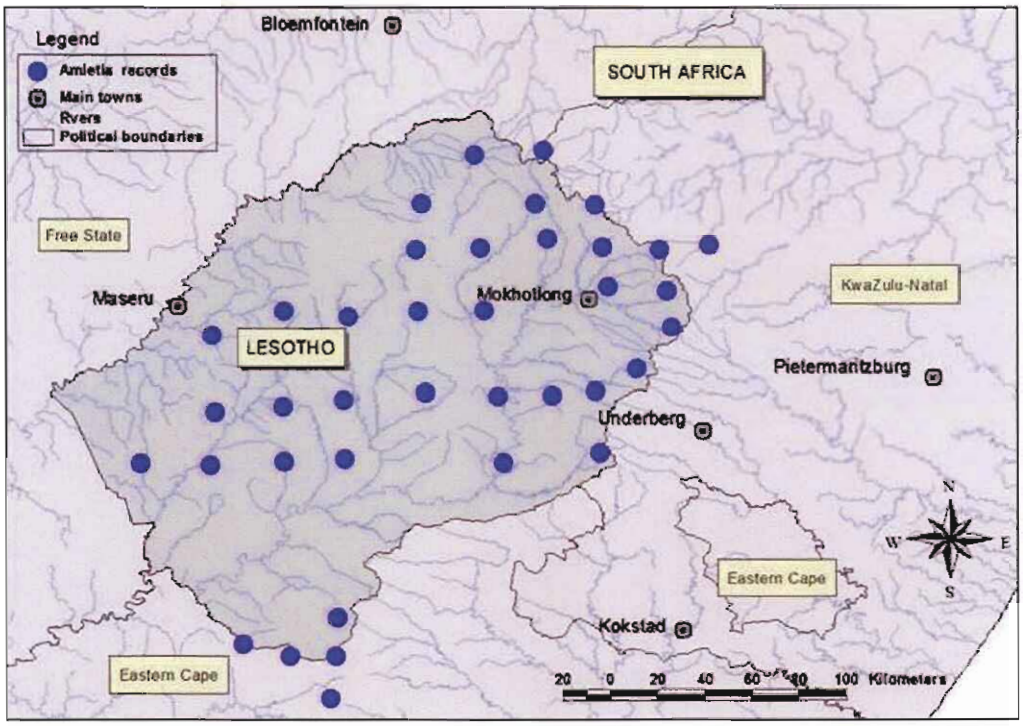

FIGURE 2.10: Distribution of *Amietia vertebralis*. Adapted from the Atlas & Red Data book (Bates, 2004).

2.3.2 Distribution of *Strongylopus hymenopus*

The distribution of *S. hymenopus* is similar to that of *A. vertebralis* in that it is endemic to the study area and is found at altitudes between 1800 – 3200m, but is restricted to the colder and wetter central and eastern highlands of Lesotho and the adjacent Drakensberg of Kwa-Zulu Natal and the Eastern Cape (Fig. 2.11) (Bates & Haacke, 2003; http://www.globalamphibians.org). It is commonly found in streams and rivers flowing eastward into South Africa, while *A. vertebralis* appears to occur in the west-flowing rivers of Lesotho. The southernmost record from Barkley East in the Eastern Cape in Figure 2.11 appears isolated and may be a case of misidentification (Michael Cunningham, personal communication, 2006).

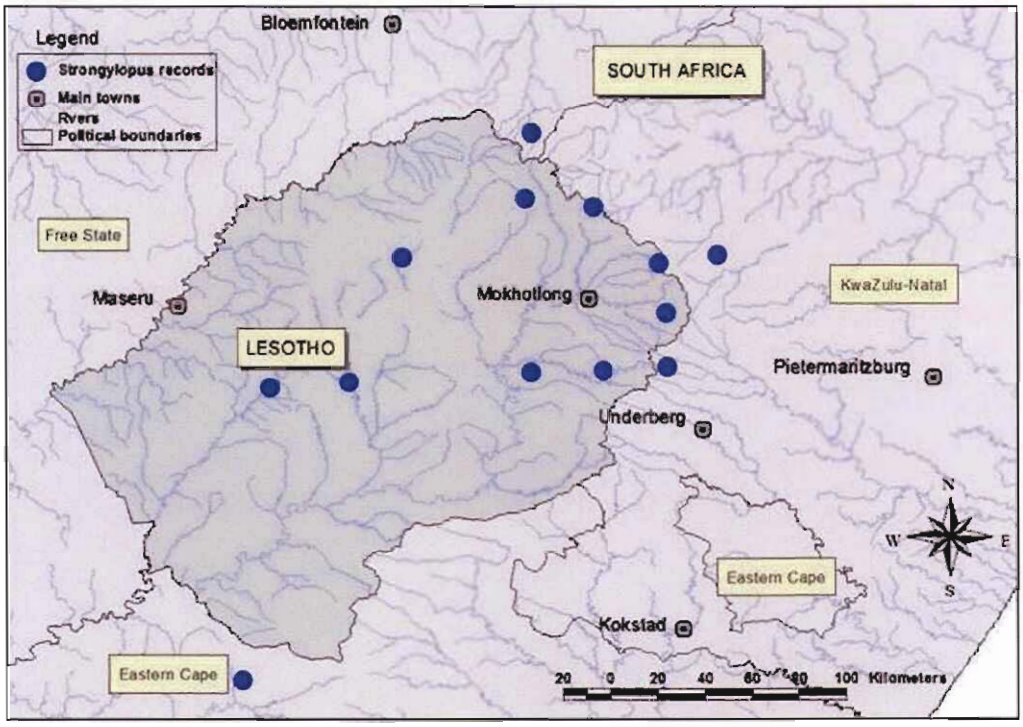

FIGURE 2.11: Distribution of *Strongylopus hymenopus*. Adapted from the Atlas & Red Data book (Minter *et al*, 2004).

2.4 Conservation status

Concern regarding global amphibian declines and extinctions has become increasingly widespread over the past few decades, with almost a third of all described species threatened (Houlahan *et al.*, 2000; Mendleson *et al.*, 2006, Minter *et al.*, 2004; Smith *et al.*, 2007). The causes for the observed declines are varied and include habitat destruction, threats from invasive species and over-exploitation (Davidson *et al.*, 2001; Vredenburg, 2004) as well the more complex threats such as pathogen outbreaks and chemical contamination (Lips, 2000; Daszak *et al.*, 2003) many of which may be exacerbated as a result of global climate change (Kiesecker *et al.*, 2001). Mountainous habitats pose extreme conditions to amphibians, and species that live at high altitudes are therefore potentially at higher risk of extinction due to elevated levels of ultraviolet-B (UV-B) radiation, shorter breeding periods, exposure to otherwise severe environmental conditions and the potential threat of invasive species as a result of climatic change. The consequences of increased UV-B exposure include declines in precipitation, reduced water depth at oviposition sites, increased water temperatures and increased susceptibility to infection by pathogens, all of which result in increased tadpole mortality (Kiesecker *et al.*, 2001).

A. vertebralis and *S. hymenopus* both have a relatively restricted range and are endemic to the Lesotho and Drakensberg highlands. *A. vertebralis* occurs, and is therefore protected, in the following nature reserves: Cathedral Peak, Drakensberg Gardens, Giant's Castle Game Reserve, the Royal Natal National Park and the Sehlabathebe National Park in Lesotho (Bates, 2002), while *S. hymenopus* occurs in the uKhahlamba-Drakensberg Transfrontier region (Channing, 2004), in particular the Royal Natal National Park. Due to the remoteness of their habitat the effects of human impact are thought to be minimal in most parts of the species' range (Bates, 2002; Minter, *et al*, 2004). Both species are thought to be abundant throughout their ranges and as such are classified in the category of "Least Concern" according to the Atlas and Red Data Book (2004) and no special conservation action is currently recommended.

However, populations of both species have been found to be infected with the widely occurring fungal disease chytridiomycosis caused by the pathogen *Batrachochytrium*

dendrobatidis (Minter *et al.*, 2004; Smith *et al.*, 2007). The disease, thought to have originated in southern Africa and spread as a result of international trade of *Xenopus laevis* commencing in the 1930s (Weldon *et al.*, 2004, 2007), currently occurs in many parts of the world and has been shown to have a drastic impact on amphibian populations wherever it is present (Weldon *et al.*, 2007). Populations of *Strongylopus hymenopus*, in particular, have been found to be heavily infected with this disease, with a prevalence of up to 38.6% (Smith, *et al*, 2007). Both adults and tadpoles can be affected by chytridiomycosis, with the repercussion that the health of entire populations is at risk. An additional observed risk to both species is the threat of predation and competition posed by the introduction of trout and other alien fish for recreational fishing into the main rivers of Lesotho (Swartz, 2005). In heavily stocked regions it is common that the frog species only occur in smaller rivers and tributaries not accessible to trout, for example, where above waterfalls, which inhibit the movement of trout (Michael Cunningham, personal communication).

A. vertebralis could also be potentially threatened in areas of Lesotho affected by the Lesotho Highlands Water Project, where the filling of the Katse and Mohale Dams may have resulted in the isolation and even extinction of some populations (Bates, 2002; Minter, *et al*, 2004). Furthermore, Lesotho and the adjacent Drakensberg highlands have been relatively poorly surveyed and as such little is known about the true extent of the species' range and their life histories (Bates & Haacke, 2003; Minter, *et al*, 2004). Determining the correct taxonomic status of these species will provide information that can be used to improve the conservation strategies used in the protection of these, and possibly other, species in this region.

2.5 General Methods

2.5.1 Fieldwork

During the course of this study several field trips were personally undertaken in the distribution area of *A. vertebralis* and *S. hymenopus*. Specimens were actively sought both for the acquirement of tissue samples (for DNA analysis) and to observe behaviour and, where possible, to record calls. Locality and altitude were recorded using GPS.

Areas personally surveyed during this study included: Cobham Nature Reserve (Polela River); Drakensberg Gardens (Mzimkulu River) – the type locality of *Rana umbraculata*; Sani Pass (Mkumizana River); Mont-aux-Sources (Bilanjil, Khubedu, Namahadi, Vimvane and Thukela rivers) and a number of regions in Lesotho including Katse, Sani, Tselhanyane, Selabathebe, Senqu and Maleba-matsoa.

For tissue samples, either a toe clipping from adults was taken (fourth toe on hind foot) or a whole tadpole or tadpole tail-tip for storage in 95% ethanol. The majority of *A. vertebralis* tissue samples for molecular analysis were provided from additional sources that included specimens from throughout the species distribution (see Appendix D).

2.5.2 Morphometrics

Specimens of both *A. vertebralis* and *S. hymenopus* from several museums, both in South Africa and abroad, as well as from private collections were examined for the morphological assessment (Table 2.1). A total of 16 external characters were measured to the nearest 0.01 mm using electronic digital callipers for a total of 268 specimens. Each measurement was repeated 3 times to minimise personal error. The average of these three measurements was used for statistical analyses (Appendix B).

TABLE 2.1: List of institutions from which specimens were examined.

Museum	**Institution Abbreviation**	**Number of *A. vertebralis* specimens**	**Number of *S. hymenopus* specimens**
British Natural History Museum (London)	BMNH	2	2
Natal Museum (Pietermaritzburg)	NM	46	7
National Museum (Bloemfontein)	NMB	19	
Port Elizabeth Museum (Bayworld, Port Elizabeth)	PEM	67	
South African Institute of Aquatic Biodiversity (Grahamstown)	SAIAB	83	
Transvaal Museum (Pretoria)	TM	24	
African Amphibian Conservation Research Group (North-West University)	AACRG		10
Michael Cunningham (University of the Free State)	MC		6
Total:		**241**	**25**

Morphological analysis was conducted using STATISTICA version 7.1 (StatSoft, 2006). The locality information, collection date, sex, details of webbing and any other pertinent information were also noted wherever possible. Methods of measurements and their analysis are described further in Chapter 3. A full list of the relevant museum abbreviations, specimen catalogue references and localities can be found in Appendix A, Table A.1

2.5.3 Molecular analysis

Regions from both mitochondrial (16S and ND2) and nuclear (RAG1 and RAG2) genes were sequenced for both *A. vertebralis* and *S. hymenopus* as well as for a number of other pyxicephalids, which served as outgroups.

Leg muscle tissue and tadpole tail tissue (either frozen or preserved in 95% ethanol) was used for DNA sequencing. Appendix D lists the samples sequenced for this study. Tissue samples were digested using proteinase K and whole genomic DNA extracted using the "salting-out" protocol. Standard PCR procedure was used to amplify the target genes and the PCR product was purified before being sent to Macrogen, Korea for sequencing. Sequences were aligned using BioEdit (North Carolina State University) and MEGA 3.1 (Kumar *et al.*, 2001). Three methods of phylogenetic analysis, namely Parsimony, Maximum Likelihood and Bayesian Inference were conducted using PAUP* (Swofford, 2000) and MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001, 2004). Various phylogenetic hypotheses were tested using the SH test. Full details regarding the methods and analysis for phylogenetic assessment are given in Chapter 4.

CHAPTER 3

MORPHOMETRIC ASSESSMENT OF *AMIETIA VERTEBRALIS* AND *STRONGYLOPUS HYMENOPUS*

3.1 Abstract

Comparison of morphological characters provides the basis for systematic assessment. For the current study 16 external morphological characters were examined for a total of 268 specimens of *Amietia vertebralis* and *Strongylopus hymenopus* as well as a number of other pyxicephalids for comparative purposes. This assessment was conducted to determine whether any clear trends within and between species could be differentiated in terms of morphology. Examination of the type specimens of both *S. hymenopus* and *A. vertebralis* revealed a number of inconsistencies and mis-identifications. The holotype of *S. hymenopus* in particular has a complicated history. Its provenance and taxonomic status is uncertain and it is suggested here that *Rana hymenopus* be considered a junior synonym of *Rana fuscigula*, since this specimen appears to most closely match the species currently known as *Amietia fuscigula*. Paratypes of *A. vertebralis* also do not match the form that it is currently associated with that name, and may instead represent the species currently referred to as *S. hymenopus*. Statistical analysis of body ratios using factor and cluster analysis provided corroboration for these observations and also confirmed that *A. vertebralis* and *S. hymenopus* are overall similar in terms of body proportions. Comparative analysis using t-tests and various effect sizes revealed significant differences between museum specimens suspected of being misidentified (including type specimens) and specimens representing these forms as currently understood. These results provide important verification for the re-classification of both *A. vertebralis* and *S. hymenopus*.

3.2 Introduction

Assessment of morphological characteristics has been the primary method used by systematists throughout history and it remains the most general criterion used to define amphibian species (Mayr & Ashlock, 1991; Vences & Wake, 2007). Collections of preserved specimens are essential to systematics since classifications are made by determining the species-specific characteristics of an organism and by comparing it to similar and related species (Mayr & Ashlock, 1991). These collections provide an essential source of biological knowledge that is basal to the undertakings of systematics through the provision of traceable information upon which biological assumptions and concepts are grounded (Kelly, 2005). Aside from providing physical material that can be preserved and studied, collections supply a permanent record of important biological information in the form of labels and additional documentation (such as photographs and call recordings) (Mayr & Ashlock, 1991; McDiarmid, 1994). In addition to its contribution to systematics, this information can be applied to many fields of biology, including biogeography, evolutionary biology and, importantly, conservation biology, which relies on the compilation of lists of species in order to create adequate protection strategies (McDiarmid, 1994). An ever increasing concern is that many species will be eliminated due to anthropogenic activity before they are discovered and one of the main goals of contemporary conservation biology is to identify and document as many new species as possible in the coming decades (Meffe & Carroll, 1997).

Ranoid (or true) frogs are one of the most diverse amphibian groups with approximately 700 species occurring throughout the world (Bossuyt *et al.*, 2006; Che *et al.*, 2007). However, the group remains one of the most neglected frog families in terms of systematics (Scott, 2005), although in recent years has been subjected to increased analysis, especially in terms of molecular research (Bossuyt *et al.*, 2006; Che *et al.*, 2007; Van der Meijden *et al.*, 2005). As a whole, the taxonomy of the family Ranidae has been revised numerous times since 1985 (Dubois, 1987, 1992, 2003; Ford & Cannatella, 1993; Frost, 1985), with the most recent revision being that of Frost *et al.* (2006). This revision has resulted in *A. vertebralis* and *S. hymenopus* being placed in the mainly southern African family Pyxicephalidae, which is only distantly related to the family Ranidae.

The suggestion by Lambiris (1991) that additional species related to *A. vertebralis* may exist, based on assessment of mouthpart morphology, as well as controversial records in the literature, has prompted the need for more extensive morphological examination of this species. For this study a number of external characters were examined for over two hundred specimens from throughout its range to determine whether any clear trends in morphological variation could be distinguished. In addition, due to the uncertainty surrounding the classification of *S. hymenopus*, specimens of this species where examined both for assessment of intraspecific diversity and for comparison with *A. vertebralis*. Examination of type specimens of both *A. vertebralis* and *S. hymenopus* was particularly important in shedding light on the taxonomy of this species and providing an explanation for much of the confusion that currently exists.

3.3 Materials and Methods

3.3.1 Specimens

Where possible all available specimens of *A. vertebralis* and *S. hymenopus* from South African museums, as well as specimens from museums abroad, personal collections and other institutions were examined for morphological assessment. A list of these specimens is provided in Appendix A (Table A.1).

3.3.2 Measurements of external characters

Sixteen external characters (Table 3.1) were examined for a total of 268 specimens. Measurements were taken using digital venier callipers with an accuracy of 0.01 mm. For all specimens each measurement was repeated three times. Each measurement was taken once for each specimen and the process repeated twice, with the average of these three measurements used for statistical analysis (Appendix B).

TABLE 3.1: The sixteen characters (abbreviations in parenthesis) measured for this study following the protocol of Lambiris (1988).

Measurement	Description
Snout-vent length (SVL):	Distance from the tip of the snout to the vent, measured with the specimen pressed lightly on a flat surface.
Snout-urostyle length (SUL):	Distance from the tip of the snout to the urostyle, when pronounced.
Tibia length (TL):	Maximum length, measured on the flexed hind limb.
Head width (HW):	Distance across the head taken at the widest point of the mouth.
Humerus length (HML):	Maximum length from the posterior of the shoulder to the flexed elbow.
Femur length (FL):	Maximum length from the flexed knee to the centre of the pelvis.
Forearm length (FAL):	Distance from the flexed elbow to the base of the thumb.
Foot length (FTL):	Maximum length of the hind foot, measured from the proximal border of the inner metatarsal tubercle to the tip of the fourth toe of the fully extended foot.
Head length (HL):	Diagonal distance from the oral angle to the tip of the snout, in its midline
Anterior tympanum-nostril (ATN):	Diagonal distance from the anterior of the tympanum to the posterior of the nostril on the same side.

Eye diameter (ED):	Greatest horizontal diameter of the eye.
Inter-nasal width (IW):	Shortest distance between the inner margin of the two nostrils.
Tympanum diameter (TD):	Horizontal diameter of the tympanum, including the tympanic ring when present.
Inter-ocular width (posterior) (IOP):	Distance between the upper eyelids.
Inter-ocular width (anterior)(IOA):	Distance between the lower eyelids.
Finger length (FL):	Distance from the proximal border of the basal sub-articular tubercle to the tip of the extended third digit of hand.

All measurements were taken on the left side of the specimen unless otherwise stated. Each specimen was also photographed from a number of different angles, and where possible, detail of webbing was photographed and noted. The locality, date of collection, sex (where determinable from external characteristics such as male nuptial pads) and any other label information were also recorded. Webbing between toes is broadly described as being either extensive (extending to tips of toes), incomplete (extending to 2nd or 3rd phalange) or trace (at base of toes or not present).

A number of museum specimens were found not to match the species to which they had been assigned upon acquisition. Such specimens were thus deemed to be misidentified and were treated separately in the statistical analysis. Details of these and suggested corrections for their identification can be found in Appendix A, Table A.2.

3.3.3 Statistical analysis

Statistical analysis was performed using STATISTICA version 7.1 (StatSoft, 2006). Initial examination of the raw data (the averages for each of the 16 variables in all 268 individuals – see appendix B) using normality histograms showed that the data was fairly normally distributed, but that the correlations between all variables were very high (> 0.9), as expected as all measurements vary with body size. This meant that the raw data was not suitable for factor or cluster analysis. Analysis of the ratios of TL/SV and HW/SV provided lower correlations between individuals and this data

was therefore used for factor analysis of the complete dataset. For additional comparative analysis between particular individuals and groups it was decided to standardise the data to create a "fingerprint" for each individual. This was done by subtracting the mean of each measurement and dividing this by its standard deviation in order that each individual be comparable with one another.

For comparative analysis of particular groups, statistical significance was tested using t-tests, which for the majority of variables showed significant differences ($p < 0.05$). Large datasets have a tendency to yield small p values and in such cases statistical significance does not necessarily imply that the result is relevant in practice (Ellis & Steyn, 2003). It was therefore important to examine effect size for differences in the means of the data. Effect size is independent of samples size and as such provides a measure of practical significance (Ellis & Steyn, 2003). For differences between means, data with effect size $|d| \geq 0.8$ is considered to be practically significant. Effect size (d) was calculated using the maximum standard deviation (s_{max}) from each sample as per the following formula:

$$d = \frac{|\bar{x}_1 - \bar{x}_2|}{s_{max}}$$

Comparative analysis was performed for the following:

- The *S. hymenopus* type specimen (BM.1978.2.28) (S) with *S. hymenopus* specimens from the AACRG and MC collections (B);
- The *A. vertebralis* PEM paratypes with both *A. vertebralis* (C) and *S. hymenopus* (B);
- Specimens from the BM labelled *A. vertebralis* specimens (T) with *A. vertebralis* (C).

3.4 Results

3.4.1 Examination of type specimens

***Amietia vertebralis* (Hewitt, 1927) type specimen and accompanying specimen from initial description: PE Museum (PEMA 1550 and 1552)**

Images of these specimens can be found in Figs. 3.1 –3.4. These specimens were collected in 1926 by a Mr Robert Essex in the vicinity of Mont-aux-Sources and labelled by Hewitt as *Rana fuscigula vertebralis*. The larger of the two specimens (PEMA 1550) with SVL of 34.5 mm (Hewitt records SVL at 38 mm so this measurement most closely matches this, taking into account the shrinkage of the specimen as a result of poor preservation) is the single type specimen designated by Hewitt, while the smaller specimen (PEMA 1552 with SVL 19.70) is one of the "five smaller specimens" referred to by Hewitt but that are not included in the type series. Since these five specimens were mentioned separately from the single type, but are likely the same species they will be dealt with here as paratypes.

The two specimens examined (the type and one smaller paratype) were unfortunately in a state of disintegration due to problems with curation. The specimens were originally held at the Albany Museum, Grahamstown, which experienced several fires in the 1940s, which destroyed museum records. They were transferred as part of a mixed collection to the PE Museum in the 1980s and only later recognised as types. Based on the shrunken and contorted bodies it is evident that at some stage these specimens became totally desiccated and as such it was not possible to obtain accurate measurements for a number of measurements. Despite their poor condition the small size, webbing pattern (extending to the first phalange, but more deeply incised than that of *A. vertebralis* (Fig. 3.3) and location (Mont-aux-Sources) suggests that these specimens more closely resemble *S. hymenopus* as it is currently known rather than *A. vertebralis* as it is currently known and this is discussed more fully below.

FIGURE 3.1: Dorsal view of the *Amietia vertebralis* type specimen (PEMA 1550) from Mont-aux-Sources.

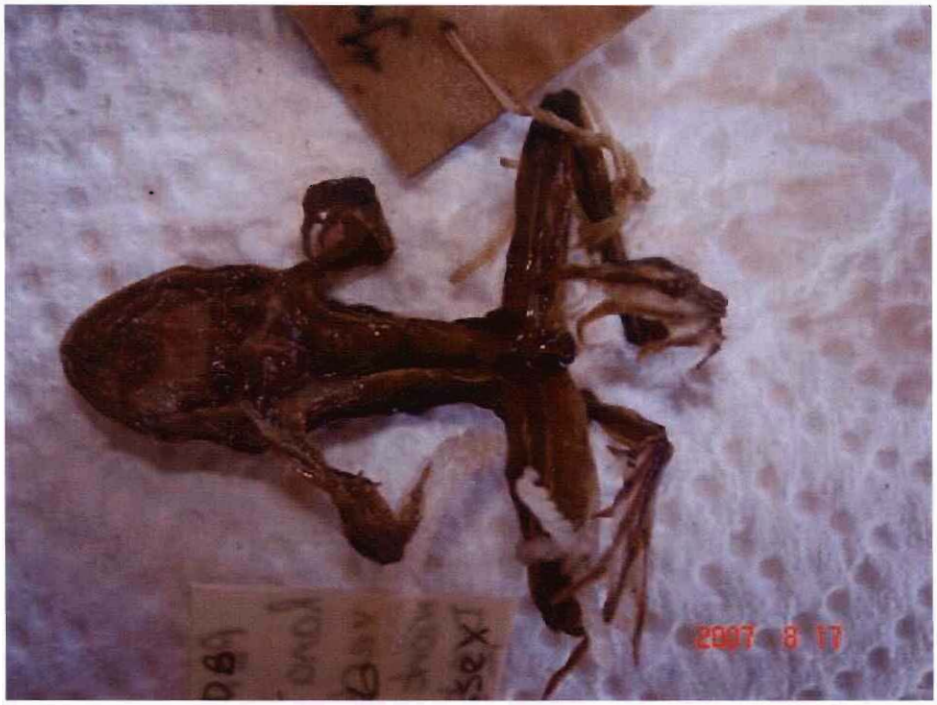

FIGURE 3.2: Ventral view of the "*Amietia vertebralis*" type specimen from Mont-aux-Sources (PEMA 1550).

FIGURE 3.3: Detail of the extensive webbing of the "*Amietia vertebralis*" type specimen from Mont-aux-Sources (PEMA 1550).

FIGURE 3.4: Dorsal view of one of the five smaller "*Amietia vertebralis*" paratype specimens from Mont-aux-Sources (PEMA 1552).

Rana umbraculata (Bush, 1952) type specimens: Transvaal Museum (TM22211 and TM22212)

Images of these specimens can be found in Figs. 3.5 – 3.12. These specimens were collected in 1951 from the Mzimkulu River in the vicinity of the Drakensberg Gardens Hotel by Robert Crass and classified as *Rana umbraculata* by Bush the following year. The female (TM 22211, original label U.N. 401) was designated by Bush as the holotype (the specimen is still labelled as the holotype) while the male (TN 22212, U.N. 408) is labelled as a co-type. Both the female and male are large with SVL of approximately 115 mm and 86 respectively. The head is wide and the nostrils are prominent and centrally positioned. The tympanum is relatively large and clear and covered by thick, tubercular skin. There is a strong supratympanic fold from the posterior corner of the eye to the mid-laternum. The canthus rostralis is completely rounded and smooth.

The skin is smooth and the colouring of the dorsum is brown with indistinct darker patches throughout and white spots on the central dorsum of the male. The venter is dirty white for both specimens, with the male having extensive thin, dark vermiculations on the limbs and gular region. The subarticular tubercles on the hands and hind feet are fairly prominent and the tips of the digits are squared off. The nuptial pads of the male are distinctive (Fig. 3.10) and the webbing of the hind feet extends to the toe tips (Fig. 3.11). The upper jaw has a row of fine, recurved teeth (Fig. 3.12) and there are three vomerine teeth on the upper surface of the mouth. In all of these features these specimens represent *A. vertebralis*, as it is currently known (hence the decision by Poynton (1964) to relegate *R. umbraculata* to synonomy).

FIGURE 3.5: Dorsal view of *Rana umbraculata* holotype (female) from Transvaal Museum (TM22211, U.N. 401).

FIGURE 3.6: Ventral view of *Rana umbraculata* holotype (female) from Transvaal Museum (TM22211, U.N. 401).

FIGURE 3.7: Detail of hand of the *Rana umbraculata* holotype (female) from Transvaal Museum (TM22211, U.N. 401).

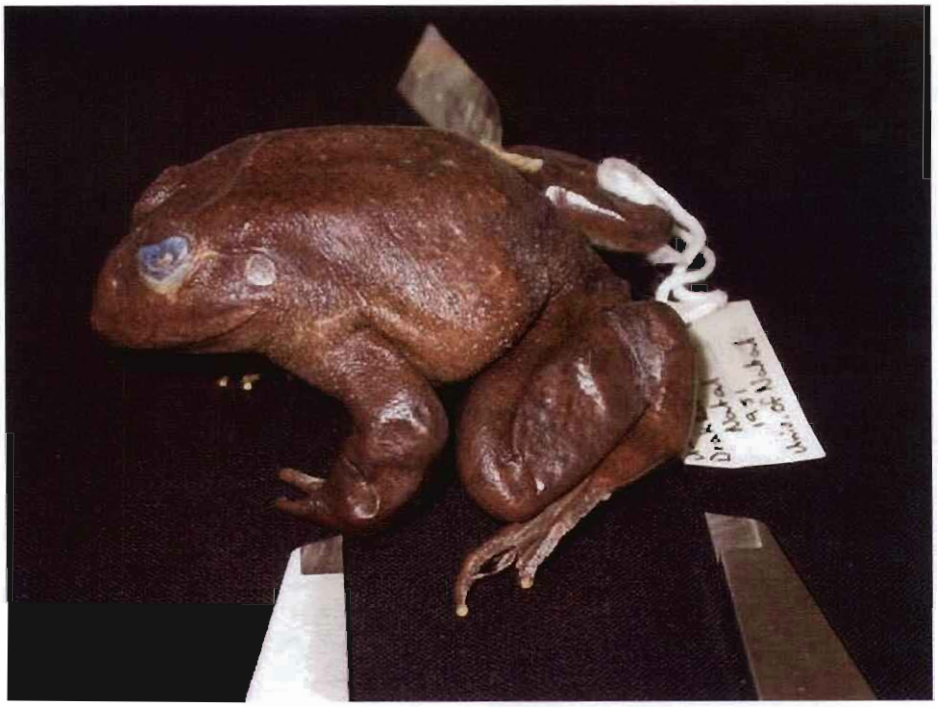

FIGURE 3.8: Side view of *Rana umbraculata* co-type (male) from Transvaal Museum (TM22212, U.N. 408).

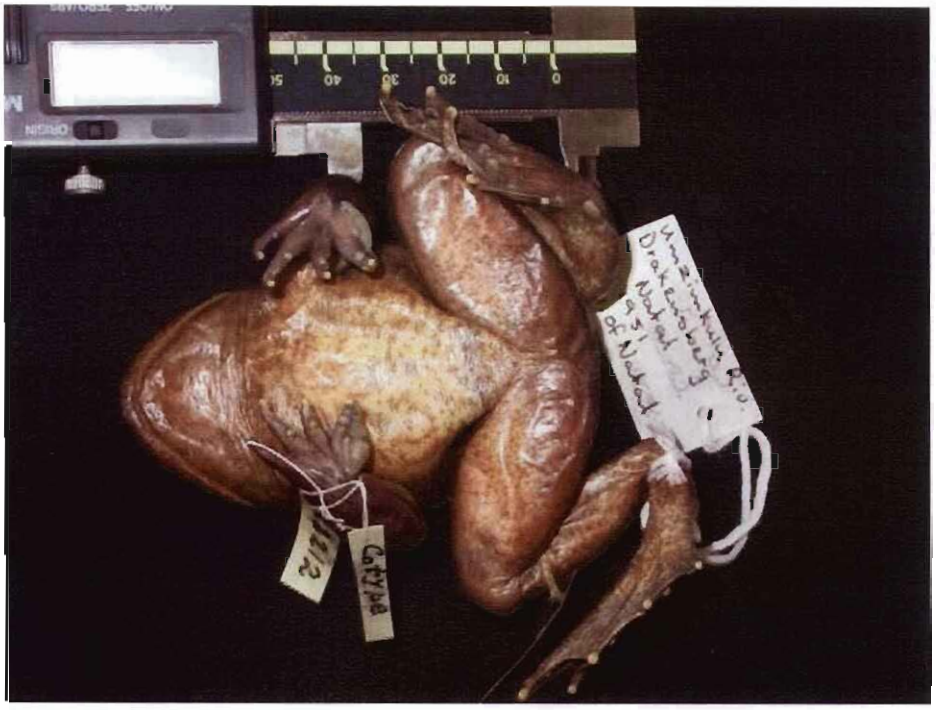

FIGURE 3.9: Ventral view of *Rana umbraculata* co-type (male) from Transvaal Museum (TM22212, U.N. 408).

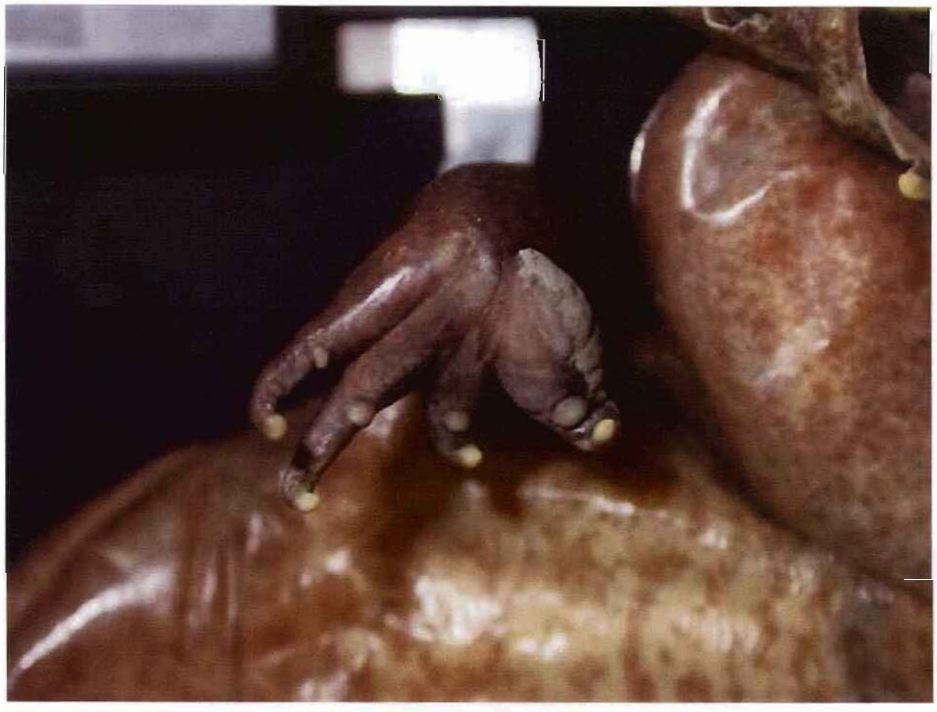

FIGURE 3.10: Detail of hand of *Rana umbraculata* co-type (male) from Transvaal Museum (TM22212, U.N. 408).

FIGURE 3.11: Detail of webbing of *Rana umbraculata* co-type (male) from Transvaal Museum (TM22212, U.N. 408).

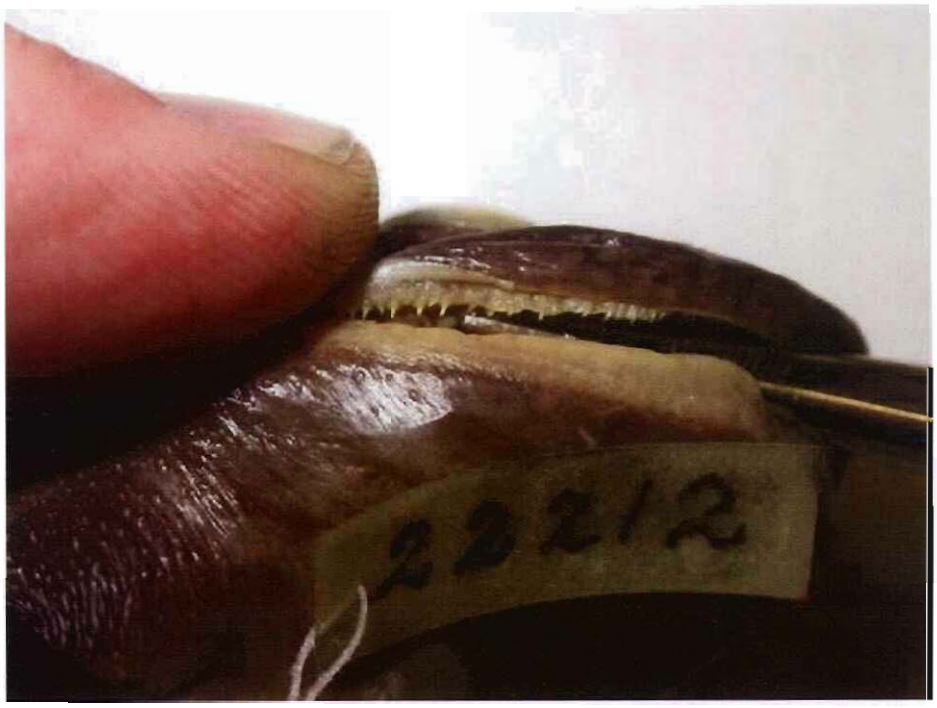

FIGURE 3.12: Detail of mouth and tooth-like projections of *Rana umbraculata* co-type (male) from Transvaal Museum (TM22212, U.N. 408).

Strongylopus hymenopus (Boulenger, 1920) holotype specimen: British Museum (BM.1947.2.28)

Images of this holotype specimen can be found in Figs. 3.13 – 3.17. Examination of this specimen revealed a number of interesting discrepancies. Firstly, the location is recorded as "South Africa" on the specimen bottle, and is thus not particularly helpful as a type locality. Furthermore, the specimen is purported, in the BM records, to have been collected by Sir Andrew Smith. However, despite Smith's extensive travels in southern Africa, his only trip to the Maluti-Drakensberg mountains was in November 1834, when he spent one day at the summit of Menyameng Pass on the western edge of the Lesotho highlands (Bates, 2005). This area is some distance from the known range of this species as it is currently understood.

There is thus no evidence that this specimen is even from Lesotho or the Drakensberg, in particular the region in which *S. hymenopus* is currently found. The record of dates is also puzzling; the species was described by Boulenger in 1920, however there is no record of the original collection date (which is likely to have been during the mid 1800s). There is a re-registration date of 1933 and a re-acquisition date to the British Museum in 1947 (following World War II when the specimens were hidden in caves to avoid bombing). This long history has raised doubt that specimen may not even be the one used by Boulonger to describe *Rana hymenopus*. However the specimen described and the one currently housed at the BM do match in the following features: the "head broader than long, much depressed; rounded snout, a little longer than the eye; tympanum very distinct, two-thirds the diameter of the eye"; slender fingers and prominent subarticular tubercles; slender hind limb; "toes slender, obtusely pointed, half-webbed, three phalanges of fourth and two of third and fifth free"; smooth skin; and the glandular fold from below the eye to the shoulder. In all likelihood, therefore, this specimen is probably the same as that used by Boulenger in 1920.

FIGURE 3.13: Dorsal view of *Rana hymenopus* holotype from the British Natural History Museum (BM.1978.2.28).

FIGURE 3.14: Ventral view of *Rana hymenopus* holotype from the British Natural History Museum (BM.1978.2.28).

FIGURE 3.15: Detail of webbing of *Rana hymenopus* holotype from the British Natural History Museum (BM.1978.2.28).

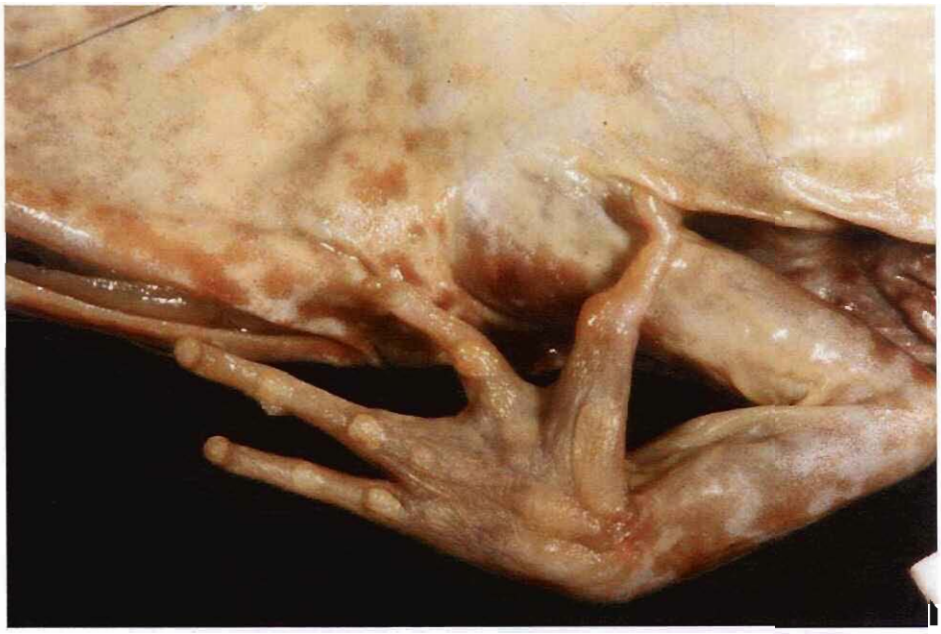

FIGURE 3.16: Detail of the fingers of *Rana hymenopus* holotype from the British Natural History Museum (BM.1978.2.28). Note the pronounced subarticular tubercles and gular mottling.

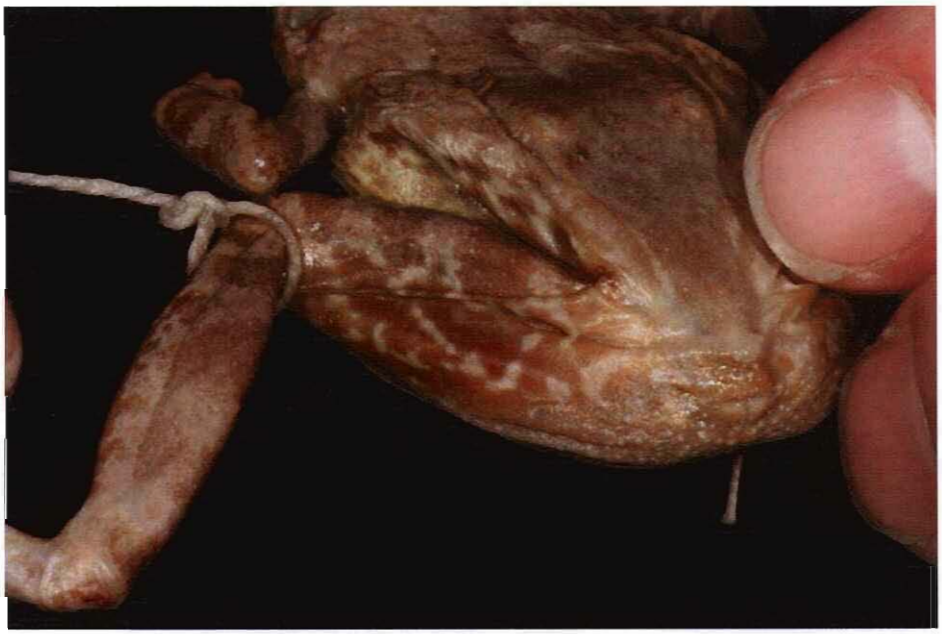

FIGURE 3.17: Detail of thigh colouration of *Rana hymenopus* holotype from the British Natural History Museum (BM.1978.2.28).

From these observations and given the lack of collection details, there is little evidence to substantiate the claim that Smith collected this specimen or that it is from southern Africa, let alone Lesotho at all. After Sir Andrew Smith, the next records of frog collections from the Maluti-Drakensberg are those by Robert Smith and JA Cottrell in 1925 and 1926, including the type collection of *A. vertebralis*. From this evidence alone it is doubtful whether this holotype is the same species as that which is currently recognised as *S. hymenopus* from the Drakensberg and this suggestion is supported by a number of morphological observations:

1. The large body size (SVL 57 mm) exceeds that of the average of observed in populations from the Drakensberg (Fig. 3.14);
2. Webbing of this specimen is incomplete, with three phalanges free of webbing (Fig. 3.15). This is inconstant with the extensive webbing (no or only one phalange free of webbing) found in specimens from the Drakensberg;
3. The bold mottled patterning on the gular area, outer thighs and the sides of the body (Figs. 3.14, 3.16 and 3.17) does not match the fine stippling patterning seen in specimens from the Drakensberg;

4. The dorsum is mainly smooth with sparsely scattered small tubercles (Fig. 3.13), which again does not fit with the rugosity typical of *S. hymenopus* from the Drakensberg;
5. The head is wide, the tympanae are very large (double the average width of that of the Drakensberg specimens);
6. The eyes fall within the profile of the head, while those of the mountain specimens protrude;
7. The snout is broad and bluntly pointed, while that of *S. hymenopus* from the Drakensberg is rounded;

The specimen appears to have been somewhat flattened (the tongue protrudes slightly from the mouth) during the fixation process and this, together with the age of the specimen may account for some of these observations, such as the smooth skin, flattened appearance and bulbous eyes. However, overall the observed differences between this specimen and *S. hymenopus* from Mont-aux-Sources (the locality in which it is most abundant) are significant enough to suggest that the holotype specimen may be a different species. More in-depth statistical analysis using the sixteen external measurements was conducted to verify these differences and is discussed below.

3.4.2 Statistical analysis

Normality histograms were constructed for each variable to test for deviations from normality. As an example, the histogram for Snout-Vent length is shown in Fig. 3.18. No evidence of outliers was found in the histograms. Pearson's correlations showed that there were high correlations ($r > 0.9$) between all variables, indicating that all measurements for large individuals were large and those for small individuals were small, as could be expected. This indicated that these variables were not suitable for further cluster or factor analysis.

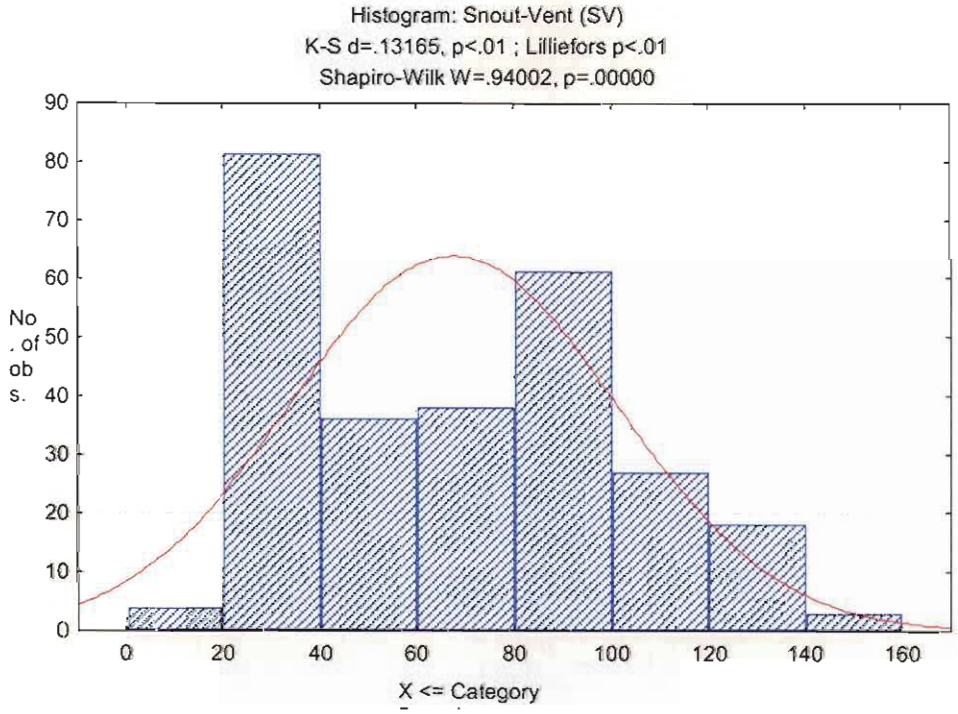

FIGURE 3.18: Histogram of Snout-Vent Length

Factor Analysis

Correlations for data based on the three most important ratios of measurements (TL/SV; HW/SV and HW/TL) were lower and were therefore better suited to multivariate analysis (Table 3.2).

TABLE 3.2: Correlations for rations of Tibia Length/Snout-Vent, Head Width/ Snout-Vent and Head Width/ Tibia Length. Marked correlations are significant at $p < 0.05$.
N=268.

Variable	TL/SV	HW/SV	HW/TL
TL/SV	1.00	-0.09	**-0.43**
HW/SV	-0.09	1.00	**0.93**
HW/TL	**-0.43**	**0.93**	1.00

Principal components analysis was performed on two of the ratios, TL/SV and HW/SV since all three measurements were included in these two ratios. The contributions of each variable of the two principal components are given in Table 3.3 and Fig. 3.19.

TABLE 3.3: Contributions of the variables to each of two independent factors.

Variable	Factor 1	Factor 2
TL/SV	0.737	0.675
HW/SW	-0.737	0.675

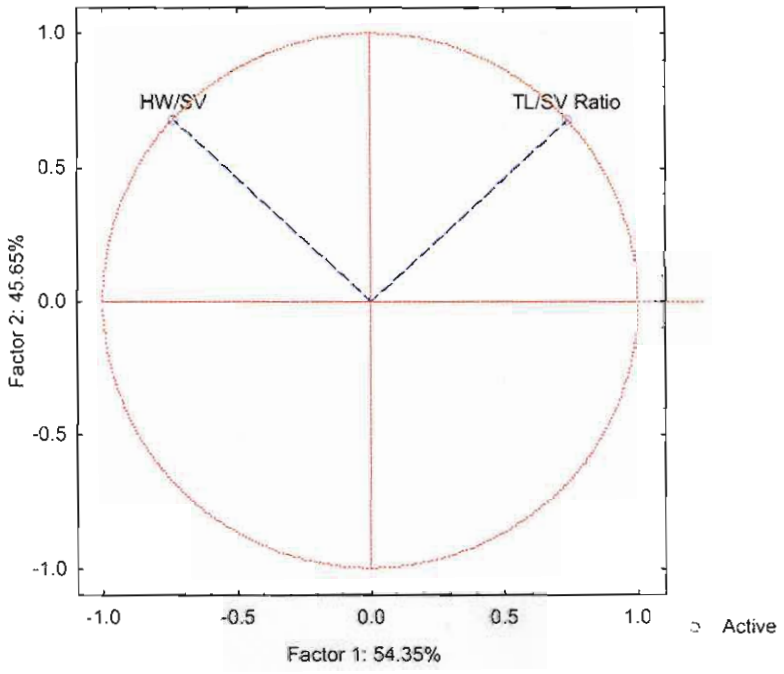

FIGURE 3.19: Projection of the variables on the factor-plane. The percentage variance accounted for by each factor is shown beside each axis.

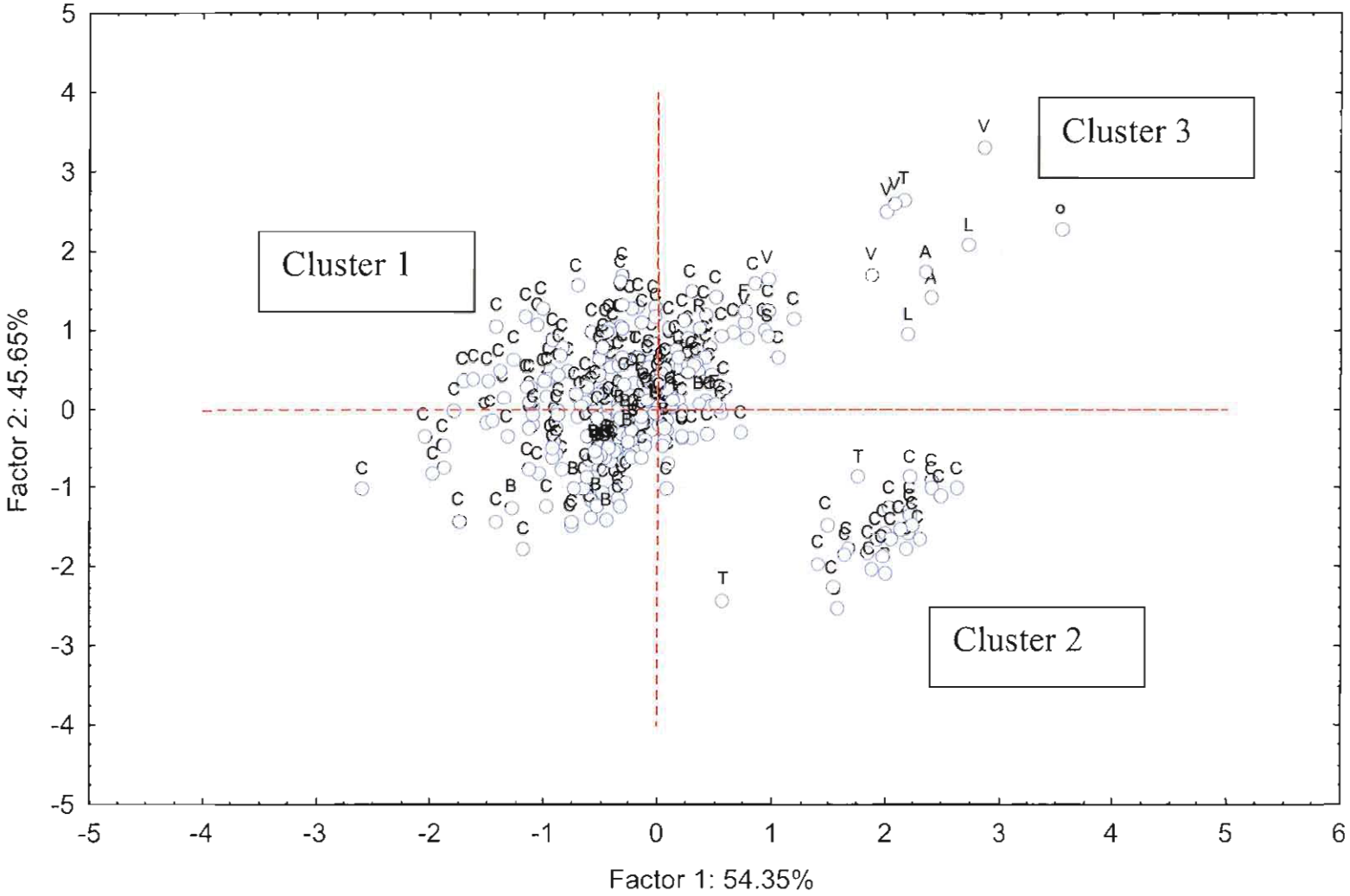

FIGURE 3.20: Factor analysis of HW/SV and TL/SV.

Key to symbols for Figure 3.20

C - *Amietia vertebralis*
T - Misidentified *Amietia vertebralis*
B - *Strongylopus hymenopus*
S - *Strongylopus hymenopus* HOLOTYPE specimen
V - Misidentified *Strongylopus hymenopus*
A - *Amietia angolensis*
F - *Amietia fuscigula*
L - *Strongylopus grayii*
o - *Strongylopus fasciatus*
R - *Rana draconensis*

The factor analysis using the ratios HW/SV and TL/SV produced three distinguishable clusters (Fig. 3.20). The largest cluster (Cluster 1) occurs to the centre and left of the axes and is comprised largely of *A. vertebralis* (C), which are mostly concentrated towards the top left of the cluster and *S. hymenopus* (B) towards the bottom and right of the cluster. The *S. hymenopus* type specimen (S) lies to the far right of this cluster, as does one of the misidentified "*S. hymenopus*" specimens from the Natal Museum.

The second cluster (Cluster 2), on the lower right of the axes is comprised of *A. vertebralis* and a few misidentified *A. vertebralis* specimens, along with the paratype *A. vertebralis* specimens from the PE museum. All of the specimens in this cluster are from the PEM collection. The third cluster (Cluster 3) is comprised of the misidentified "*S. hymenopus*" from the NM, the misidentified "*A. vertebralis*" from the BM as well as the *Amietia* sp., *S. grayii* and *S. fasciatus* specimens from the NM.

Comparative analysis

Comparative analysis was conducted to statistically examine similarity between specific individuals and groups of the same species using standardised 'fingerprint' data for each variable and the ratios HW/SV, TL/SV and HW/TL (Table 3.4). *P*-values were calculated using t-tests and effect size was calculated using the standard deviation of each sample. Table 3.4 shows the results of the comparison between the BM *S. hymenopus* holotype specimen (S) and the *S. hymenopus* specimens from the AACRG and MC collections (B).

TABLE 3.4: Comparison of the BM *S. hymenopus* type specimen (S) (n = 1) with *S. hymenopus* from AACRG and MC collection (B) (n = 16), using fingerprint data. Marked p values have significant difference ($p < 0.05$). Effect sizes > 0.8 are significant: ** = practical significance, * = visible significance.

	Mean B	Mean S	t-value	P	Std.Dev. B	Std.Dev. S	Effect Size	
Snout-Vent (SV)	2.84	2.64	2.5691	**0.021373**	0.074	0.00	2.65	**
Tibia Length (TL)	0.71	0.98	-3.5695	**0.002795**	0.074	0.00	3.68	**
Head Width (HW)	0.28	0.27	0.1187	0.907056	0.050	0.00	0.12	
Humerus Length (HL)	-0.51	-0.46	-0.7831	0.445758	0.059	0.00	0.81	**
Femur Length (FL)	0.62	0.89	-3.6607	**0.002318**	0.071	0.00	3.77	**
Forearm Length (FaL)	-0.58	-0.59	0.4299	0.673380	0.044	0.00	0.44	
Foot Length (FtL)	0.85	0.95	-0.9334	0.365396	0.105	0.00	0.96	**
Head Length (HL)	0.26	0.12	1.8817	0.079429	0.073	0.00	1.94	**
AntTym - Nostril (ATN)	-0.36	-0.54	2.9827	**0.009304**	0.058	0.00	3.07	**
Eye Diameter (ED)	-0.79	-0.86	3.4372	**0.003667**	0.021	0.00	3.54	**
Internasal Width (IW)	-0.99	-0.93	-1.9037	0.076321	0.031	0.00	1.96	**
Tympanum Length (TL)	-1.05	-1.02	-0.6893	0.501121	0.044	0.00	0.71	*
Inter Occular Post.	-0.15	-0.32	3.3086	0.004774	0.049	0.00	3.41	**
Inter Occular Ant.	-0.69	-0.67	-0.9602	0.352204	0.031	0.00	0.99	**
Finger Length (FiL)	-0.43	-0.46	0.4449	0.662742	0.066	0.00	0.46	
TL/SV	0.49	0.58	-3.4018	**0.003943**	0.024	0.00	3.51	**
HW/SV	0.39	0.39	-0.4195	0.680794	0.017	0.00	0.43	
HW/TL	0.79	0.69	2.3326	**0.034009**	0.044	0.00	2.40	**

Seven variables are significantly different ($p < 0.05$) for the t-test, and the majority are practically significant for effect size ($|d| \geq 0.8$), indicating substantial differences between the *S. hymenopus* holotype specimen from the BM and specimens collected from the Mont-aux-Sources area.

Similarly, the same tests showed significant differences between the PEM *A. vertebralis* paratypes (n = 2), from the Mont-aux-Sources region, and *A. vertebralis* (n = 233) from across the Maluti-Drakensberg highlands (Table 3.5).

TABLE 3.5: Comparison of the PEM *A. vertebralis* paratype specimens (T) (n = 2) with *A. vertebralis* (C) (n = 233), using fingerprint data. Marked p values have significant difference ($p<0.05$). Effect sizes > 0.8 are significant: ** = practical significance, * = visible significance.

	Mean C	Mean T	t-value	P	Std.Dev. C	Std.Dev. T	Effect Size	
Snout-Vent (SV)	2.74	2.51	3.3707	**0.000077**	0.094	0.011	2.39	**
Tibia Length (TL)	0.76	0.52	3.3337	**0.000997**	0.101	0.242	1.00	**
Head Width (HW)	0.41	-0.01	3.8618	**0.00146**	0.154	0.111	2.74	**
Humerus Length (HL)	-0.42	-0.55	1.2209	0.223452	0.147	0.066	0.87	**
Femur Length (FL)	0.65	0.39	2.3297	**0.020678**	0.153	0.184	1.38	**
Forearm Length (FaL)	-0.49	-0.78	5.5907	**0.000000**	0.073	0.084	3.47	**
Foot Length (FtL)	0.79	0.66	1.4923	0.136963	0.121	0.180	0.72	*
Head Length (HL)	0.33	-0.04	4.3439	**0.000021**	0.120	0.162	2.29	**
AntTym - Nostril (ATN)	-0.36				0.079			
Eye Diameter (ED)	-0.85	-0.97	1.7862	0.075376	0.066	0.000	1.79	**
Internasal Width (IW)	-1.04	-1.10	1.5742	0.116798	0.040	0.000	1.58	**
Tympanum Length (TL)	-1.08				0.042			
Inter Occular Post.	-0.24	-0.49	4.6866	**0.000005**	0.076	0.019	3.32	**
Inter Occular Ant.	-0.73	-0.88	2.7688	**0.006079**	0.077	0.135	1.13	**
Finger Length (FiL)	-0.51	-0.64	1.2067	0.228760	0.107	0.000	1.21	**
TL/SV	0.15	0.51	0.3757	0.707435	0.033	0.060	0.15	
HW/SV	0.40	0.24	2.6849	**0.007777**	0.085	0.015	1.90	**
HW/TL	0.78	0.47	2.4621	**0.014539**	0.177	0.027	1.74	**

Comparison of these paratypes specimens with the *S. hymenopus* group also showed statistically significant differences ($p < 0.05$) in both t-tests and comparison of effect size, suggesting that the PEM paratypes are also distinct from *S. hymenopus* (Table 3.6). These results may also reflect the poor condition of the *A. vertebralis* paratype specimens.

TABLE 3.6: Comparison of the PEM *A. vertebralis* paratype specimens (T) (n = 2) with *S. hymenopus* (B) (n = 16), using fingerprint data. Marked p values have significant difference ($p<0.05$). Effect sizes > 0.8 are significant: ** = practical significance, * = visible significance.

	Mean B	Mean T	t-value	P	Std.Dev. B	Std.Dev. T	Effect Size	
Snout-Vent (SV)	2.84	2.51	5.9701	**0.000020**	0.074	0.011	4.34	**
Tibia Length (TL)	0.71	0.52	2.6058	**0.019114**	0.075	0.242	0.76	*
Head Width (HW)	0.28	-0.01	7.0348	**0.000003**	0.050	0.111	2.67	**
Humerus Length (HL)	-0.51	-0.55	0.8852	0.389166	0.060	0.066	0.61	*
Femur Length (FL)	0.62	0.39	3.5698	**0.002557**	0.071	0.184	1.21	**
Forearm Length (FaL)	-0.58	-0.78	5.7597	**0.000029**	0.044	0.084	2.44	**
Foot Length (FtL)	0.85	0.66	2.2056	**0.042386**	0.105	0.180	1.02	**
Head Length (HL)	0.26	-0.04	4.8677	**0.000171**	0.073	0.162	1.83	**
AntTym - Nostril (ATN)	-0.36				0.058			
Eye Diameter (ED)	-0.79	-0.97	8.2623	**0.000001**	0.021	0.000	8.52	**
Internasal Width (IW)	-0.99	-1.10	3.2904	**0.004955**	0.031	0.000	3.39	**
Tympanum Length (TL)	-1.05				0.044			
Inter Occular Post.	-0.15	-0.49	9.4584	**0.000000**	0.049	0.020	6.91	**
Inter Occular Ant.	-0.70	-0.88	5.3789	**0.000061**	0.031	0.135	1.35	**
Finger Length (FiL)	-0.43	-0.64	3.0447	**0.008191**	0.067	0.000	3.14	**
TL/SV	0.49	0.51	-0.6053	0.553498	0.024	0.060	0.21	
HW/SV	0.39	0.24	12.1160	**0.000000**	0.017	0.015	9.01	**
HW/TL	0.80	0.48	9.8873	**0.000000**	0.044	0.027	7.27	**

Comparison of the BM *A. vertebralis* specimens (BM.1978.1254 and BM.1978.1240) with the *A. vertebralis* group showed that many of the variables were not significantly different and that effect size, although practically significant for some variables was not as high as that of the other comparisons, thus making it more difficult to completely reject that these are not *A. vertebralis* specimens (Table 3.7).

TABLE 3.7: Comparison of the BM *A. vertebralis* specimen (T) (n = 1) with *A. vertebralis* (C) (n = 233), using fingerprint data. Marked p values have significant difference ($p<0.05$). Effect sizes > 0.8 are significant: ** = practical significance, * = visible significance.

	Mean T	Mean C	t-value	P	Std.Dev. T	Std.Dev. C	Effect Size	
Snout-Vent (SV)	2.43	2.74	-3.32	**0.001052**	0.000	0.094	3.33	**
Tibia Length (TL)	1.17	0.77	4.04	**0.000073**	0.000	0.101	4.05	**
Head Width (HW)	0.27	0.41	-0.92	0.361010	0.000	0.154	0.92	**
Humerus Length (HL)	-0.50	-0.42	-0.56	0.573961	0.000	0.147	0.56	*
Femur Length (FL)	0.81	0.65	1.04	0.300144	0.000	0.153	1.04	**
Forearm Length (FaL)	-0.45	-0.49	0.50	0.624433	0.000	0.073	0.49	
Foot Length (FtL)	1.10	0.79	2.50	**0.013280**	0.000	0.121	2.50	**
Head Length (HL)	0.33	0.33	-0.04	0.970453	0.000	0.120	0.04	
AntTym - Nostril (ATN)	-0.50	-0.36	-1.71	0.089242	0.000	0.079	1.71	**
Eye Diameter (ED)	-0.91	-0.85	-0.92	0.358985	0.000	0.066	0.92	**
Internasal Width (IW)	-1.05	-1.04	-0.28	0.781167	0.000	0.040	0.28	
Tympanum Length (TL)	-1.03	-1.08	1.17	0.244243	0.000	0.042	1.17	**
Inter Occular Post.	-0.35	-0.24	-1.56	0.120102	0.000	0.076	1.56	**
Inter Occular Ant.	-0.75	-0.73	-0.27	0.803993	0.000	0.077	0.25	
Finger Length (FiL)	-0.55	-0.51	-0.38	0.705238	0.000	0.107	0.38	
TL/SV	0.51	0.52	4.64	**0.000006**	0.033	0.095	0.92	**
HW/SV	0.24	0.40	0.32	0.751206	0.085	0.015	0.44	
HW/TL	0.48	0.79	-0.80	0.425063	0.177	0.140	0.24	

3.5 Discussion

3.5.1 Diagnostic characters of *Amietia vertebralis*

At the outset of this dissertation the need to assign clear diagnostic characters to *A. vertebralis* was emphasised. This is important considering that this species does resemble *S. hymenopus* in multiple ways, and has, in some cases, resulted in confusion between the two species, especially when comparing similar-sized individuals. The suggestion of additional related species of *A. vertebralis* also necessitated further investigation since this assumption was made based on the examination of laryngeal morphology of only a few individuals (Lambiris, 1991). In this study external morphology was examined for a total of 241 *A. vertebralis* specimens. A number of these were found to be mis-identified (and were therefore analysed as such) however the majority shared overwhelming morphological similarity.

Examination of the PEM type and paratype specimens for *A. vertebralis* (PEMA 1550 and 1552) was made difficult because of their extremely poor condition, however a number of characteristics suggest that they are *S. hymenopus* specimens rather than *A. vertebralis* (using these names as they are currently recognised). Firstly, their locality is recorded as a stream near the summit of Mont-aux-Sources (on the South African side of the border). Current observations show that *S. hymenopus* is abundant in this area, while *A. vertebralis* occurs some distance from this area and only on the Lesotho side. The size of these two specimens is also consistent with that of *S. hymenopus*. The webbing is extensive, with only one phalange free of webbing, and toe- and fingertips are slender and tapered, rather than thick and squared-off as is found in *A. vertebralis*.

The TM *Rana umbraculata* holotype (TM22211) and accompanying specimen (TM22212) are well preserved and therefore provide a good reference for what is now considered *A. vertebralis* (the names were synonymised in 1964 by Poynton).

A full description of this *A. vertebralis* was given in the previous chapter, but the following characteristics can be considered as descriptive of this form and should be used for comparative purposes:

1. Large body size of adults (SVL up to 160 mm);
2. Body shape – broad at the shoulders and tapering at the pelvis;
3. Robust forearms, particularly in males;
4. Wide head and broad mouth. The upper jaw contains a row of maxillary teeth;
5. Presence of an umbraculum;
6. Position of the eyes and nostrils and the relatively large tympanum;
7. Smooth skin of the dorsum;
8. Occurrence of white vertebral spots (often seen in males);
9. Dark vermiculations of the venter, which can vary in both thickness and extent;
10. Extensive webbing to the toe tips, and which is not deeply incised;
11. Squared-off fingers and toes with well-pronounced subarticular tubercles.

3.5.2 Diagnostic characters of *Strongylopus hymenopus*

Examination of the British Museum holotype specimen of *S. hymenopus* raised a number of questions as to its identity. The validity of the taxonomy of this species is therefore dubious and the implications of this are discussed more fully in Chapter 5, where a full redescription of *S. hymenopus* is given. For these reasons it was decided not to base the following description of *S. hymenopus* on the holotype specimen. The following characteristics can be described as diagnostic for *S. hymenopus* as it is currently known and are based on the specimens from the AACRG and MC collections from the Mont-aux-Sources region:

1. Stocky body shape, with very muscular ventrum and robust forearms;
2. Relatively wide head and central position of nostrils;
3. Small tympana (on average half the width of the eye diameter);
4. Rigous dorsal skin;
5. Stippling pattern on throat and edge of thighs;
6. Extensive webbing: with a minimum of one phalange free of webbing or extending to toe tips, but more deeply incised than that of *A. vertebralis*;
7. Thin, tapering digits, with poorly pronounced subarticular tubercles.

3.5.3 Statistical Analysis

Factor Analysis

The factor analysis using the ratios of head width/snout-vent and tibia length/snout-vent produced three main clusters (Figure 3.20). The largest cluster (to the centre and left of the axes) is comprised mostly of *A. vertebralis* (C) from the SAIAB collection, the NM collection, the NMB collection, the TM collection and some from the PEM collection. These specimens are from localities from throughout the species' range and include large variations in size (with SVL ranging from < 20 mm to > 143 mm). The points for these specimens occur mostly towards the top left and centre of the cluster, but some are scattered throughout the cluster. All of the *S. hymenopus* specimens (B) from the AACRG and MC collections also occur towards the bottom left of this cluster. This indicates that certain body dimensions of *S. hymenopus* and *A. vertebralis* are very similar. This is reflected by a number of observations, including

the overall shape of the body (flattened and stocky), the wide head and rounded snout, the robust forearms and thighs and the position of the eyes and tympanum on the head. It is therefore not surprising that these two species cluster closely together and explains why, in some instances, they have been confused with one another, especially with regard to specimens of similar size (i.e. juvenile *A. vertebralis* that are similar in size to *S. hymenopus* adults). The BM holotype specimen of *S. hymenopus* (S) falls to the far right in the top quadrant of this cluster, but not in the vicinity of the other *S. hymenopus* specimens. This indicates that it does share similar body proportions to both *S. hymenopus* and *A. vertebralis*, but there is not strong support for it being identical to *S. hymenopus* collected in the vicinity of Mont-aux-Sources. The V point that occurs to the far right and top of this cluster is specimen BM.1978.1235 – one of the *S. hymenopus* specimens from the BM believed to be misidentified. Its position here provides support for the suggestion that it is likely a species of *Amietia* (previously *Afrana*).

The second cluster, towards the bottom right, is comprised of *A. vertebralis* specimens that are all from the PEM collection, including PEMA1550 and PEMA1552 (the poorly preserved type and paratype specimens). Again these specimens are from a fairly wide distribution, including the Sani, Mokhotlong and Sehonghong Rivers. Aside from the type specimens, all of these specimens were collected in 1988 and there is significant variation in SVL among specimens (approximately 30 mm to 120 mm) and there are both females and males in the sample. In addition, all specimens are recorded as having extensive webbing and exhibit the other characteristics common to *A. vertebralis*. This grouping may indicate a separate species, although this seems unlikely since the group includes specimens from locations identical to those in the main cluster. Their separate grouping may be a result of preservation or error in measurement technique, resulting in some kind of distortion, since no specimens from any of the other collections occur in this group. In addition, most of these specimens have been dissected, although this should not have caused any significant changes to their dimensions. In one case a specimen is missing an entire foot, but is still very obviously *A. vertebralis* (Figure 3.21)

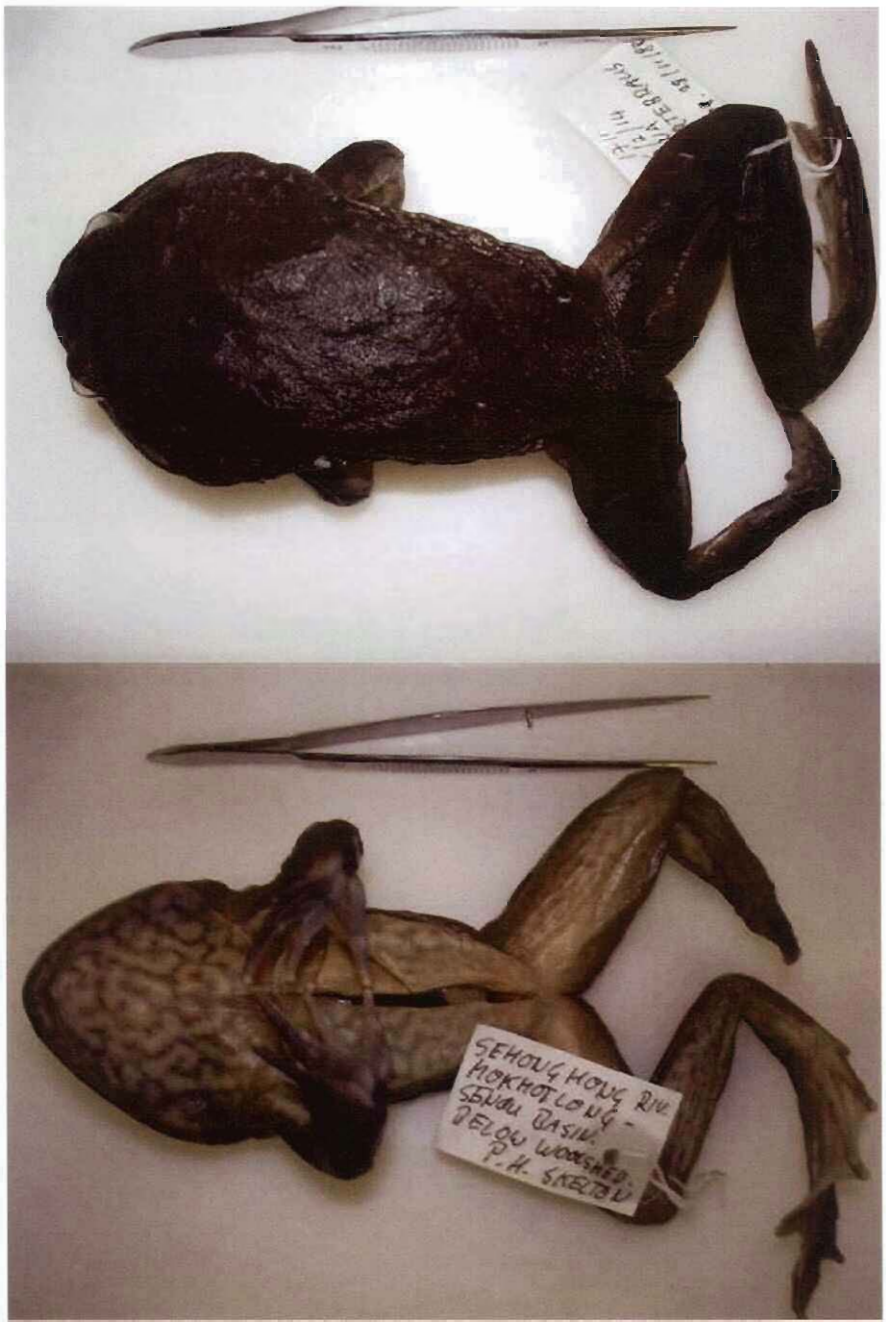

FIGURE 3.21: Example of *A. vertebralis* specimen (PEMA 1711) from the second cluster. Note the missing foot. Preservation technique may have caused distortion resulting in these PEM specimens clustering together.

The *A. vertebralis* type and paratype (V) occur to the left of this cluster, but are not very close to each other, with one occurring in the top left of the cluster and the other in the bottom left. These specimens were in exceedingly poor condition (Figs. 3.1 – 3.4) and it was therefore impossible to obtain accurate measurements for many of the

dimensions. Both specimens were also relatively small (SVL 19.70 mm and 34.54 mm respectively) and literally were desiccated skin over bone, making it difficult to draw any clear conclusions about them. From the cluster analysis they fall closest to *A. vertebralis* and *S. hymenopus* indicating that overall they share similar dimensions to those species. For the reasons mentioned above they appear to most closely resemble *S. hymenopus*.

The third cluster is comprised of the misidentified *S. hymenopus* from the NM as well as the *Amietia* and *Strongylopus* species from the NM that were included for comparative purposes. This confirms that the NM specimens (V) have been incorrectly identified and that they are probably *Amietia* species. One of the *A. vertebralis* specimens from the BM (BM.1978.1240) (T) also falls in this cluster, confirming the suspicion that this is most likely a species of *Amietia*. The *S. grayii* (L) and *S. fasciatus* (o) specimens occur to the right of the cluster and are clearly distinguishable from the *S. hymenopus* specimens (B) in cluster 2.

This factor analysis is useful as it reveals a number of patterns in terms of morphology:

- *A. vertebralis* and *S. hymenopus* share similar body proportions, but are distinguishable from one another;
- *S. hymenopus* is significantly different from other *Strongylopus* species;
- Those specimens suspected of being misidentified on the basis of a variety of observations (discussed in the previous section) cluster separately from the main cluster of *A. vertebralis* and *S. hymenopus* thus confirming these suspicions

Comparative data

The results of the comparative data using t-tests and effect size, support both the general observations as discussed above and the patterns obtained from the cluster analysis. The comparison of the British Museum *S. hymenopus* holotype specimen with the rest of the *S. hymenopus* group (Table 3. 4) is of particular importance as it clearly shows substantial differences between the type and specimens obtained from the Mont-aux-Sources area. This therefore provides additional evidence to suggest that the *S. hymenopus* holotype is not *S. hymenopus* as it is currently known from the Drakensberg.

In addition, comparison of the PEM *A. vertebralis* paratypes and the *A. vertebralis* group (Table 3.5) revealed significant differences for both t-tests and effect size, thus indicating that it is unlikely that these specimens are truly *A. vertebralis*. Furthermore, comparison of these specimens with the *S. hymenopus* group (Table 3.6) produced lower significant differences between variables, indicating that the dimensions of these specimens are more closely correlated with those of *S. hymenopus* than *A. vertebralis*. Some measurements were significantly different, but as mentioned above, the poor condition of these specimens make it difficult to draw any clear conclusions about them.

Finally, the comparison between the BM *A. vertebralis* specimens and the rest of the *A. vertebralis* group (Table 3.7) was also significantly different for some variables, but overall most variables were shown not to be significantly different. Effect size, however, was visibly significant for most variables. This suggests that these specimens do resemble *A. vertebralis* in a number of ways, however taking the general observations into account it is difficult to conclude that they are unequivocally *A. vertebralis*.

3.5.4 Adaptation to a high altitude environment

Both *A. vertebralis* and *S. hymenopus* are adapted to the harsh environs of the high altitude conditions in which they occur. These regions experience extreme fluctuations in temperature (both on a yearly and daily basis) and high wind velocities. The previous chapter describes the frogs' adaptations to there severe environmental conditions in detail, but it is worth noting here the possession of the umbraculum, which is thought to shield the eye from high levels of UV-B radiation experienced at high altitudes (Channing, 2001). Other adaptations include resistance to extreme temperature fluctuations and opportunistic breeding patterns. Both species also share characteristics that have been observed in montane anurans elsewhere in the world, for example, the almost entirely aquatic lifestyle and mostly diurnal activity are also found in *Rana temporaria* in the Spanish Pyrenees (Miguel Vences, personal communication). Also of interest is that the larvae of *R. temporaria* exhibit a high degree of morphological plasticity depending on ecological conditions. This may explain the high variance in mouthpart morphology observed by Lambiris (1991) and documented by Bates (2002).

Thus, overall the statistical analysis of the morphological data supports the suggestions made from examination of type specimens, and those specimens considered to be incorrectly identified. These findings provide sufficient confirmation of the need to consider re-classification of both species, and this is further supported by the molecular analysis as is discussed in the following chapter.

CHAPTER 4

THE PHYLOGENY OF *AMIETIA VERTEBRALIS* AND *STRONGYLOPUS HYMENOPUS* USING MOLECULAR ANALYSIS

4.1 Abstract

The phylogenetic placement of *Amietia vertebralis* and *Strongylopus hymenopus* is contentious and this has contributed to uncertainty regarding the taxonomic status of both species. For this study both mitochondrial (16S and ND2) and nuclear (RAG1 and RAG2) gene fragments were sequenced to infer the relationships of *A. vertebralis* and *S. hymenopus* among related genera, as well as to assess geographic variation within each species. This was crucial for testing the hypothesis that more than one species of *A. vertebralis* exists. Three methods of phylogenetic reconstruction were applied, namely, parsimony, maximum likelihood and Bayesian analysis. Specific phylogenetic hypotheses were tested using likelihood-based SH tests. Analysis of both individual gene fragments and combined datasets revealed similar results, with the combined data set of 16S and RAG1-AmpF1 providing the most decisive data. Very little genetic variation was detected among *A. vertebralis* from throughout its range thereby dispelling the suggestion of additional species and instead suggesting a recent genetic bottleneck. Despite a smaller sample size for *S. hymenopus*, there appears to be significantly more genetic variation within this species than in *A. vertebralis*. A significant outcome of this study was the discovery that *S. hymenopus* was found not to be monophyletic with *Strongylopus*, but rather to be a sister species to *A. vertebralis* and this clade was shown to be closest to *Amietia*. These findings support the conclusions obtained from morphological analysis, that is, that both *A. vertebralis* and *S. hymenopus* have both been misplaced in the current genera and thus require taxonomic revision.

4.2 Introduction

Anurans have one of the most conserved body plans of all the vertebrate groups. Thus, despite their probable adaptive properties, conspicuous morphological features are often used for anuran systematics (Bossuyt & Milinkovitch, 2000). Morphological homoplasy among different adaptive clades therefore often obscures the phylogenetic relationships among anurans (Hoegg *et al.*, 2004). Advances in the use of molecular techniques in the last two decades has meant that knowledge from DNA sequences is starting to exceed that of other biological functions (Hall, 2004; Shaffer & McKnight, 1996).

Such knowledge has been valuable in revealing homologies not apparent from morphological examination (Williams, 1993) and has been especially useful for identifying cryptic species of amphibians (Frost *et al.*, 2006; Hanken, 1999; Vences *et al.*, 2004; Vences & Wake, 2007). It was thus deemed necessary for the present study to compare molecular data to that of the morphological assessment of *A. vertebralis* and *S. hymenopus* in order to resolve the systematics of this group. Such analysis has not previously been done on these species, and the combination of both mitochondrial and nuclear DNA analysis is still rare among South African amphibian systematics.

The use of multiple genes strengthens the power of phylogenetic analyses by providing more potentially informative nucleotide sites for and by accessing variation at different levels since the evolutionary rate of different genes often varies (Cummings *et al.*, 1995; Makokha, 2007). Here, both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) were used to infer phylogenies including *A. vertebralis* and *S. hymenopus*. MtDNA markers are particularly useful in systematic and population studies, especially among closely related taxa (Avise 2000; Moritz, *et al.*, 1987) and where population data is limited due to small samples sizes (Vences & Wake, 2007).

4.2.1 The use of Mitochondrial DNA in molecular analysis

Since animal mtDNA is maternally inherited and non-recombining, the entire molecule shares the same pattern of common descent (Hillis *et al.*, 1996). It is therefore useful for discriminating between species and constructing phylogenetic hypotheses. The mitochondrial genome is usually a small circular, supercoiled, covalently closed double helix and very different to the chromosomes of the eukaryotic nucleus (Boore, 1999). The replication of these organellar genomes is independent of DNA replication in the nucleus, however many functions of the organelles are coded for and regulated by the nuclear DNA (Madigan *et* al., 1997). Almost all animal mitochondrial genomes contain thirty-seven genes that encode the ribosomal RNAs, twenty-two transfer RNAs and thirteen proteins of the respiratory chain, such as cytochromes, dehydrogenases and ATPases (Boore, 1999; Moritz *et al.*, 1987). A large non-coding region contains the controlling elements for replication and transcription (Boore, 1999). In general, mitochondrial sequences evolve rapidly, however their gene rearrangements are relatively stable. It is these unique rearrangements between major groups that make mtDNA useful for resolving deep phylogenies (Boore, 1999; Macey, 1997).

Here, two regions of mtDNA, 16S ribosomal RNA (16S) and NADH dehydrogenase subunit 2 (ND2) are used for phylogenetic analysis. The use of genes that encode ribosomal RNA are especially useful for inferring phylogenies because they are easily accessible, have a wide range of evolutionary rates and as such can provide resolution across a large time scale (Hillis *et al.*, 1996). The ND2 gene evolves at a relatively rapid rate and is therefore useful for reconstructing relationships at the population level where variation is expected to be low (Macey *et* al., 2001; Ashton & de Queiroz 2001). 16S, on the other hand, shows a greater range of evolutionary rates, in different sections, including slowly evolving regions and is therefore useful for analysing deeper phylogenetic relationships (Macey *et al.*, 2001; Ashton & de Queiroz 2001; Swart 2006). 16S has been proposed as a universal DNA barcoding marker for amphibians, due to the consistent pattern of variation found in certain regions of the gene between species (Vences *et al.*, 2005). In addition, the constrained protein sequence of mtDNA coding genes, makes it easy to determine homology across

sequence sites and to interpret the pattern of nucleotide substitution as most changes occur as silent substitutions at third codon positions.

4.2.2 The use of nuclear genes in molecular assessment

The mitochondrial genome of animals is inherited as a single unit and different mitochondrial genes are therefore not independent indicators of organismal phylogeny (Moore, 1995). In some cases, mitochondrial genes have been found to provide limited resolution, especially at deeper nodes (Hoegg *et al.*, 2004). The use of nuclear loci, although still less common than mtDNA, is applicable to assessments of diversity both within and among species, and for this reason it is useful to combine mtDNA data with that of nuclear sequences (Hillis *et al.*, 1996). The RAG1 and RAG2 gene cluster is a highly conserved region of the nuclear genome of jawed vertebrates. These recombination activation genes encode the proteins for antigen receptors unique to the adaptive immune system of jawed vertebrates (Fugmann, *et al.*, 2006). Combination of these multiple gene loci is likely to provide higher resolution of phylogenetic relationships than analysis of singular molecular markers. In addition, the random nature of gene-lineage sorting due to genetic drift results in phylogenetic variation, and conflict, among genes.

4.2.3 Defining species

One of the original motivations for this study was the suggestion that multiple species of *A. vertebralis* exist (Lambiris, 1991). A species refers to the basic unit of biodiversity and is therefore also the easiest unit by which biodiversity can be quantified (de Queiroz, 2005; Meffe & Carroll, 1997). However, defining what constitutes a species has been one of the most important and long-standing debates amongst biologists. Coined by Mayr (1957) as "the species problem", the use of a variety of different species "concepts" could result in the recognition of different numbers of species. A species concept refers to the biological definition of the word "species" and the criteria upon which this definition is based (Mayr, 2001). Today there are over twenty species concepts, with the biological, evolutionary and phylogenetic concepts being amongst the most widely used (de Queiroz, 2005; Hey, 2006). Clearly, the implications of this problem are far-reaching, and is pertinent to systematics.

With the general conceptual shift in the priorities of systematics from that of classifying organisms to that of testing hypotheses regarding lineage boundaries and phylogenetic relationships, it is becoming apparent that the adoption of a unified species concept is necessary to the future of taxonomy. De Queiroz (2005) argues that all of the rival species concepts have in common the basic idea that a species is essentially a separately evolving lineage segment and that the defining properties of species concepts (for example, reproductive isolation, distinct adaptive zones and monophyly) are secondary. Thus the unified species concept treats all separately evolving population level lineages as species. This unified species concept is applicable to this study since we are concerned with the monophyly of the taxa concerned, and as has been discussed, taxonomy based purely on morphological characters is often insufficient for anuran systematics.

4.2.4 Phylogenetic hypotheses

Monophyletic lineages are minimal groups of organisms that include all descendants of a common ancestor. Determining monophyly of a sampled group is important to many evolutionary studies (Huelsenbeck & Crandall, 1997). In order to test the various phylogenetic hypotheses that could explain the evolutionary relationships of *A. vertebralis* and *S. hymenopus*, both in relation to each other and the rest of the Pyxicephalidae family, it was necessary to test a number of hypotheses using likelihood. These hypotheses include:

1. Are there multiple species of *A. vertebralis* (as suggested by van Dijk (1966) and Lambiris (1987,1991)?
2. Does *S. hymenopus* belong within the genus *Strongylopus*?
3. What is the relationship between *S. hymenopus* and *A. vertebralis*?
4. What is the relationship between *A. vertebralis* and *Amietia* (previously *Afrana*)? Do they form a monophyletic assemblage?
5. Where do *A. vertebralis* and *S. hymenopus* fit into the African Pyxicephalid clade (*sensu* Frost *et al.* 2006)?

The aims of this study, therefore, were firstly to assess intraspecific diversity, especially that of *A. vertebralis*, in order to ascertain the possible existence of multiple species within this taxon. This was done by sequencing DNA from many individuals sampled from throughout the species range. Secondly, to assess interspecific diversity to determine the relationship of *A. vertebralis* with *S. hymenopus* and to determine their position within the family Pyxicephalidae so that hypotheses of relationships could be generated. Three methods of phylogenetic inference were used, namely, Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). Together with the morphometric assessment, these results could be used to resolve the classification problems currently associated with both species.

4.3 Materials and Methods

4.3.1 Samples

A total of 125 samples of *A. vertebralis* (86 samples) and *S. hymenopus* (26 samples) from various localities throughout their range, as well as samples of from number of Pyxicephalid outgroup species (13 samples), were sequenced for genetic analysis. The majority of the *A. vertebralis* samples were provided by SAIAB (South African Institute of Aquatic Biodiversity, Grahamstown) and include specimens from a broad geographic area, while the remaining samples were provided by a variety of additional sources (details of these samples and their localities can be found in Appendix D). Fig. 4.1 shows the geographical distribution of these samples. Where possible, these samples were accompanied by specimens included in the morphometric analysis. Additional sequences for outgroup taxa (other Pyxicephalids) were obtained from Genbank, and Braae's phylogenetic study of *S. grayii* (2006). Sequences of *Pyxicephalus adspersus* (the Giant Bullfrog) were used for the outgroup (Genbank Accession number AF206472.1).

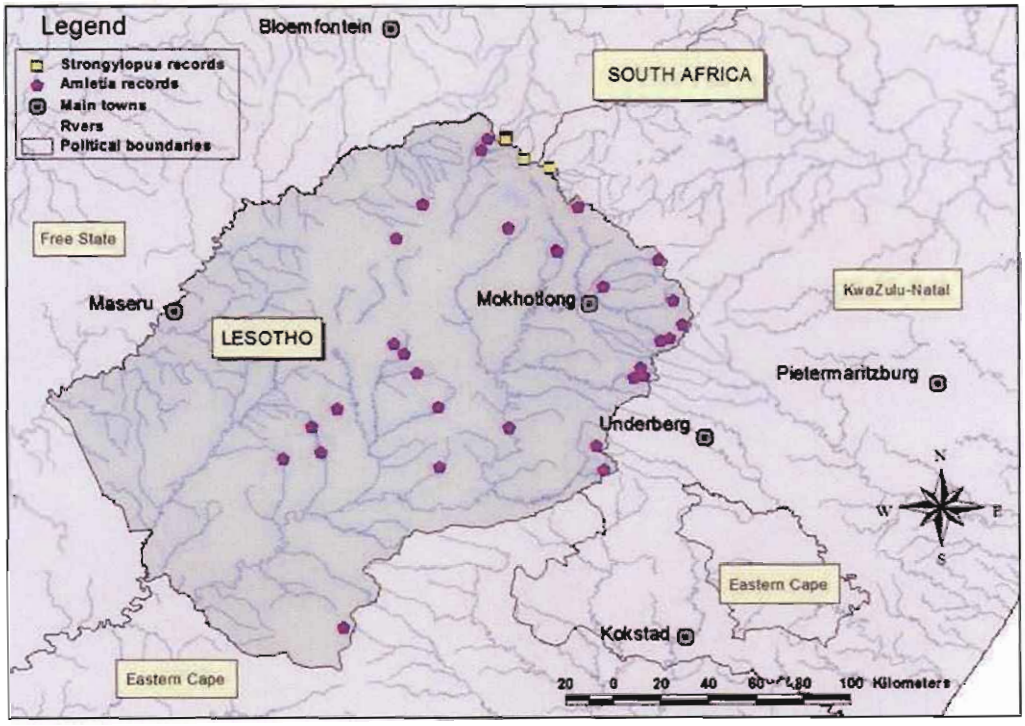

FIGURE 4.1: Geographical distribution of tissue samples used for sequencing.

4.3.2 Extraction, amplification and sequencing

Tissue samples were taken from leg muscle and toe clippings of adults or from tadpole tails and were either frozen or stored in 95-100% ethanol. Whole genomic DNA was extracted using proteinase K and the "salting out" extraction method. Effectiveness of the extractions was verified using electrophoresis on 1% agarose gels. Two mitochondrial gene fragments 16S rRNA (472 base pairs) and ND2 (1028 bp for the combined dataset) and two nuclear gene fragments (RAG1 and RAG2, with a total of 1600 and 781 characters respectively) were selected for sequencing. Double-stranded PCR was used to amplify the target gene fragments using the primers shown in Table 4.1.

Primer	Gene	Sequence	Reference
16Sar	16S	5'-CGCCTGTTTATCAAAAACAT-3'	Cunningham *et al.* 1992
16Sbr	16S	5'-CCGGTCTGAACTCAGATCACGT-3'	Cunningham *et al.* 1992
VMet	ND2	5'-GCTAAACAAGCTTTCGGGCCCATACC-3'	Cunningham & Cherry, 2004
VTrp	ND2	5'-CTCCTGCTTAGGGCTTTGAAGGC-3'	Cunningham & Cherry, 2004
AmpF1	RAG1	5'-ACAGGATATGATGARAAGCTTGT-3'	
AmpR2	RAG1	5'-GGTGYTTYAACACATCTTCCATYTCRTA-3'	
AmpR1	RAG1	5'-AACTACGCTGCATTKCCAATRTCACA-3'	
Lung35F	RAG2	5'-GGCCAAAGAGRTCYTGTCCIACTGG-3'	

TABLE 4.1: List of primers used in this study

PCR reactions were carried out in 25 µl quantities, each consisting of 14.4µl ddH_20, 2.5 µl 10 x reaction buffer (including 1.5 mM $MgCl_2$ per reaction), 1.0 µl (0.2mM) of each of the four nucleotides (Promega), 2.0 µl (0.8mM) of each primer, 0.1 µl (0.5 units) *Taq* polymerase (NEB or Go Taq, Promega) and 3.0 µl (~ 100ng) template DNA (from extractions). Samples were loaded into the thermocycler at 80 °C. The PCR programme for mtDNA included an initial denaturation step at 94 °C for 1 min 30 sec, followed by 34 cycles of 94 °C for 45 sec, annealing at 55 °C for 45 sec and 72 °C for 1 min 30 sec. One extension cycle was performed at 94 °C for 45 sec and 72 °C for 5 min after which the product was held at 10 °C. For nDNA the programme was slightly different: initial denaturing step of 94 °C for 2 min 20 sec, followed by 39 cycles of 50 °C for 50 sec, 72 °C for 3 min and 72 °C for 10 min. This was followed by a final run of 94 °C for 45 sec and 72 °C for 5 min and a holding temperature of 8 °C. Temperatures and reaction components were adjusted to improve specificity when

necessary. PCR product was visualised using 1% agarose gel electrophoresis stained with ethidium bromide and viewed under UV trans-illumination. PCR product was either purified using the Qiagen PCR clean-up kit (Qiagen, Valencia, USA), or sent to Macrogen Corp. (Seoul, Korea) for purification and sequencing. Sequencing was done using the Applied Biosystems (Foster City, USA) automatic sequencer (ABI3730XL) using the appropriate primer.

4.3.3 Sequence alignment

Randomly selected sequences were checked for identity using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequence chromatograms were initially assessed and edited using the program BioEdit (North Carolina State University) and then aligned using Clustal W in MEGA 3.1 (Kumar, *et al.*, 2004) using the default alignment parameters. Where necessary, adjustments to the sequence alignments were made manually after visual inspection, in order to increase nucleotide identity around gaps. Alignment of the mtDNA sequences was relatively straightforward and required the introduction of only a few gaps. The RAG sequences were somewhat more complicated due to coding problems, which may have been present due to the presence of introns and other non-coding regions, and therefore required additional manipulation. From these initial alignments, simple Neighbour-Joining guide trees were generated to give an overall idea of what could be expected from further model-based analysis (Fig. C.1, Appendix C, provides an example). This distance-based method (Saitou & Nei, 1987) calculates the distance between each pair of taxa and, by successively joining the most similar taxon groups, finds a tree that matches the observed divergence. It does not guarantee finding the tree with the smallest overall distance, but the approximation is generally good and provides a fast means of getting a general overview of the data (Felsenstein, 2004; Hall, 2004).

4.3.4 Phylogenetic analysis

Three methods of phylogenetic inference were implemented to assess the relationships between *A. vertebralis*, *S. hymenopus* and other Pyxicephalids, in particular *Amietia* and *Strongylopus*. Separate analysis was conducted for each of the gene fragments as well as for combinations of various genes where it was possible to match taxa (the combination of 16S and RAG1-AmpF1 provided the strongest

matching). The methods used were Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). Homogeneity of the combined datasets was tested using the incongruence-length difference test (Farris *et* al., 1995) implemented in PAUP* (as the "partition homogeneity" test, Swofford, 2000). Using the distance matrices generated in PAUP* all identical sequences and any anomalies (for example Genbank samples believed to be misidentified) were removed from the dataset so that further analysis time could be reduced.

Parsimony

Parsimony looks for trees with the minimum number of evolutionary changes and is based on the assumption that the most likely tree requires the fewest number of changes to explain the data. Multiple equal length trees are generated, of which a strict consensus tree is saved. All MP analysis was conducted using PAUP* 4.0 using unweighted heuristic searches with the tree-bisection-reconnection (TBR) branch swapping algorithm, ACCTRANS character optimisation and random addition of taxa and 100 replicates with 1 tree held for each replicate. Nodal support was assessed by 10000 bootstrap pseudoreplicates, with up to 1000 trees held at each step.

Maximum Likelihood

A molecular evolutionary model of sequence change and parameters for this model, used in likelihood and Bayesian analyses, were estimated using MODELTEST 3.7 (Posada & Crandell, 1998). This programme identifies the best-fitting model of DNA substitution from 56 possible models. ML uses this model of evolution to find the tree that maximises the likelihood of observing the data. Heuristic searches were used with 10 replicates. Robustness of ML trees was tested using 100 bootstrap replicates with random taxon addition.

Bayesian Inference

Bayesian posterior probabilities were calculated using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001, 2004), which uses a Metropolis-coupled Markov chain Monte Carlo algorithm to explore the very large set of possible trees (Laget & Simon 1999). Four independent searches were performed, with the number of generations being calculated separately for each dataset. Each search ran four Markov chains (one cold, three heated) initiated from random trees with trees saved every 100

generations. The burn-in (those initial trees acquired before the average standard deviation of split frequencies approaches zero) was determined, and the trees prior to the stable log likelihood value were excluded by examination the average standard deviation of split frequencies (usually 10% of the number of trees). The remaining trees were used to construct a 50%, right majority rule consensus tree, indicating the posterior probabilities for the nodes.

Hypothesis Testing

Parsimony and maximum likelihood are methods that both search for optimal trees, under a set of evolutionary assumptions (by minimising conflict among aligned nucleotide sites or by maximising the fit to the data respectively, given a fully specified model of sequence change). Even if the assumptions made using these methods are reasonable, there is no guarantee that these trees reflect the actual historical relationships among taxa, as sequence change along the true tree may have followed a more complicated path than in the optimised reconstructions. It is therefore important to determine, given these assumptions and data, whether it is possible to reject some potential relationships as statistically unlikely, especially those that may affect taxonomic classification.

The best trees found under alternative hypotheses of relationships, outlined below, were compared with the maximum likelihood tree using likelihood based SH-tests (Shimodaira & Hasegawa, 1999) implemented in PAUP* 4.0b8 (Swofford, 2000). Topological hypotheses of relationships were specified *a-priori*, as constraints in likelihood searches. A constraint hypothesis forces the monophyly of a specified group on the analysis without specifying other relationships either within or outside this group, such that there are numerous fully resolved trees consistent with any particular constraint. Constraint hypotheses are each represented as a minimally resolved 'bush', with a single clade comprising the taxa in question and with all other taxa emerging from a basal polytomy.

The specific hypotheses tested here were (1) that *Strongylopus*, including *S. hymenopus*, is a monophyletic group (Fig. 4.2); (2) that *Amietia* (previously *Afrana*) and *A. vertebralis* form a clade (Fig. 4.4); (3) that *A. vertebralis* and *S. hymenopus* are

sister taxa (Fig. 4.6); (4) that *A. vertebralis*, *Amietia* and *S. hymenopus* form a monophyletic group (Fig. 4.8), excluding other *Strongylopus*. The unconstrained likelihood search described above was used as the null hypothesis (hypothesis 0, Fig. 4.2). Table 4.2 below summarises compatibility among these hypotheses. Several hypotheses may be simultaneously satisfied on a single tree, so long as each pair-wise combination of these hypotheses is potentially compatible (e.g. Hypotheses 0+1+2 are mutually compatible on some trees; hypotheses 2 & 3 are each compatible with 4 but 2+3+4 is not a viable combination because hypotheses 2 & 3 conflict). Hypothesis testing was performed for the combined dataset (16S and Rag1-AmpF1, using *Pyxicephalus adspersus* as outgroup) using the GTR+G model of evolution with Resampling Estimated Log Likelihood (RELL) approximation of tree scores and differences based on 1000 bootstrap pseudoreplicate datasets.

Hypothesis	0	1	2	3	4
0 (null)	-				
1	+	-			
2	+	±	-		
3	+	×	×	-	
4	+	×	±	±	-

TABLE 4.2: Compatibility among constraint hypotheses
+ = complete compatibility, ± = compatibility in some trees, × = incompatible hypotheses

Hypothesis 0 (Null): No assumptions are made about any taxon relationships.

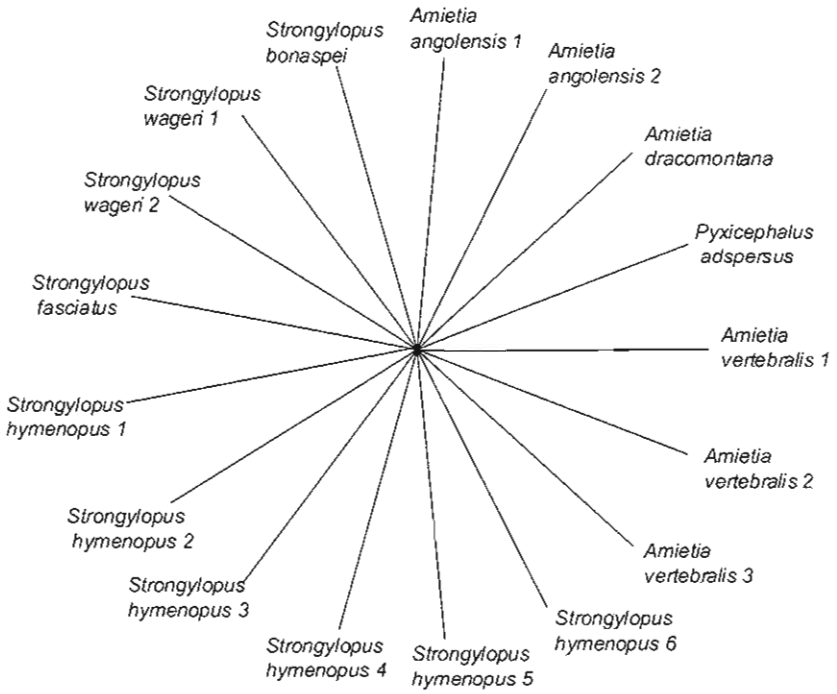

FIGURE 4.2: The null hypothesis - an unconstrained bush, compatible with all possible trees

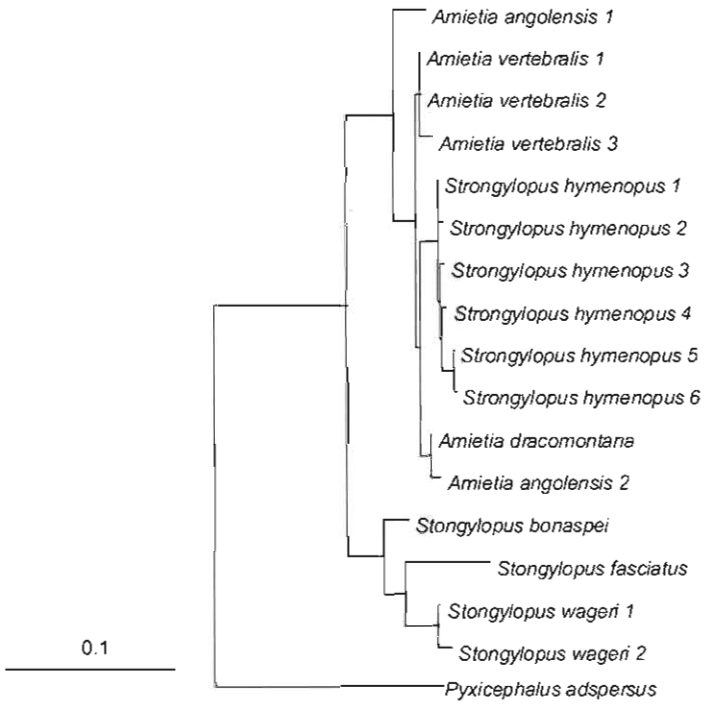

FIGURE 4.3: The optimal tree under the null hypothesis (-lnL = 3827). This is the unconstrained ML tree shown in Fig. 4.14.

Hypothesis 1: *Strongylopus* is monophyletic, including *S. hymenopus*

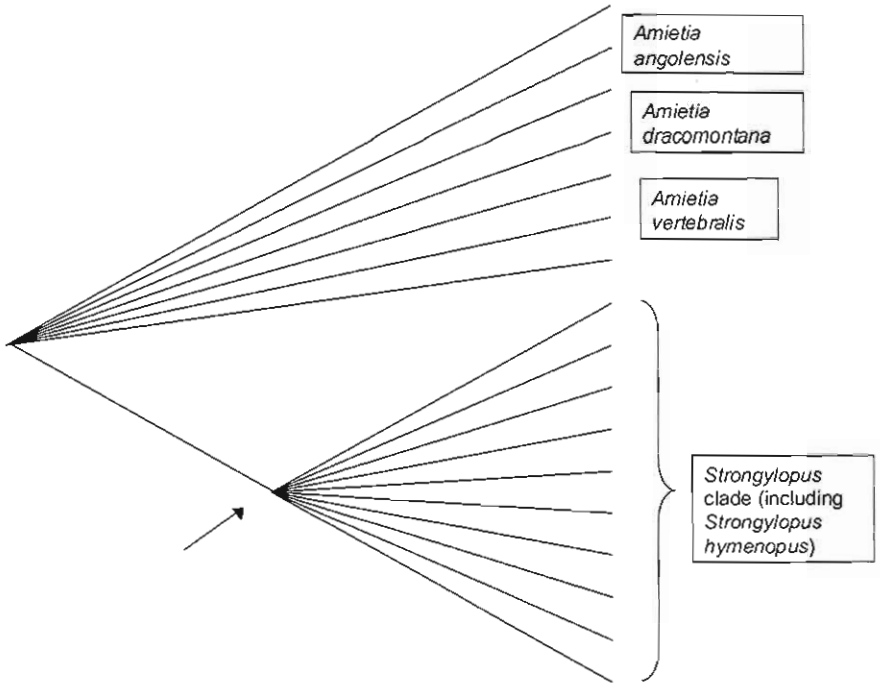

FIGURE 4.4: *Strongylopus* (including *S. hymenopus*) are constrained to monophyly. No relationships are specified within *Strongylopus* or between this clade and other taxa.

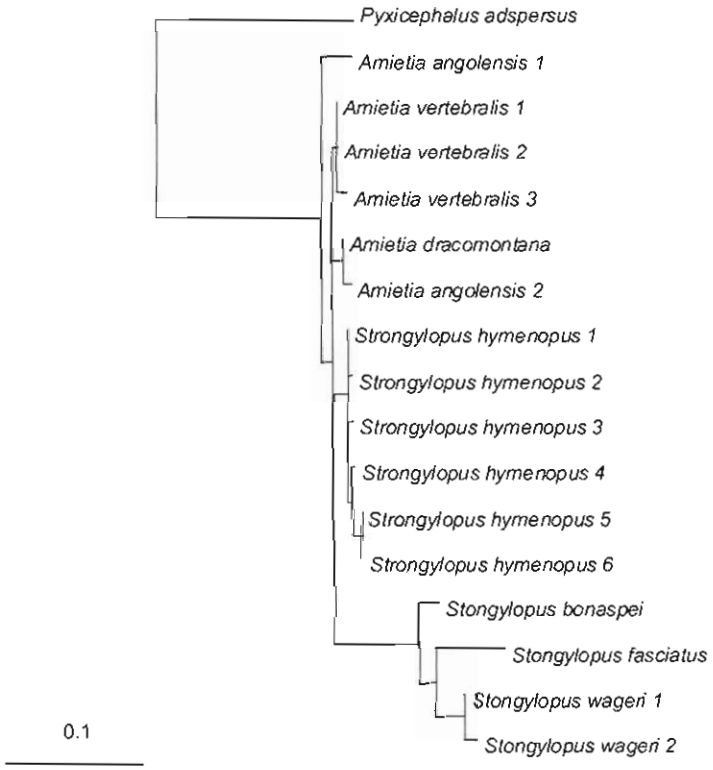

FIGURE 4.5: The best likelihood tree with *Strongylopus*, as currently classified, forming a clade (-lnL = 3856).

Hypothesis 2: *Amietia* and *A. vertebralis* are monophyletic

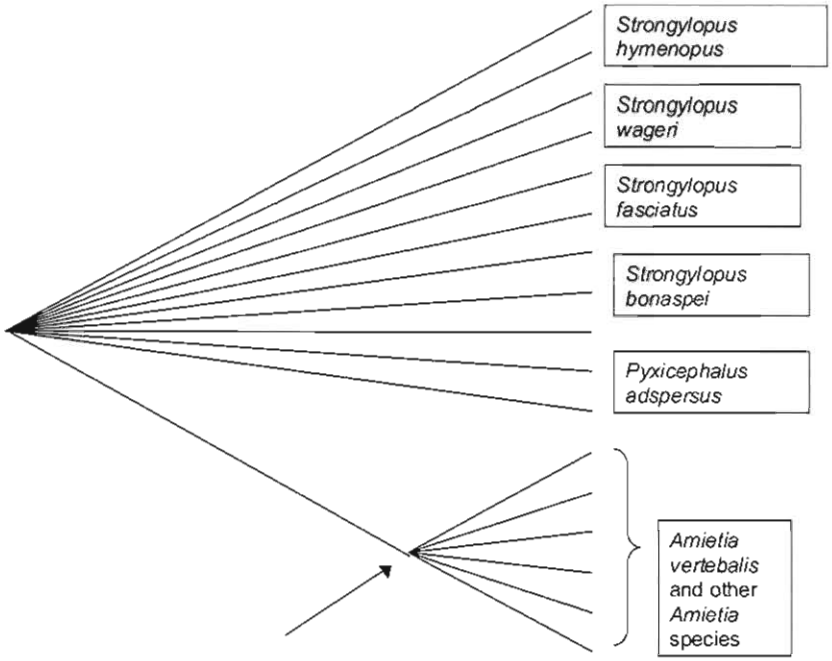

FIGURE 4.6: *Amietia* (previously *Afrana*) and *Amietia vertebralis* are constrained to form a clade.

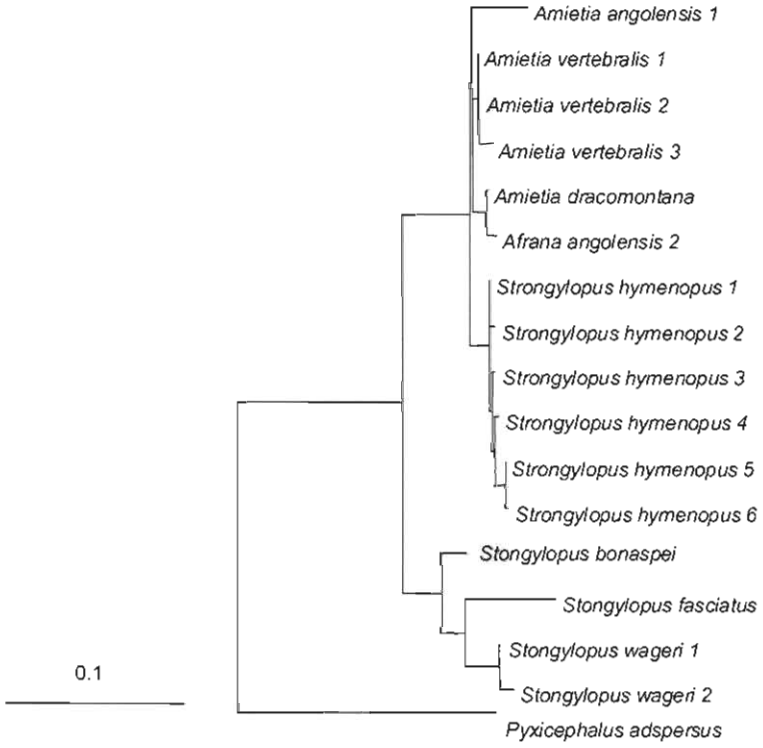

FIGURE 4.7: The best likelihood tree with *Amietia* and *A. vertebralis* forming a clade (-lnL = 3838).

Hypothesis 3: *A. vertebralis* and *S. hymenopus* are monophyletic

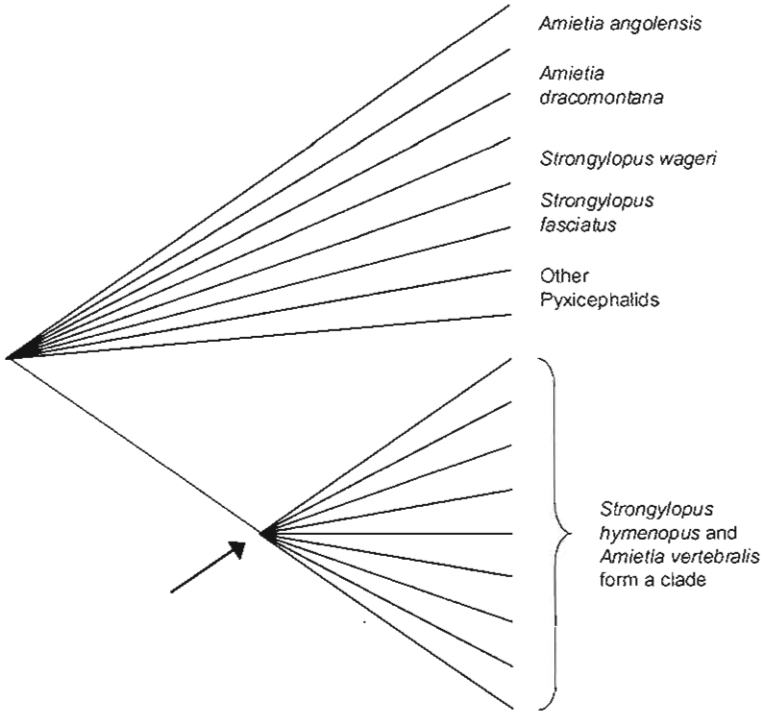

FIGURE 4.8: Constraint forcing *Strongylopus hymenopus* and *Amietia vertebralis* to monophyly, excluding *Amietia* and other *Strongylopus*. This hypothesis differs the most from current classification.

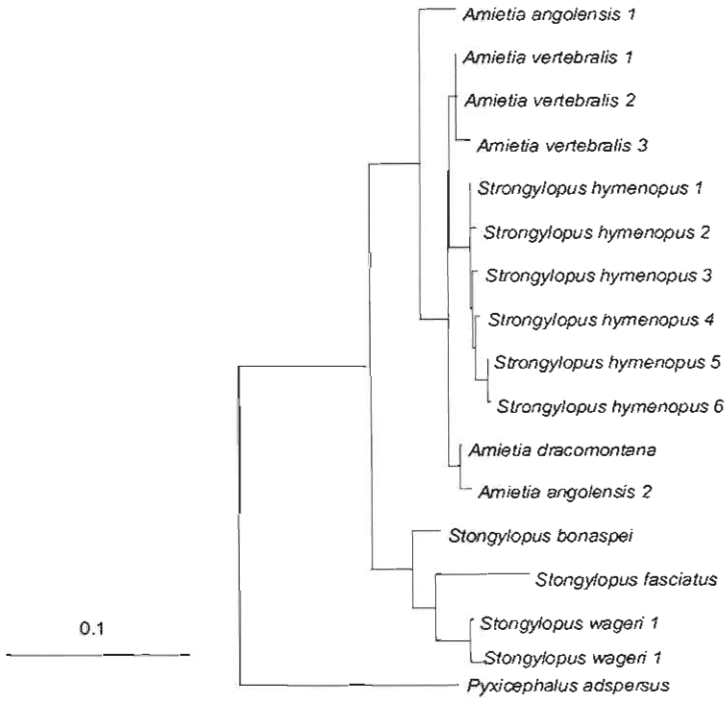

FIGURE 4.9: The best likelihood tree with *A. vertebralis* and *S. hymenopus* as sister groups (-lnL = 3831).

Hypothesis 4: *A. vertebralis*, *S. hymenopus* and *Amietia* are monophyletic, excluding other *Strongylopus*

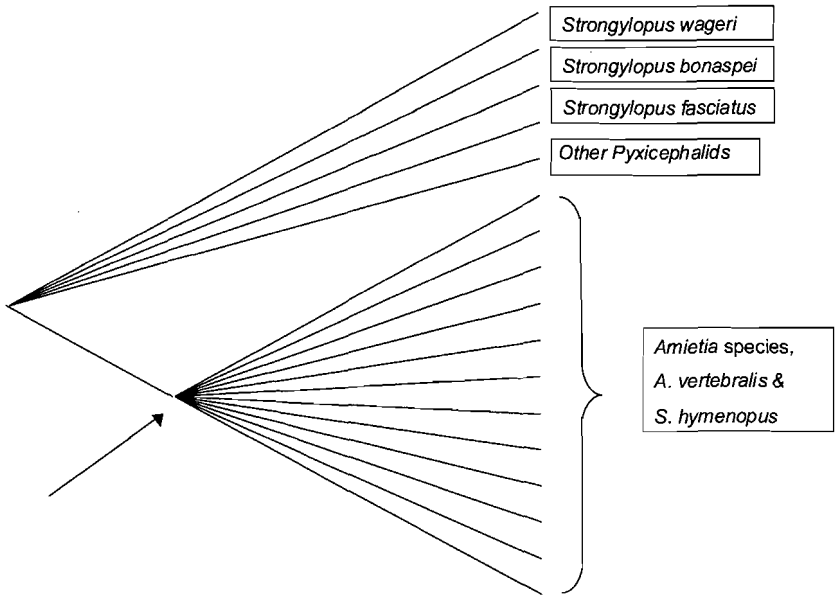

FIGURE 4.10: Constraint forcing monophyly of *Amietia* (previously *Afrana*), *A. vertebralis* and *S. hymenopus*, excluding other *Strongylopus*.

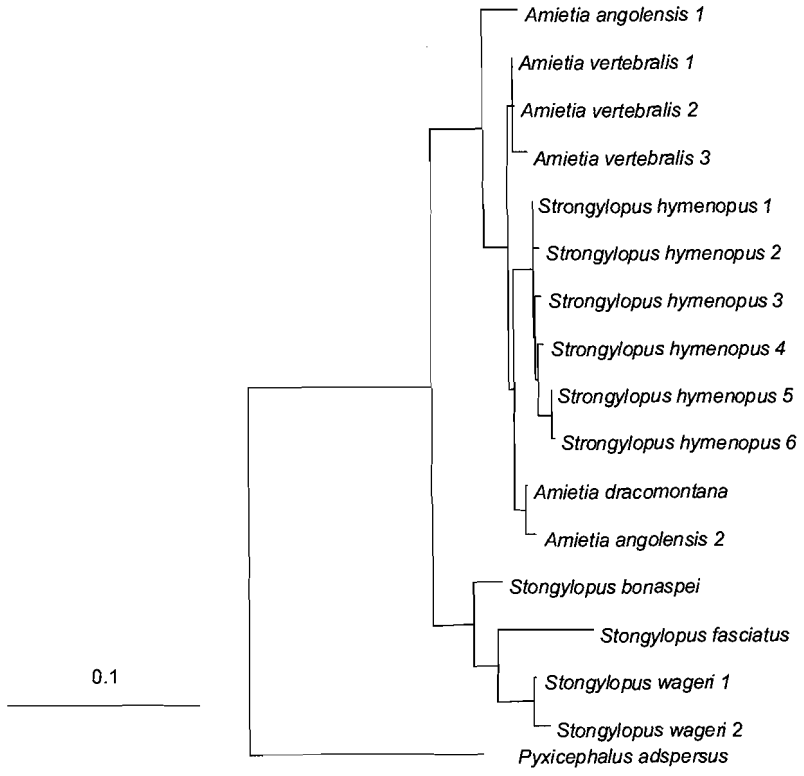

FIGURE 4.11: The best likelihood tree consistent with hypothesis 4. (-lnL = 3827). This tree is identical to the maximum likelihood tree (Fig. 4.14).

4.4 Results

4.4.1 Phylogenetic results

Both the combined and separate datasets yielded results with similar tree topologies and each analysis method produced phylogenies supporting the same clades.

Analysis of separate gene fragments

63 individuals were sequenced for 16S (472 characters). Following initial construction of neighbour-joining trees it was evident that there was very little variation within *A. vertebralis* (Appendix E, Fig. E.1). A total of 35 redundant taxa were excluded from the dataset following examination of pair-wise distance matrices. 29 of these were *A. vertebralis* sequences with the dominant allele represented by sample G1 and 6 were *S. hymenopus* sequences with the dominant allele represented by sample J6. Of the 472 characters, 316 were constant and 80 were parsimony-informative. The strict consensus of 129 of the best trees is shown in Fig. 4.12. All three methods of phylogenetic inference produced similar trees, with strong support for *A. vertebralis*, *S. hymenopus* and *Amietia* forming a monophyletic clade, separating *S. hymenopus* from other *Strongylopus* species.

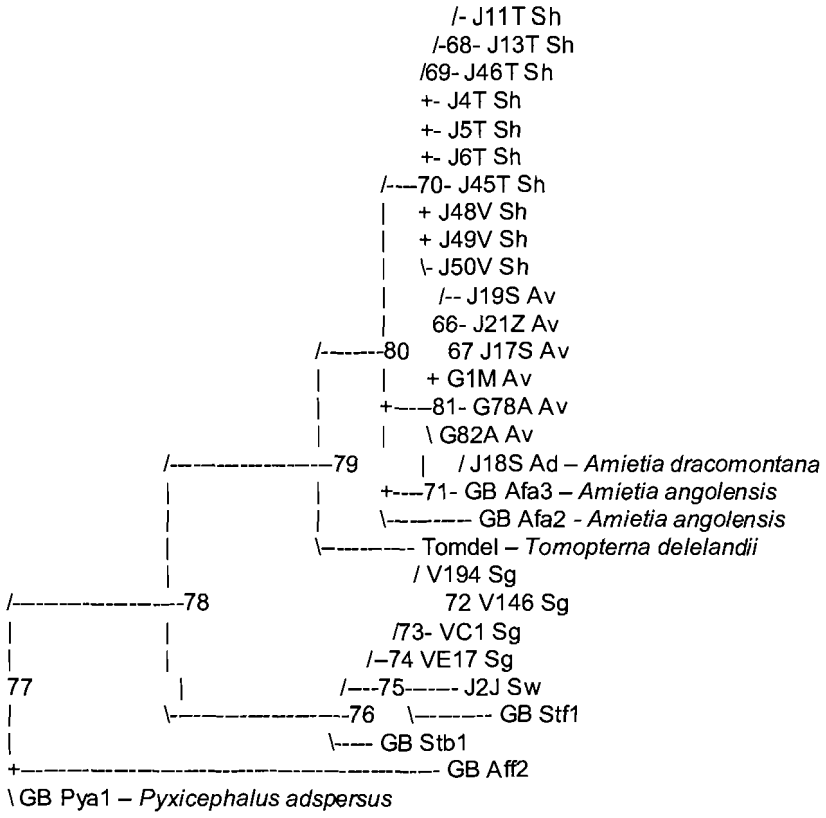

FIGURE 4.12: The strict consensus tree from Parsimony analysis for the 16S dataset with *Pyxicephalus adspersus* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

For the separate analysis of RAG1-AmpF1 48 individuals were included, of which 29 were retained for further analysis after removal of identical sequences. Of 776 characters, 598 were constant and 178 were variable of which 85 were parsimony-informative. MP analysis generated 144 trees, the strict consensus of which is shown in Fig. E.2. Bootstrap support for each clade is high (> 58%), with the *Amietia* grouping between *A. vertebralis* and *S. hymenopus*. Furthermore, *S. hymenopus* is not monophyletic with the other *Strongylopus* species used in this analysis. For ML analysis the HKY+G model was selected with parameter values estimated by ModelTest. The single ML tree for RAG1-AmpF1 analysis (-Ln likelihood = 2373) is shown in Fig. E.3. ML analysis produced the same results as MP analysis.

Only 15 samples were available for analysis of ND2 (following exclusion of 5 redundant sequences) and as such only MP analysis was performed. *Tomopterna delalandii* served as the outgroup for this analysis. Of 775 characters, 417 were constant and 238 were parsimony-informative. The strict consensus phylogram is shown in Fig. E.4.

Analysis of the combined datasets

Trees for the combined dataset of 16S and RAG1-AmpF1 are presented below. Trees from other datasets are presented in Appendix D. In total, 17 individuals were included in the combined dataset for 16S and RAG1-AmpF1 (following the deletion of 4 redundant taxa). The outgroup selected for this dataset was *Pyxicephalus adspersus* from Genbank (GB_Pya1 - Genbank Accession number AF206472.1). Of a total of 1248 characters, 937 were constant, 169 were parsimony-uninformative and 142 were parsimony-informative. The 16S fragment contributed 76 of the parsimony-informative characters, while the AmpF1 fragment contributed 66.

Unweighted parsimony recovered only 3 trees, the strict consensus of which (tree length 409) is shown in Fig. 4.13. All major nodes in the MP tree have high support values (minimum 70%). There is very strong bootstrap support (100%) for *S. hymenopus* forming a clade with *A. vertebralis* and *Amietia.*

Hierarchical likelihood tests implemented in Modeltest (Posada & Crandell, 1998) selected the GTR+G model as best fitting the combined data. This model includes three parameters specifying base frequencies, six rate parameters for different nucleotide substations and one parameter for the shape of a gamma distribution specifying rate variation among sites used in the aligned sequences. The phylogram for ML analysis (likelihood score 3827) is shown in Fig. 4.14. Again, bootstrap support is high (minimum 70%), with 95% bootstrap support for the combined clade of *A. vertebralis*, *Amietia* and *S. hymenopus*. Fig. 4.15 shows the 50% majority rule consensus tree for Bayesian analysis, excluding the burn-in of 9000 trees. This tree gives a posterior probability value of 100% for the above clade.

Analysis of other combinations of gene fragments suffered from limited taxon sampling due to inconsistencies in sample selection and sequencing. Only 7 samples from four species were available for the combined analysis of 16S and ND2 including *Tomopterna delalandii* as the outgroup. Of a total of 1247 characters, 899 were constant, with 159 being parsimony-informative. The consensus tree from MP analysis is shown in Fig. E.5. *A. vertebralis* groups most closely with samples assigned to *Amietia dracomontana* (bootstrap support of 95%).

The MP tree for the combined analysis of RAG1 (AmpF1 & AmpF2) is shown in Fig. E.6. *Amietia vandijki* was used as the outgroup. Of 1597 characters, 1428 were constant and 70 were-parsimony informative. Again, *A. vertebralis*, *S. hymenopus* and *Amietia* are shown to be monophyletic and separate from *Strongylopus wageri*.

In a combined analysis of RAG1 (AmpF1 and AmpF2) and RAG2 (Lung35F) only 5 taxa were available following the exclusion of 5 identical sequences, with *S. wageri* as the outgroup. Of a total of 2384 characters, 2013 were constant and 54 were parsimony-informative. The MP 50% majority rule consensus tree is shown in Fig. E.7. *A. vertebralis* and *S. hymenopus* are shown to be monophyletic (90% bootstrap support).

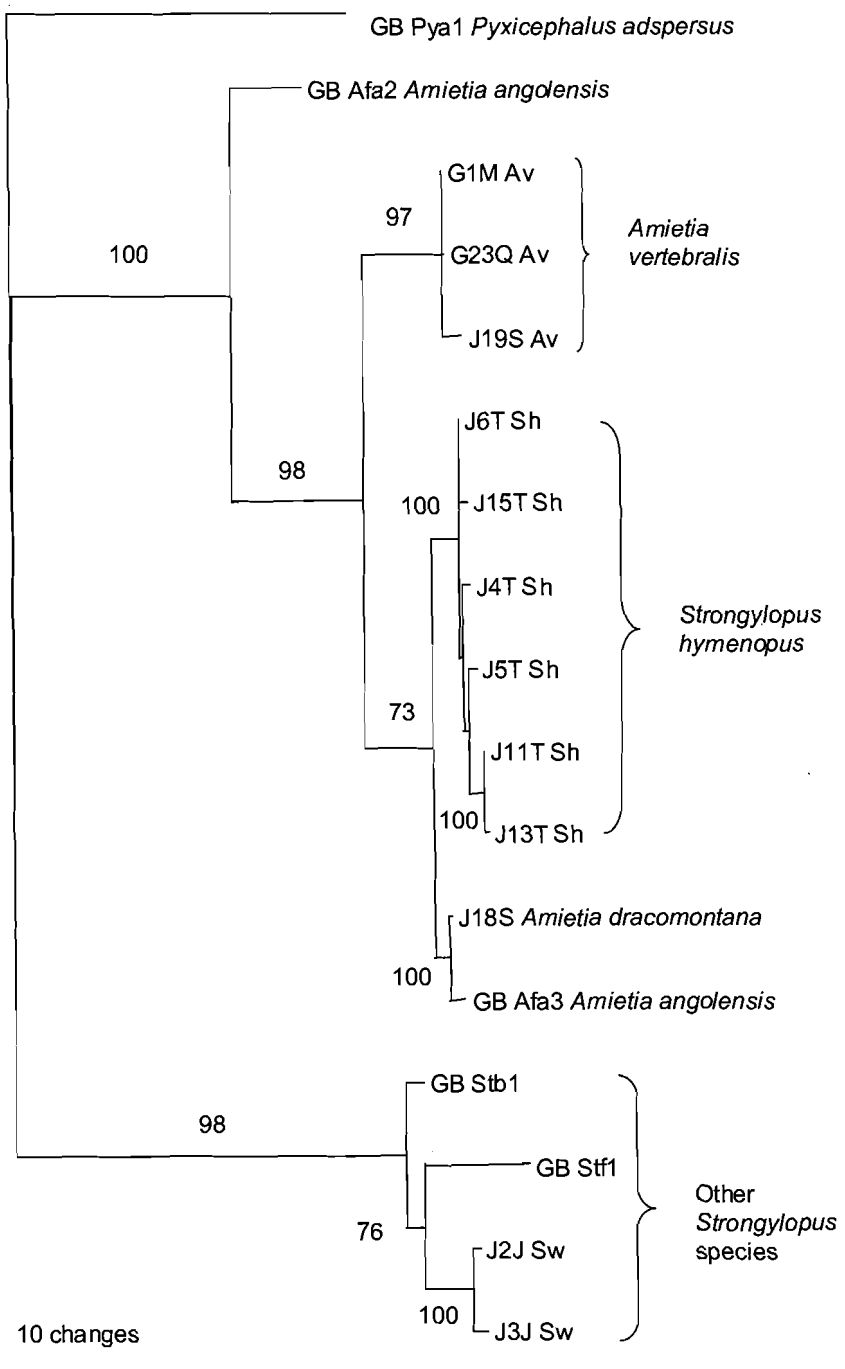

FIGURE 4.13: The strict consensus tree from parsimony analysis for the combined dataset 16S and RAG1-AmpF1 with *Pyxicephalus adspersus* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

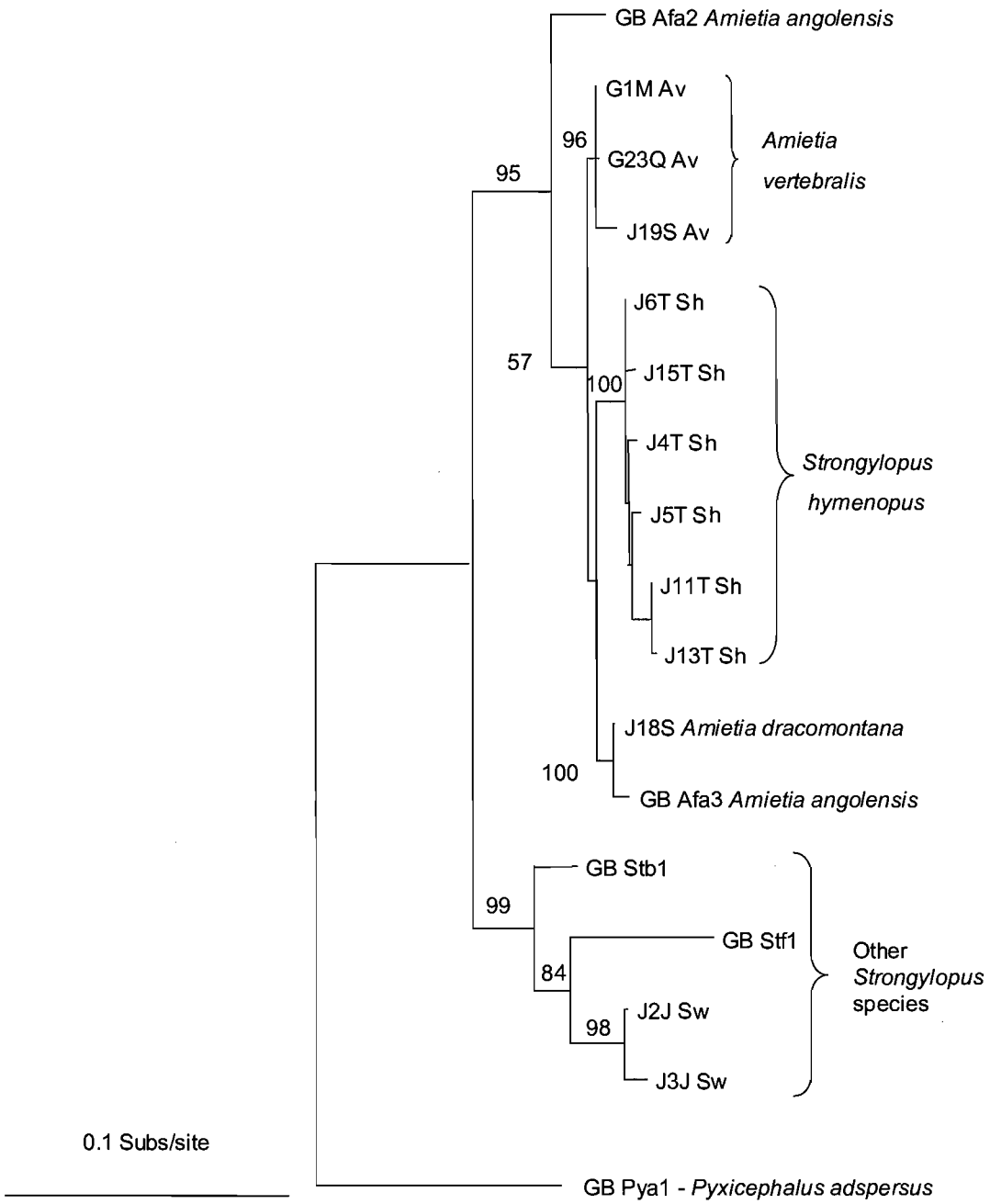

FIGURE 4.14: The single Maximum Likelihood tree (-Ln likelihood = 3827) from analysis of the combined 16S and RAG1-AmpF1 dataset under the GTR+G model of DNA substitution, with parameter values estimated by ModelTest (-Ln likelihood = 3827.03). Branch lengths are proportional to the number of substitutions per site. Bootstrap values are shown at the major nodes. Individual reference numbers correspond to those in Appendix D.

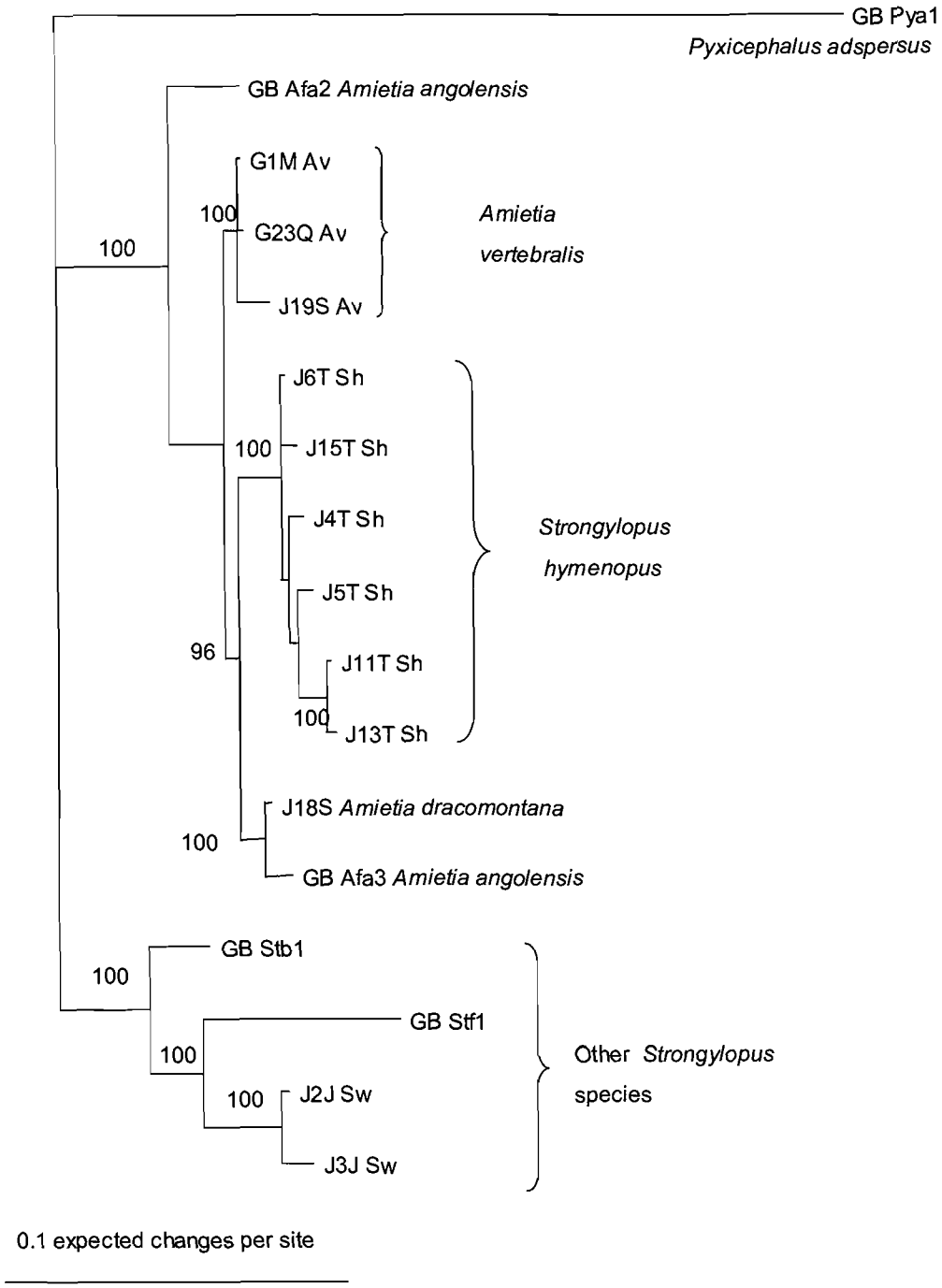

FIGURE 4.15: The 50% majority rule consensus tree from Bayesian analysis of the combined dataset 16S and RAG1-AmpF1. Numbers at nodes are the posterior probability values. Individual reference numbers correspond to those in Appendix D.

4.4.2 Hypotheses testing

The best likelihood trees under each of the five specified phylogenetic hypotheses are shown in Figs. 4.3: null hypothesis – unconstrained maximum likelihood tree, 4.5: *Strongylopus* is monophyletic, 4.7: *A. vertebralis* and *Amietia* are monophyletic, 4.9: *A. vertebralis* and *S. hymenopus* are monophyletic and 4.11: *A. vertebralis*, *S. hymenopus* and *Amietia* monophyletic. SH-tests (Shimodaira & Hasegawa, 1999) were used to statistically compare these trees.

Table 4.3 below shows the likelihoods for each tree, the difference in likelihood between constrained trees and the unconstrained maximum likelihood tree, and the P-value for the SH-test, which is the estimated probability of observing the difference in likelihood values between the ML tree and the actual phylogeny of these species, for the given model and observed sequence data.

TABLE 4.3: SH-Test comparison of the likelihoods for 5 phylogenetic hypotheses (* $P < 0.05$).

Tree	-ln L	Diff -ln L	P
0	3827.03	(best)	
1	3856.46	29.44	0.008*
2	3837.63	10.60	0.179
3	3830.82	3.79	0.570
4	3827.03	0.00	1.000

Forcing monophyly of *Strongylopus* on the analysis resulted in an unusually shaped tree with the basal group consisting of *A. vertebralis*, *S. hymenopus* and *Amietia*, and with other *Strongylopus* species emerging on a long branch out of this cluster, only distantly and weakly related to *S. hymenopus* (Fig. 4.5). Furthermore, the best tree under hypothesis 2 had a much higher likelihood score than the unconstrained ML tree, resulting in a very low probability ($P \leq 0.01$). This hypothesis can therefore be rejected which strongly indicates that *S. hymenopus* is not monophyletic with the *Strongylopus* genus.

None of the other hypotheses were rejected, and the best tree with *A. vertebralis*, *S. hymenopus* and *Amietia* being monophyletic was identical to the unconstrained maximum likelihood tree (Fig 4.11). In each case, except hypothesis 2, the best constrained trees showed these three taxa as monophyletic, with other *Strongylopus* species falling into a separate clade. Relationships within the *A. vertebralis*, *S. hymenopus* and *Amietia* clade varied among analyses, and very short branches separated these taxa. Interestingly, the two *Amietia angolensis* do not group together. These sequences were obtained from GenBank (Accession numbers DQ347318.1 and DQ019493.1) and this result indicates that they could be cases of mis-identification or unrecognised taxonomic diversity.

4.5 Discussion

This study uses both nuclear and mitochondrial DNA sequences to assess the phylogenetic relationships of *Amietia vertebralis* and *Strongylopus hymenopus*. Although it was not possible to generate a complete concatenated data set comprised of all four gene fragments (16S, ND2, RAG1 and RAG2), due to inadequate sampling (of *S. hymenopus*) and inconsistencies in analysis, the results obtained are strongly supported by both the separate and combined datasets. Constrained searches (Figures 4.2 to 4.11) revealed similar topologies under four of the five hypotheses, with *A. vertebralis*, *S. hymenopus* and *Amietia* forming a clade separate from other *Strongylopus* species. These patterns were also consistent across all three methods of phylogenetic inference (i.e. parsimony, maximum likelihood and Bayesian inference).

4.5.1. The question of multiple species of *Amietia vertebralis*

The speculative suggestion that morphological variation among *A. vertebralis* indicates the existence of additional species (Lambiris 1987, 1991; van Dijk, 1966) is not supported by the results of this molecular assessment, which indicates only one species. Morphological polymorphisms have been observed in the tadpoles of other species, including some from high altitude habitats, for example the Mexican spadefoot toad, *Scaphiopus couchii* (see Pfennig & Murphy, 2000) and *Rana temporaria* in the Spanish Pyrenees (Miguel Vences, personal communication), and these appear to be related to competitive feeding strategies (Hunter, 2006). It is unlikely, however, that such polymorphisms could result in speciation, as they would have very little influence on the reproductive behaviour of adults. Given the results of the present study, the possibility of morphological variation in relation to trophic polymorphisms in tadpoles of *A. vertebralis* requires quantitative analysis.

An alternative, and more probable, explanation for the proposed variation is that taxonomic confusion, which is reflected in the inconsistent descriptions and inadequate diagnosis, has resulted in subsequent misidentification and assignment of various other species to *A. vertebralis*. This is consistent with the results of the morphological assessment of preserved specimens, which were found to include multiple specimens.

4.5.2 Intraspecific variation

The lack of genetic variation observed among *A. vertebralis* populations from throughout their distribution provides sufficient evidence that only one species of this taxon exists, and suggests a relatively recent reduction in population size, with the result that the current population is represented by one dominant allele over a large area which includes substantial barriers to dispersal. This implies population expansion over many generations from a single, small refugial population resulting from a population bottleneck. Bottlenecks occur when the number of individuals in a population declines, potentially resulting in loss of genetic variability (Cox, 1997). Bottlenecks that are short-term may have insignificant effects on heterozygosity. However bottlenecks that persist over a number of generations may have a more deleterious impact on variability (Cox, 1997).

This appears to be the case with *A. vertebralis* and may be the result of the most recent ice age which reached its peak about 18 000 years ago and ended roughly 10 000 years ago (McCarthy & Rubidge, 2005). This low level of intraspecific genetic variation within *A. vertebralis* throughout its entire range strongly suggests that there is only one species present, and this provides sufficient evidence to reject the suggestion by Lambiris (1991) of possible additional species, based on his analysis of tadpole mouthparts.

It is interesting to note that, despite inadequate sampling of *S. hymenopus* from a limited geographical area, there is more variation within the *S. hymenopus* populations than in the much larger sample of *A. vertebralis* from a much wider geographical range. It would therefore be of interest to conduct more extensive sampling within populations of *S. hymenopus* to more accurately assess intraspecific variation and potential geographical structure.

4.5.3 Interspecific relationships

One of the most important revelations of the molecular analysis is that *S. hymenopus* is not monophyletic with other *Strongylopus*. This finding is corroborated by the numerous morphological and behavioural aspects discussed above that separate this species from the other *Strongylopus* species. For example, *Strongylopus* are

characterised by very long limbs and very little webbing, while *S. hymenopus* has a compact build and extensive webbing. In addition, there are a number of lifestyle features common to other *Strongylopus* species, which are not exhibited by *S. hymenopus*. All three methods of phylogenetic inference show strong support for *A. vertebralis* being a sister species to *S. hymenopus* and together forming a clade with *Amietia* (although the specific relationships within this clade remain unresolved). *S. hymenopus* and *A. vertebralis* also share a number of important morphological and behavioural adaptations to the high-altitude, aquatic lifestyle. These features may now be interpreted as the results of shared ancestry rather than convergence in a similar environment.

These findings are important in clarifying the phylogenetic relationships of these species and as such can be used to resolve some of the taxonomic problems associated with them. The nomenclatural implications that have arisen as a result of this study are discussed below. For reasons discussed in further detail below, the suggestion is made here that frogs currently assigned to *Strongylopus hymenopus,* should be assigned the name *Rana vertebralis* Hewitt (1927), while *Amietia vertebralis* as currently recognised is referred to as *Rana umbraculata* Bush (1952). Since the taxon *Rana* is no longer a valid name for African ranids (and species in these genera have been found to be monophyletic) both taxa should be assigned to the genus *Amietia*. These taxonomic changes require a re-assessment of the validity and content of both *Amietia* and *Afrana*.

CHAPTER 5

A RE-DESCRIPTION OF *STRONGYLOPUS HYMENOPUS*

The records from the British Museum regarding the holotype specimen of *Strongylopus hymenopus* are astoundingly incomplete. There is no record of the collection date and the locality is recorded only as "South Africa". The earliest firm date associated with the specimen is the re-registration date of 1933. Sir Andrew Smith, who supposedly collected the specimen, conducted his trip to southern Africa during the 1830s, meaning that the specimen spent at least eighty years in preservation before being assessed by Boulenger in 1920. There is therefore no clear evidence to confirm that the specimen is from Lesotho or the Drakensberg at all. Boulenger (1919) mentions in his original notes that the specimen was recorded in Andrew Smith's catalogue of 1858, but this is the only link of the specimen to Smith's collection. From these notes it is clear that Boulenger had trouble placing the specimen in relation to the other African ranids, comparing it to both *Rana grayii* and *Tomopterna natalensis*. He also brings attention to the fact that the locality of the specimen is unknown and that only one specimen was available.

From the morphological assessment conducted for this study it is apparent that the type specimen is dissimilar to *S. hymenopus* individuals from the Drakensberg in multiple ways. These features include, the incomplete webbing, the smooth dorsal skin, the marbling pattern on the edge of the thighs and throat (which is very different to the small stippling pattern seen on *S. hymenopus* as it is currently known) and the much larger tympanum (double the relative size of that of the other *S. hymenopus* specimens). Although webbing can be a highly variable character within genera due to environmental adaptation (Scott, 2005), this variability does not seem to be applicable to *S. hymenopus* since all populations occur within a small range, areas of which experience very similar environmental conditions. These characteristics and what little is known of the history of this specimen suggest that the holotype cannot be

considered conspecific with populations in the northern Drakensberg currently considered to be *S. hymenopus*. From the morphological characteristics observed in this study this holotype specimen most closely resembles *Amietia fuscigula* from the Cape lowlands, where Smith did much of his collecting, although this requires further comparative analysis and implies that the correct identity of this specimen remains indeterminate. The suggestion is made here that the name of this holotype be made *incertae sedis* until further assessment is carried out with regard to its identity and further investigation into the possibility of the existence of a separate species from the Menyameng region in Lesotho (although this seems unlikely).

In addition to the British Museum holotype, a number of other "*S. hymenopus*" specimens that were examined do not match those recently located in the field. Details of these and suggested corrections for their identification can be found in Appendix A, Table A.2 and images are given in Appendix C (Plates 1 and 2). Such mis-identifications have resulted in much confusion in field guides (for example, Passmore & Carruthers, 1995) and inconsistency among authors & accounts (Bates & Haacke, 2003; Channing, 2001; Wager, 1965; Lambiris, 1988, 1991; Minter *et al.*, 2004). Many of these inconsistencies are based on compound samples and un-referenced observations, and include pictures of mis-identified specimens (Passmore & Carruthers, 1995) and distribution records that do not match current observations, for example the record of *S. hymenopus* from Fort Hooke in the Eastern Cape. In some cases there is a clear mix-up of *S. hymenopus* and *A. vertebralis*, for example Wager (1965) uses a picture of *S. hymenopus* to represent *Rana vertebralis*, while Passmore & Carruthers (1995) suggest two forms of *A. vertebralis*, one from Sani Pass (that shows a genuine specimen) and one from Mont-aux-Sources (that shows *S. hymenopus*). For these reasons it is essential that the frogs from the Drakensberg that are currently referred to as *S. hymenopus* be re-described. The following is a description of *S. hymenopus* based on specimens recently (2006/7) collected from the Vemvane stream in the vicinity of Mont-aux-Sources (a locality in which this species is abundant):

Colouring: The dorsal colour varies from very dark shale in some specimens to lighter brown in others. The underside is much paler (creamy-white to golden-yellow). There are usually three bands discernable on the segments of the hind legs, with the dark and

pale bands being roughly equal in width. There are no distinguishing markings on the ventrum other than a small degree of stippling on the border of the mouth and gular area and on the edges of the thighs. The tadpoles exhibit similar patterns in colouration, with the golden ventrum and stippled tail.

Build: The body is flattened, compact and muscular, with the stomach muscles clearly defined. The forearms of males are particularly robust.

Head: The head is relatively wide. The tympanum is small, approximately half the width of the eye diameter. A supratympanic fold is present. The nostrils are raised and centrally positioned. The eyes are bulbous and raised and fall within the perimeter of the head.

Skin texture: The dorsum is rigose and tubular, while the underside is mostly very smooth with some roughness in the cloacal area.

Webbing: The webbing of the hind feet is extensive, extending to the distal phalange. The webbing exhibits a similar stippling pattern as that which occurs on the borders of the thighs and gular region.

Digits: The fingers and toes are thin and tapering, and the subarticular tubercles are indistinct.

The differences between this *S. hymenopus* and others of the genus *Strongylopus* raise questions as to the generic placement of this species. For example, all other *Strongylopus* species have comparably slender bodies and attenuated limbs and especially long toes as opposed to *S. hymenopus*, which has a stocky, robust body shape and does not exhibit the characteristic long toes seen in other species. Furthermore, the webbing of other *Strongylopus* species is minimal (with at least three phalanges free of webbing in *S. wageri*), while that of *S. hymenopus* is extensive. They also differ considerably in life history characteristics, with *S. hymenopus* being the only species in the genus to have a predominantly aquatic life style. These observations, together with the statistical and phylogenetic assessment, imply that *S. hymenopus* requires a complete taxonomic revision and this is discussed below.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 Evaluation of present study

The objective of this dissertation was to provide a thorough systematic review of the Aquatic River Frog, *Amietia vertebralis* and the Berg Stream Frog, *Strongylopus hymenopus* through the use of both morphometric and molecular analysis. Such a review was made necessary due to inconsistencies in museum records and published literature, contentious observations in the field and suggestions by several authors that questioned the taxonomy of both species. The use of both morphological and molecular data for systematic studies inevitably provides an advantage over using just one approach (Hillis *et al.*, 1996; Scott, 2005). The combination of these methods has enabled the objectives of this study to be achieved by providing clear diagnostic characters for both species and by elucidating their phylogenetic relationships at several levels, both of which can be applied in clarifying their taxonomic status.

6.1.1 Morphometric assessment

Examination of preserved specimens of both species, including the type specimens revealed numerous inconsistencies and even mis-identifications (especially with respect to *S. hymenopus*). The cause of much of the taxonomic confusion becomes clear with the discovery that the type specimens for both *A. vertebralis* and *S. hymenopus* do not resemble the species to which these names are currently applied. In the case of *S. hymenopus* there is very little reason (both from the morphology and the history regarding the specimen) to believe that the British Museum holotype is *S. hymenopus* as it is currently known from the Drakensberg. That the holotype locality is recorded only as "South Africa" adds to the uncertainty and, in part, explains the subsequent misidentification of many specimens. It appears that this holotype specimen could be *Amietia fuscigula* from the Western Cape (which is currently also thought to potentially be a separate species from *A. fuscigula* that occurs in the eastern

parts of the country) (Michael Cunningham, personal communication). Similarly, the PEM *A. vertebralis* paratypes, despite being in very poor condition, appear to more closely resemble *S. hymenopus* rather than *A. vertebralis*. That their locality is Mont-aux-Sources further substantiates this suggestion since *A. vertebralis* does not occur at this site.

Statistical analysis provided additional support for the initial morphological assessment. Factor analysis revealed three clear groupings based on various body length ratios. These groupings showed that:

1. *A. vertebralis* and *S. hymenopus* share similar body proportions;
2. *S. hymenopus* is distinctly different from other *Strongylopus* species;
3. Those specimens suspected of being misidentified (including type specimens) grouped separately from *A. vertebralis* and *S. hymenopus*, with most of them being most similar to *Amietia* (previously *Afrana*).

More in-depth comparative statistical analysis using "fingerprint" data and body ratios for each individual confirmed these findings. Most importantly, the *S. hymenopus* holotype specimen differed significantly from *S. hymenopus* as currently understood and the *A. vertebralis* paratypes more closely resemble *S. hymenopus* than *A. vertebralis*.

6.1.2 Phylogenetic relationships

A summary of the main findings of the phylogenetic analysis is given below. These results are based on the Parsimony, Maximum Likelihood and Bayesian Inference phylograms generated from the various datasets used for the molecular analysis:

1. There is very little genetic variation within *A. vertebralis*;
2. *Strongylopus hymenopus* is not monophyletic with the *Strongylopus* genus;
3. *A. vertebralis* and *S. hymenopus* are sister species;
4. *A. vertebralis* and *S. hymenopus* are monophyletic with *Amietia* (previously *Afrana*) within Pyxicephalidae.

Both mitochondrial and nuclear loci were analysed for this study, with the combined dataset of 16S and RAG1-AmpF1 providing the best results. All three methods of phylogenetic inference (MP, ML and BI) produced similar trees, thus providing strong support for the above-mentioned conclusions. The overwhelming lack of genetic variation in populations of *A. vertebralis* from throughout its range provides strong support for there being only one species of *A. vertebralis*. This therefore dismisses the hypothesis that additional species of *A. vertebralis* may exist. The dominance of a single allele throughout all populations of this species suggests a relatively recent (circa 20 000 years ago) genetic bottleneck. Importantly, the results also clearly indicate that *S. hymenopus* is not monophyletic with *Strongylopus*. This is strongly corroborated by the morphological evidence and one questions the reasoning behind the initial decision to place this species within this genus.

The molecular results also provide strong support for *A. vertebralis* and *S. hymenopus* being sister species. Again this is supported by the morphological and life history characteristics that are common to both species. That both species are endemic to the high-altitudes of Lesotho and the Drakensberg may be the result of speciation events brought about due to climatic changes during the Plio-Pleistocene (Mucina & Rutherford, 2006). Observations in the field suggest that the two species are largely sympatric, with *S. hymenopus* being restricted to the north-eastern regions of the Drakensberg on the South African border, while *A. vertebralis* has a broader distribution throughout the central and eastern regions of Lesotho. Furthermore, *S. hymenopus* appears to occur mostly in the rivers that flow eastward into South Africa and *A. vertebralis* is dominant in the larger rivers that flow westerly into Lesotho. From these observations it is reasonable to assume that the location points for *S. hymenopus* in the Eastern Cape and south-central region of Lesotho on the SAFAP map (Figure 2.11) are cases of mis-identification (corrections for these are given in Appendix A).

The phylogenetic analysis also provides strong evidence for *A. vertebralis* being a sister species to *S. hymenopus*, and together forming a clade with *Amietia* within the group Pyxicephalidae. The nomenclatural implications of these findings are discussed below.

6.1.3 Nomenclatural implications

The results from both the morphometric and molecular analysis provide clear evidence that the classification of both *Amietia vertebralis* and *Strongylopus hymenopus* requires revision. The following changes are suggested:

The correct name for *Amietia vertebralis* is *Amietia umbraculata*

The taxon *Amietia vertebralis* as it is currently known is comprised of one species only and this should be renamed *Amietia umbraculata.* This is because the holotype of *Rana fuscigula vertebralis* in fact corresponds with what is currently known as *Strongylopus hymenopus*. The earliest available name for what is currently known as *Amietia vertebralis* is thus *Amietia umbraculata*, taken from the description of the specimens that correspond with this species, i.e. *Rana umbraculata,* Bush, 1952 (the genus *Rana* has subsequently been changed to *Afrana* (sensu Dubois, 1992) and most recently, to *Amietia* (sensu Frost, 2006)). That this species no longer seems to occur in its type locality (vicinity of Drakensberg Gardens, Mzimkulu river) may be due to a number of reasons, the most obvious of which is that trout have been introduced to the area and this has lead to the extirpation of the species from this area. It may also be possible that the climatic conditions in the area have changed significantly, forcing *Amietia umbraculata* to higher altitudes.

The correct name for *Strongylopus hymenopus* is *Amietia vertebralis*

Evidence gleaned from the literature, examination of the holotype specimen and the morphometric and molecular analysis, suggests that *Strongylopus hymenopus* as we currently know it should be changed to *Amietia vertebralis*. The following supports this: Firstly, the initial description by Boulenger (1920) of *Rana hymenopus* is based on a single specimen that does not match the description of *S. hymenopus* as currently known (which may be an undescribed form of *A. fuscigula* from the Western Cape). It is therefore recommended that *Rana hymenopus* be considered *incertae* sedis until further assessment of the specimen and the area from which it is purported to have come from can be made. Secondly, the description of *Rana vertebralis* by Hewitt (1927) matches that of "*Strongylopus hymenopus*" observed in the field. Thirdly, the type specimen (SVL 38 mm) and five smaller paratypes of *A. vertebralis* most closely resemble *S. hymenopus* specimens from Mont-aux-Sources, and finally, the type

locality (Mont-aux-Sources) of *Rana vertebralis* used in the initial description matches that of the distribution of "*Strongylopus hymenopus*" as seen in the field ("*Amietia vertebralis*" as we currently know it does not occur there). Furthermore, the phylogenetic data clearly indicate that this species does not belong to *Strongylopus* but to *Amietia* (sensu Frost). *Amietia vertebralis* is thus the first available name for the currently known *S. hymenopus*, and according to the International Code for Nomenclature, takes seniority.

Generic placement

The genus *Rana* is no longer applicable to the African clade in which both species occur since all African frogs belonging to this genus were subsequently placed in the genus *Afrana* (Dubois, 1992). In 2006 Frost *et al.* placed all species previously in the genus *Afrana* into *Amietia* (Dubois, 1987) and as such both taxa investigated in this study belong to the genus *Amietia*. The relationships within this clade and thus generic placement of these species requires further investigation.

6.2 Future work

6.2.1 Acoustics

The advertisement call of males is one of the most important criteria for defining frog species. Even if no morphological differences are found between individuals within a population, but distinctive calls are observed, then more than one reproductively isolated species is assumed to be involved (Vences & Wake, 2007). At present very little call information and recordings have been obtained for *Amietia umbraculata* (currently *A. vertebralis*) and *Amietia vertebralis* (currently *S. hymenopus*) and as such it would be important to obtain proper recordings of these calls from throughout their ranges. Both species are highly aquatic and are known to call underwater, although this can be heard from above. Additional knowledge regarding these calls will provide important information that can be used for distinguishing species.

6.2.2 Behavioural studies

Due to the remoteness of their location, in-depth knowledge of the lifestyle habits of both species remains insufficient. Both are evidently adapted to very cold conditions and both tadpoles and adults have been observed swimming under ice during the winter months. However it remains unclear as to whether they are truly active throughout the year. In addition, the stimulus for mating is possibly connected to the spring rains, although amplecting pairs of *Amietia vertebralis* (currently *S. hymenopus*) have been seen as early as July. It would thus be of interest to have a better understanding of this apparently opportunistic behaviour. A number of discrepancies have also been observed with regard to the tadpoles of *Amietia vertebralis* (currently *S. hymenopus*) (Lambiris, 1987, Du Preez & Weldon, unpublished work) and this in itself may provide scope for additional taxonomic study.

6.2.3 Further phylogenetic analysis

In terms of sampling, this study was certainly biased with respect to *Amietia umbraculata* (currently *A. vertebralis*). For this species there were ample samples from a large number of localities throughout the species distribution. However,

although *Amietia vertebralis* (currently *S. hymenopus*) has a much smaller distribution, sampling of this species from throughout its range was inadequate to accurately assess interspecific diversity. More thorough sampling from throughout this species' range is therefore necessary to fully assess patterns of diversity. Interestingly, despite this skewed sampling, there appears to be more diversity within *Amietia vertebralis* (currently *S. hymenopus*) than the much more widely distributed *Amietia umbraculata* (currently *A. vertebralis*). It would thus be of interest to obtain additional samples and conduct more in-depth population studies of this species. Furthermore, the phylogenetic results obtained in the present study show that *Amietia umbraculata* ("*A. vertebralis*"), *Amietia vertebralis* ("*S. hymenopus*") and *Amietia* (previously *Afrana*) form a clade, however the exact relationships within this clade remain unclear. Further phylogenetic analysis is therefore necessary to elucidate the relationships within this clade. In addition, more in-depth analysis on the phylogeography of the two species would give an indication of the geographical structure of the genetic variation.

6.3 Conservation implications

Accurate taxonomic knowledge is necessary to ensure adequate conservation efforts (Lötters, *et al*, 2004). The revelation of cryptic or additional species through phylogenetic analysis can result in the need for revision of conservation strategies (for example, if a small population of a new species is discovered it will require specialised conservation treatment). In this case there remain only two species in question, despite the name changes. However, as high-altitude species, *Amietia umbraculata* and *Amietia vertebralis* do face increased risk of extinction for a number of reasons. In general, the environment in which they survive is extreme and any disturbances brought about as a result of climatic change may severely alter this habitat. More specifically, although they are distributed in a relatively remote area of southern Africa, these species are still at risk from increasing habitat destruction, especially as the Highlands Water Project continues. They are also increasingly threatened by invasive species, in particular trout, which have been introduced for recreational purposes.

Both species are currently listed under the Red List Category of "Least Concern", in view of their relatively wide distribution and presumed large population (http://www.globalamphibians.org). This is accurate with respect to *A. umbraculata*, but *A. vertebralis* appears to have a much more restricted distribution than is reflected in the literature. Small populations are inherently at higher risk of extinction, and this in addition to the high prevalence of chytridiomycosis infection recorded in this species indicates the need to conduct more thorough monitoring of populations and possibly the introduction of specific conservation measures and parasite management.

CHAPTER 7

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APPENDIX A

TABLE A.1: List of museum specimens used for morphometric analysis. Species names are those that appear on the label of specimen or with museum records and may not reflect true identity of the specimen. Suggested corrections for misidentified specimens (*) are given in Table A.2. All specimens examined were post-metamorphic. Museum abbreviations: AACRG = African Amphibian Conservation Research Group, Potchefstroom. BM = British Museum, London. MC = Michael Cunningham, personal collection. NM = Natal Museum, Pietermaritzburg. NMBA = National Museum, Bloemfontein. PEMA = Port Elizabeth Museum. JR & DT specimens are from SAIAB collection. TM = Transvaal Museum, Pretoria.

Species	Specimen Ref	Location	Collection date
*A. vertebralis**	BM.1978.1254	Summit of Drakensberg, Natal	1946
*A. vertebralis**	BM.1978.1240	Mont-aux-Sources	1978
A. vertebralis	NM06297	Underberg	1976
A. vertebralis	NM06354	Organ Pipes	1977
A. vertebralis	NM06355	Organ Pipes	1977
A. vertebralis	NM06358	Organ Pipes	1977
A. vertebralis	NM06349	Organ Pipes	1977
A. vertebralis	NM06356	Lesotho, Giant's Castle	1958
A. vertebralis	NM06348	Lesotho, Organ Pipes Pass	1977
A. vertebralis	NM06351	Organ Pipes	1977
A. vertebralis	NM06357	Lesotho, Organ Pipes Pass	1977
A. vertebralis	NM06359	Organ Pipes	1977
A. vertebralis	NM06347	Lesotho, Organ Pipes Pass	1977
A. vertebralis	NM06353	Lesotho, Organ Pipes Pass	1977
A. vertebralis	NM06352	Lesotho, Organ Pipes Pass	1977
A. vertebralis	NM0630	Organ Pipes	
A. vertebralis	NM05596	Tugela	1944
A. vertebralis	NM00981	KZN, Umzimkulu River, Underberg	1951
A. vertebralis	NM00982	KZN, Umzimkulu River, Underberg	1951
A. vertebralis	NM02621	Drakensberg Gardens Forestry Reserve	1951
A. vertebralis	NM05598	Tugela	1944
A. vertebralis	NM05587	Giant's Castle	1958
A. vertebralis	NM02616	KZN, Royal Natal National Park, MAS	1955
A. vertebralis	NM02615	Unknown	
A. vertebralis	NM05601	Tugela	1944
A. vertebralis	NM05599	Tugela	1944
A. vertebralis	NM05592	Giant's Castle	1958
A. vertebralis	NM05597	Tugela	1944
A. vertebralis	NM02617	KZN, Tugela	1944
A. vertebralis	NM05588	Giant's Castle	1958
A. vertebralis	NM05600	Tugela	1944
A. vertebralis	NM05591	Organ Pipes	1977
A. vertebralis	NM05593	KZN, Natal National Park, MAS	1954
A. vertebralis	NM05602	Unknown	
A. vertebralis	NM02618	Giant's Castle	1956
A. vertebralis	NM05589	Lesotho, Giant's Castle	1958
A. vertebralis	NM05590	Giant's Castle	1958
A. vertebralis	NM02619	Giant's Castle	1956
A. vertebralis	NM00980	Giant's Castle	1956
A. vertebralis	NM02614	Unknown	
A. vertebralis	NM02608	Drakensberg Gardens Forestry Reserve	1951
A. vertebralis	NM05594	Giant's Castle	1958

A. vertebralis	NM02609	Drakensberg Gardens Forestry Reserve	1951
A. vertebralis	NM02613	Drakensberg Gardens Forestry Reserve	1951
A. vertebralis	NM02620	Drakensberg Gardens Forestry Reserve	1951
A. vertebralis	NM00973	Cathedral Peak	1952
A. vertebralis	NM00974	Cathedral Peak	1952
A. vertebralis	NM02607	Lekhalabaletsi River	1949
A. vertebralis	NMBA4799	Malibamatso River, near Thaba-Li-Mele.	1991
A. vertebralis	NMBA4798	Malibamatso River, near Thaba-Li-Mele.	1991
A. vertebralis	NMBA4800	Malibamatso River, near Thaba-Li-Mele.	1991
A. vertebralis	NMBA4804	Mothae River Phofung, Lesotho	1991
A. vertebralis	NMBA4604	Junction of Mokhotlong & Senqu River	1989
A. vertebralis	NMBA4803	Malibamatso River, near Thaba-Li-Mele.	1991
A. vertebralis	NMBA4605	Junction of Mokhotlong & Senqu River	1989
A. vertebralis	NMBA5802	Malibamatso River, near Thaba-Li-Mele.	1991
A. vertebralis	NMBA4815	Mothae River Phofung, Lesotho	1991
A. vertebralis	NMBA4806	Mothae River Phofung, Lesotho	1991
A. vertebralis	NMBA6288	Btw Marakbei & Ha Cassamo, Lesotho	1991
A. vertebralis	NMBA5906	3km S of Sethamana, Linakeng Area	1992
A. vertebralis	NMBA4807	Mothae Riv, Phofung, Lesotho	1991
A. vertebralis	NMBA4811	Mothae Riv, Phofung, Lesotho	1991
A. vertebralis	NMBA4809	Mothae Riv, Phofung, Lesotho	1991
A. vertebralis	NMBA5905	3km S of Sethamana, Linakeng Area	1992
A. vertebralis	NMBA4810	Mothae Riv, Phofung, Lesotho	1991
A. vertebralis	NMBA4808	Mothae Riv, Phofung, Lesotho	1991
A. vertebralis	NMBA4812	Upper reaches Bokong Riv, Lesotho	
A. vertebralis	PEMA1664	Thaba Phutso River, Kotisepmola pass	1988
*A. vertebralis**	PEMA1552	Locality: Mont-Aux-Sources.	
A. vertebralis	PEMA1666	Thaba Phutso River, Kotisepmola pass	1988
*A. vertebralis**	PEMA1550	Locality: Mont-Aux-Sources.	
A. vertebralis	PEMA1688	Sehonghong Riv. Senqu Basin.	1988
A. vertebralis	PEMA1692	Mokhotlong Riv. Senqu Basin.	1988
A. vertebralis	PEMA1683	Mangaung Riv. Sani-Linakeng Basin	1988
A. vertebralis	PEMA1682	Mangaung Riv. Sani-Linakeng Basin	1988
A. vertebralis	PEMA1629	Sani River, Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1662	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1696	Sani River, Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1670	Sani River, Sani top	
A. vertebralis	PEMA1646	Sehonghong Riv. Senqu Basin.	1988
A. vertebralis	PEMA1641	Sehonghong Riv.Mokhotlong-Senqu Basin	1988
A. vertebralis	PEMA1671	Sani River, Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1633	Sani River, Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1647	Sehonghong Riv. Senqu Basin.	1988
A. vertebralis	PEMA1710	Sehonghong Riv. Mokhotlong-Senqu Basin	1988
A. vertebralis	PEMA1668	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1643	Sehonghong Riv.Mokhotlong-Senqu Basin	1988
A. vertebralis	PEMA1674	Sanqubethu Riv. Mokhotlong-Senqu basin.	1988
A. vertebralis	PEMA1665	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1701	Sani Riv. Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1628	Sani Riv. Linakeng-Senqu Basin, Sani Flats	1988
A. vertebralis	PEMA1626	Sehonghong Riv. Mokhotlong-Senqu Basin	1988
A. vertebralis	PEMA1698	Sani Riv. Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1655	No locality info	
A. vertebralis	PEMA1640	Sani Riv. Linakeng-Senqu Basin	
A. vertebralis	PEMA1703	Sani Riv. Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1667	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1671	Sani River Valley	
A. vertebralis	PEMA1695	Sani River, Linakeng-Senqu Basin	1988
A. vertebralis	PEMA1689	Sehonghong Riv. Senqu-Orange Basin	1988
A. vertebralis	PEMA1687	Sehonghong Riv. Senqu-Orange Basin	1988
A. vertebralis	PEMA1661	Thaba Phutso River, Kotisepmola pass	1988

A. vertebralis	PEMA1663	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1711	Sehonghong Riv. Mokhotlong-Senqu Basin.	1988
A. vertebralis	PEMA1700	Sani River, Linakeng-Senqu Basin, above bridge	1988
A. vertebralis	PEMA1680	Sani River Valley	1989
A. vertebralis	PEMA1686	Sehonghong Riv. Senqu-Orange Basin	1988
A. vertebralis	PEMA1657	Sani Riv. Linakeng-Senqu Basin. Mangaung confl.	1988
A. vertebralis	PEMA1684	Sehonghong Riv. Senqu-Orange Basin	1988
A. vertebralis	PEMA1691	Mokhotlong Riv. Senqu Basin. Above Senqu Bethu	1988
A. vertebralis	PEMA1654	Sani Riv. Linakeng-Senqu Basin. Sani Top	1988
A. vertebralis	PEMA1627	Sani Riv. Linakeng-Senqu Basin. Sani Flats	1988
A. vertebralis	PEMA1678	Mangaung Riv. Sani-Linakeng Basin.	1988
A. vertebralis	PEMA1693	Sani Riv. Linakeng-Senqu Basin. Mangaung confl.	1988
A. vertebralis	PEMA1630	Sani Riv. Linakeng-Senqu Basin. Sani Flats	1988
A. vertebralis	PEMA1699	Sani Riv. Linakeng-Senqu Basin. Above Bridge	1988
A. vertebralis	PEMA1690	Mokhotlong Riv. Senqu Basin. Above Senqu Bethu	1988
A. vertebralis	PEMA1648	Sehonghong Riv. Mokhotlong-Senqu Basin.	
A. vertebralis	PEMA1712	Sehonghong Riv. Mokhotlong-Senqu Basin.	1988
A. vertebralis	CDNC5124	Eastern Cape - Naude's Nek, Bell River	
A. vertebralis	CDNC5165	Eastern Cape - Naude's Nek, Bell River	
A. vertebralis	PEMA1648	Lesotho, Sehonghong River, Senqu-orange-basin	
A. vertebralis	PEMA1636	Lesotho, Sehonghong River, Senqu-orange-basin	
A. vertebralis	PEMA1644	Lesotho, Sehonghong River, Senqu-orange-basin	
A. vertebralis	PEMA1645	Lesotho, Sehonghong River, Senqu-orange-basin	
A. vertebralis	PEMA1651	Lesotho, Sehonghong River, Senqu-orange-basin	
A. vertebralis	PEMA1672	Sani River, Linakeng-Senqu Basin, Mangaung	
A. vertebralis	PEMA1694	Sani River, Linakeng-Senqu Basin, Mangaung	
A. vertebralis	PEMA1705	Sani River, Linakeng-Senqu Basin, Mangaung	
A. vertebralis	PEMA1704	Sani River, Linakeng-Senqu Basin, Mangaung	1988
A. vertebralis	PEMA1635	Sani River, Linakeng-Senqu Basin, Mangaung	1988
A. vertebralis	PEMA1656	Tributory Sani Linakeng Basin at Road Drift	
A. vertebralis	PEMA1669	Thaba Phutso River, Kotisepmola pass	1988
A. vertebralis	PEMA1677	Sani River, Linakeng-Senqu Basin, Mangaung	1988
A. vertebralis	JR16.1	Mashai	2000
A. vertebralis	JR16.2	Mashai	2000
A. vertebralis	JR16.3	Mashai	2000
A. vertebralis	JR16.4	Mashai	2000
A. vertebralis	JR16.5	Mashai	2000
A. vertebralis	JR14A.1	Mangaung	2000
A. vertebralis	JR14A.2	Mangaung	2000
A. vertebralis	JR14A.3	Mangaung	2000
A. vertebralis	JR14A.4	Mangaung	2000
A. vertebralis	JR14A.5	Mangaung	2000
A. vertebralis	JR14A.6	Mangaung	2000
A. vertebralis	JR14A.7	Mangaung	2000
A. vertebralis	JR14A.8	Mangaung	2000
A. vertebralis	JR16.6	Mashai	2000
A. vertebralis	JR16.7	Mashai	2000
A. vertebralis	JR16.8	Mashai	2000
A. vertebralis	DT8.1	Bafali	2000
A. vertebralis	DT8.2	Bafali	2000
A. vertebralis	DT8.3	Bafali	2000
A. vertebralis	DT8.4	Bafali	2000
A. vertebralis	DT8.5	Bafali	2000
A. vertebralis	DT16.1	Qabane	2000
A. vertebralis	DT16.2	Qabane	2000
A. vertebralis	DT16.3	Qabane	2000
A. vertebralis	DT16.4	Qabane	2000
A. vertebralis	DT16.5	Qabane	2000
A. vertebralis	JR4.1	Lesobeng	2000
A. vertebralis	JR4.2	Lesobeng	2000

A. vertebralis	JR4.3	Lesobeng	2000
A. vertebralis	JR4.4	Lesobeng	2000
A. vertebralis	JR4.5	Lesobeng	2000
A. vertebralis	DT17A.1	Tsatsana	2000
A. vertebralis	DT17A.2	Tsatsana	2000
A. vertebralis	DT17A.3	Tsatsana	2000
A. vertebralis	DT17A.4	Tsatsana	2000
A. vertebralis	DT17A.5	Tsatsana	2000
A. vertebralis	DT17A.6	Tsatsana	2000
A. vertebralis	JR12.2	Mokhotlong	2000
A. vertebralis	JR13.2	Redi	2000
A. vertebralis	JR9.1	Maletsunyane	2000
A. vertebralis	JR9.2	Maletsunyane	2000
A. vertebralis	JR9.3	Maletsunyane	2000
A. vertebralis	JR15.2	Sani tributary	2000
A. vertebralis	JR15.3	Sani tributary	2000
A. vertebralis	JR15.4	Sani tributary	2000
A. vertebralis	DT9.1	Langa-le-balele	2000
A. vertebralis	DT9.2	Langa-le-balele	2000
A. vertebralis	DT9.3	Langa-le-balele	2000
A. vertebralis	DT9.4	Langa-le-balele	2000
A. vertebralis	JR11B.1	Lower Moremoholo	2000
A. vertebralis	JR11B.2	Lower Moremoholo	2000
A. vertebralis	JR11B.3	Lower Moremoholo	2000
A. vertebralis	JR3.1	Mantsonyane	2000
A. vertebralis	JR3.2	Mantsonyane	2000
A. vertebralis	JR3.3	Mantsonyane	2000
A. vertebralis	DT11.1	Sani	2000
A. vertebralis	DT11.2	Sani	2000
A. vertebralis	DT11.3	Sani	2000
A. vertebralis	DT11.4	Sani	2000
A. vertebralis	DT11.5	Sani	2000
A. vertebralis	DT5	Makhaleng	2000
A. vertebralis	JR5B	Tshelanyane	2000
A. vertebralis	DT10.1	Mohlesi	2000
A. vertebralis	JR15.1	Sani tributary	2000
A. vertebralis	JR5A.1	Tshelanyane	2000
A. vertebralis	JR5A.2	Tshelanyane	2000
A. vertebralis	JR5A.3	Tshelanyane	2000
A. vertebralis	DT2.1	Tenane	2000
A. vertebralis	JR21.1	Senqunyane	2000
A. vertebralis	JR21.2	Senqunyane	2000
A. vertebralis	JR7.1	Ketane	2000
A. vertebralis	JR16.9	Mashai	2000
A. vertebralis	JR16.10	Mashai	2000
A. vertebralis	JR10.1	Senqu	2000
A. vertebralis	JR10.2	Senqu	2000
A. vertebralis	JR16.11	Mashai	2000
A. vertebralis	JR16.12	Mashai	2000
A. vertebralis	JR16.13	Mashai	2000
A. vertebralis	JR8.1	Maletsunyane	2000
A. vertebralis	TM79374	Ngwangwane River	2000
A. vertebralis	TM13954	Source of the Thukela, Mont-aux-Sources	2000
A. vertebralis	TM30373	Malutseuyane Falls.	1965
A. vertebralis	TM13952	Lesotho, Source of Thukela, Mont-aux-Sources	1930
A. vertebralis	TM21357	Lesotho, Tugela River, Mont-aux-Sources	1944
A. vertebralis	TM13961	Lesotho, Source of Thukela, Mont-aux-Sources	1930
A. vertebralis	TM13953	Lesotho, Source of Thukela, Mont-aux-Sources	1930
A. vertebralis	TM13957	Lesotho, Source of Thukela, Mont-aux-Sources	1930
A. vertebralis	TM26154	No label info	

A. vertebralis	TM21356	Tugela, Mont-aux-Sources 1944	
A. vertebralis	TM13960	Lesotho, Source of Thukela,, Mont-aux-Sources	1930
A. vertebralis	TM21355	Lesotho, Source of Thukela,, Mont-aux-Sources	1944
A. vertebralis	TM13955	Lesotho, Source of Thukela,, Mont-aux-Sources	1930
A. vertebralis	TM21358	Lesotho, Source of Thukela,, Mont-aux-Sources	1944
A. vertebralis	TM30059	Lesotho, Sani River Btw Sani Pass & Makhotlong	1963
A. vertebralis	TM56436	3km NW Thaba Tseka, Lesotho	1982
A. vertebralis	TM21354	Unknown	
A. vertebralis	TM13959	Lesotho, Source of Thukela,, Mont-aux-Sources	1930
A. vertebralis	TM21724	Unknown	
A. vertebralis	TM13963	Lesotho, Source of Thukela,, Mont-aux-Sources	1930
A. vertebralis	TM30262	Khabos	1964
A. vertebralis	TM13956	Lesotho, Source of Thukela,, Mont-aux-Sources	1944
A. vertebralis	TM30264	Khabos	1965
A. vertebralis	TM30299	Blue Mountain Pass	1964
S. hymenopus	AACRG647	Mont-aux-Sources	2006
S. hymenopus	AACRG343	Mont-aux-Sources	2006
S. hymenopus	AACRG340	Mont-aux-Sources	2006
S. hymenopus	AACRG336	Mont-aux-Sources	2006
S. hymenopus	AACRG341	Mont-aux-Sources	2006
S. hymenopus	AACRG342	Mont-aux-Sources	2006
S. hymenopus	AACRG339	Mont-aux-Sources	2006
S. hymenopus	AACRG337	Mont-aux-Sources	2006
S. hymenopus	AACRG338	Mont-aux-Sources	2006
S. hymenopus	AACRG335	Mont-aux-Sources	2006
S. hymenopus	MC1	Mont-aux-Sources. Chain ladders	
S. hymenopus	MC2	Mont-aux-Sources. Chain ladders	
S. hymenopus	MC3	Mont-aux-Sources. Chain ladders	
S. hymenopus	MC4	Mont-aux-Sources. Chain ladders	
S. hymenopus	MC5	Mont-aux-Sources. Chain ladders	
S. hymenopus	MC6	Mont-aux-Sources. Chain ladders	
*S. hymenopus**	BM.1978.2.28	South Africa Collected: Sir A Smith	1800s
*S. hymenopus**	BM.1978.1235	Sani Pass	1978
*S. hymenopus**	NM00651	No locality info	1938
*S. hymenopus**	NM2774	Cathedral Peak	1944
*S. hymenopus**	NM2773	Cathedral Peak	1943
*S. hymenopus**	NM904	Mont-aux-Sources	1946
*S. hymenopus**	NM2772	Cathkin Peak	1943
A. angolensis	NM7761	Goedgedunk, R69 bridge, Xamu River	1997
A. angolensis	M7764	Cathedral Peak Nature Reserve, Ndumeni Stream	1997
A. fuscigula	NM7315	Spioenkop Public Resort	1985
A. fuscigula	NM7535	Tributary of Lozi River	
S. grayii	NM6994	Nelsons Kop (20km E of Van Reenen)	
S. grayii	NM6992	Port Elizabeth	1979
S. fasciata	NM6396	Pietermaritzburg, Wylie Park	1977
R. draconensis	NM734	no locality info	

TABLE A.2: Suggested corrections for mis-identified museum specimens

Museum Identification	Museum Specimen reference	Collection notes	Examination notes	Suggested correction
Amietia vertebralis	BM.1978.1254	Rana vertebralis Skull removed Location: Summit of Drakensberg 1946	1 phalange free of webbing Small body size rugose dorsum with elongate tubercles Leg banding consistant with *S. hymenopus*	*Strongylopus hymenopus*
Amietia vertebralis	BM.1978.1240	Rana vertebralis Location: Mont-aux-Sources (~ 2450 m) 1978	Dorsum smooth with small tubercles Relatively long legs and head Collected well below plateua	*Amietia fuscigula*
Amietia vertebralis	PEMA1550	*A. vertebralis* paratypes. Location: Mont-aux-Sources No date	Very poor condition Small body size Extensive webbing	*Strongylopus hymenopus*
Amietia vertebralis	PEMA1552	*A. vertebralis* paratypes. Location: Mont-aux-Sources No date	Very poor condition Small body size Extensive webbing	*Strongylopus hymenopus*
Strongylopus hymenopus	BM.1978.2.28	*S. hymenopus* Holotype. Location: South Africa Re-registration: 1933	3 phalanges free of webbing Large body size Wide head Large tympana Smooth dorsum	Possibly *Amietia fuscigula* (Cape lowlands)
Strongylopus hymenopus	BM.1978.1235	Skull removed Location: Sani Pass 1966	3 phalanges free of webbing Large body size Head narrow Dorsum very rugose Tympana set forward	Possibly *Amietia dracomontana*
Strongylopus hymenopus	NM00651	No locality info 1938	3 phalanges free of webbing on outer IV. Digits are tubular. Relatively long limbs, dark bands broader than light bands, 5-6 bands per segment Tapering head. Robust forearms and thighs Squared off snout	Possibly *A. angolensis* or *A. fuscigula*

			Dorsum tubercular. Appears to have moderately mottled gular and perhaps mottling behind thighs. Eyes don't seem to project beyond the profile (may be preservation)	
Strongylopus hymenopus	NM2772	Cathkin Peak 1943	3 phalanges free of webbing on outer IV. Squared off snout. Dark banding very broad, about 5-6 bands per segment. Bulbous eyes projecting beyond body line, also close together dorsally. Large, bulbous subcarpal tubercles	Possibly *A. angolensis* or *A. fuscigula*
Strongylopus hymenopus	NM2774	Cathedral Peak 1944	Leg-bands: thick (dark) & thin (light), about 4 bands per segment. 2.5 phalanges free of webbing on outer IV digit. Dorsum irregularly rugose	Possibly *A. angolensis* or *A. fuscigula*
Strongylopus hymenopus	NM2773	Cathedral Peak 1943	As above	Possibly *A. angolensis* or *A. fuscigula*
Strongylopus hymenopus	NM904	Mont-aux-Sources 1946	As above	Possibly *A. angolensis* or *A. fuscigula*

APPENDIX B

TABLE B.1: Raw data of the average of the three measurements taken for each specimen (n = 268) for each of the 16 variables examined.

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	BM.1978.1254	32.87	32.87	17.59	14.81	8.14	17.83	7.70	19.00								7.35	0.54	0.45	0.84	Extensive	Summit of Drakensberg, Natal
Amietia vertebralis	BM.1978.1240	44.11	44.11	29.50	18.95	9.99	25.31	10.58	28.62	19.69	10.03	5.23	3.63	3.82	11.73	7.16	9.48	0.67	0.43	0.64	1 free (outer)	Mont-aux-Sources
Amietia vertebralis	NM06297	53.00	52.82	29.51	22.40	15.64	28.77	13.59	28.17	21.22	11.89	7.56	4.99	2.69	14.86	8.37	12.63	0.56	0.42	0.76	Extensive	Underberg
Amietia vertebralis	NM06354	30.93	30.75	17.94	12.55	8.06	17.41	7.36	17.63	12.46	7.59	5.15	3.17	2.15	8.11	4.40	8.20	0.58	0.41	0.70	Extensive	Organ Pipes
Amietia vertebralis	NM06355	35.86	35.43	18.57	13.69	9.60	17.49	6.85	20.39	13.86	7.81	5.33	3.28	2.52	9.62	5.66	8.19	0.52	0.38	0.74	Extensive	Organ Pipes
Amietia vertebralis	NM06358	30.48	30.42	16.30	12.86	9.21	15.86	6.80	16.93	12.61	7.02	4.39	2.96	2.35	8.53	4.72	7.51	0.53	0.42	0.79	Extensive	Organ Pipes
Amietia vertebralis	NM06349	31.93	31.64	18.29	13.07	9.13	16.76	7.01	17.88	12.62	7.44	3.99	2.75	2.04	8.75	4.37	7.79	0.57	0.41	0.71	Extensive	Organ Pipes
Amietia vertebralis	NM06356	36.84	36.22	18.52	13.14	8.37	16.97	6.88	19.56	12.94	8.05	4.34	2.79	2.26	8.92	4.58	8.47	0.50	0.36	0.71	Extensive	Lesotho, Giant's Castle
Amietia vertebralis	NM06348	31.51	30.45	16.36	11.46	8.10	15.24	6.77	13.65	11.22	6.99	3.84	2.57	1.46	7.71	3.90	6.87	0.52	0.36	0.70	Extensive	Lesotho, Organ Pipes Pass
Amietia vertebralis	NM06351	31.46	31.42	17.22	12.07	9.20	16.59	7.16	18.39	12.07	7.55	4.53	2.94	2.21	8.58	4.08	7.80	0.55	0.38	0.70	Extensive	Organ Pipes
Amietia vertebralis	NM06357	40.24	40.24	19.36	14.60	9.99	18.64	7.27	18.37	14.18	8.10	4.73	2.74	2.40	10.92	5.93	8.34	0.48	0.36	0.75	Extensive	Lesotho, Organ Pipes Pass
Amietia vertebralis	NM06359	30.69	30.69	18.08	12.81	9.21	16.45	6.94	14.81	12.13	7.80	4.39	2.75	2.42	9.06	4.56	7.46	0.59	0.42	0.71	Extensive	Organ Pipes
Amietia vertebralis	NM06347	31.94	31.94	16.76	11.45	9.09	16.08	7.29	16.46	11.51	6.73	4.41	2.24	2.22	7.54	4.08	6.57	0.52	0.36	0.68	Extensive	Lesotho, Organ Pipes Pass
Amietia vertebralis	NM06353	27.11	27.11	16.06	10.83	8.89	14.34	5.87	13.33	11.54	6.21	4.18	1.74	2.40	8.08	3.28	7.29	0.59	0.40	0.67	Extensive	Lesotho, Organ Pipes Pass

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	NM06352	30.23	30.23	17.33	11.46	9.28	16.57	6.98	15.55	12.07	6.92	4.42	2.40	2.13	7.60	3.85	7.72	0.57	0.38	0.66	Extensive	Lesotho, Organ Pipes Pass
Amietia vertebralis	NM0630	36.56	36.56	17.99	12.40	9.60	15.69	6.89	16.23	12.60	6.67	4.60	2.93	2.53	9.11	3.50	6.71	0.49	0.34	0.69	Extensive	
Amietia vertebralis	NM05596	20.96	20.96	9.02	7.71	6.17	9.64	5.31	9.71	7.94	5.34	3.06	2.59	1.47	6.07	3.36	6.18	0.43	0.37	0.85		Tugela
Amietia vertebralis	NM00981	104.12	107.35	56.97	55.40	25.34	57.57	23.51	56.47	46.57	24.38	10.82	7.56	5.13	31.72	17.56	24.76	0.55	0.53	0.97	Extensive	KZN, Umzimkulu River,
Amietia vertebralis	NM00982	86.53	85.41	46.33	38.95	20.98	45.42	23.42	45.53	35.71	20.52	10.95	7.57	4.10	25.48	14.08	20.01	0.54	0.45	0.84	Extensive	KZN, Umzimkulu River.
Amietia vertebralis	NM02621	103.46	101.84	44.92	41.63	24.18	47.11	18.59	44.25	38.36	21.79	11.99	6.01	5.32	26.08	14.07	23.56	0.43	0.40	0.93	Extensive	Drakensberg Gardens Forestry
Amietia vertebralis	NM05598	34.07	34.07	17.59	12.54	8.53	16.91	6.39	18.89	13.86	6.89	4.24	3.03	2.22	9.38	4.77	8.21	0.52	0.37	0.71		Tugela
Amietia vertebralis	NM05587	42.38	42.38	20.81	16.29	11.36	20.95	9.45	22.00	13.72	8.71	5.13	2.61	1.67	10.34	5.48	10.13	0.49	0.38	0.78		Giant's Castle
Amietia vertebralis	NM02616	35.98	35.29	16.28	12.99	9.76	16.07	8.84	17.47	12.78	7.96	5.05	2.44	2.40	9.19	4.93	7.72	0.45	0.36	0.80		KZN, Royal Natal National Park
Amietia vertebralis	NM02615	26.79	26.79	14.98	11.30	7.22	13.98	6.05	16.57	10.98	6.31	3.53	2.51	1.88	7.41	4.44	7.07	0.56	0.42	0.75		Unknown
Amietia vertebralis	NM05601	35.05	34.82	17.36	13.53	9.45	17.00	8.11	18.68	12.50	7.66	4.57	2.52	1.98	8.98	4.95	8.31	0.50	0.39	0.78		Tugela
Amietia vertebralis	NM05599	29.61	29.61	15.48	12.15	7.20	14.35	6.36	16.82	11.29	7.02	4.10	2.58	1.88	8.58	5.11	7.23	0.52	0.41	0.78		Tugela
Amietia vertebralis	NM05592	29.10	29.10	15.32	11.65	8.43	14.15	6.34	15.86	11.19	6.85	4.33	2.41	1.90	8.38	3.99	7.77	0.53	0.40	0.76		Giant's Castle
Amietia vertebralis	NM05597	49.07	48.07	22.93	18.12	13.26	23.05	10.14	22.68	17.92	10.46	5.56	3.04	3.03	11.40	6.62	10.05	0.47	0.37	0.79	Extensive	Tugela
Amietia vertebralis	NM02617	34.76	33.92	15.76	13.45	12.89	15.22	8.13	16.36	12.81	8.02	4.93	2.74	2.65	9.23	4.81	7.80	0.45	0.39	0.85	Extensive	KZN, Tugela
Amietia vertebralis	NM05588	34.97	34.97	17.88	13.63	10.98	17.04	7.22	17.54	12.63	7.61	3.96	2.76	2.31	9.01	4.45	8.92	0.51	0.39	0.76		Giant's Castle

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	NM05600	32.16	32.16	16.44	12.11	10.89	15.72	8.02	17.86	11.70	6.86	4.27	2.17	2.02	8.52	4.57	5.36	0.51	0.38	0.74	Extensive	Tugela
Amietia vertebralis	NM05591	32.49	32.49	17.47	12.51	8.30	16.10	6.26	17.96	11.55	6.76	4.01	2.26	2.38	8.47	4.40	7.27	0.54	0.39	0.72	Extensive	Organ Pipes
Amietia vertebralis	NM05593	19.19	19.19	9.85	8.52	5.99	9.88	3.32	7.82	7.57	4.43	2.59	1.91	1.53	5.18	2.86	4.59	0.51	0.44	0.86		KZN, Natal National Park, MAS
Amietia vertebralis	NM05602	29.13	29.13	15.36	11.31	8.14	14.22	5.31	13.37	11.82	6.68	3.81	2.41	2.61	8.52	4.79	6.38	0.53	0.39	0.74		Unknown
Amietia vertebralis	NM02618	24.19	24.19	11.94	9.28	6.97	11.50	4.09	9.79	9.15	5.13	3.49	2.02	1.60	6.52	3.38	4.73	0.49	0.38	0.78		Giant's Castle
Amietia vertebralis	NM05589	19.25	19.25	9.17	6.88	5.24	8.69	3.60	8.89	7.49	4.58	2.80	2.06	1.43	5.41	2.77	3.61	0.46	0.36	0.75		Lesotho, Giant's Castle
Amietia vertebralis	NM05590	18.14	18.14	8.85	8.45	4.78	8.59	3.48	8.19	8.14	4.84	2.47	1.86	1.24	5.93	2.77	3.96	0.49	0.47	0.96		Giant's Castle
Amietia vertebralis	NM02619	20.40	20.40	10.08	8.03	5.37	9.72	3.80	10.13	8.16	5.27	3.35	1.88	1.49	5.86	3.24	4.39	0.49	0.39	0.80		Giant's Castle
Amietia vertebralis	NM00980	123.02	121.04	54.23	57.64	35.08	60.31	25.11	52.03	49.20	26.65	12.35	7.89	7.25	31.57	17.90	20.93	0.44	0.47	1.06	Extensive	Giant's Castle
Amietia vertebralis	NM02614	73.65	69.89	40.40	29.48	21.57	39.11	15.48	38.88	27.31	15.29	8.66	4.51	4.45	19.53	10.28	14.36	0.55	0.40	0.73	Extensive	Unknown
Amietia vertebralis	NM02608	117.13	115.08	54.96	56.09	32.27	53.08	24.12	53.34	49.95	26.35	12.50	8.26	7.55	30.75	17.60	24.74	0.47	0.48	1.02	Extensive	Drakensberg Gardens Forestry
Amietia vertebralis	NM05594	90.48	90.48	47.24	40.80	25.97	49.35	21.48	46.25	40.34	18.63	10.67	5.77	5.29	24.93	12.52	19.86	0.52	0.45	0.86	Extensive	Giant's Castle
Amietia vertebralis	NM02609	73.51	70.44	36.22	29.52	22.43	37.17	14.21	35.51	25.75	15.54	9.95	5.21	4.71	19.53	8.93	16.48	0.49	0.40	0.82	Extensive	Drakensberg Gardens Forestry
Amietia vertebralis	NM02613	60.26	60.26	31.68	23.89	18.88	31.83	12.07	29.16	22.46	12.53	7.36	4.10	3.71	15.20	7.88	10.78	0.53	0.40	0.75	Extensive	Drakensberg Gardens Forestry
Amietia vertebralis	NM02620	97.56	94.81	45.24	40.58	28.37	46.82	22.38	43.58	38.34	21.50	11.21	6.16	5.06	26.40	12.78	16.25	0.46	0.42	0.90	Extensive	Drakensberg Gardens Forestry
Amietia vertebralis	NM00973	106.92	105.24	51.48	48.51	23.64	50.28	21.81	49.47	44.03	24.64	11.29	6.48	5.79	29.17	16.21	18.44	0.48	0.45	0.94	Extensive	Cathedral Peak

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	NM00974	95.03	94.24	50.68	46.72	19.68	46.71	20.62	50.42	40.19	24.51	11.52	6.56	5.72	27.52	14.51	21.57	0.53	0.49	0.92	Extensive	Cathedral Peak
Amietia vertebralis	NM02607	115.50	110.24	58.91	62.80	29.56	51.48	28.75	65.38	53.92	30.60	11.74	8.17	6.42	31.72	18.38	26.64	0.51	0.54	1.07	Extensive	Lekhalabalet si River
Amietia vertebralis	NMBA4799	95.84	92.94	48.02	38.48	26.80	51.12	19.47	50.19	35.03	19.41	9.43	5.70	5.24	22.36	11.67	19.34	0.50	0.40	0.80	Extensive	Malibamatso River, near Thaba-Li-
Amietia vertebralis	NMBA4798	103.32	99.72	48.77	42.01	27.31	49.32	22.04	51.96	37.20	21.27	9.81	5.71	5.16	23.68	13.81	19.69	0.47	0.41	0.86	Extensive	Malibamatso River, near Thaba-Li-
Amietia vertebralis	NMBA4800	81.18	81.18	39.14	31.59	23.57	40.60	16.79	41.68	30.13	15.56	7.79	5.07	5.03	19.55	10.71	16.93	0.48	0.39	0.81	Extensive	Malibamatso River, near Thaba-Li-
Amietia vertebralis	NMBA4804	49.29	48.15	25.57	19.12	10.70	25.90	9.83	26.74	18.18	11.02	5.86	3.47	3.30	13.06	7.39	11.30	0.52	0.39	0.75	Extensive	Mothae River Phofung, Lesotho
Amietia vertebralis	NMBA4604	31.80	31.80	16.08	12.26	6.47	14.67	5.62	16.38	13.15	8.11	4.24	2.41	1.87	9.06	5.54	6.68	0.51	0.39	0.76		Junction of Mokhotlong & Senqu
Amietia vertebralis	NMBA4803	35.76	33.94	17.08	13.01	6.89	16.39	6.88	16.98	12.92	8.06	4.72	2.35	2.20	9.88	5.42	6.73	0.48	0.36	0.76	Extensive	Malibamatso River, near Thaba-Li-
Amietia vertebralis	NMBA4605	26.32	25.60	12.47	9.72	5.54	12.98	4.92	12.85	10.14	6.89	3.93	2.33	1.71	8.02	4.40	5.68	0.47	0.37	0.78	Extensive	Junction of Mokhotlong & Senqu
Amietia vertebralis	NMBA5802	28.39	27.21	12.89	10.32	5.35	13.37	4.93	12.67	10.73	6.07	3.93	2.11	1.92	7.96	4.44	5.17	0.45	0.36	0.80	Extensive	Malibamatso River, near Thaba-Li-
Amietia vertebralis	NMBA4815	29.63	29.63	14.10	10.57	6.11	14.28	4.91	13.80	11.33	7.01	4.31	2.59	1.82	8.43	4.89	6.10	0.48	0.36	0.75		Mothae River Phofung, Lesotho
Amietia vertebralis	NMBA4806	24.63	24.63	12.21	9.42	5.34	11.71	4.70	13.01	9.73	6.47	3.73	2.67	1.67	7.37	4.76	5.42	0.50	0.38	0.77	Extensive	Mothae River Phofung, Lesotho
Amietia vertebralis	NMBA6288	20.50	20.50	9.63	7.82	3.96	9.77	4.29	10.61	8.47	6.02	3.31	2.41	1.95	6.45	4.27	4.30	0.47	0.38	0.81	Extensive	Btw Marakbei & Ha
Amietia vertebralis	NMBA5906	41.50	41.50	23.01	17.26	9.28	22.20	9.51	22.42	18.19	10.92	6.39	3.35	3.01	12.98	5.89	9.25	0.55	0.42	0.75	Extensive	Cassamo, 3km S of Sethamana,
Amietia vertebralis	NMBA4807	55.61	54.50	29.85	21.05	12.42	28.42	11.55	28.44	21.59	12.31	6.55	4.11	3.30	15.24	8.07	11.19	0.54	0.38	0.71	Extensive	Linakeng Mothae Riv, Phofung, Lesotho
Amietia vertebralis	NMBA4811	33.16	31.85	17.71	11.37	7.12	16.56	5.84	17.20	12.43	7.69	4.54	2.73	2.17	9.11	4.92	7.09	0.53	0.34	0.64	Extensive	Mothae Riv, Phofung, Lesotho

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	NMBA4809	31.53	31.53	16.30	11.64	6.42	15.60	6.19	16.97	12.15	7.96	4.90	3.07	2.19	9.00	4.98	6.68	0.52	0.37	0.71	Extensive	Mothae Riv, Phofung, Lesotho
Amietia vertebralis	NMBA5905	35.88	35.88	18.31	14.52	7.42	18.89	7.23	17.20	14.74	8.31	5.64	3.27	2.58	10.73	6.08	7.88	0.51	0.40	0.79	Extensive	3km S of Sethamana, Linakeng
Amietia vertebralis	NMBA4810	33.58	33.58	18.02	12.31	6.73	17.22	6.26	17.18	12.00	7.95	5.13	3.31	2.26	2.26	10.21	5.97	0.54	0.37	0.68	Extensive	Mothae Riv, Phofung, Lesotho
Amietia vertebralis	NMBA4808	51.61	51.61	27.80	19.20	10.84	25.34	9.90	27.26	20.54	11.71	6.28	3.90	3.17	14.06	7.82	10.78	0.54	0.37	0.69	Extensive	Mothae Riv, Phofung, Lesotho
Amietia vertebralis	NMBA4812	123.94	123.94	58.43	59.16	25.35	56.11	28.44	59.38	50.75	24.76	11.90	8.35	4.87	32.28	18.86	24.75	0.47	0.48	1.01	Extensive	
Amietia vertebralis	PEMA1664	75.62	73.87	39.03	29.42	16.11	37.46	15.31	39.18	27.96	15.94	9.33	5.69	4.75	20.04	9.39	14.16	0.52	0.39	0.75	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1552	19.70	19.70	9.15	7.74	4.54	8.74	3.34	10.04	7.81	#DIV/0!	#DIV/0!	2.07	#DIV/0!	4.99	3.62	#DIV/0!	0.46	0.23	0.50	Extensive	
Amietia vertebralis	PEMA1666	70.46	70.46	36.45	28.41	13.22	36.07	12.59	37.48	28.09	14.95	8.08	5.01	4.41	17.93	9.68	14.14	0.52	0.40	0.78	Extensive	Thaba Phutso River, Kotisepmola pass
Amietia vertebralis	PEMA1550	34.54	34.54	18.96	12.17	8.66	17.53	6.77	19.78	11.67	#DIV/0!	4.64	#DIV/0!	#DIV/0!	8.91	4.60	7.51	0.55	0.25	0.46	Extensive	
Amietia vertebralis	PEMA1688	29.80	29.80	14.53	11.54	5.75	14.70	5.48	13.43	11.79	7.50	4.68	2.61	2.29	8.69	4.70	5.96	0.49	0.39	0.79	Extensive	Sehonghong Riv. Senqu Basin. Bridge
Amietia vertebralis	PEMA1692	86.45	84.15	48.06	42.09	21.00	44.59	19.37	50.20	39.82	21.38	10.05	5.53	6.07	23.99	13.02	16.85	0.56	0.49	0.88	Extensive	Mokhotlong Riv. Senqu Basin. Above
Amietia vertebralis	PEMA1683	71.65	70.52	39.37	29.89	16.74	34.42	15.87	41.11	29.53	16.23	8.75	4.78	4.82	19.81	10.82	14.68	0.55	0.42	0.76	Extensive	Manguang Riv. Sani-Linakeng
Amietia vertebralis	PEMA1682	58.46	56.77	30.99	23.65	11.16	29.16	12.20	31.02	23.62	13.63	7.31	3.91	4.07	16.51	8.38	12.90	0.53	0.40	0.76	Extensive	Manguang Riv. Sani-Linakeng
Amietia vertebralis	PEMA1629	66.26	66.26	36.24	27.20	13.81	33.87	13.76	37.05	27.66	15.12	7.71	4.46	4.13	18.71	9.94	13.85	0.55	0.41	0.75	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1662	77.84	74.53	40.14	30.36	16.16	38.77	15.61	40.82	29.99	16.00	8.31	4.77	5.14	18.60	10.31	15.64	0.52	0.39	0.76	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1696	84.01	82.86	45.64	35.36	17.45	40.42	16.71	47.91	33.78	19.00	9.26	5.24	5.28	21.65	12.23	18.64	0.54	0.42	0.77	Extensive	Sani River, Linakeng-Senqu Basin.

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	PEMA1670	129.00	125.35	62.41	63.03	25.71	61.01	26.78	67.31	55.16	29.05	12.61	7.71	6.95	31.98	18.98	27.07	0.48	0.49	1.01	Extensive	
Amietia vertebralis	PEMA1646	94.27	92.20	46.56	38.46	20.89	45.09	18.87	50.32	37.92	19.41	10.08	5.18	5.63	23.92	11.93	18.53	0.49	0.41	0.83	Extensive	Sehonghong Riv. Senqu Basin. Bridge
Amietia vertebralis	PEMA1641	91.13	89.01	46.01	38.79	21.15	46.11	19.71	47.65	37.44	19.11	10.02	5.42	5.05	23.98	12.47	16.48	0.50	0.43	0.84	Extensive	Sehonghong Riv.Mokhotlo ng-Senqu
Amietia vertebralis	PEMA1671	95.61	93.89	47.47	41.84	19.53	44.49	19.95	51.19	40.23	21.37	10.33	5.71	5.70	24.95	13.49	18.34	0.50	0.44	0.88	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1633	82.14	81.13	43.12	34.04	18.35	40.34	17.92	47.92	33.60	17.92	8.59	4.57	5.48	21.09	11.40	16.76	0.52	0.41	0.79	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1647	81.31	79.10	43.58	34.13	16.98	40.17	17.24	45.55	31.86	17.69	8.44	4.68	4.73	20.11	11.17	17.25	0.54	0.42	0.78	Extensive	Sehonghong Riv. Senqu Basin. Bridge
Amietia vertebralis	PEMA1710	47.65	47.12	25.08	18.92	9.79	22.77	9.60	25.39	19.48	10.81	6.05	3.23	2.80	13.14	7.38	10.55	0.53	0.40	0.75	Extensive	Sehonghong Riv.
Amietia vertebralis	PEMA1668	90.01	89.16	45.74	38.48	16.56	41.60	18.65	46.33	35.90	19.22	9.30	5.40	5.11	24.03	13.23	20.23	0.51	0.43	0.84	Extensive	Mokhotlong-Thaba Phutso River,
Amietia vertebralis	PEMA1643	85.30	84.07	46.62	35.78	21.17	42.32	19.09	48.62	33.83	18.11	9.22	5.17	5.62	22.34	11.88	17.06	0.55	0.42	0.77	Extensive	Kotisepmola Sehonghong Riv.Mokhotlo ng-Senqu
Amietia vertebralis	PEMA1674	41.86	41.86	21.27	15.59	8.40	21.05	7.17	21.50	14.38	9.57	5.10	3.22	1.93	10.42	6.10	7.98	0.51	0.37	0.73	Extensive	Sanqubethu Riv.
Amietia vertebralis	PEMA1665	62.57	61.84	35.77	26.30	12.15	33.39	12.83	29.89	26.85	13.46	7.29	4.37	4.01	16.66	8.90	13.59	0.57	0.42	0.74	Extensive	Mokhotlong-Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1701	59.23	59.23	31.34	23.88	11.04	28.44	11.19	30.59	24.43	13.78	6.89	4.09	3.63	16.22	9.14	11.46	0.53	0.40	0.76	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1628	62.58	62.58	34.81	27.38	12.50	31.35	12.35	33.99	26.05	14.49	7.72	4.37	4.12	18.00	9.75	13.57	0.56	0.44	0.79	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1626	80.99	80.99	43.61	35.28	18.80	44.19	17.65	47.87	33.61	18.73	9.07	4.83	4.98	21.75	10.97	16.71	0.54	0.44	0.81	Extensive	Sehonghong Riv.
Amietia vertebralis	PEMA1698	58.44	58.44	30.75	23.27	11.00	28.53	11.16	30.74	23.96	13.88	7.16	4.02	3.89	15.74	8.65	12.67	0.53	0.40	0.76	Extensive	Mokhotlong-Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1655	65.42	65.42	37.11	30.49	13.78	30.87	13.79	35.06	29.36	15.38	7.67	4.07	4.12	17.82	10.44	15.62	0.57	0.21	0.37	Extensive	
Amietia vertebralis	PEMA1640	66.55	66.55	37.55	29.33	13.65	35.93	13.86	39.09	28.87	15.86	7.55	4.45	4.15	18.05	10.81	15.97	0.56	0.21	0.36	Extensive	

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	PEMA1703	55.65	55.65	30.08	23.05	10.24	27.40	11.04	30.15	23.20	12.11	6.76	3.97	3.48	14.70	7.99	12.04	0.54	0.18	0.34	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1667	80.14	80.14	40.43	33.12	16.76	40.92	15.59	41.13	33.22	17.13	8.29	4.92	5.08	21.41	10.96	15.58	0.50	0.21	0.41	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1671	65.88	65.88	35.92	30.01	14.00	34.34	13.72	35.40	29.53	14.86	7.76	4.57	4.64	19.10	11.11	15.15	0.55	0.21	0.39	Extensive	
Amietia vertebralis	PEMA1695	68.60	67.45	36.66	28.93	11.97	32.24	13.00	36.89	28.77	15.11	8.16	5.15	4.28	18.53	10.05	14.48	0.53	0.17	0.33	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1689	28.33	28.33	15.01	11.47	5.57	14.47	5.63	15.11	12.30	7.36	4.48	2.19	1.89	8.40	3.95	6.83	0.53	0.20	0.37	Extensive	Sehonghong Riv. Senqu-Orange
Amietia vertebralis	PEMA1687	33.76	33.76	19.27	12.63	6.51	17.25	6.58	17.87	13.20	7.65	4.53	2.79	2.21	9.00	5.44	7.16	0.57	0.19	0.34	Extensive	Sehonghong Riv. Senqu-Orange
Amietia vertebralis	PEMA1661	39.48	39.48	22.25	16.39	7.71	21.00	7.69	20.74	15.94	9.50	5.58	3.16	2.36	11.41	6.23	8.99	0.56	0.20	0.35	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1663	64.68	64.68	35.61	26.22	13.02	33.88	13.86	35.20	25.36	14.10	7.31	4.35	4.38	17.68	9.17	13.62	0.55	0.20	0.37	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1711	95.87	95.87	50.03	46.51	22.28	44.56	19.36	51.62	42.63	21.81	9.45	5.92	5.11	25.62	15.01	22.15	0.52	0.23	0.45	Extensive	Sehonghong Riv. Mokhotlong
Amietia vertebralis	PEMA1700	71.51	71.51	38.23	29.71	14.16	35.36	13.49	38.56	28.76	15.52	7.73	4.80	4.27	18.94	9.64	16.32	0.53	0.20	0.37	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1680	41.03	41.03	21.89	16.30	7.75	20.48	8.20	22.42	16.14	9.43	5.39	3.48	2.68	12.31	7.15	8.06	0.53	0.19	0.35	Extensive	Sani River Valley
Amietia vertebralis	PEMA1686	40.85	40.85	21.32	16.67	7.79	20.38	7.92	21.14	16.56	9.08	5.27	3.02	2.50	11.21	6.14	12.62	0.52	0.19	0.37	Extensive	Sehonghong Riv. Senqu-Orange
Amietia vertebralis	PEMA1657	66.26	66.26	36.32	27.88	13.02	32.76	13.95	35.04	26.44	15.74	7.00	4.52	3.97	16.99	9.75	15.08	0.55	0.20	0.36		Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1684	30.74	30.74	15.93	12.32	6.29	15.70	6.11	15.54	12.02	7.46	4.07	2.83	2.05	8.71	5.00	6.69	0.52	0.20	0.39	Extensive	Sehonghong Riv. Senqu-Orange
Amietia vertebralis	PEMA1691	30.16	30.16	16.98	12.10	6.77	16.36	5.62	17.27	12.27	8.19	4.55	2.70	1.99	7.05	9.42	5.34	0.56	0.22	0.40	Extensive	Mokhotlong Riv. Senqu Basin. Above
Amietia vertebralis	PEMA1654	47.56	47.56	25.76	19.74	8.36	25.61	9.18	25.83	18.64	12.01	6.09	3.64	2.83	13.11	7.56	9.63	0.54	0.18	0.32	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1627	54.07	54.07	27.93	21.59	9.62	26.08	10.42	29.03	21.44	12.96	6.33	4.07	3.31	14.36	8.00	9.88	0.52	0.18	0.34	Extensive	Sani Riv. Linakeng-Senqu Basin.

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	PEMA1678	45.42	45.42	24.75	19.36	8.59	23.32	8.81	25.93	19.72	11.49	6.62	3.87	3.46	13.49	7.31	10.91	0.55	0.19	0.35	Extensive	Manguang Riv. Sani-Linakeng
Amietia vertebralis	PEMA1693	55.42	55.42	28.73	23.31	9.25	26.86	10.96	29.60	23.05	12.90	6.84	4.32	3.87	15.61	8.63	12.74	0.52	0.17	0.32	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1630	59.62	59.62	32.19	24.39	11.48	30.04	11.91	32.21	24.31	13.72	6.82	4.09	4.50	15.73	8.70	13.01	0.54	0.19	0.36	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1699	82.12	79.39	40.38	34.43	13.69	37.96	15.09	44.54	34.34	18.30	8.70	4.77	5.03	21.80	11.55	19.51	0.49	0.17	0.34	Extensive	Sani Riv. Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1690	73.83	70.21	38.74	29.56	13.37	34.85	13.65	38.41	30.89	17.25	8.54	4.74	4.51	19.91	10.32	13.97	0.52	0.18	0.35	Extensive	Mokhotlong Riv. Senqu Basin. Above
Amietia vertebralis	PEMA1648	97.75	95.27	48.77	43.17	17.95	46.76	19.50	48.62	42.70	22.64	9.15	5.94	5.70	24.89	14.65	19.69	0.50	0.18	0.37	Extensive	
Amietia vertebralis	PEMA1712	114.13	114.13	58.78	56.37	23.00	57.83	23.82	58.07	49.97	28.36	11.91	7.09	6.60	30.49	18.42	25.41	0.52	0.20	0.39	Extensive	Sehonghong Riv. Mokhotlong-
Amietia vertebralis	CDNC5124	143.59	136.12	60.86	60.52	#DIV/0!	52.24	36.82	58.40	51.51	32.70	12.88	7.42	6.38	32.14	20.89	15.93	0.42	0.42	0.99	Extensive	
Amietia vertebralis	CDNC5165	139.32	136.67	60.66	65.35	#DIV/0!	53.90	34.52	60.35	53.67	33.56	12.75	8.03	7.60	33.98	18.77	18.42	0.44	0.47	1.08	Extensive	
Amietia vertebralis	PEMA1648	107.66	106.08	53.33	51.98	#DIV/0!	52.41	28.07	52.44	45.05	28.94	11.44	6.40	7.08	30.29	16.68	14.67	0.50	0.48	0.97	Extensive	
Amietia vertebralis	PEMA1636	137.89	133.30	69.19	70.47	#DIV/0!	65.56	33.19	64.94	55.58	35.45	12.90	8.00	7.64	33.96	21.21	19.50	0.50	0.51	1.02	Extensive	
Amietia vertebralis	PEMA1644	123.45	120.60	60.95	55.67	#DIV/0!	56.53	24.11	63.20	45.07	28.62	10.61	6.53	7.28	29.72	16.94	15.12	0.49	0.45	0.91	Extensive	
Amietia vertebralis	PEMA1645	72.65	71.77	42.88	32.30	#DIV/0!	38.51	18.00	38.61	30.28	17.90	8.37	4.42	4.63	19.28	10.04	8.37	0.60	0.44	0.75	Extensive	
Amietia vertebralis	PEMA1651	101.28	99.37	51.89	48.21	#DIV/0!	50.22	22.66	52.50	41.06	25.17	9.56	6.42	5.64	26.15	16.31	13.55	0.51	0.48	0.93	Extensive	
Amietia vertebralis	PEMA1672	93.00	91.30	45.30	40.82	#DIV/0!	44.02	19.91	46.76	34.23	21.25	9.72	5.35	4.93	23.42	13.32	13.00	0.49	0.44	0.90	Extensive	
Amietia vertebralis	PEMA1694	63.28	62.17	33.22	26.90	#DIV/0!	32.51	13.52	33.67	23.87	14.38	7.40	3.41	3.44	17.47	9.25	10.43	0.53	0.43	0.81	Extensive	
Amietia vertebralis	PEMA1705	84.64	82.96	45.00	34.91	#DIV/0!	43.83	19.71	45.92	30.00	17.05	8.83	5.69	5.45	21.29	11.51	9.88	0.53	0.41	0.78	Extensive	

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	PEMA1704	80.31	78.81	41.18	34.21	#DIV/0!	40.12	17.71	42.11	29.36	17.56	8.65	4.82	4.61	20.73	11.36	11.10	0.51	0.43	0.83	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1635	85.72	83.11	44.44	39.09	#DIV/0!	40.78	18.09	45.18	31.63	21.10	8.69	5.65	4.95	21.34	12.04	10.82	0.52	0.46	0.88	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	PEMA1656	85.18	83.03	44.72	37.00	#DIV/0!	42.31	18.90	45.55	30.81	19.08	8.76	5.67	5.20	21.32	11.78	10.35	0.53	0.43	0.83	Extensive	
Amietia vertebralis	PEMA1669	89.32	87.80	43.14	35.81	#DIV/0!	42.61	18.14	42.72	32.26	20.74	9.49	5.29	5.10	21.97	11.80	11.93	0.48	0.40	0.83	Extensive	Thaba Phutso River, Kotisepmola
Amietia vertebralis	PEMA1677	71.27	68.76	36.25	28.30	#DIV/0!	33.92	14.13	35.25	23.89	15.23	7.55	4.24	3.90	18.21	9.25	9.65	0.51	0.40	0.78	Extensive	Sani River, Linakeng-Senqu Basin.
Amietia vertebralis	JR16.1	87.54	86.56	49.33	45.25	16.36	47.98	19.19	52.18	43.55	22.59	9.99	5.99	5.92	26.66	14.63	18.66	0.56	0.52	0.92	Extensive	Mashai
Amietia vertebralis	JR16.2	110.52	109.71	57.43	54.72	21.60	54.64	23.45	60.78	52.45	28.77	11.31	7.78	5.96	31.55	17.62	24.95	0.52	0.50	0.95	Extensive	Mashai
Amietia vertebralis	JR16.3	126.66	125.68	66.03	66.39	25.24	60.69	30.34	67.29	62.82	31.91	12.26	8.01	7.93	34.64	19.31	25.03	0.52	0.52	1.01	Extensive	Mashai
Amietia vertebralis	JR16.4	143.79	143.79	64.48	69.42	28.77	14.38	31.05	71.55	68.84	35.42	13.54	8.93	7.79	37.75	21.81	29.51	0.45	0.48	1.08	Extensive	Mashai
Amietia vertebralis	JR16.5	109.58	108.04	54.38	51.27	19.46	50.24	23.59	58.08	51.48	27.87	11.00	6.50	6.40	31.02	17.46	20.77	0.50	0.47	0.94	Extensive	Mashai
Amietia vertebralis	JR14A.1	101.28	98.38	48.28	42.67	21.19	46.92	21.75	55.56	42.73	24.08	10.62	5.61	5.53	27.08	13.51	17.93	0.48	0.42	0.88	Extensive	Manguang
Amietia vertebralis	JR14A.2	94.36	92.25	46.38	39.53	19.56	45.70	20.25	49.99	38.91	20.54	9.50	5.76	4.75	24.77	12.96	17.18	0.49	0.42	0.85	Extensive	Manguang
Amietia vertebralis	JR14A.3	72.12	71.75	37.47	29.68	15.41	35.33	14.25	39.22	30.32	16.03	8.78	5.21	4.40	19.54	10.15	14.11	0.52	0.41	0.79	Extensive	Manguang
Amietia vertebralis	JR14A.4	102.80	102.26	52.12	49.81	20.47	46.14	19.44	51.77	42.23	25.81	9.67	7.39	5.31	27.04	15.75	20.00	0.51	0.48	0.96	Extensive	Manguang
Amietia vertebralis	JR14A.5	97.14	96.01	46.67	41.75	19.85	44.73	18.35	50.03	40.75	23.25	10.50	6.51	5.78	27.04	13.63	18.40	0.48	0.43	0.89	Extensive	Manguang
Amietia vertebralis	JR14A.6	87.75	79.36	41.87	33.76	17.16	39.66	15.88	42.61	33.31	18.63	9.37	5.35	4.05	21.27	11.44	16.12	0.48	0.38	0.81	Extensive	Manguang
Amietia vertebralis	JR14A.7	82.54	81.88	45.12	36.19	20.97	38.44	19.41	47.31	35.52	19.67	8.76	6.03	4.82	21.44	11.77	14.80	0.55	0.44	0.80	Extensive	Manguang

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	JR14A.8	78.03	77.03	42.52	35.24	16.48	38.82	16.33	39.39	33.49	19.29	8.68	5.46	4.44	22.01	11.57	14.80	0.54	0.45	0.83	Extensive	Manguang
Amietia vertebralis	JR16.6	136.15	133.57	65.34	71.10	28.40	59.09	28.94	70.13	67.10	35.19	13.30	8.21	8.26	38.62	20.02	27.22	0.48	0.52	1.09	Extensive	Mashai
Amietia vertebralis	JR16.7	92.20	89.17	46.37	38.88	18.17	46.14	18.49	48.69	38.13	20.81	10.12	5.46	4.79	24.58	12.19	17.38	0.50	0.42	0.84	Extensive	Mashai
Amietia vertebralis	JR16.8	88.04	83.56	46.47	37.79	20.51	44.99	18.24	47.23	37.25	19.19	9.98	6.95	4.39	22.92	11.65	16.23	0.53	0.43	0.81	Extensive	Mashai
Amietia vertebralis	DT8.1	121.82	119.50	64.42	64.94	24.45	60.28	25.58	64.71	57.84	30.83	11.85	8.32	6.41	32.96	19.73	27.57	0.53	0.53	1.01	Extensive	Bafali
Amietia vertebralis	DT8.2	140.82	139.99	63.01	70.55	26.56	60.77	29.58	67.17	64.89	34.66	13.38	9.10	8.35	40.50	22.82	26.87	0.45	0.50	1.12	Extensive	Bafali
Amietia vertebralis	DT8.3	123.03	122.03	61.89	59.00	25.68	54.72	24.43	62.97	54.85	29.27	12.35	8.65	6.86	31.58	18.87	25.47	0.50	0.48	0.95	Extensive	Bafali
Amietia vertebralis	DT8.4	100.60	98.20	49.62	40.25	22.13	44.13	18.89	50.27	39.40	22.24	11.94	6.74	5.52	27.21	14.05	15.37	0.49	0.40	0.81	Extensive	Bafali
Amietia vertebralis	DT8.5	87.79	84.85	47.31	37.55	19.36	18.64	18.53	46.73	37.27	21.57	8.60	5.27	5.81	23.19	13.02	16.15	0.54	0.43	0.79	Extensive	Bafali
Amietia vertebralis	DT16.1	103.16	101.81	56.01	49.63	19.37	53.06	21.89	55.05	46.51	6.86	10.66	7.68	5.42	27.42	15.63	22.71	0.54	0.48	0.89	Extensive	Qabane
Amietia vertebralis	DT16.2	99.07	95.61	50.47	41.69	18.92	47.38	20.19	51.90	41.51	24.11	8.91	7.29	5.75	24.92	14.41	21.08	0.51	0.42	0.83	Extensive	Qabane
Amietia vertebralis	DT16.3	87.73	87.34	50.88	39.84	22.40	43.41	17.83	48.98	37.50	22.98	10.21	6.73	5.42	24.53	13.16	18.83	0.58	0.45	0.78	Extensive	Qabane
Amietia vertebralis	DT16.4	83.15	82.06	44.72	36.64	19.31	41.51	17.42	46.31	34.93	20.35	8.24	6.99	5.45	21.53	11.99	17.08	0.54	0.44	0.82	Extensive	Qabane
Amietia vertebralis	DT16.5	78.61	77.96	43.65	35.77	18.85	38.73	16.66	42.74	33.84	20.56	9.08	5.41	4.70	21.34	11.96	15.75	0.56	0.46	0.82	Extensive	Qabane
Amietia vertebralis	JR4.1	138.78	134.59	72.40	74.41	32.00	67.69	29.95	72.35	65.27	36.86	12.68	8.93	7.00	37.52	20.78	29.80	0.52	0.54	1.03	Extensive	Lesobeng
Amietia vertebralis	JR4.2	80.52	79.03	45.08	38.45	17.25	41.40	17.30	41.48	37.31	22.75	8.57	6.31	5.33	22.20	12.94	17.93	0.56	0.48	0.85	Extensive	Lesobeng
Amietia vertebralis	JR4.3	88.34	85.76	48.89	43.19	16.32	44.93	19.29	49.78	41.80	24.00	9.62	6.73	4.25	24.99	13.87	19.12	0.55	0.49	0.88	Extensive	Lesobeng

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	JR4.4	80.67	78.11	44.92	37.98	17.16	42.24	17.00	46.46	34.99	20.52	8.24	5.65	4.64	21.68	11.87	17.40	0.56	0.47	0.85	Extensive	Lesobeng
Amietia vertebralis	JR4.5	84.59	82.77	45.65	41.10	19.71	43.33	18.56	48.83	37.95	21.95	9.09	6.73	4.70	23.73	12.78	17.41	0.54	0.49	0.90	Extensive	Lesobeng
Amietia vertebralis	DT17A.1	88.20	86.88	46.29	40.30	20.53	44.41	19.89	46.95	38.48	22.69	9.80	6.20	4.58	24.02	13.07	17.37	0.52	0.46	0.87	Extensive	Tstasana
Amietia vertebralis	DT17A.2	96.41	95.61	46.17	43.66	18.85	46.45	18.57	48.14	42.46	24.64	10.04	6.01	5.21	25.95	14.24	19.58	0.48	0.45	0.95	Extensive	Tstasana
Amietia vertebralis	DT17A.3	79.41	76.39	39.40	33.30	16.45	38.97	15.61	42.19	31.65	18.53	9.56	5.65	4.46	20.47	11.05	13.77	0.50	0.42	0.85	Extensive	Tstasana
Amietia vertebralis	DT17A.4	85.35	82.96	45.93	40.89	18.74	43.90	17.44	49.12	37.96	22.57	10.15	7.46	5.36	24.14	12.95	18.37	0.54	0.48	0.89	Extensive	Tstasana
Amietia vertebralis	DT17A.5	89.99	89.59	47.52	40.90	16.41	46.89	18.75	47.40	39.42	23.23	9.37	6.66	5.86	23.16	13.08	17.85	0.53	0.45	0.86	Extensive	Tstasana
Amietia vertebralis	DT17A.6	82.85	81.31	43.73	39.08	13.91	42.51	17.22	45.85	37.89	22.71	8.63	5.87	5.07	22.41	12.85	17.41	0.53	0.47	0.89	Extensive	Tstasana
Amietia vertebralis	JR12.2	90.56	90.56	46.24	40.27	24.49	39.65	18.44	50.05	40.98	23.60	11.29	6.33	4.95	24.36	14.21	16.31	0.51	0.44	0.87	Extensive	
Amietia vertebralis	JR13.2	84.59	81.82	42.93	40.31	17.22	40.05	17.05	45.19	39.23	22.80	9.45	5.80	4.67	24.65	13.26	18.25	0.51	0.48	0.94	Extensive	Redi
Amietia vertebralis	JR9.1	129.59	129.06	63.91	66.03	25.47	61.40	29.18	65.10	56.84	36.16	13.03	9.42	7.00	36.13	20.92	28.10	0.49	0.51	1.03	Extensive	
Amietia vertebralis	JR9.2	133.71	131.91	65.44	63.12	24.84	56.81	25.35	63.56	59.57	35.05	13.13	9.57	6.88	33.99	20.44	23.25	0.49	0.47	0.96	Extensive	
Amietia vertebralis	JR9.3	110.64	108.94	56.12	49.01	24.93	51.79	21.66	58.33	48.05	28.48	11.51	7.56	5.77	28.82	16.11	18.11	0.51	0.44	0.87	Extensive	
Amietia vertebralis	JR15.2	97.02	94.40	51.90	42.38	22.23	47.20	20.97	53.79	41.37	22.67	10.38	6.06	4.98	24.70	12.85	18.30	0.53	0.44	0.82	Extensive	
Amietia vertebralis	JR15.3	89.57	87.73	47.67	41.03	19.78	43.93	19.64	50.10	40.61	23.06	8.73	6.49	4.77	23.97	13.90	17.52	0.53	0.46	0.86	Extensive	
Amietia vertebralis	JR15.4	65.71	62.88	34.54	27.14	12.76	29.12	13.37	33.48	27.23	15.98	7.57	5.27	4.49	16.28	9.35	14.62	0.53	0.41	0.79	Extensive	
Amietia vertebralis	DT9.1	131.08	131.08	63.78	66.74	25.99	61.41	27.95	66.56	62.51	33.86	13.28	7.63	5.87	35.05	20.08	23.97	0.49	0.51	1.05	Extensive	

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	DT9.2	130.37	129.13	65.35	62.57	27.02	60.82	27.35	62.39	59.15	33.95	12.61	7.30	6.83	34.94	21.08	25.90	0.50	0.48	0.96	Extensive	
Amietia vertebralis	DT9.3	102.92	98.96	51.93	45.66	23.85	52.37	27.12	54.38	43.82	24.36	9.94	6.84	5.61	26.45	15.07	18.28	0.50	0.44	0.88	Extensive	
Amietia vertebralis	DT9.4	89.41	87.25	46.60	36.91	20.71	42.91	17.76	48.78	35.47	20.49	9.14	5.57	4.89	23.39	12.60	15.32	0.52	0.41	0.79	Extensive	
Amietia vertebralis	JR11B.1	122.17	119.99	60.39	59.19	25.08	56.59	26.75	61.19	54.50	30.01	12.13	7.53	7.84	33.31	18.92	24.38	0.49	0.48	0.98	Extensive	
Amietia vertebralis	JR11B.2	103.06	103.06	50.65	46.17	21.44	48.30	22.80	54.26	44.55	26.56	10.06	6.50	6.50	29.07	13.84	18.42	0.49	0.45	0.91	Extensive	
Amietia vertebralis	JR11B.3	97.50	96.38	49.82	44.33	23.65	47.44	20.75	55.37	41.72	23.86	10.66	6.65	5.00	26.69	13.47	18.60	0.51	0.45	0.89	Extensive	
Amietia vertebralis	JR3.1	126.15	125.33	60.97	65.36	24.48	64.83	26.28	66.14	59.94	35.73	11.68	8.58	6.16	35.10	21.17	25.25	0.48	0.52	1.07	Extensive	
Amietia vertebralis	JR3.2	99.54	97.58	49.95	44.19	21.24	51.74	19.99	52.93	43.31	25.27	10.64	6.73	5.72	26.88	14.14	18.98	0.50	0.44	0.88	Extensive	
Amietia vertebralis	JR3.3	103.16	100.28	47.74	43.66	26.33	47.49	21.47	51.22	41.99	24.67	10.54	6.87	6.01	25.44	14.18	16.34	0.46	0.42	0.91	Extensive	
Amietia vertebralis	DT11.1	113.66	112.15	59.09	57.44	22.66	57.53	22.95	60.79	52.89	29.53	11.39	7.91	6.28	31.74	16.55	25.41	0.52	0.51	0.97	Extensive	Sani
Amietia vertebralis	DT11.2	76.58	75.89	41.59	36.73	15.71	39.34	15.26	43.60	35.50	19.49	8.14	5.92	4.79	21.80	11.07	17.02	0.54	0.48	0.88	Extensive	Sani
Amietia vertebralis	DT11.3	107.61	107.61	59.36	53.32	22.36	59.32	23.45	58.74	50.83	27.83	10.54	7.01	6.52	30.10	17.16	22.72	0.55	0.50	0.90	Extensive	Sani
Amietia vertebralis	DT11.4	131.34	131.34	61.12	66.12	28.80	63.58	26.01	66.38	64.98	35.19	11.77	8.64	7.51	34.91	20.09	27.24	0.47	0.50	1.08	Extensive	Sani
Amietia vertebralis	DT11.5	100.60	98.65	51.85	49.34	19.58	49.22	20.32	54.05	45.93	25.08	10.29	6.56	6.58	27.58	15.98	22.40	0.52	0.49	0.95	Extensive	Sani
Amietia vertebralis	DT5	50.05	47.60	26.54	19.95	8.85	23.89	9.58	24.60	20.29	12.67	5.92	4.15	2.74	12.91	7.05	8.92	0.53	0.40	0.75	Extensive	Makhaleng
Amietia vertebralis	JR5B	61.30	61.30	34.75	25.96	13.21	34.06	13.17	34.00	25.78	15.14	6.14	4.87	3.57	16.10	8.43	10.73	0.57	0.42	0.75	Extensive	
Amietia vertebralis	DT10.1	78.31	77.31	39.25	33.34	15.61	38.29	13.90	41.00	34.50	19.21	8.25	4.81	4.97	21.19	10.79	16.05	0.50	0.43	0.85	Extensive	Mohlesi

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	JR15.1	110.50	106.36	52.37	46.42	25.95	52.07	20.52	54.84	45.15	25.37	9.49	6.36	5.56	27.11	14.42	17.08	0.47	0.42	0.89	Extensive	
Amietia vertebralis	JR5A.1	80.30	80.30	44.24	38.04	21.28	42.21	18.00	48.06	37.23	22.60	9.51	6.25	5.13	24.50	12.35	14.53	0.55	0.47	0.86	Extensive	
Amietia vertebralis	JR5A.2	69.25	69.25	38.95	31.36	16.10	38.41	15.92	39.61	31.54	17.88	7.66	5.56	4.59	18.59	10.63	12.27	0.56	0.45	0.81	Extensive	
Amietia vertebralis	JR5A.3	44.51	44.51	24.40	18.54	8.59	22.05	8.60	23.14	17.69	11.19	6.31	3.25	3.51	13.12	6.79	8.48	0.55	0.42	0.76	Extensive	
Amietia vertebralis	DT2.1	79.41	79.41	42.65	38.48	16.90	41.32	17.79	42.28	37.47	22.31	8.79	6.86	5.43	22.30	12.89	15.87	0.54	0.48	0.90	Extensive	Tenane
Amietia vertebralis	JR21.1	107.09	102.89	56.24	50.15	24.17	52.91	22.71	52.63	45.10	26.57	10.12	6.84	6.01	28.30	16.19	22.45	0.53	0.47	0.89	Extensive	
Amietia vertebralis	JR21.2	90.36	88.71	52.01	42.50	23.01	49.67	21.29	51.40	37.22	23.54	9.73	5.85	5.68	24.73	14.37	17.38	0.58	0.47	0.82	Extensive	
Amietia vertebralis	JR7.1	98.78	93.96	55.37	50.72	20.26	47.97	21.62	54.19	45.76	26.56	9.14	7.07	5.43	26.43	16.29	22.45	0.56	0.51	0.92	Extensive	Ketane
Amietia vertebralis	JR16.9	51.28	49.12	27.87	21.17	9.87	25.40	9.41	27.31	20.88	12.43	6.70	3.84	2.56	14.68	7.41	9.31	0.54	0.41	0.76	Extensive	Mashai
Amietia vertebralis	JR16.10	80.75	79.23	42.11	35.27	19.37	39.55	18.71	45.92	34.58	20.60	8.88	6.17	4.28	21.83	12.10	12.18	0.52	0.44	0.84	Extensive	Mashai
Amietia vertebralis	JR10.1	86.97	85.54	49.65	38.94	18.28	44.47	18.57	50.32	38.10	21.69	9.55	5.75	4.75	23.40	12.41	16.44	0.57	0.45	0.78		Senqu
Amietia vertebralis	JR10.2	89.21	88.47	47.83	38.02	22.10	45.24	19.36	50.22	38.82	21.97	9.15	5.79	6.01	23.22	11.79	17.24	0.54	0.43	0.79		Senqu
Amietia vertebralis	JR16.11	76.36	74.39	41.95	33.34	18.00	39.02	17.08	44.32	32.80	20.74	10.12	5.32	5.60	22.50	10.82	14.11	0.55	0.44	0.79		Mashai
Amietia vertebralis	JR16.12	73.51	71.44	40.19	31.53	16.63	35.67	16.97	39.24	30.97	18.63	8.29	4.83	4.29	20.64	9.83	12.83	0.55	0.43	0.78		Mashai
Amietia vertebralis	JR16.13	35.26	35.26	18.72	14.81	6.50	18.40	6.50	17.74	14.13	8.74	5.27	2.78	2.45	10.30	5.79	5.14	0.53	0.42	0.79		Mashai
Amietia vertebralis	JR8.1	97.38	97.38	49.14	44.28	24.07	47.79	21.04	48.95	43.40	26.45	11.40	6.29	5.61	28.64	14.66	16.70	0.50	0.45	0.90		
Amietia vertebralis	TM79374	106.62	105.87	50.10	48.84	20.16	47.31	22.00	52.99	44.03	27.68	11.27	7.13	5.27	28.57	15.66	21.76	0.47	0.46	0.97	Extensive	

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	TM13954	28.29	28.29	15.09	11.96	6.93	14.59	5.95	14.95	11.32	7.49	4.28	2.46	2.01	8.55	4.66	5.68	0.53	0.42	0.79	Extensive	
Amietia vertebralis	TM30373	104.08	104.08	50.47	47.85	20.49	53.02	20.79	52.60	41.49	25.33	9.60	5.89	4.26	25.75	14.70	21.95	0.48	0.46	0.95	Extensive	Malutseuyane Falls.
Amietia vertebralis	TM13952	27.37	27.37	13.79	10.46	5.61	13.33	5.53	14.91	10.48	6.58	3.93	2.35	1.71	7.38	3.86	5.20	0.50	0.38	0.76	Extensive	Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM21357	34.63	33.07	16.76	12.62	7.36	15.48	6.50	19.12	12.45	7.67	3.77	2.21	2.28	8.84	4.43	5.93	0.48	0.36	0.75	Extensive	Lesotho, Tugela River, Mont-aux-
Amietia vertebralis	TM13961	24.35	24.35	12.75	10.21	5.27	12.12	4.56	13.29	9.53	6.43	3.63	2.05	1.86	7.35	3.96	5.66	0.52	0.42	0.80	Extensive	Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM13953	23.71	23.71	12.12	9.48	5.65	11.73	5.42	12.33	8.11	5.94	3.61	1.74	1.62	6.86	3.72	4.94	0.51	0.40	0.78	Extensive	Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM13957	28.58	27.15	14.15	10.85	5.75	12.98	5.36	14.28	10.19	6.64	3.90	2.28	1.69	7.46	4.30	6.71	0.50	0.38	0.77	Extensive	Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM26154	43.35	40.37	20.50	15.19	8.12	19.14	6.59	21.36	13.77	8.59	4.63	2.82	2.31	9.87	5.66	7.89	0.47	0.35	0.74	Extensive	
Amietia vertebralis	TM21356	29.45	28.34	14.66	11.03	5.65	14.32	5.68	15.84	11.38	7.44	3.73	2.35	1.99	7.83	3.98	6.36	0.50	0.37	0.75		Tugela, Mont-aux-Sources
Amietia vertebralis	TM13960	32.98	32.98	17.45	13.27	8.34	16.95	6.79	17.74	12.16	7.54	4.49	2.47	2.37	8.93	4.55	6.78	0.53	0.40	0.76		Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM21355	36.24	36.24	18.95	14.66	7.99	17.25	7.23	19.77	12.98	8.38	4.63	2.31	2.39	9.71	4.99	7.03	0.52	0.40	0.77		Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM13955	21.88	21.88	11.24	8.87	4.95	10.95	4.35	11.29	8.61	5.70	3.73	2.09	1.58	6.58	3.13	4.72	0.51	0.41	0.79		Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM21358	21.17	21.17	10.38	8.37	4.15	10.35	4.28	10.60	8.37	5.49	3.05	2.06	1.61	6.38	3.38	3.71	0.49	0.40	0.81		Lesotho, Source of Tugela. MAS
Amietia vertebralis	TM30059	71.25	69.11	34.91	28.21	12.94	28.88	14.75	34.52	29.85	16.63	8.99	4.48	5.06	18.51	9.61	14.94	0.49	0.40	0.81		Lesotho, Sani River
Amietia vertebralis	TM56436	95.57	94.33	45.63	40.87	25.31	47.96	17.95	50.46	35.92	23.10	10.38	5.92	5.20	25.32	13.08	19.91	0.48	0.43	0.90		Btw Sani 3km NW Thaba Tseka.
Amietia vertebralis	TM21354	42.95	42.95	21.54	16.84	9.51	19.60	7.60	24.28	16.21	10.19	4.96	3.12	3.01	10.83	5.62	9.47	0.50	0.39	0.78		Unknown
Amietia vertebralis	TM13959	26.46	26.46	13.03	10.39	5.02	12.37	4.74	14.21	11.12	6.79	3.80	2.11	1.75	7.91	4.26	5.03	0.49	0.39	0.80		Lesotho, Source of Tugela. MAS

APPENDIX B

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Amietia vertebralis	TM21724	33.35	32.99	15.87	12.99	7.15	15.45	6.74	16.71	13.57	7.62	4.73	2.19	2.67	8.56	4.74	7.33	0.48	0.39	0.82		Unknown
Amietia vertebralis	TM13963	24.57	24.57	12.00	9.68	4.80	11.63	4.47	13.33	9.24	5.93	3.53	1.80	1.69	7.00	3.96	5.07	0.49	0.39	0.81		Lesotho, Source of Tugela, MAS Khabos
Amietia vertebralis	TM30262	119.74	117.94	49.15	59.06	22.53	47.72	21.34	56.32	50.58	30.68	12.13	7.22	5.94	31.47	18.56	25.80	0.41	0.49	1.20		
Amietia vertebralis	TM13956	27.59	26.87	13.85	10.97	5.70	13.42	5.15	14.14	10.57	6.91	3.57	2.18	1.75	7.43	4.52	5.38	0.50	0.40	0.79		Lesotho, Source of Tugela, MAS Khabos
Amietia vertebralis	TM30264	73.64	70.88	38.44	30.57	16.51	36.53	16.09	40.92	28.58	17.98	8.41	3.96	4.04	19.33	10.78	17.13	0.52	0.42	0.80		
Amietia vertebralis	TM30299	76.94	75.83	40.55	36.54	15.03	40.72	17.89	41.12	31.82	19.37	8.07	4.74	4.33	20.25	10.55	17.06	0.53	0.47	0.90		Blue Mtn Pass
Strongylopus hymenopus	647	42.76	41.82	19.98	15.50	8.87	19.65	8.23	20.80	14.19	8.31	5.02	2.86	3.00	10.85	5.92	8.59	0.47	0.36	0.78	Extensive	MAS
Strongylopus hymenopus	343	29.97	29.11	16.02	11.51	6.78	14.44	5.51	15.60	11.08	7.12	4.03	2.42	2.02	8.63	4.77	6.85	0.53	0.38	0.72	Extensive	
Strongylopus hymenopus	340	24.26	23.83	11.95	9.65	4.94	11.26	4.38	12.50	9.60	5.93	3.38	2.20	1.89	7.05	3.87	5.67	0.49	0.40	0.81	Extensive	
Strongylopus hymenopus	336	23.31	23.31	11.79	9.29	4.93	11.74	4.41	13.30	9.57	5.82	3.27	2.39	1.77	7.18	4.16	6.04	0.51	0.40	0.79	Extensive	
Strongylopus hymenopus	341	22.60	22.60	11.72	9.01	4.92	11.23	4.35	12.94	8.95	5.87	3.36	1.75	1.42	6.94	3.95	4.28	0.52	0.40	0.77	Extensive	
Strongylopus hymenopus	342	28.02	28.02	14.44	10.76	5.77	14.26	5.08	15.19	10.60	6.73	3.90	2.44	1.99	7.89	4.41	6.33	0.52	0.38	0.75	Extensive	
Strongylopus hymenopus	339	30.51	30.15	14.88	12.12	6.07	14.29	5.99	15.79	11.68	6.92	3.90	2.61	2.26	8.37	4.87	6.26	0.49	0.40	0.81	Extensive	
Strongylopus hymenopus	337	24.42	24.42	12.67	9.80	5.06	12.53	4.72	13.43	10.33	6.19	3.51	2.56	1.89	7.54	3.82	5.75	0.52	0.40	0.77	Extensive	
Strongylopus hymenopus	338	31.92	31.92	16.20	13.07	5.81	15.29	5.85	17.95	12.15	6.94	3.92	2.33	2.29	8.81	4.85	7.30	0.51	0.41	0.81	Extensive	
Strongylopus hymenopus	335	30.85	30.85	15.65	12.76	5.95	14.99	5.95	16.90	11.78	7.19	4.13	2.55	2.21	8.57	4.60	6.43	0.51	0.41	0.82	Extensive	
Strongylopus hymenopus	MC1	44.82	44.82	20.80	15.59	9.40	19.59	8.13	22.91	15.14	10.63	5.38	2.90	3.65	12.37	6.16	8.17	0.46	0.35	0.75	Extensive	

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Strongylopus hymenopus	MC2	27.05	27.05	12.64	10.45	5.36	12.57	5.06	13.74	10.27	6.33	3.68	2 71	2.01	7.85	4.54	6.64	0.47	0.39	0.83	Extensive	
Strongylopus hymenopus	MC3	23.84	23.84	11.59	9.48	4.96	10 75	4.50	11.67	9.55	6.03	3.61	2.82	1.87	7.26	4.31	5.78	0.49	0 40	0.82	Extensive	
Strongylopus hymenopus	MC4	26.16	26.16	13.09	10.30	4.76	12.13	4.78	14.08	10.53	6.49	3.58	2.24	2.04	8.12	4.28	5.99	0.50	0.39	0.79	Extensive	
Strongylopus hymenopus	MC5	20.87	20.87	9.26	8.44	4.21	9.28	3.99	9.57	8.48	5.80	3.19	2.18	1.68	6.55	3.18	4.92	0.44	0.40	0.91	Extensive	
Strongylopus hymenopus	MC6	24.80	24.80	12.09	9.97	5.32	11.10	4.84	13.47	10.16	5.81	3.50	2.11	1.87	6.84	4.05	5.68	0.49	0.40	0.82	Extensive	
Strongylopus hymenopus (TYPE)	BM.1978.2.28	55.33	54.22	32.05	22.12	11.76	30.73	9.87	31.55	19.89	10.68	6.06	5.10	3.85	13.79	8.87	11.81	0.58	0.40	0.69	3 free (outer & inner)	South Africa
Strongylopus hymenopus??	BM.1978.1235	55.05	53.77	33.11	24.29	11.91	28.04	11.14	30.33	20.89	11.88	7.61	5.18	3.93	15.57	10.03	12.44	0.60	0.44	0.73	3 free (outer & inner)	Sani Pass
Strongylopus hymenopus??	NM00651	45.43	45.43	32.31	19.38	11.72	30.12	10.51	30.13	18.67	10.09	6.78	4.00	3.71	13.08	8.08	10.58	0.71	0.43	0.60	3 free (outer & inner)	No locality info
Strongylopus hymenopus??	NM2774	46.10	42.31	30.44	19.81	10.66	25.68	8.80	29.27	17.80	9.94	5.66	4.86	3.41	13.03	8.29	9.28	0.66	0.43	0.65	3 free (outer & inner)	Cathedral Peak
Strongylopus hymenopus??	NM2773	35.20	35.20	23.40	15.17	7.85	19.92	7.50	21.45	14.68	7.46	4.71	3.75	2.44	10.52	6.98	8.09	0.66	0.43	0.65	3 free (outer & inner)	Cathedral Peak
Strongylopus hymenopus??	NM904	28.92	28.92	16.72	12.22	6.86	14.84	5.36	15.25	12.05	7.15	4.61	3 22	2.04	9.09	5.60	6.45	0.58	0.42	0.73	3 free (outer & inner)	Mount-Aux-Sources
Strongylopus hymenopus??	NM272	57.95	57.95	36.58	22.67	12.33	33.46	12.02	35.26	25.70	12.26	7.47	5.86	4.30	16.12	9.29	12.51	0.63	0.39	0.62	3 free (outer & inner)	Cathkin Peak
Afrana angolensis	NM7761	56.36	54.63	36.00	19.44	13.09	31.47	10.46	26.71	21.74	12.16	7.88	3.52	4.88	12.65	6.80	11.87	0.64	0.34	0.54	1 free inner	Goedgedunk, R69 bridge, Xamu River
Afrana angolensis	M7764	35.39	33.83	22.89	12.95	7.16	21.20	6.83	22.68	12.94	8.23	4.88	2.74	2.38	9.18	5.61	8.44	0.65	0.37	0.57	1 free inner	Cathedral Peak Nature Reserve
Afrana fuscigula	NM7315	64.31	64.31	37.45	27.64	13.60	37.44	13.77	36.34	28.18	15.53	8.20	4.44	6.87	15.39	9.78	15.89	0.58	0.43	0.74	Extensive	Spioenkop Public Resort
Afrana fuscigula	NM7535	68.77	67.02	37.08	25.99	9.73	32.44	12.19	36.81	28.11	14.93	8.46	4.62	6.07	14.96	9.89	14.57	0.54	0.38	0.70	Extensive	
Strongylopus grayii	NM6994	34.47	34.47	23.06	12.55	6.17	20.10	6.70	23.58	13.42	7.79	4.52	2.90	2.56	8.78	5 48	8.68	0.67	0 36	0.54	4 phalanges free	

Species	Specimen ID	Snout-Vent (SV)	Snout-Urostyle (SU)	Tibia Length (TL)	Head Width (HW)	Humerus Length (HL)	Femur Length (FL)	Forearm Length (FaL)	Foot Length (FtL)	Head Length (HL)*	Anterior Tympanum - Nostril	Eye Diameter (ED)	Internasal Width (IW)	Tympanum Length (TL)	Inter Ocular Posterior	Inter Ocular Anterior	Finger Length	TL/SV	HW/SV	HW/TL	Webbing	Location
Strongylopus grayii	NM6992	29.64	29.64	18.33	9.77	5.06	17.20	5.95	18.88	12 58	5.59	3.93	2.27	1.85	6.76	4.63	7.02	0.62	0.33	0.53	4 phalanges free	Port Elizabeth
Strongylopus fasciata	NM6396	34.27	34.27	24.00	11.28	7.41	21.91	7.09	29.86	15.52	7.83	4.56	3.59	2.17	8.47	5.85	9.99	0.70	0.33	0.47	4 phalanges free	Pietermaritzburg, Wylie Park
Rana dracoensis	NM734	53.22	51.64	30.02	23.41	10.18	27.61	10.54	30.34	20.33	11.64	6.66	3.87	3.31	14.52	8.89	8.91	0.56	0.44	0.78	3 phalanges free	

APPENDIX C

Mis-identified *S. hymenopus* specimens (Natal Museum)

PLATE 1: Dorsal view of misidentified *S. hymenopus* specimens (NM2774). Suggested correction is *Amietia angolensis* or *A. fuscigula*.

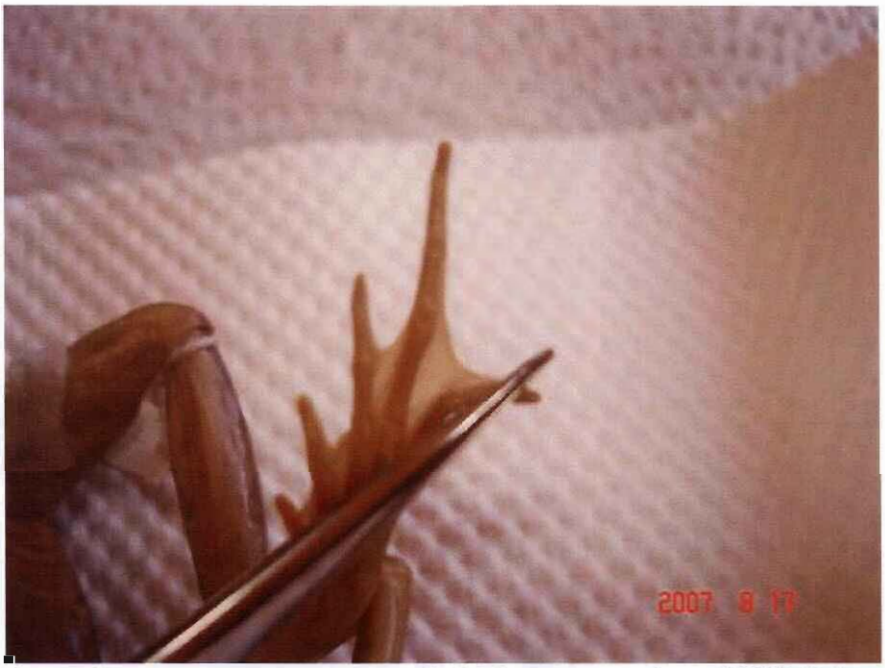

PLATE 2: Detail of webbing of misidentified *S. hymenopus* specimens (NM2774). Suggested correction is *Amietia angolensis* or *A. fuscigula*.

APPENDIX D

TABLE D.1: List of specimens from which tissue was obtained for molecular analysis. Tissues obtained from tadpoles are indicated by *. Specimens also used for molecular assessment are indicated by **. Collection abbreviations: JB = Jeanne Berkeljon, personal collection. MC = Michael Cunningham, personal collection. SAIAB = South African Institute of Aquatic Biodiversity collection.

Taxon	Collection	Site No.	Extraction No.	Locality	16S	ND2	RAG1	RAG2
A. vertebralis **	SAIAB	DT1	G01	Mohlaka Liboba	✓		✓	
A. vertebralis **	SAIAB	DT2	G02	Tenane	✓			
A. vertebralis **	SAIAB	DT4A	G03	Matsoku	✓			
A. vertebralis **	SAIAB	DT4B	G04	Matsoku	✓	✓		
A. vertebralis **	SAIAB	DT5	G05	Makhaleng		✓	✓	✓
A. vertebralis **	SAIAB	DT6.1	G06	Maletsunyane		✓		
A. vertebralis **	SAIAB	DT8.1	G08	Bafali		✓		
A. vertebralis **	SAIAB	DT8.2	G09	Bafali	✓		✓	
A. vertebralis **	SAIAB	DT8.5	G12	Bafali		✓		
A. vertebralis **	SAIAB	DT9.1	G13	Langa-le-balele		✓		
A. vertebralis **	SAIAB	DT9.2	G14	Langa-le-balele		✓		
A. vertebralis **	SAIAB	DT9.4	G16	Langa-le-balele	✓			
A. vertebralis **	SAIAB	DT10	G17	Mohlesi	✓			
A. vertebralis **	SAIAB	DT11.1	G18	Sani			✓	
A. vertebralis **	SAIAB	DT16.1	G23	Qabane	✓	✓	✓	
A. vertebralis **	SAIAB	DT16.5	G27	Qabane	✓			
A. vertebralis **	SAIAB	DT17A.1	G28	Tsatsane			✓	
A. vertebralis **	SAIAB	DT17A.2	G29	Tsatsane	✓			
A. vertebralis **	SAIAB	DT17A.4	G31	Tsatsane	✓			
A. vertebralis **	SAIAB	DT17A.5	G32	Tsatsane	✓			
A. vertebralis **	SAIAB	DT17A.6	G33	Tsatsane	✓			
A. vertebralis **	SAIAB	JR3A	G34	Mantsonyane	✓		✓	
A. vertebralis **	SAIAB	JR3B	G35	Mantsonyane	✓			
A. vertebralis **	SAIAB	JR4.1	G37	Lesobeng	✓			

A. vertebralis **	SAIAB	JR4.2	G38	Lesobeng				
A. vertebralis **	SAIAB	JR4.3	G39	Lesobeng			✓	
A. vertebralis **	SAIAB	JR5A	G44	Tsehlanyane	✓			
A. vertebralis **	SAIAB	JR5A	G45	Tsehlanyane				
A. vertebralis **	SAIAB	JR5B	G46	Tsehlanyane	✓			
A. vertebralis **	SAIAB	JR7	G47	Ketane			✓	
A. vertebralis **	SAIAB	JR8.1	G48	Maletsunyane			✓	
A. vertebralis **	SAIAB	JR8.2	G49	Maletsunyane				
A. vertebralis **	SAIAB	JR9.1	G50	Maletsunyane				
A. vertebralis **	SAIAB	JR9.2	G51	Maletsunyane				
A. vertebralis **	SAIAB	JR9.3	G52	Maletsunyane				
A. vertebralis **	SAIAB	JR10.1	G53	Senqu	✓		✓	
A. vertebralis **	SAIAB	JR10.2	G54	Senqu	✓			
A. vertebralis **	SAIAB	JR11B.1	G55	Moremoholo	✓			
A. vertebralis **	SAIAB	JR11B.2	G56	Moremoholo				
A. vertebralis **	SAIAB	JR11B.3	G57	Moremoholo				
A. vertebralis **	SAIAB	JR12.1	G58	Mokhotlong				
A. vertebralis **	SAIAB	JR12.2	G59	Mokhotlong				
A. vertebralis **	SAIAB	JR13.1	G60	Redi			✓	
A. vertebralis **	SAIAB	JR13.2	G61	Redi				
A. vertebralis **	SAIAB	JR14.1	G62	Manguang				
A. vertebralis **	SAIAB	JR14.2	G63	Manguang				
A. vertebralis **	SAIAB	JR14.3	G64	Manguang	✓			
A. vertebralis **	SAIAB	JR14.4	G65	Manguang				
A. vertebralis **	SAIAB	JR14.5	G66	Manguang				
A. vertebralis **	SAIAB	JR14.6	G67	Manguang				
A. vertebralis **	SAIAB	JR14.7	G68	Manguang				
A. vertebralis **	SAIAB	JR14.8	G69	Manguang				
A. vertebralis **	SAIAB	JR15.1	G70	Sani tributary				

Taxon	Collection	Site No.	Extraction No.	Locality	16S	ND2	RAG1	RAG2
A. vertebralis **	SAIAB	JR15.3	G72	Sani tributary				
A. vertebralis **	SAIAB	JR15.4	G73	Sani tributary				
A. vertebralis **	SAIAB	JR16.1	G74	Mashai	✓		✓	
A. vertebralis **	SAIAB	JR16.2	G75	Mashai				
A. vertebralis **	SAIAB	JR16.3	G76	Mashai	✓			
A. vertebralis **	SAIAB	JR16.4	G77	Mashai	✓			
A. vertebralis **	SAIAB	JR16.5	G78	Mashai	✓			
A. vertebralis **	SAIAB	JR16.6	G79	Mashai	✓			
A. vertebralis **	SAIAB	JR16.7	G80	Mashai	✓			
A. vertebralis **	SAIAB	JR16.8	G81	Mashai	✓			
A. vertebralis **	SAIAB	JR16.9	G82	Mashai	✓			
A. vertebralis **	SAIAB	JR16.11	G83	Mashai	✓			
A. vertebralis **	SAIAB	JR16.12	G84	Mashai	✓			
A. vertebralis **	SAIAB	JR17	G85	Thamathu				
A. vertebralis **	SAIAB	JR18	G86	Tsoelikane				
A. vertebralis **	SAIAB	JR22	G87	Morotong				
A. vertebralis **	SAIAB	JR22B.1	G88	Morotong				
A. vertebralis **	SAIAB	JR22B.2	G89	Morotong	✓			
A. vertebralis	JB	SP0016	G92	Tsehlanyane				
A. vertebralis	JB	SP0038	G93	Tsehlanyane				
A. vertebralis *	JB	SP0031	G94	Tsehlanyane	✓			
A. vertebralis *	JB	SP0009	G95	Tsehlanyane				
A. vertebralis *	JB	SP0039	G96	Tsehlanyane				
A. vertebralis *	JB	SP0028	G97	Tsehlanyane	✓			
A. vertebralis *	MC	MH0649	J16	Kwasongada R	✓	✓	✓	
A. vertebralis	MC	QQ0016	J17	Trib of upper Tsatsane	✓		✓	✓
A. vertebralis	MC	MH0667	J19	Tsatsane	✓		✓	
A. vertebralis	MC	MH0398	J20	Kgotjwane R				
A. vertebralis	MC	MH0242	J21	Koakoatsi R	✓			
A. vertebralis	JB	SP0048	J35	Sani Top, Sani River	✓	✓	✓	
A. vertebralis	JB	SP0049	J36	Sani Top, Sani River			✓	
A. vertebralis	JB	SP0040	J37	Sani Top, Sani River			✓	

S. hymenopus *	JB	SP0017	A01	Vemvane				
Taxon	**Collection**	**Site No.**	**Extraction No.**	**Locality**	**16S**	**ND2**	**RAG1**	**RAG2**
S. hymenopus *	JB	SP0030	A02	Vemvane, MAS				
S. hymenopus *	JB	SP0015	A03	Vemvane, MAS			✓	
S. hymenopus *	JB	SP0005	A04	Vemvane, MAS	✓	✓		
S. hymenopus *	JB	SP0043	A05	Vemvane, MAS				
S. hymenopus *	MC	MH1211	J04	Vemvane Stream, nr Chain Ladders	✓		✓	
S. hymenopus	MC	MH0433	J05	Vemvane wetland, near Namahali camp	✓		✓	
S. hymenopus *	MC	MH1323	J06	Vemvane Stream, nr Chain Ladders	✓		✓	✓
S. hymenopus *	MC	MPU100	J07	Upper Tugela R	✓		✓	
S. hymenopus *	MC	MH1041	J08	Vemvane Stream, nr Chain Ladders	✓		✓	
S. hymenopus *	MC	MH0445	J09	Vemvane Stream, nr Chain Ladders	✓		✓	
S. hymenopus	MC	MH0438	J10	Vemvane wetland, near Namahali camp				
S. hymenopus	MC	MH0443	J11	Vemvane wetland, near Namahali camp	✓		✓	
S. hymenopus	MC	MH0426	J12	Vemvane wetland, near Namahali camp				
S. hymenopus *	MC	MH1060	J13	Vemvane Stream, nr Chain Ladders	✓	✓	✓	
S. hymenopus	MC	MH1096	J14	Vemvane Stream, nr Chain Ladders	✓		✓	
S. hymenopus *	MC	MH1097	J15	Vemvane Stream, nr Chain Ladders	✓	✓	✓	
S. hymenopus	MC	MH0455	J40	SE trib of Khubelu R		✓	✓	
S. hymenopus *	MC	QQ0097	J41	Vemvane wetland			✓	
S. hymenopus	MC	MPU124	J45	Upper Tugela R, Mont-au-Sources Plateau	✓		✓	
S. hymenopus	MC	MPU127	J46	Vemvane	✓		✓	

S. hymenopus *	MC	QQ0109	J47	Vemvane		✓	✓	
Taxon	**Collection**	**Site No.**	**Extraction No.**	**Locality**	**16S**	**ND2**	**RAG1**	**RAG2**
S. hymenopus *	MC	QQ0121	J48	Vemvane	✓	✓	✓	
S. hymenopus *	MC	QQ0068	J49	Vemvane wetland	✓	✓	✓	
S. hymenopus *	MC	QQ0123	J50	Vemvane wetland	✓		✓	
S. hymenopus *	MC	MH0483	J51	Kokoatsoan R, on plateau behind the Saddle	✓		✓	
Strongylopus wageri *	JB	SP0001	J01	Mkamazama, Sani Pass			✓	✓
Strongylopus wageri *	JB	SP0002	J02	Mkamazama, Sani Pass	✓		✓	
Strongylopus wageri *	JB	SP0003	J03	Mkamazama, Sani Pass	✓		✓	
Strongylopus grayii	MC	MH0545	J39	Upper Namahadi			✓	✓
Strongylopus bonaespei	MC	MH0854	O5	Hottentots-Holland plateau				
Afrana dracomontana *	MC	QQ0009	J18	Trib of upper Tsatsane	✓	✓	✓	✓
Afrana dracomontana *	JB	SP0050	J33	Sani Top, Sani River		✓	✓	
Afrana dracomontana *	JB	SP0007	J34	Sani Top, Sani River		✓	✓	
Afrana angolensis	MC	QQ0110	O1	Kgotjwane tributary				
Afrana fuscigula	MC	QQ0139	O2	N8 at R26 (Wepener Rd) intersection			✓	✓
Afrana vandijki *	MC	MH0107	O3	Waboomsberg Gt Swartberg			✓	✓
Afrana fuscigula	MC	MH0190	O4	Leopards Camp, Maanskynkop			✓	
Tomopterna cryptotis	MC	QQ0133	O6	Oranje, R711 Fouriesburg-Clarens			✓	✓

APPENDIX E

TABLE E.1: Abbreviations for localities of sequenced samples. This code follows the extraction code and species code e.g. G1Mav = sample G1 from Mohlaka of *Amietia vertebralis*.

Code	Location
A	Mashai
B	Bafali
C	Lesobeng
D	Senqu
E	Tenane
F	Mangaung
G	Morotong
H	Kwasingada
I	Mohlesi
J	Sani
K	Khubelu
L	Langa-le-balele
M	Mohlaka
N	Ketane
O	Liboda
P	Moremoholo
Q	Qabane
R	Maletsunyane
S	Tsatsana
T	Tugela
U	Matsoku
V	Vemvane
W	Makhaleng
X	Tsehlanyane
Y	Mantsonyane
Z	Koakoatsi
1	Redi
2	Upper Namahali

Species codes:

Ad = *Amietia dracomontana*
Afa = *Amietia angolensis*
Aff = *Amietia fuscigula*
Av = *Amietia vertebralis*
Pya = *Pyxicephalus adspersus*
Sb = *Strongylopus bonaspei*
Sg = *Strongylopus grayii*
Sh = *Strongylopus hymenopus*
Sw = *Strongylopus wageri*

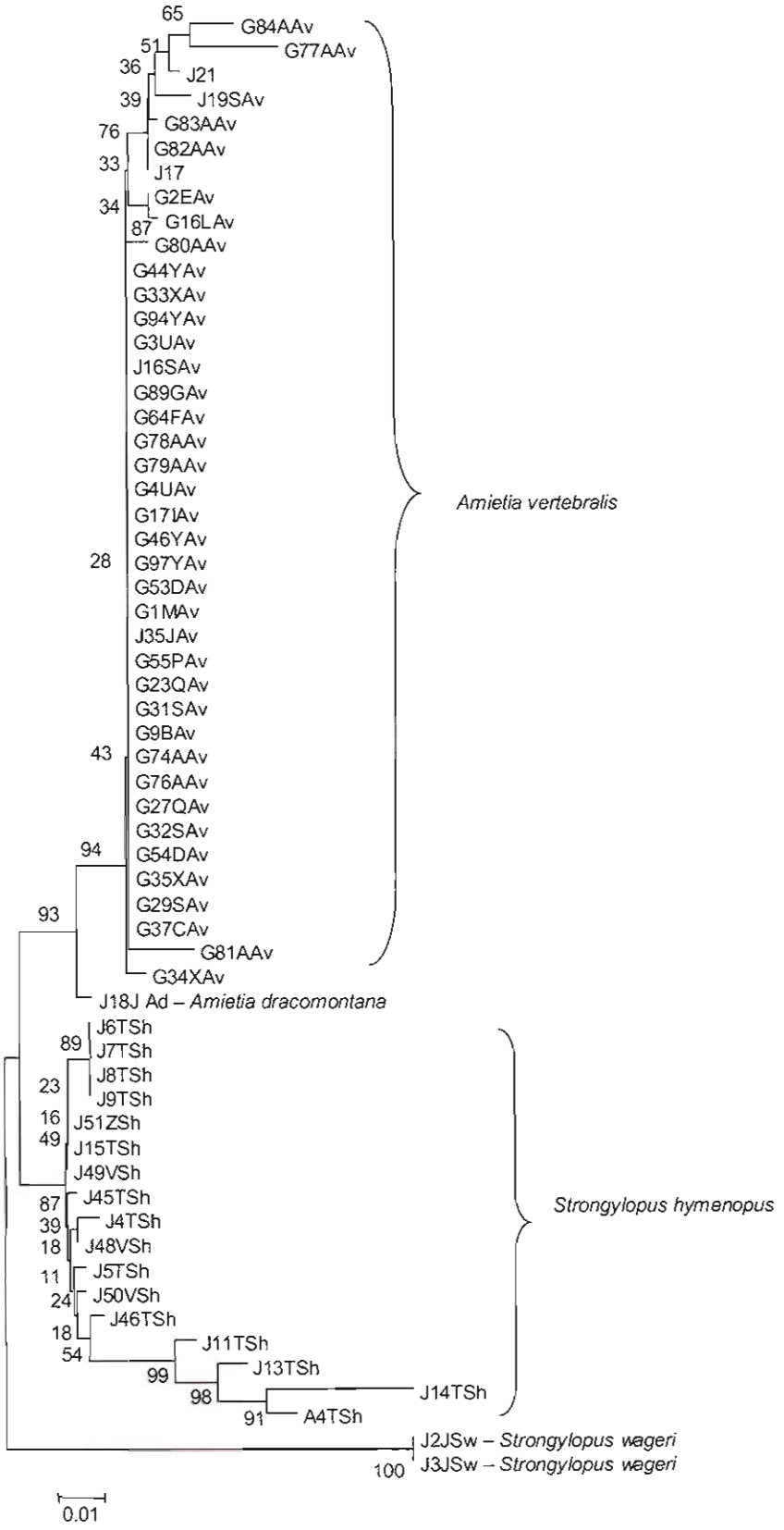

FIGURE E.1: Initial Neighbour-Joining Tree for 16S showing bootstrap values. Note the significant lack of variation among *A. vertebralis* samples.

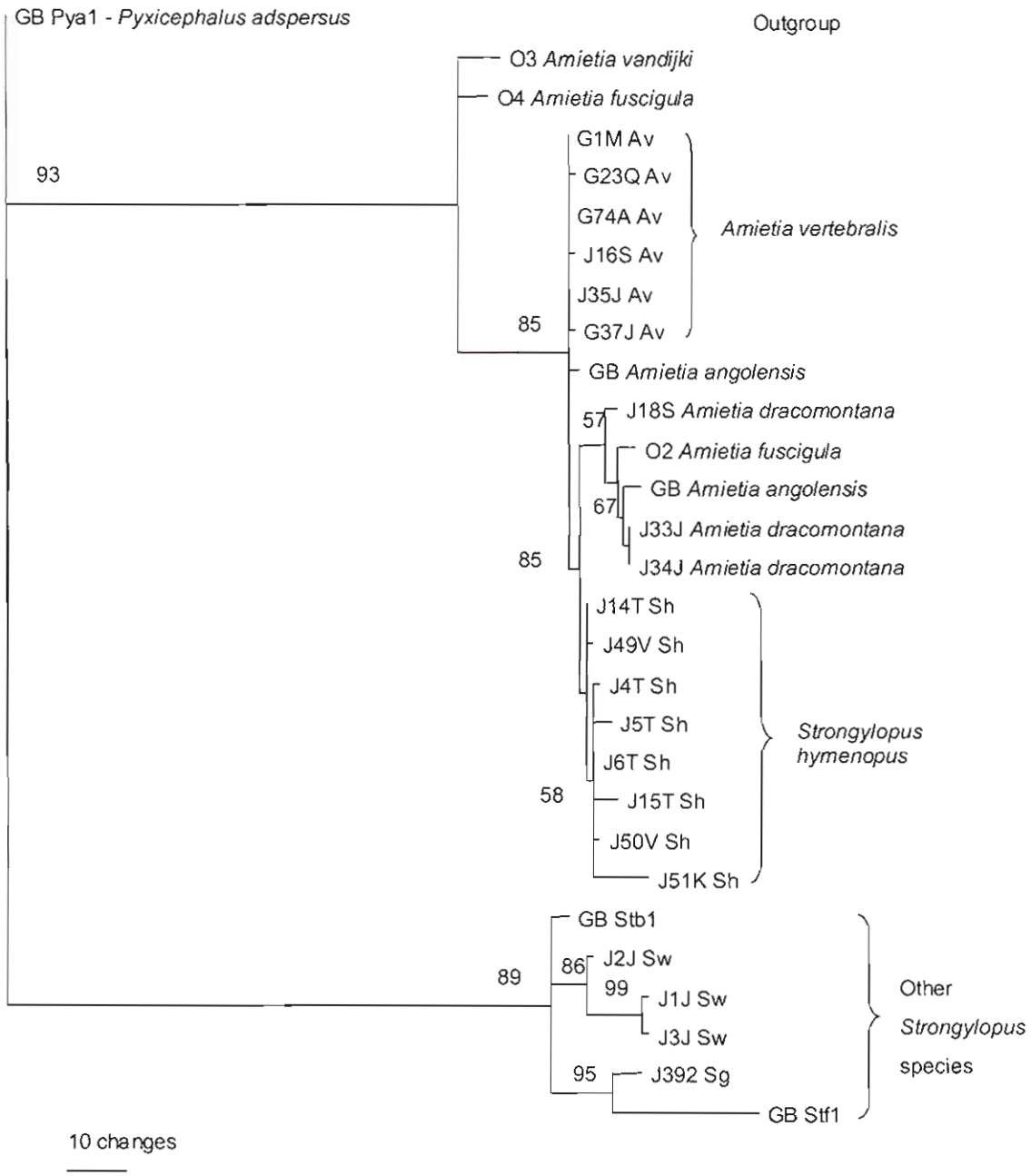

FIGURE E.2: The strict consensus tree from parsimony analysis of RAG1-AmpF1 with *Pyxicephalus adspersus* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

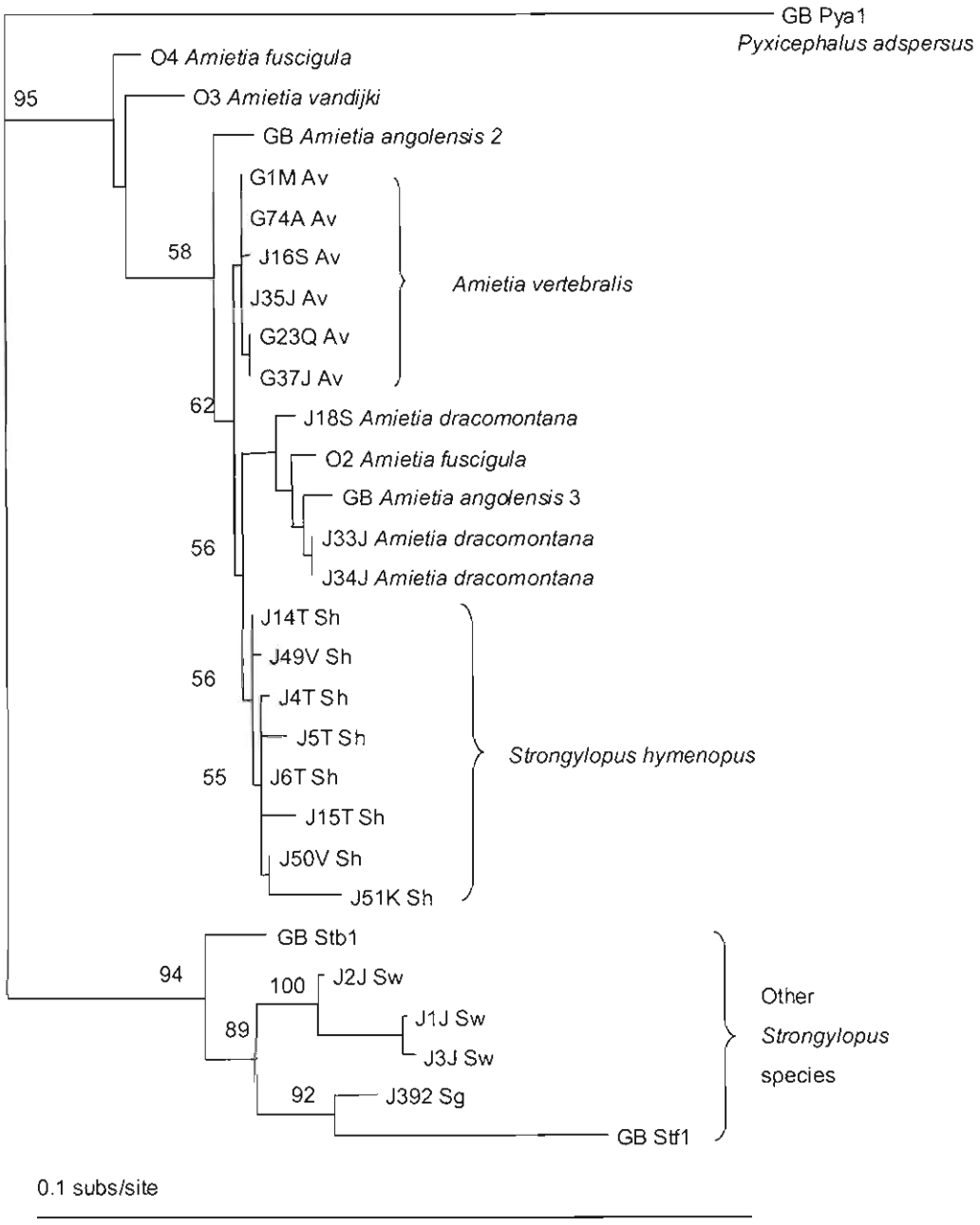

FIGURE E.3: The single Maximum Likelihood tree from analysis of RAG1-AmpF1 with *Pyxicephalus adspersus* as the outgroup using the HKY+G model of DNA substitution, with parameter values estimated by ModelTest (-Ln likelihood = 2373). Branch lengths are proportional to the number of substitutions per site. Bootstrap values are shown at the major nodes. Individual reference numbers correspond to those in Appendix D.

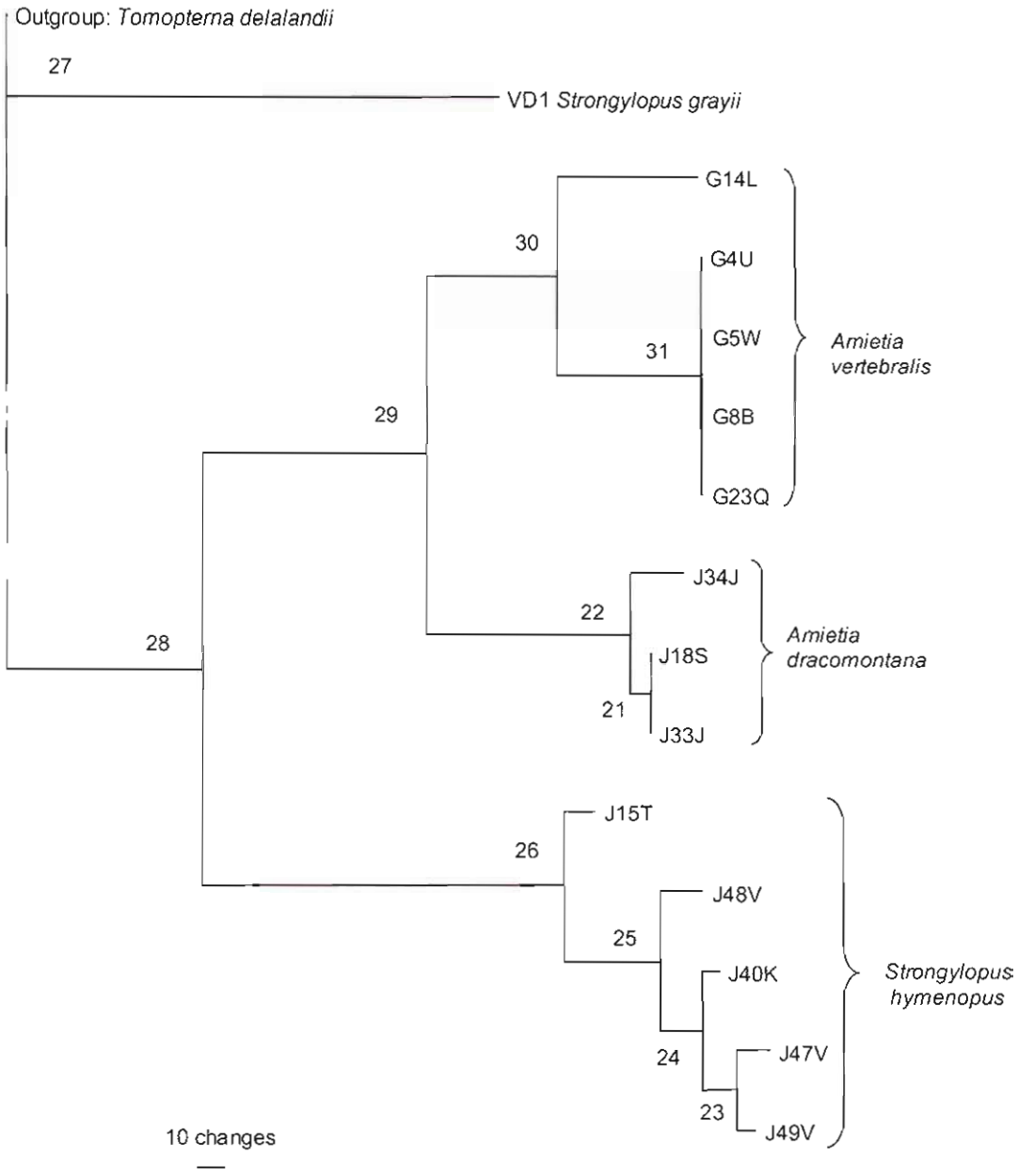

FIGURE E.4: The bootstrap 50% majority rule consensus tree from Parsimony analysis of ND2-VMet with *Tomopterna delalandii* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

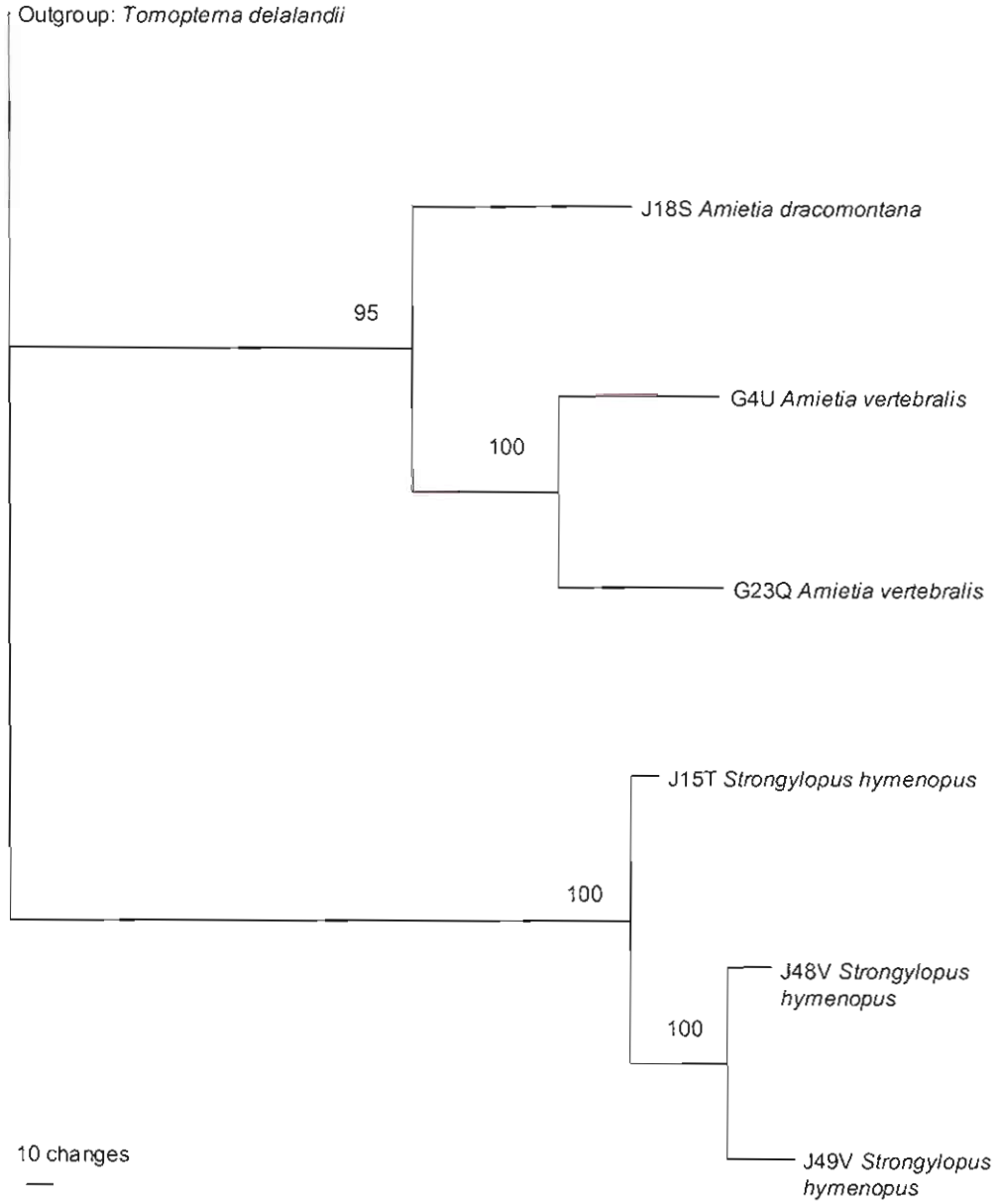

FIGURE E.5: The strict consensus tree from parsimony analysis of the combined dataset 16S and ND2-VMet with *Tomopterna delalandii* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

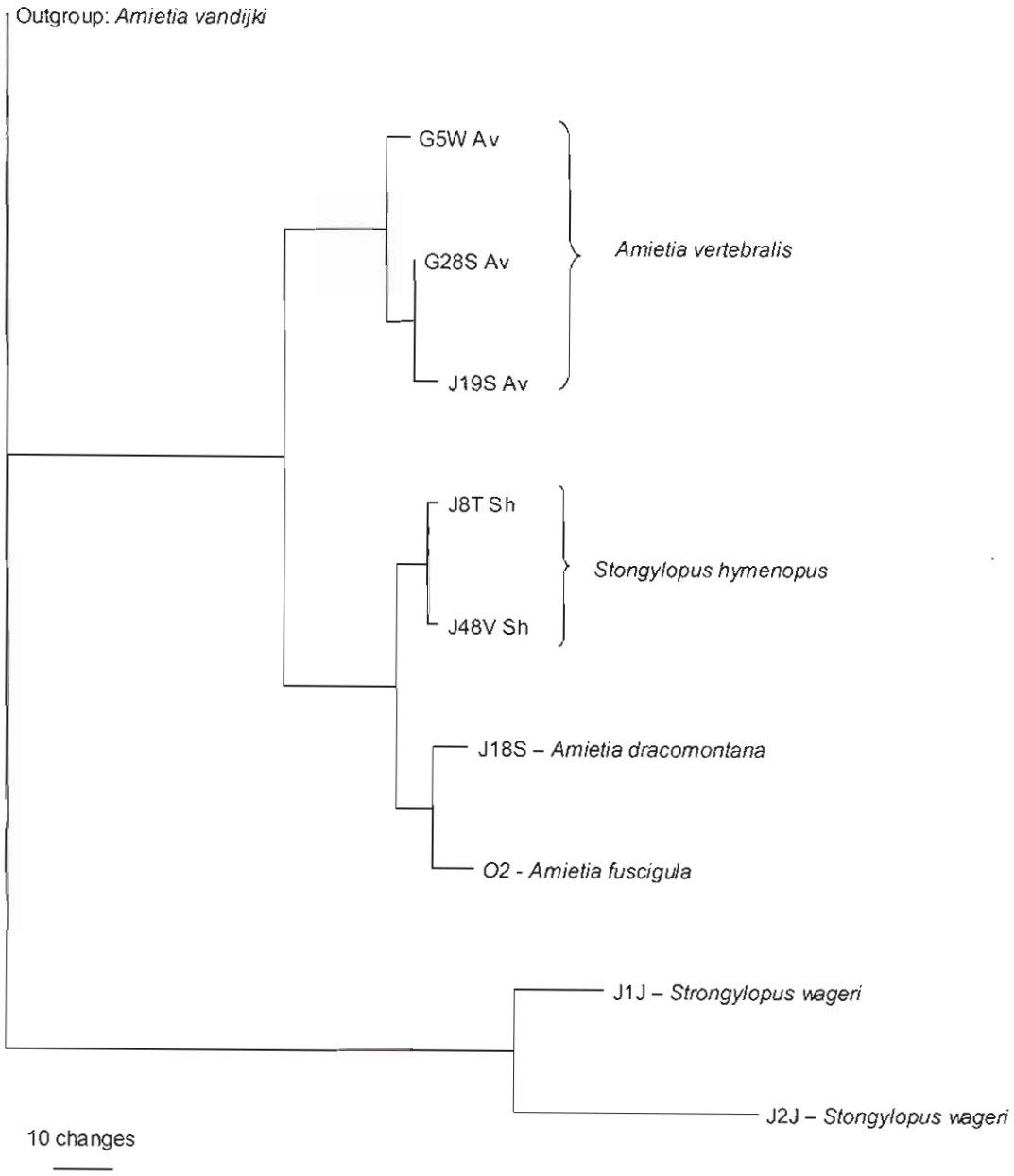

FIGURE E.6: The strict consensus tree from parsimony analysis of the combined RAG1 (AmpF1&AmpF2) fragments with *Amietia vandijki* as the outgroup. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.

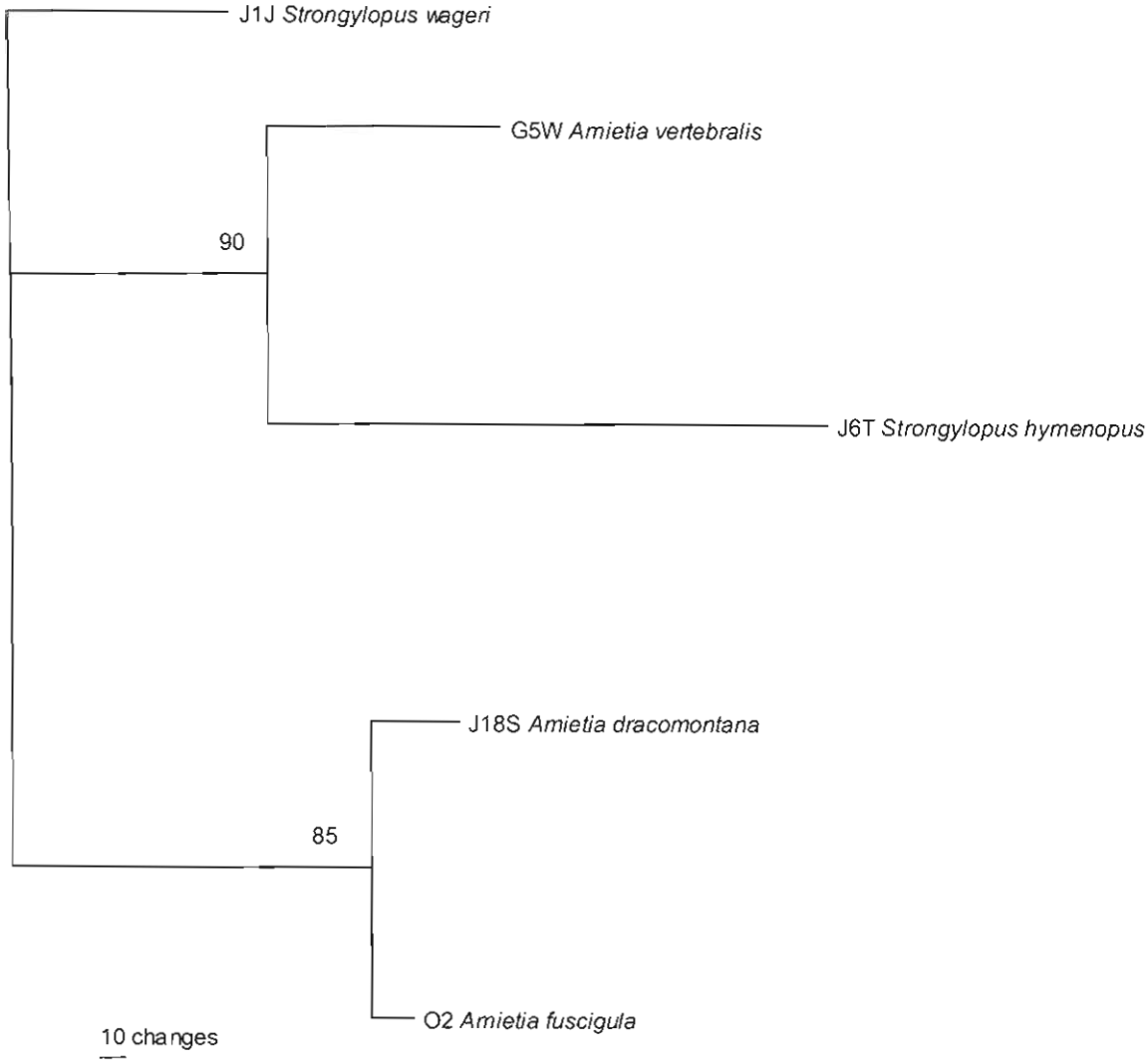

FIGURE E.7: The bootstrap 50% majority rule consensus tree from parsimony analysis of the combined dataset RAG1 & RAG2 with *Strongylopus wageri* as the outgroup. Bootstrap values are shown at the major nodes. Branch lengths are proportional to the number of unambiguous changes in the original sequence data. Individual reference numbers correspond to those in Appendix D.