Occurrence of lungfish in the Brisbane River, Queensland, Australia dates back to 3850 yr BP

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RH: Ancient lungfish in the Brisbane River

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Abstract

Bone fragments collected from the Platypus Rock Shelter in southeast Queensland, on the banks of the Brisbane River, can be compared with bone from the living Australian lungfish, *Neoceratodus forsteri*, and suggest that this species, which was widely distributed in Queensland in Pliocene and Pleistocene deposits, was also found in the Brisbane River as recently as 3850 years before the present, based on current ^{14}C dates. The fragments have dimensions and morphology consistent with parts of lungfish jaws and palatal bones, and differ from the bones of teleost fish of comparable size that live in the Brisbane River. Unfortunately, attempts to extract mitochondrial DNA from the bones have not been successful due to very low levels of endogenous DNA. The presence of morphologically identifiable lungfish bones suggests that the Brisbane River has, and always did have, a population of lungfish that belong in this river and were not translocated. The Brisbane River is separated from the Mary and Burnett Rivers by mountain ranges, and the Rockshelter is 90 kilometres away from the nearest tributary of the Mary River. Using a morphological analysis of carbon-dated midden site skeletal material, we show that lungfish were present in the Brisbane River over three thousand years ago, and may always have been there, despite attempts to translocate lungfish to this habitat. This finding is significant because lungfish are now seriously at risk in all of their present habitats from human interference in the environment and the resulting loss of biodiversity. Confirmation that the Brisbane River contains a population of lungfish, and always has done, increases the need for protection of this endangered species.

1. Introduction

Australia is one of three continents with a population of living lungfish, and the Australian lungfish, *Neoceratodus forsteri* (Krefft 1870), differs from the lungfishes of Africa and South America. It is also the species most restricted in habitat, being confined to a small corner of southeast Queensland, and the one most at risk of extinction (Bancroft 1928; Frentiu et al. 2001; Kemp 2011). The Australian lungfish had a wide distribution in eastern Australia in the Pliocene and Pleistocene, including what is now the Condamine River (Kemp 1997a). In historical times, this contracted to coastal rivers of southeast Queensland, with the first lungfish described coming from the Burnett River (Krefft 1870). A second paper (Gunther 1871) described lungfish from the Mary River, approximately 200 km south of the Burnett River. A continuing debate, fuelled by the translocation activities of O'Connor (1897, 1902), has addressed the issue of whether a population of lungfish has always existed in the Brisbane River, south of the Mary River and similar in water

quality, flora and fauna to the Mary and Burnett Rivers. Most authors assume that the original distribution of lungfish was confined to the Mary and Burnett Rivers (Arthington 2009; James *et al.* 2010), but evidence from DNA analysis suggests that this is not the case. The Brisbane River has been shown to contain a separate and independent population using Random Amplified DNA Fragment analysis when compared to the Burnett and Mary River fish (Lissone 2001; Lissone *et al.* 2003).

An additional indication that lungfish may have been in the Brisbane River before the first scientific descriptions of the species comes from an Aboriginal midden site in a cave on the Brisbane River at Northbrook, now submerged under the waters of Lake Wivenhoe. The cave is known as the Platypus Rock Shelter, and was occupied from about 5000 to about 538 years before the present (Hall 1990; Hall *et al.* 1988). Several bone fragments among the material in the cave, excavated before Lake Wivenhoe was built in 1984 (Hall *et al.* 1988) are consistent in size, shape and structure with bones of the jaws and palate from modern lungfish specimens, suggesting that lungfish were present in the Brisbane River in historical times. The material is preserved in the collections of the Queensland Museum. This contribution compares the bone fragments with skeletal specimens from a modern lungfish, and describes an attempt to extract mitochondrial DNA from the bones.

2. 1. Materials

A number of ancient bone samples were kindly provided by the Queensland Museum. Three of these fragments, S465-1, S465-2 and S465-3, were well enough preserved to compare with prepared lungfish bones, to confirm identification as parts of the lungfish skeleton (Fig. 1). Comparative material comes from a specimen of a modern lungfish, from the Brisbane River (QM I 26018), in the collections of the Queensland Museum.

One of the samples, S465-1, came from stratigraphic unit SU6, and has been dated to 3870 $\pm$ 170 Conventional Radiocarbon Age BP, based on current ^{14}C dates (Hall *et al.* 1988). The remaining two fragments come from stratigraphic unit SU7a, which has been dated less precisely,

to between 2479 and 4327 Conventional Radiocarbon Age BP (Hall *et al.* 1988). These units are all at the lowest level of the cave deposits.

Numerous stone tools have been analysed from the Platypus Rockshelter, suggesting significant technological changes over the duration of human occupancy of the site (Hiscock and Hall, 1988). The Rockshelter deposit included large numbers of bones of small macropods and possums, and the remains of snakes (carpet pythons) and lizards (water dragons). Vertebrae and head plates of catfish were also present, with some remnants of other bony fish common in riverine environments, as well as a few fragments of amphibians. The material that includes animal remains has been sorted, but none of the specimens have been categorised in a systematic way, and we were not permitted to examine all of the collection.

2.2. Site of Platypus Rockshelter

The Platypus Rockshelter is situated in cliffs close to the mid Brisbane River at Northbrook (Fig.1). The area is at least 90 kilometres from the nearest tributary of the Mary River large enough to have a population of lungfish, and at least 150 kilometres from the Burnett River. Both the Mary and Burnett Rivers are separated from the catchment of the Brisbane River by mountain ranges (Fig 1.) and any connection between the three systems is impossible during the times under consideration. Prior to white settlement, before roads existed, Aboriginal peoples would have travelled around the country on foot (Morwood 1986).

2.3. Methods

Identification of bones: Bones that appeared to belong to lungfish were selected from a number of specimens that had been identified as remnants of aquatic vertebrates. Because the bone fragments were fragile, examination was limited to visual inspection only. The specimens were then photographed and compared with bones of living lungfish.

Ancient DNA (aDNA) extraction: Approximately 20mg of shavings were collected from each lungfish bone by scalpel, and extracted overnight with rotation at 56°C in 0.25M EDTA, 0.01% Triton X100 with ~2mg of proteinase K. The extracts were then clarified by the addition of HCl and then purified by silica column binding using a Qiagen Dneasy® Blood and Tissue Kit. A

final purification was carried out by centrifugation of the 30µl extract through 300µl 1 of dry Sephacryl S200HR.

PCR amplification: 2µl of purified aDNA was amplified in a 10 µl reaction containing 50mM Tris-Cl pH 8.8, 20mM (NH₄)₂SO₄, 2.5mM MgCl₂, 1mg/ml BSA, 200uM of each dNTP, 0.5µM of each primer (below) and 0.3U of Platinum Taq polymerase (Invitrogen).

Primers:

LfAt6.8274F+F (5'-agtcgacgcttctagcttAACTGATACAACCATTAACCAAGGA)

LfAt6.8346R (5'-ATAAGGAATAGCATTATGGCGGTTAG).

The primers used were designed to amplify a small region of the mitochondrial ATPase6 region that contained single nucleotide polymorphisms (SNPs) capable of distinguishing between haplotypes Hatp 1-2 and Hatp 3-4 derived from Brisbane River fish (Loh, pers. com.), or haplotypes A-F, and G from several Queensland localities (Frentiu *et al.* 2001). A generic primer (F; shown in lower case) was added to primer LfAt6.8274F+F to allow direct sequencing of any PCR products. PCR reaction mixes were amplified using the following cycling programme: 94°C for 2min then 45 x (94°C 20sec, 56°C 60sec). Amplified products were visualized by agarose gel electrophoresis and ethidium bromide staining.

3. Results

Lungfish (Pisces: Sarcopterygii: Dipnoi) have cellular bone, with layers of mineralised tissue containing numerous osteocytes enclosed in lacunae (Kemp 2003). Bones of lungfish jaws have an external surface without a superficial cortical layer, and most show the trabecular structure of the bone on the surface. None of the fragments resemble the bones of other species of fish of similar size found in the Brisbane River, and the sizes of the fragments are consistent with lungfish bones (Fig. 2). The fragments were examined for the presence of characters typical of lungfish bones, such as the shape of the fragment, the lack of an external layer of cortical bone, exposed trabeculae on the surface, and traces of articular facets. Dimensions of all of the fragments, in particular the thickness of the specimens, varied from 1.5-2.5mm, as is found in adult lungfish bones from

modern material. The Rock Shelter fragments can be superimposed on the bones of the jaws and palate derived from a living adult Australian lungfish (Fig. 2A-C). The fragments may have come from one individual, or possibly two.

One fragment, S-465-1, had curved margins and a smooth surface, as are present in the medial region of the parasphenoid (Fig. 2A). This bone, in living lungfish, is found in the roof of the mouth, and is smooth on the palatal surface. Anteriorly the bone is broad and flat, medially it is narrow, and posteriorly it grows a little wider to support the base of the chondrocranium. The fragment is consistent with the medial region of the parasphenoid bone of a lungfish, and the curvature of the fragment matches the curvature of medial region of the parasphenoid of the modern lungfish.

The lower tooth bearing bone of a lungfish has a long anterior process that meets the matching bone in the mid line (Fig. 2B). Trabeculae are present on the external surface of this bone, but less obvious than those on the upper bone. The articular facet is shallow and supported by strong connective tissue fibrils, which leave little trace on the bone (Kemp 2013a). A second fragment of a jawbone, S 465-2, is consistent in shape and size with the extremity of the anterior process, and carries faint marks of trabeculae on the surface (Fig. 2B).

The bone that supports the upper tooth plate has a medial articulation with the matching bone, and a posterior process (Fig. 2C). This process is curved, and shows distinctive trabeculae. It has no articular facet, and is supported by the lateral extensions of the parasphenoid bone. The jaw articulation in lungfish is cartilaginous (Gunther 1871). The fragment labeled S465-3 is shaped like the posterior process of an upper jawbone. It has distinctive trabeculae and no articular facet (Fig. 2C). This fragment is consistent in shape and dimensions with part of the upper jawbone.

Despite a number of attempts on multiple extractions, no amplified DNA products were obtained for any of the samples. The possibility that PCR inhibitors were present in the aDNA extracts was determined by ‘spiking’ a PCR reaction containing modern lungfish DNA (known to be successful for PCR amplification) with each of the ancient lungfish aDNAs. The modern lungfish DNA sample amplified successfully in the presence of all ancient DNA extracts suggesting

the absence of any PCR inhibitors. Amplifications were carried out at high cycle numbers (42 - 45) to increase our chances of amplifying from very low concentrations of mitochondrial DNA.

4. Conclusion / Discussion

Lungfish have a skeleton that is based partly on cartilage and partly on bone (Gunther 1871). Bones of the jaws and palate are heavily ossified. However, as the jaw articulation in this species is entirely cartilaginous, the bones serve only as supports for chondral elements of the skull. There is no ossified jaw articulation of the bones of *N. forsteri*. This alone makes them distinct from the bones of other fish (Bjerring 1999; Gregory 1959), and from amphibian bones (de Beer 1937). Several of the bone fragments are consistent in size, shape and thickness with the bones of the jaws and the parasphenoid of a lungfish, and can be superimposed on these bones, suggesting that they may have originated from lungfish caught and eaten by people who lived in the cave. The presence of lungfish bones in the oldest sediments of the Platypus Rockshelter, over 3000 years before the present, suggests that the Brisbane River system had a population of lungfish at that time. Survival of this population has been confirmed by analysis of Random Amplified DNA Fragments from Brisbane River fish, which differ from those found in Burnett and Mary River fish (Lissone 2001; Lissone *et al.* 2003).

Most sites that include evidence of Aboriginal occupation or food gathering and consuming activities in southeast Queensland are coastal. These middens contain the remains of marine animals, particularly shellfish (Hall, 1984). Lungfish live in freshwater, and would not be found in a marine environment. Apart from the Platypus Rockshelter there are two other cave sites, the Maidenwell and Gatton Rockshelters, in southeast Queensland near the Brisbane River (Morwood 1986). Both are remote from the coast.

The Maidenwell Rockshelter lies among the hills of the Great Dividing Range that separates the Brisbane and Burnett River catchments from the rivers of the interior (Morwood 1986). Nearby creeks join the Burnett River. Two charcoal samples from Maidenwell were dated, suggesting that the site was occupied 1210 years BP plus or minus 100 years and 4300 years BP, plus or minus 70

years. Few bones are present at Maidenwell, and they are not in good condition. Macropods, possums, bandicoots and rodents could be identified, but no fish remains are recorded.

The Gatton Rockshelter lies near a small impermanent tributary of Lockyer Creek, too small to have lungfish, that ultimately joins the Brisbane River. This site was extensive, suggesting many years of occupation, and three samples give dates of 1090 plus or minus 70 years BP, 3030 plus or minus 90 years BP and 3820 plus or minus 120 BP. Faunal material from the site is well preserved and plentiful. Remains recorded from the Gatton Rockshelter include the bones of dingoes, large and small macropods, koalas, bandicoots, possums, rodents, snakes, lizards and birds, as well as traces of perch and freshwater mussels. This represents most of the animals that would have been present around the Rockshelter in summer. Lilley (1984:27) suggests that groups of Aborigines in areas like this would have gathered near major rivers during winter, possibly at the roughly contemporaneous Platypus Rockshelter, and travelled to smaller tributaries in summer.

The possibility that the lungfish fragments in the Platypus Rockshelter came from fish caught in the Mary or the Burnett River is unlikely because of the separation of the river systems by mountain ranges and the considerable distances involved (Fig. 1). Nor is it likely that the bones came from a raptor or other predator fishing in the Mary or Burnett River. Quite apart from the distances involved, lungfish are benthic and hide under submerged water plants (Kemp 1987) and therefore are unlikely to be caught easily by a dingo or a bird of prey. Adult lungfish are also large and heavy, and carrying a big fish for 90 or 150 kilometres is improbable. The most parsimonious explanation for the presence of lungfish bone fragments among the oldest levels of a cave deposit occupied intermittently by humans is that the lungfish formed at least a small part of the diet of the people who lived in the cave.

Previous researchers have suggested that lungfish are at risk because the genetic diversity of the species is low (Frentiu *et al.* 2001). This is not the only threat to the survival of the Australian lungfish, as was recognised nearly a hundred years ago (Bancroft 1912, 1928). Without suitable refuges in their natural habitats, few small lungfish survive to be recruited to the adult population. Since all of the rivers that have a population of lungfish have now mostly been converted into

reservoirs with water levels that fluctuate and prevent the establishment of macrophytes that provide refuges for young lungfish (Kemp 2011), the situation has deteriorated. Although lungfish have continued to spawn in the reservoirs, the adults have not been able to feed on the small molluscs that provide essential nutrients such as volatile fatty acids, because these animals live close to the shore among macrophytes, and the eggs produced as a result fail to develop normally. Every juvenile raised from eggs collected in Lake Wivenhoe on the Brisbane River and in Lake Samsonvale on the Pine River has died in the laboratory, and late stage embryos and hatchlings found in these two reservoirs were equally abnormal (Kemp 2011, 2013b).

The original translocations of *N. forsteri* from the Mary River to other potential habitats was funded by the Royal Society of Queensland, and carried out by O'Connor (1897, 1902). Before the translocations were performed, it was not certain if the new habitats actually contained lungfish already. More to the point, nearly half of the lungfish caught by O'Connor for translocations died as a result of injuries sustained during capture, while they were being kept before moving to new habitats, or during transportation (O'Connor 1897, 1902). Several of the prospective habitats received only a few lungfish, perhaps not enough to establish a new population (Kemp 1987). In the case of the Brisbane River, lungfish were actually placed in a farm dam that only communicates with the river in times of flood. The five fish that survived the journey were not put in the Brisbane River (O'Connor 1902), and may never have escaped from the dam.

There is no reliable written evidence for the existence of *N. forsteri* in the Brisbane River system prior to the activities of O'Connor, despite a short description of a fossil tooth plate found, along with the jaw of a fossil lizard, in a well on a property at Eight Mile Plains, now part of the city of Brisbane (de Vis 1894). The specimen was misidentified as "*Ceratodus forsteri*", but actually belongs to a related fossil species, *Metaceratodus palmeri* (Kemp 1997b), known from Pleistocene and Pliocene deposits at Chinchilla and King Creek on the western side of the Great Dividing Range. The specimen may have been reworked, found elsewhere and deposited in the well at some later date.

The extraction and amplification methods used in this work have been used routinely on a number of ancient bones in our laboratory and have been very useful for the amplification of aDNA from poorly preserved bone samples (Huynen *et al.* 2003). The inability to amplify any DNA from the ancient lungfish samples provided suggests that there is very little aDNA present in the sample, and if present is likely to be there as very small fragments, possibly less than 50 bp in length, the target limit at which our primers would have successfully amplified. As aDNA survives best in thick bones from cool, dry, dark environments (Lindahl 1993; Kumar *et al.* 2000; Marota *et al.* 2002; Smith *et al.* 2003; Zinc and Nerlich 2003; Leney 2006; Allentoft *et al.* 2012), it is perhaps not surprising that we were unable to recover aDNA from the relatively small, thin lungfish bones from subtropical Queensland. The age of the fragments, and the conditions under which they were preserved may also have affected the chances for DNA to be preserved. The Platypus Rockshelter consists of two shallow caves halfway up a rocky outcrop on the banks of the Brisbane River near Northbrook in southeast Queensland. The environment of the caves was affected by seepage, and would have been quite damp at times (Hall *et al.* 1988). It was occupied intermittently from “over 5000 years ago to the recent past” (Hall *et al.* 1988). Although there is some disturbance of the deposits, it appears that materials were deposited sequentially on the floor of the cave, and the possible lungfish bones are in two of the oldest layers, stratigraphic units 6 and 7a (Hall *et al.* 1988), so have been exposed to the vicissitudes of the occupied cave environment for a long time. It is therefore not surprising that DNA was apparently absent from the bones.

Previous aDNA extraction and amplification of two catfish vertebrae and a “fish plate” specimen from Platypus Cave however, returned a 50% success rate (Hlinka *et al.* 2002). Lungfish bone differs from the material successfully amplified by Hlinka *et al.* (2002) in fine structure, but how this affects DNA preservation is unknown. Furthermore, the nested amplifications carried out by Hlinka *et al.* (2002) are known to be very sensitive, and as a result can be prone to spurious amplifications. The modified extraction and amplification methods used in this work have been optimised for the detection of target DNAs present at less than 10 copies (Huynen *et al.* 2003). Moreover, as we targeted a fragment of only ~50 bp in length, the approximate lower limit of PCR

amplification, the results suggest that very little if any lungfish DNA has remained in these samples. Ancient DNA analyses on samples from relatively warm climates such as those in Australia continues to present challenges, and may require the discovery of samples trapped in Australian micro-environments that are more amenable to the long term survival of DNA. Unfortunately, the Platypus Rockshelter is the only ancient site in southeast Queensland that includes possible lungfish bone.

Although the attempts to amplify DNA from the bone fragments were not successful, morphological analysis of the skeletal fragments suggest that lungfish were present in the Brisbane River system 3870 years before the present time. An independent study analysing Random Amplified Fragments from Brisbane River fish compared to Burnett and Mary River fish (Lissone 2001; Lissone *et al.* 2003) confirms that the Brisbane River has its own population of lungfish.

The Australian lungfish is poorly adapted to survive environmental changes (Kemp 2011, 2013b), and, although not endangered when the Rockshelter was occupied, is now under threat of extinction. Further complications for the Australian lungfish arise because the few remaining habitats, natural or translocated, are now seriously degraded by human activities, such as the proliferation of towns, farms and factories on the banks of the rivers, and the building of huge water impoundments across rivers, separating populations of lungfish permanently and reducing the quality of the habitat as well. Since lungfish of the Brisbane River are not a translocated population, but belong in this river, the Brisbane River should be included with the Mary and Burnett Rivers as one of the ancestral homes of this iconic, and now endangered, species, and given effective government protection as a result.

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from the living lungfish. Material was collected with the permission of the Queensland Department of Primary Industries and Fisheries, permit #92060. Assistance from all sources is acknowledged with many thanks.

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Figure legends

Figure 1. Map showing the courses of the Brisbane River, with the Pine River and the nearest tributaries of the Mary and Burnett Rivers included, before the building of large water impoundments across the rivers. Broken lines indicate the mountain ranges that separate the catchments of the different river systems.

Br – Brisbane River, Bu – Burnett River, M – Mary River, N – site of the Platypus Rockshelter at Northbrook, P – Pine River. Scale bar = 30 kilometres.

Figure 2. Bones recovered from the Platypus Rock Shelter site and alignment with actual lungfish bones. A. S465-1, a possible parasphenoid fragment aligned with an intact parasphenoid. B. S465-2, a possible lower jaw fragment aligned with a lower bone. C. S465-3, a possible upper jaw fragment aligned with an upper bone. Modern lungfish bones from QM I 26018, from the Brisbane River, southeast Queensland. a anterior process of parasphenoid, as anterior symphysis of lower jaw, d dentition, m medial process of parasphenoid, p posterior process of parasphenoid, pa

posterior articular supports of jaws, pp pterygopalatine process, s smooth surface of parasphenoid, sq support for quadrate on parasphenoid bone, su sulcus on underside of lower jaw, tr trabeculae. Scale bars 1cm and 2 mm.