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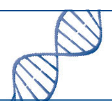

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An integrative approach to species delineation incorporating different species concepts: a case study of *Limnadopsis* (Branchiopoda: Spinicaudata)

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The unambiguous delineation and identification of species remain central problems in systematic and taxonomic studies. Species delineation depends on the data utilized and the species concept applied. In recent years, morphology-based species delineation has been complemented by DNA sequence data, leading to an integrative taxonomy. Such integrative approaches, however, are hampered by the partial incongruence of the various data types with certain species concepts. In this study, we delineated Australian *Limnadopsis* species employing one mitochondrial (cytochrome *c* oxidase subunit I, COI) and one nuclear (elongation factor 1α, EF1α) marker and a morphological character apparently part of the specific mate recognition complex, and therefore potentially indicative of reproductive isolation. By integrating the data over various species concepts (e.g. the 'biological', 'Hennigian', 'recognition', 'phylogenetic' and 'evolutionary' species concepts), the delineation of most species becomes straightforward and unambiguous. Conflicts are particularly interesting as they reveal different aspects of speciation considering the various species concepts. Our study emphasizes the benefits of a truly integrative approach to taxonomy. By combining molecular data with morphological characters indicative of reproductive isolation, it is possible to delineate species integrating not only different data types, but also different underlying species concepts. Overall, 11 *Limnadopsis* species could be delineated, including all eight currently recognized species, and three so far undescribed species. Most species were congruently delineated under all species concepts. A strict application of the evolutionary species concept, however, would have further split *L. parvispinus* into two species on the basis of the COI data. In addition, *Limnadopsis tatei* is consistently split into two sympatrically occurring species under all applied species concepts. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **104**, 575–599.

ADDITIONAL KEYWORDS: Australia – clasper – DNA barcoding – integrative taxonomy – specific mate recognition system.

INTRODUCTION

Traditionally, the delineation of species, as well as their taxonomy, has been based predominantly on morphological characters. The strength of this approach is evident, considering the vast number of over 1.7 million described species to date (The World Conservation Union, 2010). However, it has become obvious that morphological characters alone often fail to delineate all species actually present within taxonomic groups. In particular, closely related species are often morphologically too alike to be unambiguously delineated. Species delineation is further impaired if the morphological characters studied are variable within the species, and character combinations overlap between species. In these cases, a clear distinction between intra- and interspecific variation of characters is hardly possible. Therefore, the advent of molecular data seemingly promised a revolution in taxonomy: species identification and delineation based purely on DNA sequence data without the need to refer to any morphological character, an approach that has been named 'DNA taxonomy' (Tautz *et al.*,

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2002, 2003). The so-called 'DNA barcoding' introduced by Hebert *et al.* (2003a) differs from the previous approach in so far as it focuses on the assignment of unidentified individuals to known species, and proposes a threshold value of 3% genetic distance [for the most commonly used barcode gene: cytochrome *c* oxidase subunit I (COI)] to delineate species (Hebert *et al.*, 2003a). This threshold facilitates the detection of unknown or cryptic species (Hebert *et al.*, 2003a, 2004) based on the assumption that a 'barcoding gap' separates lower intraspecific from higher interspecific genetic distances, but this general use of a universal fixed threshold value to separate species has been questioned (Meyer & Paulay, 2005; Wiemers & Fiedler, 2007). A fixed threshold value does not accommodate high intraspecific genetic distances for old species or low interspecific genetic distances for recent speciation events (Meier *et al.*, 2006). In addition, Meier *et al.* (2006) showed that, in a set of three sequences, two pairwise distances can be below the 3% threshold, whereas the third pairwise distance can be above the threshold, rendering species delineation based on a fixed threshold ambiguous.

To overcome these problems, several authors have argued for an 'integrative taxonomy', combining molecular, morphological and any other available data to delineate and identify species (Dayrat, 2005; Will, Mishler & Wheeler, 2005; Padial & de la Riva, 2010). Several studies have already applied such integrative approaches (Ballard, Chernoff & James, 2002; Wiens & Penkrot, 2002; Laamanen, Petersen & Meier, 2003; Page, Choy & Hughes, 2005; Sanders, Malhotra & Thrope, 2006; Roe & Sperling, 2007; Alström *et al.*, 2008; Tan *et al.*, 2010), but the integration of the various data is not straightforward. The different types of data may lead to conflicting conclusions regarding the delineation of species.

An integrative approach relies on the consistent application of species concepts to all the available data (Agapow *et al.*, 2004; Tan *et al.*, 2008). The classification of a group of individuals or populations to one or several species requires the explicit use of a species concept. As all data types used in integrative approaches differ in their relevance for the various species concepts, species boundaries may differ for each data type (Padial & de la Riva, 2010) and species concept (Laamanen *et al.*, 2003; Tan *et al.*, 2008). Such differences do not impede species delineation; instead, they enable biologists to take different lines of argumentation into account, leading to well-founded conclusions regarding the delineation of the studied species.

In our study, we apply six different species concepts to delineate species: the biological species concept (BSC) (Mayr, 1942, 2000), the Hennigian species concept (HSC) (Meier & Willmann, 2000), the recognition species concept (RSC) (Paterson, 1993b), the evolutionary species concept (ESC) (Wiley & Mayden, 2000), the phylogenetic species concept (PSC) *sensu* Mishler & Theriot (2000) and the PSC *sensu* Wheeler & Platnick (2000).

The most popular species concept among biologists is still the BSC, emphasizing reproductive isolation as the defining element. The BSC requires that the species occur in sympatry or at least parapatry to test the presence of reproductive isolation mechanisms in nature, which is not the case in allopatric populations (or only artificially in captivity). Similarly, the HSC is based on reproductive isolation between contemporary populations, but adds the historical aspect to the BSC, demanding the extinction of the stem species during each speciation event (Meier & Willmann, 2000). The RSC also belongs to this group of species concepts, because it requires a 'common fertilization system' for each species, which enables the recognition of mates of the same species (Paterson, 1993b).

Probably, the most commonly (and most intuitively) applied species concept in molecular systematics and phylogeography is the ESC, originally introduced by Wiley (1981). Wiley & Mayden (2000) define species as 'an entity composed of organisms which maintains its identity from other such entities through time and over space, and which has its own independent evolutionary fate and historical tendencies'. Although the definition appears vague, distinct genetic lineages deduced from phylogenetic analyses may be assumed to have an 'independent evolutionary fate' and their own 'historical tendencies'. A certain genetic distance from other lineages might implicitly be the crucial point when such lineages are considered as species.

This comes very close to the PSC *sensu* Mishler & Theriot (2000), who defined species as 'the smallest monophyletic groups worthy of formal recognition . . .'. In both cases, species are recognized on the basis of the results of phylogenetic analyses (only monophyletic lineages can be considered as having an independent evolutionary fate) and some kind of genetic (or morphological) distance to other lineages. The exact extent of the latter ('worthy of formal recognition'), however, remains uncertain.

The PSC *sensu* Wheeler & Platnick (2000) differs remarkably from the other species concepts, because it does not require apomorphies or a phylogenetic analysis to characterize species. It defines species 'as the smallest aggregation of (sexual) populations or (asexual) lineages diagnosable by a unique combination of character states'. For molecular markers, a 'unique combination of character states' can already be assumed based on the alignment of the sequences before any phylogenetic analyses, and might be given by a certain genetic distance between sequences.

Attempts have been made to define a species concept which encompasses the differing aspects of

the various species concepts, e.g. Mayden's (1999) hierarchy of species concepts and the 'unified concept of species' introduced by de Queiroz (2005), which both accept the ESC as the primary concept. The defining elements of the other concepts (reproductive isolation, monophyly, diagnosability) are seen as properties allowing the delineation of lineages, which are then regarded as species (Naomi, 2011). Under these concepts, lineages could be considered to be separate species even if they fuse again into a single species after secondary contact (de Queiroz, 2005). In our view, this approach neglects important ontological differences which are integral parts of the particular species concepts, e.g. whether or not complete reproductive isolation is the defining element of species. However, even if such a unifying species concept is applied, the different lines of evidence still need to be weighed against each other to decide whether certain lineages should be considered or not as separate species. Ultimately, the species delineation under such a unifying species concept would depend on the preferred criteria, and would thus be identical to the delineation based on the individual species concepts.

In this study, we apply an integrative approach for species delineation on the Australian endemic clam shrimp taxon *Limnadopsis* (Spinicaudata: Limnadiidae). Currently, eight *Limnadopsis* species are recognized (Richter & Timms, 2005, Timms, 2009), which all inhabit temporary water bodies in Australia. Recent molecular studies have confirmed the monophyly of *Limnadopsis* (Schwentner *et al*., 2009; Weeks *et al*., 2009) and have indicated the presence of further species (Weeks *et al*., 2009). In Spinicaudata, morphology-based taxonomy has been hampered by the extensive intraspecific plasticity of most morphological characters (e.g. Marinček & Petrov, 1998). Therefore, it is important to focus on morphological characters which are likely to exhibit distinct differences among different species. Such species-specific differences are generally expected for male genitalia and other male structures which take active parts in copulation (Eberhard, 1985). The male genitalia of spinicaudatans are not very differentiated; they are mere openings which are pressed against the female genital openings during insemination (Dumont & Negrea, 2002), but male spinicaudatans have an important secondary sexual character: the first two pairs of thoracopods are modified into claspers. The male uses these claspers to hold on to the female's carapace during mating. In several other taxa, similar secondary sexual characters are supposed to be important elements of the specific mate recognition systems (SMRS; Paterson, 1993a). A well-known example from another branchiopod taxon is the second antenna of the males of anostracan species. The complex ornamentation of these antennae fits to the amplexial groove of the females in a type of lock-and-key fashion (Rogers, 2002). Furthermore, males in Ostracoda also feature clasping structures which are assumed to facilitate mate recognition, but here the mechanisms are not yet well understood (Martens, 2000). Although the clasper–carapace interaction in spinicaudatans has not been studied in detail, there are some indications that mate recognition is at least partially dependent on this interaction. Males of *Eulimnadia texana* seem unable to discriminate between males and receptive and unreceptive hermaphrodites (females are lacking in this species) prior to physical contact (Knoll, 1995; Medland, Zucker & Weeks, 2000). However, once males clasp a carapace, they are able to make this differentiation and hold on to receptive hermaphrodites much longer than to others (Weeks & Benvenuto, 2008). During this process, males of *E. texana* (Weeks & Benvenuto, 2008) and *Limnadopsis parvispinus* (M. Schwentner, per. observ.) move their claspers along the female's (or hermaphrodite's) carapace, clasping to various parts of the carapace. The tip of the movable finger is always in direct contact with the inside of the female's carapace, and is thus the ideal position for any species-specific structure. Here, all *Limnadopsis* species exhibit 'scales' (probably derived setae; Timms, 2009), whereas all other Limnadiidae have a sucker-like projection at the tip of the movable finger (e.g. Olesen, Martin & Roessler, 1996). The shape of these scales at the tip of the movable finger serves as the morphological character in this study. As we assume a role in the mate recognition process, individuals with distinct differences in this character can be directly interpreted as belonging to different species on the basis of the RSC (which is defined by the recognition process), as well as the BSC and the HSC (if individuals cannot recognize each other as mates, reproductive isolation is established). How the mating partners recognize each other is speculative. It may be merely by mechanical stimuli caused by the physical contact. Such stimuli have been reported from other arthropods (e.g. sepsid flies; Eberhard, 2001) and the respective structures need to differ in small details only (Eberhard, 1993).

In the following, we test the delineation of *Limnadopsis* species following an integrative taxonomy approach that integrates different types of data (one mitochondrial and one nuclear genetic marker and a morphological character) as well as various species concepts.

MATERIAL AND METHODS

Collection and identification of specimens

Most of the specimens were collected during several field trips between 1998 and 2011 by the authors (see

Table 1 for collection details). Adult specimens were collected with hand nets and fixed directly in 100% ethanol or in RNAlater (Qiagen). Some individuals were fixed in formalin and stored in 70% ethanol; these specimens were not available for DNA sequencing. In addition, sediment was collected from the surface of a few dry pools to later hatch and rear specimens in the laboratory. About 100 g of sediment were incubated in the laboratory with distilled water in a 2-L glass aquarium with constant aeration, 27 °C and a 16-h : 8-h light : dark cycle. Juveniles were fed with an algae suspension (Hobby-Liquizell®) and fixed as young adults in 100% ethanol. If hatching failed, resting eggs were collected from the sediment using a stereomicroscope (Olympus SZ51) and identified on the basis of Pabst & Richter (2004) and Timms (2009). Adult specimens were identified on the basis of the descriptions and key included in Timms (2009).

DNA extraction, amplification and sequencing

It was attempted to sequence all studied specimens for the barcoding region (Hebert *et al*., 2003a) of the COI gene. Based on the genetic lineages revealed by the resulting dataset, a few specimens of each lineage were chosen to further sequence a partial sequence of elongation factor 1α (EF1α). Tissue samples were taken from the muscle connecting the carapace halves. Genomic DNA was extracted with either the DNeasy Blood and Tissue Kit (Qiagen), following the directions of the manufacturer, or a modified HotSHOT protocol (Montero-Pau, Gómez & Muñoz, 2008), with a final volume of 60 μL. The resting eggs were crushed directly in HotSHOT lysis buffer and the final volume was reduced to 40 μL to increase the DNA concentration.

The COI and EF1α double-stranded sequence fragments were polymerase chain reaction (PCR) amplified with a TGradient Thermocycler (Biometra). The PCR comprised 15 μL Taq PCR Master Mix [Qiagen; contains Taq polymerase, 1.5 mM $MgCl_2$ and 200 μM of each deoxynucleoside triphosphate (dNTP)], 3 μL of each primer (10 μM) and 3–4.5 μL template DNA, and was topped up to a total volume of 30 μL with purified water. All primers are listed in Table 2. To successfully amplify the barcoding region of the COI gene, several primer combinations were required, always combining one LCO and one HCO primer. The primer combinations LCO1490/HCO709 and LCO2/outout were the most successful. The PCR programme for the COI fragment consisted of an initial denaturation step of 1 min at 94 °C, followed by 38 amplification cycles (94 °C for 1 min, 46 °C for 30 s, 70 °C for 1 min) and a final elongation step of 5 min at 70 °C. The 38 amplification cycles for the EF1α fragment consisted of 94 °C for 1 min, 51 °C for 30 s and 72 °C for 1 min. Amplification success was determined using electrophoresis on 1.5% agarose/TAE gel containing 0.01% ethidium bromide. PCR product purification was performed either by using magnetic beads (Agencourt AMPure, Beckman Coulter) or by cutting out visible bands following the directions of the QIAquick Gel Extraction Kit (Qiagen). Final elution was carried out in 30 μL or 25 μL, respectively.

The purified PCR products were sequenced with the same primers as used in the amplification. The COI fragments were sequenced unidirectionally with the respective LCO primer (the sequencing reaction was repeated with the respective HCO primer if the resulting sequence contained ambiguous bases), whereas the EF1α fragments were sequenced bidirectionally. Most sequencing was performed with the DCTS Quick Start Kit (Beckman Coulter) on an automated sequencer (CEQTM 800 from Beckman Coulter) following the manufacturer's instructions. Some samples were sequenced by the Qiagen Sequencing Service (Qiagen, Germany). Sequencing errors were eliminated using the program Sequencher 4.1.4 (Gene Codes Corporation). The final sequences were submitted to GenBank (accession numbers HQ717722–HQ717795 and JF966697–JF966728; Table 1; Benson *et al*., 2008).

Alignment, genetic distances and phylogenetic analyses

The alignments of all corrected sequences were performed for each gene fragment separately using ClustalW (Thompson, Higgins & Gibson, 1994), as implemented in the program BioEdit 7.0.9.0 (Hall, 1999). The alignments were checked for pseudogenes or numts (nuclear mitochondrial pseudogenes) following the directions of Song *et al*. (2008). As pseudogenes are not functional, they are expected to accumulate indels in the nucleic acid sequence and stop codons in the amino acid sequence over time, which implies that recent pseudogenes may not be detected with this procedure. The sequences of COI and EF1α were transcribed into the corresponding amino acid sequences with the program MEGA4 (Tamura *et al*., 2007), using the implemented genetic codes 'Invertebrate Mitochondrial' for COI and 'Standard' for EF1α. The numbers of variable and parsimony informative sites were determined with MEGA4.

Two different approaches for the phylogenetic analysis were used: Maximum Parsimony analysis and Bayesian analysis. As a result of the large number of sequences, identical COI sequences were excluded from the analyses to minimize the calculation times. Specimens with sequences identical to those included in the analyses are shown in Figure 1. *Limnadia* sp. A (P.84142) was chosen as outgroup.

Table 1. List of all specimens used in this study with their respective GenBank accession numbers and detailed collection locations

Species	Specimen number	Clasper	COI	EF1α	Location
Limnadopsis birchii (Baird, 1860)	P.84143	X			Australia, QLD, Pan near Cooburra Waterhole, Currawinya Nat. Park, .ii.1998
Limnadopsis birchii (Baird, 1860)	P.84144	X			Australia, QLD, Pan near Cooburra Waterhole, Currawinya Nat. Park, .ii.1998
Limnadopsis birchii (Baird, 1860)	P.84145	X			Australia, QLD, Pan near Cooburra Waterhole, Currawinya Nat. Park, .ii.1998
Limnadopsis birchii (Baird, 1860)	P.84146	X			Australia, QLD, Pan near Cooburra Waterhole, Currawinya Nat. Park, .ii.1998
Limnadopsis birchii (Baird, 1860)	P.84147		HQ717755		Australia, NSW, Muella Vegetated Pool 4, MS, 29°30′00.7″S, 144°54′59.6″E, 31.iii.2009
Limnadopsis birchii (Baird, 1860)	P.84148		HQ717756	HQ717725	Australia, QLD, poplar box pool on western boundary fence, RS, 28°56′S, 144°55.7′E, 10.vi.2007
Limnadopsis birchii (Baird, 1860)	P.84149		HQ717757		Australia, QLD, poplar box pool on western boundary fence, RS, 28°56′S, 144°55.7′E, 10.vi.2007
Limnadopsis birchii (Baird, 1860)	P.84150		HQ717758		Australia, QLD, poplar box pool on western boundary fence, RS, 28°56′S, 144°55.7′E, 10.vi.2007
Limnadopsis birchii (Baird, 1860)	P.84151		FJ830343	HQ717726	Australia, QLD, Mid Blue Lake, RS, 28°53′S, 144°57′E, 09.vi.2007
Limnadopsis birchii (Baird, 1860)	P.84152	X	HQ717759	HQ717727	Australia, QLD, vegetated swamp, former beach of Lake Buchanan, 21°32′11.88″S, 145°47′49.08″E, 26.ii.2008
Limnadopsis birchii (Baird, 1860)	ST5	X			Australia, QLD, Currawinya Nat. Park, pan near Coomburra waterhole, .ii.1998, private coll. SR
Limnadopsis minuta Timms, 2009	P.84153	X			Australia, NT, Keep River Nat. Park, 15°57′S, 129°03′E, 08.ii.1986
Limnadopsis multilineata Timms, 2009	P.84154	X			Australia, WA, pool near Kalumbaru, 22.ii.1995
Limnadopsis occidentalis Timms, 2009	P.84155	X			Australia, WA, CB56 Carnarvon, 27°31′29″S, 115°05′14″E, 15.iii.1995
Limnadopsis occidentalis Timms, 2009	P.84156	X			Australia, WA, Tardun CBC Swamp, 28°43′S, 115°49′E, 26.vii.1999
Limnadopsis occidentalis Timms, 2009	P.84157	X			Australia, WA, Tardun CBC Swamp, 28°43′S, 115°49′E, 26.vii.1999
Limnadopsis paradoxa Timms, 2009	P.84158	X	JF966701		Australia, WA, East Lake Bryde, 33°22′S, 118°54′E, 21.iii.2006

Table 1. *Continued*

Species	Specimen number	Clasper	COI	EF1α	Location
Limnadopsis paradoxa Timms, 2009	P.84159		JF966702		Australia, WA, Pool 4 on Sieda Farm, 33°13′39″S, 121°47′32″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84160		HQ717747	HQ717728	Australia, WA, Pool 3 on Sieda Farm, 33°13′21″S, 121°47′20″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84161		JF966703		Australia, WA, Pool 3 on Sieda Farm, 33°13′21″S, 121°47′20″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84162		JF966704		Australia, WA, Pool 3 on Sieda Farm, 33°13′21″S, 121°47′20″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84163		FJ830346	HQ717729	Australia, WA, Pool 3 on Sieda Farm, 33°13′21″S, 121°47′20″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84164	X	JF966705		Australia, WA, Pool 3 on Sieda Farm, 33°13′21″S, 121°47′20″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84165		JF966706		Australia, WA, Pool 4 on Sieda Farm, 33°13′39″S, 121°47′32″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84166		JF966707		Australia, WA, Pool 4 on Sieda Farm, 33°13′39″S, 121°47′32″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84167	X	HQ717748		Australia, WA, Pool 4 on Sieda Farm, 33°13′39″S, 121°47′32″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84168		JF966708		Australia, WA, Pool 4 on Sieda Farm, 33°13′39″S, 121°47′32″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84169	X	HQ717760		Australia, WA, pool on Plunketts Farm, 33°04′23″S, 121°52′53″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84170	X	HQ717750		Australia, WA, pool on Plunketts Farm, 33°04′23″S, 121°52′53″E, 12.03.2007
Limnadopsis paradoxa Timms, 2009	P.84171		JF966709		Australia, WA, pool on Plunketts Farm, 33°04′23″S, 121°52′53″E, 12.iii.2007

Limnadopsis paradoxa Timms, 2009	P.84172		JF966710		Australia, WA, pool on Plunketts Farm, 33°04′23″S, 121°52′53″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84173		HQ717761		Australia, WA, pool near Truslove Salt Lake, 33°20′50″S, 121°46′5″E, 16.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84174		JF966711		Australia, WA, pool near Truslove Salt Lake, 33°20′50″S, 121°46′5″E, 16.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84175		HQ717749		Australia, WA, pool near Truslove Salt Lake, 33°20′50″S, 121°46′5″E, 16.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84176		JF966712		Australia, WA, Pool on Nolan's Farm, 33°06′23″S, 121°45′50″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84177		HQ717762		Australia, WA, Pool on Nolan's Farm, 33°06′23″S, 121°45′50″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84178		JF966713		Australia, WA, Pool on Nolan's Farm, 33°06′23″S, 121°45′50″E, 12.iii.2007
Limnadopsis paradoxa Timms, 2009	P.84179		JF966714		Australia, WA, Pool on Nolan's Farm, 33°06′23″S, 121°45′50″E, 12.iii.2007
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	Egg10		HQ717743		Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″E, 1998
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.84180		FJ830344	HQ717730	Australia, NSW, Titanic Blackbox Swamp, BS, 29°26′S, 144°47′E, 07.vi.2007
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.84181	X			Australia, NSW, Lower Crescent Pool, BS, 29°32′40″S, 144°51′30″E, 1998
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.84182	X	HQ717763		Australia, NSW, Lower Crescent Pool, BS, 29°32′40″S, 144°51′30″E, 1998
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.80896	X	HQ717751		Australia, NSW, Muella Vegetated Pool 3, MS, 29°30′12.0″S, 144°55′37.4″E, 31.iii.2009

Table 1. *Continued*

Species	Specimen number	Clasper	COI	EF1α	Location
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.80897	X	HQ717752		Australia, NSW, Muella Vegetated Pool 3, MS, 29°30′12.0″S, 144°55′37.4″E, 31.iii.2009
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.80898	X	HQ717753		Australia, NSW, Muella Vegetated Pool 3, MS, 29°30′12.0″S, 144°55′37.4″E, 31.iii.2009
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.84183		HQ717764	HQ717731	Australia, QLD, Number 33 Blackbox Swamp, RS, 28°54′S, 144°58′E, 09.vi.2007
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.84184		HQ717765		Australia, QLD, poplar box pool on western boundary fence, RS, 28°56′S, 144°55.7′E, 10.vi.2007
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	Egg44		HQ717744		Australia, NSW, first pool east of Mossgiel, 33°17′43.2″S, 144°43′08.8″E, 23.i.10
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	Egg46		HQ717745		Australia, NSW, first pool east of Mossgiel, 33°17′43.2″S, 144°43′08.8″E, 23.i.10
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.85022	X	JF966716		Australia, NSW, pool on granite, 11 km north of Berridale, 36°16′23.7″S, 148°48′13.6″E, 14.iii.10
Limnadopsis cf. *parvispinus* Henry, 1924 'Paroo'	P.85023		JF966717		Australia, NSW, pool on granite, 11 km north of Berridale, 36°16′23.7″S, 148°48′13.6″E, 14.iii.10
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84186	X	HQ717767		Australia, QLD, vegetated swamp (Y17), former beach of Lake Buchanan, 21°32′06.6″S, 145°47′50.7″E, 26.ii.2008

Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84187		HQ717768	HQ717733	Australia, QLD, vegetated swamp (Y17), former beach of Lake Buchanan, 21°32′06.6″S, 145°47′50.7″E, 26.ii.2008
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84188	X	HQ717769		Australia, QLD, vegetated swamp (Y17), former beach of Lake Buchanan, 21°32′06.6″S, 145°47′50.7″E, 26.ii.2008
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84189		HQ717770		Australia, QLD, vegetated swamp (Y17), former beach of Lake Buchanan, 21°32′06.6″S, 145°47′50.7″E, 04.iv.2009, reared from sediment
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84190		HQ717771		Australia, QLD, vegetated swamp (Y17), former beach of Lake Buchanan, 21°32′06.6″S, 145°47′50.7″E, 04.iv.2009, reared from sediment
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84191		HQ717772		Australia, QLD, vegetated swamp (Y7), former beach of Lake Buchanan, 21°34′34.1″S, 145°48′10.2″E, 24.ii.2008
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.84192		HQ717773		Australia, QLD, Y12, former beach of Lake Buchanan, 21°32′02.8″S, 145°48′15.6″E, 04.iv.2009
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.85014	X	JF966715		Australia, QLD, Y2, former beach of Lake Buchanan, 21°30′55.2″S, 145°48′20.8″E, 04.iv.2009, reared from sediment
Limnadopsis cf. *parvispinus* Henry, 1924 'Buchanan'	P.85029		JF966718		Australia, QLD, Y31, former beach of Lake Buchanan, 21°30′41.7″S, 45°48′09.5″E, 05.iv.2009, reared from sediment
Limnadopsis pilbarensis Timms, 2009	P.84193	X			Australia, WA, Burrup Peninsula, 17.viii.2005
Limnadopsis pilbarensis Timms, 2009	P.84194	X			Australia, WA, Burrup Peninsula, 22.viii.2005
Limnadopsis cf. *tatei* 'Titanic'	P.84195	X	HQ717774		Australia, NSW, Titanic Blackbox Swamp, BS, 29°26′S, 144°47′E, 07.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84196		HQ717775		Australia, NSW, Titanic Blackbox Swamp, BS, 29°26′S, 144°47′E, 07.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84197		HQ717776		Australia, QLD, Number 33 Blackbox Swamp, RS, 28°54′S, 144°58′E, 09.vi.2007

Table 1. *Continued*

Species	Specimen number	Clasper	COI	EF1α	Location
Limnadopsis cf. *tatei* 'Titanic'	P.84198		FJ830345		Australia, QLD, Number 33 Blackbox Swamp, RS, 28°54′S, 144°58′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84199	X	HQ717777	HQ717734	Australia, QLD, small gilgai pool, RS, 28°51′S, 144°57.6′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84200		HQ717778		Australia, QLD, small gilgai pool, RS, 28°51′S, 144°57.6′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84201	X	HQ717779	HQ717735	Australia, QLD, small gilgai pool, RS, 28°51′S, 144°57.6′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84202		HQ717780		Australia, QLD, small gilgai pool, RS, 28°51′S, 144°57.6′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.84203	X	HQ717781	HQ717736	Australia, QLD, small gilgai pool, RS, 28°51′S, 144°57.6′E, 09.vi.2007
Limnadopsis cf. *tatei* 'Titanic'	P.85864		JF966719		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85865		JF966720		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85866		JF966721		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85867	X	JF966722	JF966697	Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85868	X	JF966723		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85869	X	JF966724	JF966698	Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85870	X	JF966725	JF966699	Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85871		JF966726		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85872		JF966727		Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Titanic'	P.85873	X	JF966728	JF966700	Australia, SA, vegetated stony dug out 34 km north of Marla, 27°05′26.8″S, 133°28′16.2″E, 10.iii.2011
Limnadopsis cf. *tatei* 'Carter's'	P.84204		HQ717782		Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 31.iii.2009, reared from sediment
Limnadopsis cf. *tatei* 'Carter's'	P.84205		HQ717783	HQ717737	Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 31.iii.2009, reared from sediment
Limnadopsis cf. *tatei* 'Carter's'	P.84206		HQ717784		Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 19.i.2010

Limnadopsis cf. *tatei* 'Carter's'	P.84207		HQ717785		Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 19.i.2010
Limnadopsis cf. *tatei* 'Carter's'	P.84208	X	HQ717786	HQ717738	Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 19.i.2010
Limnadopsis cf. *tatei* 'Carter's'	P.84209	X	HQ717787	HQ717739	Australia, NSW, Carter's Swamp, MS, 29°26′05″S, 144°58′52″, 19.i.2010
Limnadopsis cf. *tatei* 'Carter's'	P.84210	X	HQ717788	HQ717740	Australia, NSW, Grassy Pool north of Yantabulla, 29°19′04.8″S, 145°00′31.5″E, 20.i.2010
Limnadopsis cf. *tatei* 'Carter's'	P.84211		HQ717789		Australia, NSW, Grassy Pool north of Yantabulla, 29°19′04.8″S, 145°00′31.5″E, 20.i.2010
Limnadopsis sp. 'Lagoon'	P.82579	X		HQ717722	Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Lagoon'	P.82580	X	HQ717754	HQ717723	Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Lagoon'	P.85177		HQ717792	HQ717741	Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Lagoon'	P.85178	X	HQ717793		Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Lagoon'	P.85179		HQ717794	HQ717742	Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Lagoon'	P.85180		HQ717795		Australia, QLD, Eoleptestheria lagoon, 25°12′54.2″S, 149°34′34.5″E, 13.ii.2010
Limnadopsis sp. 'Roskos'	P.85175	X	HQ717790		Australia, NSW, Roskos Paleolake, BS, 29°27′42.9″S, 144°48′12.5″E, 19.ii.2010
Limnadopsis sp. 'Roskos'	P.85176		HQ717791		Australia, NSW, Roskos Paleolake, BS, 29°27′42.9″S, 144°48′12.5″E, 19.ii.2010
Limnadopsis sp. 'Roskos'	Egg51		HQ717746		Australia, NSW, first pool east of Mossgiel, 33°17′40.7″S, 144°42′54.3″E, 23.i.2010
Limnadia sp. A	P.84142		FJ830341	HQ717724	Australia, NSW, Quandong Gilgai Swamp, BS, 29°26′S, 144°51′E, 07.vi.2007

Specimens are marked with an 'X' if their claspers were studied. All individuals whose specimen numbers include 'P.' were deposited at the Australian Museum Sydney.

BS, Bloodwood Station; COI, cytochrome *c* oxidase subunit I; EF1α, elongation factor 1α; MS, Muella Station; NSW, New South Wales; NT, Northern Territory; QLD, Queensland; RS, Rockwell Station; SA, South Australia; WA, Western Australia.

Table 2. List of all primers utilized for polymerase chain reactions and sequencing

Name	Sequence 5′–3′	Reference
COI		
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. (1994)
LCO2	TCNACHAAYCATAAAGAYATTGGAAC	New primer by L. Krebes and R. Bastrop (pers. comm.)
LCO3	TCNACHAAYCATAAAGAYATTGGTAC	Krebes *et al*. (2010)
HCOoutout	GTAAATATATGNTGNGCTC	Folmer *et al*. (1994)
HCO-MZ1-rev	CTTTVATDCCNGTVGGSACWGCRATAATYAT	Krebes *et al*. (2010)
HCO709	AATNAGAATNTANACTTCNGGGTG	Blank *et al*. (2008)
EF1α		
HaF2For1	GGGYAAAGGWTCCTTCAARTATGC	Richter, Olesen & Wheeler (2007) [American Museum of Natural History (AMNH) laboratory]
2R53ST	CAGGAAACAGCTATGACGCGAACTT GCAAGCAATGTGAGC	Richter *et al*. (2007) (AMNH laboratory)

COI, cytochrome *c* oxidase subunit I; EF1α, elongation factor 1α.

The Maximum Parsimony analysis was calculated using Winclada (Nixon, 1999) implementing Nona (Goloboff, 1999). An heuristic search was carried out with 100 replications, 1000 starting trees and a maximum of 1000 trees to keep. The search strategy was 'Multiple TBR + TBR'. Node support was assessed with 1000 bootstrap replications with 10 search replications and 100 starting trees per replication. The Bayesian analysis was carried out with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with four runs, six chains and 6×10^6 generations, every 1200th of which was sampled. The first 10% of sampled trees were discarded as 'burn-in'. Of the remaining 4500 trees, a majority rules consensus tree was calculated. The posterior probability was used as node support. For the separate analyses of COI and EF1α, the evolutionary model GTR + I was implemented as determined by MrModeltest (Posada & Crandall, 2001; Nylander, 2004) using the Akaike information criterion. The concatenated alignment of both gene fragments was partitioned according to the genes, and the evolutionary model GTR + I + G as suggested by Nylander *et al*. (2004) was implemented. All trees were visualized and processed with FigTree v1.2 (Rambaut, 2006).

The genetic distance between COI sequences is a common tool for the delineation of genetically distinct lineages. Commonly, genetic distances are calculated by applying a model of genetic evolution, and reported as the mean genetic distances within and between lineages, instead of giving the full range of distances. This has been shown to artificially increase the barcoding gap (Meier, Zhang & Ali, 2008). We decided to calculate uncorrected *p* distances for COI and EF1α; these are the percentage differences between sequences without the assumption of an additional model. Instead of a mean, we reported the range of intra- and interspecific distances to obtain a more detailed overview of the genetic distance distribution. Genetic distances were calculated with MEGA4 (Tamura *et al*., 2007). Furthermore, we applied the 'Cluster' algorithm implemented in Species Identifier 1.7.8 (Meier *et al*., 2006) to group the sequences into clusters. In these clusters, each sequence is linked to at least one other sequence by a genetic distance below a certain threshold value, eliminating the problem that, of three sequences, two pairwise distances can be below a threshold value whereas the third pairwise distance can be above the threshold. Threshold values in the range 1–10%, with 1% increments, were employed.

Scanning electron microscopy (SEM) imaging

Forty-three adult *Limnadopsis* males (Table 1) were chosen to study the clasper morphology. Both right claspers of these specimens were dissected, leaving the claspers interconnected. For a few specimens, the two left claspers were dissected as well. The claspers were cleaned in an ultrasonic water bath (Elma®) for 5 s. Prior to critical point drying (Emitech, K850), they were transferred to 100% acetone. The critical point-dried claspers were glued onto a fine pin and sputter coated with gold. The pin was then mounted on a specimen holder as described by Pohl (2010). Most SEM images were taken with a DSM 906A (Zeiss) at the Electron Microscopy Centre at the University of Rostock. A few specimens were scanned at the Australian Museum Sydney using an EVO LS15 (Zeiss) with backscatter. The specimens scanned at the Australian Museum were mounted on SEM stubs.

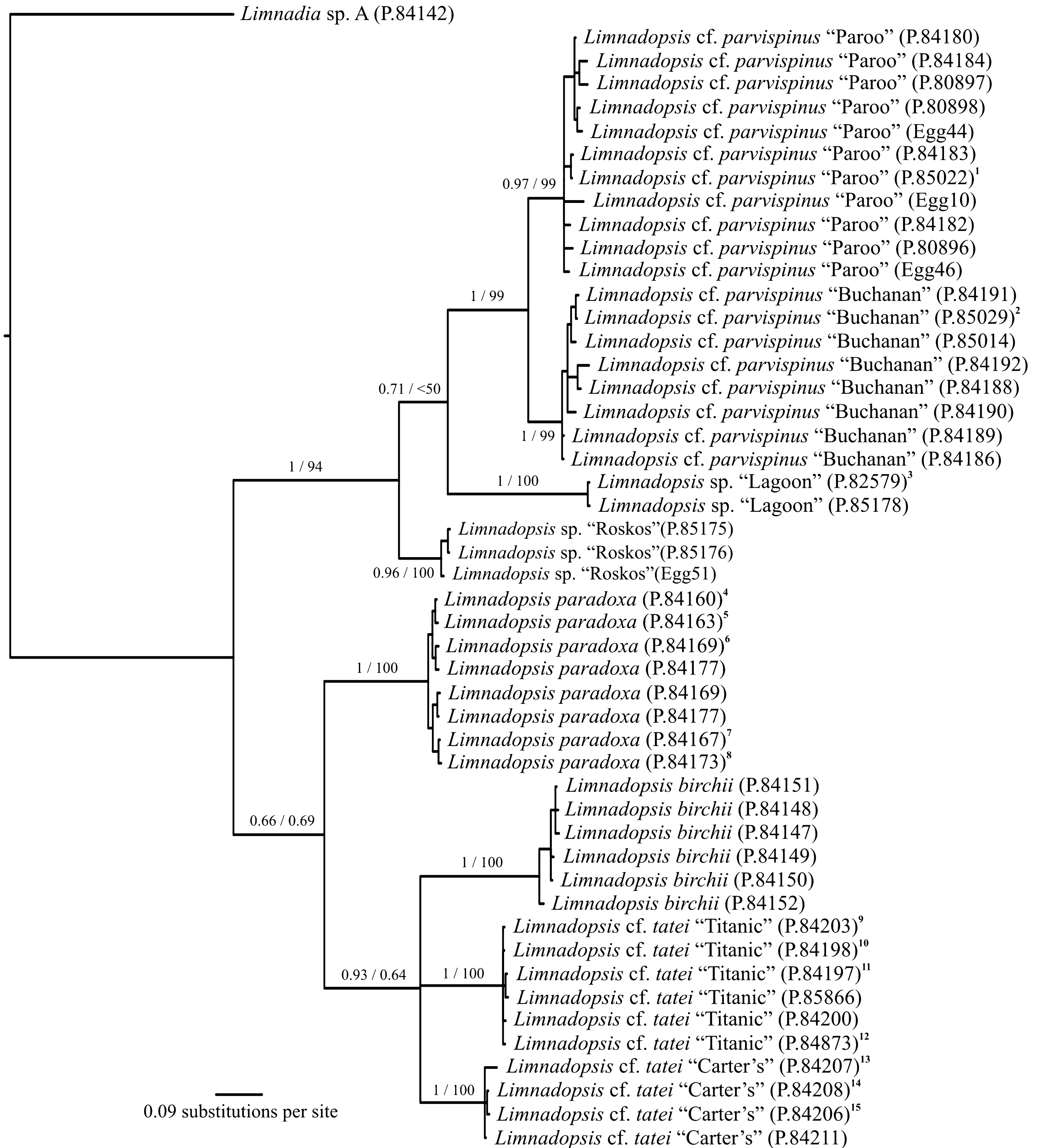

Figure 1. Bayesian inference majority rule tree based on the cytochrome *c* oxidase subunit I (COI) dataset. For each branch, the posterior probabilities of the Bayesian analysis and bootstrap support values of the Maximum Parsimony analysis are shown. Support values within putative species are not given. If several individuals had identical COI sequences, the respective COI sequence was included only once in the analyses. Individuals with identical sequences are: 1, P.85023; 2, P.84187; 3, P.82580, P.85177 and P.85180; 4, P.84158, P.84159, P.84161, P.84164, P.84166, P.84176 and P.84179; 5, P.84162; 6, P.84172; 7, P.84165, P.84168, P.84171 and P.84178; 8, P84174; 9, P.84196, P.84199 and P.84201; 10, P.84195; 11, P. 84202 and P.85864; 12, P.85865, P.85867, P.85868, P.85869, P.85870, P.85871 and P.85872; 13, P.84204 and P.84205; 14, P.84210; 15, P.84209.

RESULTS

Initial identification of species

Based on Timms (2009), most specimens could be identified to belong to one of the eight previously described *Limnadopsis* species. Specimens of two locations could not be assigned to any described species; they were termed *Limnadopsis* sp. 'Lagoon' and *Limnadopsis* sp. 'Roskos' in the following.

Alignments and delineation of monophyletic lineages

The alignment of the partial COI sequence data had a length of 627 bp with an AT content of 61.2%; 213 sites were variable, 192 of which were parsimony informative. The alignment of the partial EF1α sequences had a total length of 795 bp; 59 positions were variable, 41 of which were parsimony informative. The AT content was 43%. Both alignments contained no indels and the transcribed amino acid sequences showed no stop codon or any accumulation of amino acid substitutions in any sequence. Therefore, we found no indication for the presence of pseudogenes or numts in our data.

Both the Maximum Parsimony and Bayesian analysis of the COI dataset recovered eight main distinct monophyletic lineages (Fig. 1), splitting what we have identified as *L. tatei* and *L. parvispinus* into two distinct lineages each. The recovered lineages are (Fig. 1): *L.* cf. *parvispinus* 'Paroo', *L.* cf. *parvispinus* 'Buchanan', *L.* sp. 'Roskos', *L.* sp. 'Lagoon', *L. paradoxa*, *L. birchii*, *L.* cf. *tatei* 'Titanic' and *L.* cf. *tatei* 'Carter's'. All of these lineages are supported by bootstrap support values ≥ 99 and posterior probabilities ≥ 0.96 (Fig. 1). These main monophyletic lineages correspond to the clusters recovered by Species Identifier for a 3% threshold (values ≤ 2% recovered additional clusters and values ≥ 7% collapsed both *L.* cf. *parvispinus* clusters into one). The uncorrected COI *p* distances within each of the eight main lineages are within the range of 0–4.2% (Table 3), whereas the distances among main lineages are in the range 6.8–18.6% (usually > 10%), thus exhibiting a clear gap between the intra- and interlineage genetic distances. Notably, the two highest intralineage COI distances (4.2% and 3.5%) occur within each of the *L.* cf. *parvispinus* lineages, and the lowest interlineage distance (6.8%) occurs between the two *L.* cf. *parvispinus* lineages.

The analyses of EF1α also recovered the lineages of *L. paradoxa*, *L. birchii*, *L.* cf. *tatei* 'Carter's', *L.* cf. *tatei* 'Titanic' and *L.* cf. *parvispinus* as monophyletic, but partially with low support values (Fig. 2). *Limnadopsis* sp. 'Lagoon' was not recovered as monophyletic with respect to *L.* cf. *parvispinus*. The uncorrected

Table 3. Uncorrected *p* distances within (along the diagonal) and between the main genetic lineages of *Limnadopsis*. Cytochrome *c* oxidase subunit I (COI) distances are above the diagonal and elongation factor 1α (EF1α) distances are below the diagonal. All distances are percentages

	L. cf. *parvispinus* 'Buchanan'	*L.* cf. *parvispinus* 'Paroo'	*L. paradoxa*	*L. birchii*	*L.* cf. *tatei* 'Titanic'	*L.* cf. *tatei* 'Carter's'	*L.* sp. 'Lagoon'	*L.* sp. 'Roskos'
L. cf. *parvispinus* 'Buchanan'	0.0–3.5 n.a.	6.8–9.9	15.3–17.7	16.5–17.9	16.3–17.7	16.2–17.3	12.9–14.0	10.4–13.0
L. cf. *parvispinus* 'Paroo'	0.8–1.2	0.0–4.2 0.0	15.5–18.6	16.9–18.6	16.7–18.2	15.3–17.4	12.4–14.2	11.0–13.4
L. paradoxa	2.8–3.0	1.9–2.1	0.0–1.5 0.1	11.9–12.8	12.6–14.3	12.0–13.3	15.4–16.3	14.1–15.1
L. birchii	2.7	1.6–1.7	1.2–1.4	0.2–2.2 0.0	10.4–11.9	11.0–12.4	17.4–18.1	17.1–18.3
L. cf. *tatei* 'Titanic'	2.4–2.7	1.6–2.0	1.1–1.8	0.8–1.2	0.0–0.7 0.0–0.4	9.6–10.6	17.2–17.7	15.1–16.2
L. cf. *tatei* 'Carter's'	3.0–3.1	2.0–2.4	1.4–1.7	0.7–0.9	0.8–1.4	0.0–1.8 0.0–0.1	16.1–17.2	14.4–15.5
L. sp. 'Lagoon'	2.2–2.4	0.9–2.0	3.3–4.5	3.0–3.8	2.8–3.8	3.1–4.3	0.0–0.2 2.0–2.4	12.3–12.8
L. sp. 'Roskos'	n.a.	n.a	n.a	n.a.	n.a.	n.a.	n.a.	0.2–1.2 n.a.

n.a., not calculated as sequences are missing.

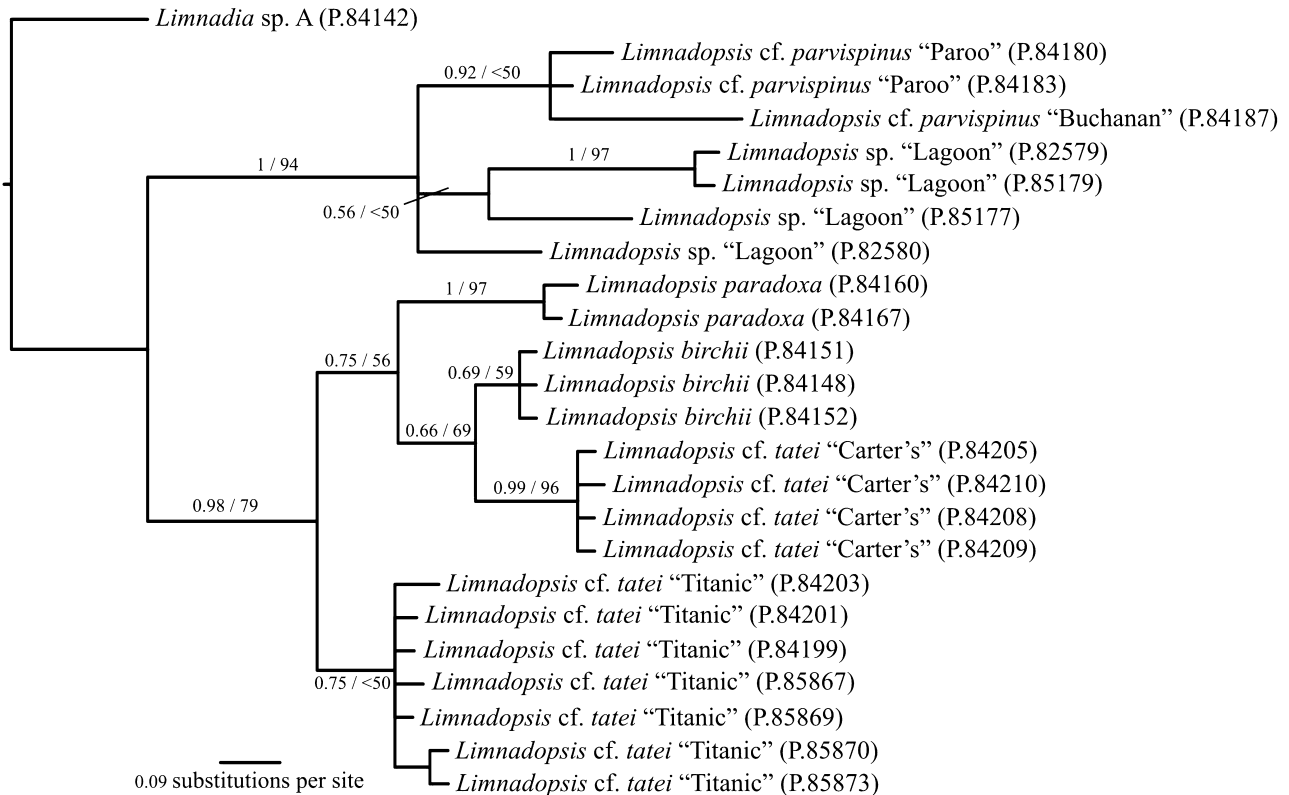

Figure 2. Bayesian inference majority rule tree based on the elongation factor 1α (EF1α) dataset. For each branch, the posterior probabilities of the Bayesian analysis and bootstrap support values of the maximum parsimony analysis are shown. Support values within putative species are not given.

EF1α *p* distances were lower than the COI distances (Table 3): within lineages 0.0–2.4% (all lineages except *L.* sp. 'Lagoon' have distances $\leq 0.4\%$) and among lineages 0.8–4.5%. Thus, a clear gap separating intralineage from interlineage EF1α distances is not present. Only if *L.* sp. 'Lagoon' is excluded a gap of 0.4–0.8% separating intralineage from interlineage distances is present.

Clasper morphology

The terminology used for the description of the claspers is based on Olesen *et al*. (1996). The structures at the tip of the movable finger are referred to as 'scales' following Richter & Timms (2005). The spatial orientation of the claspers needs to be explained in detail, as arrangements are not intuitive (compare with Fig. 3A, B). All images of the claspers and their components are upside down for a more intuitive visualization. Therefore, the ventral part is always shown on top of the dorsal part.

All studied specimens have typical spinicaudatan claspers (Fig. 3) comprising the same basic components: The movable finger bears round scales on the surface that is opposing the apical club (Fig. 3E). Further ventrally at the very tip of the movable finger, one to four scales are present which differ markedly from the other scales of the movable finger (Figs 3A, B, 4 and 5). These scales exhibit a great variability in shape and number (Table 4). Even among the four claspers of a single individual, the scales are not identical; in particular, the number of scales varies among the claspers within several individuals. Despite the variation present within species [as identified on the basis of Timms (2009)], most species can be differentiated from others on the basis of the characteristics of the scales (Table 4); this includes individuals of both lineages of *L.* cf. *tatei* (*L.* cf. *tatei* 'Titanic' and *L.* cf. *tatei* 'Carter's'), which can be differentiated on the basis of the presence/absence of a ledge ventrally on the scale. The delineation of the two *L.* cf. *parvispinus* lineages ('Paroo' and 'Buchanan') from one another and from *L.* sp. 'Lagoon' is problematic. There is no characteristic that differentiates among both *L.* cf. *parvispinus* lineages. This can be partly attributed to the great variation present in these lineages. *Limnadopsis* sp. 'Lagoon' (Fig. 5O, P) differs slightly in number, size and arrangement of

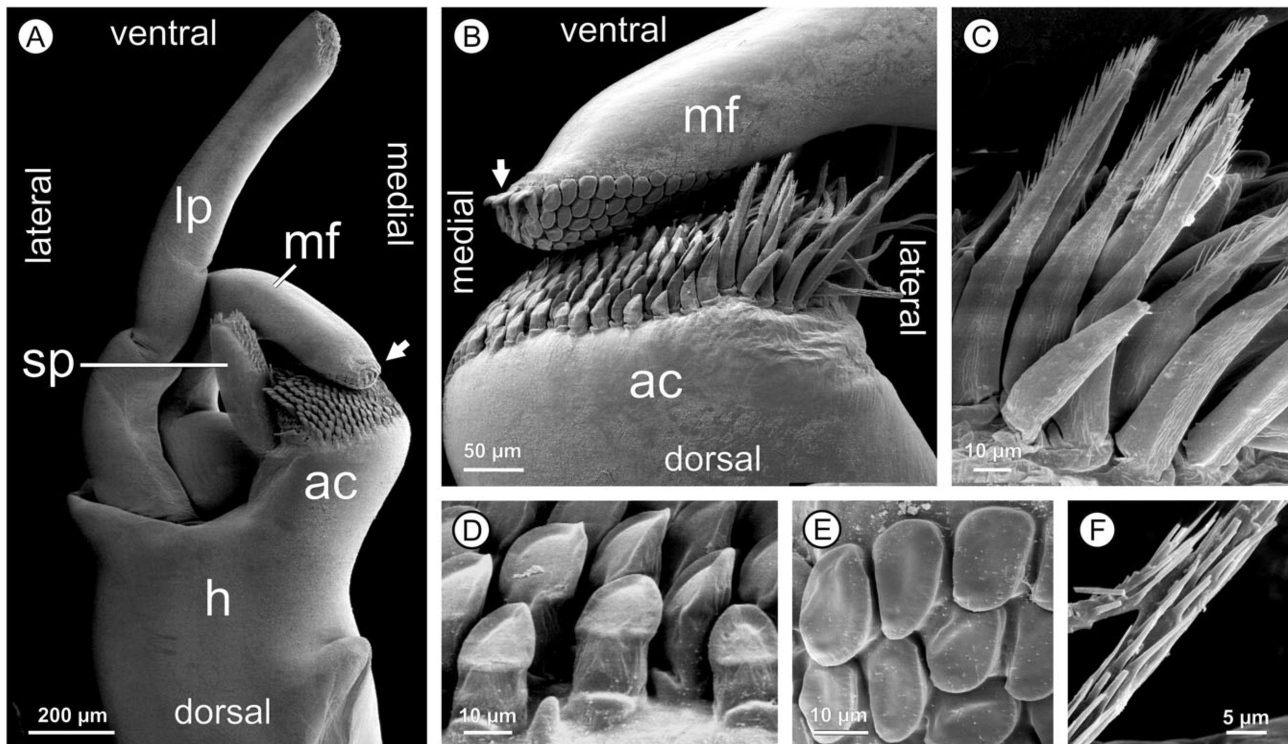

Figure 3. The clasper of *Limnadopsis*, shown for *Limnadopsis* cf. *parvispinus* 'Paroo' P.84181 as an example: A, posterior view; B, anterior view; C, setae on apical club, anterior view; D, teeth-like setae on apical club; E, flat, broad scales on movable finger facing the apical club; F, detail of setae on apical club. The arrows in (A) and (B) indicate the scales on the tip of the movable finger. ac, apical club; h, hand; lp, large palpus; mf, movable finger; sp, small palpus.

scales from *L.* cf. *parvispinus* (Fig. 5I–N, Table 4). In addition, the scales of *L.* sp. 'Roskos' and *L. occidentalis* are very similar; the scales of the latter, however, are shorter and thinner.

DISCUSSION

SUCCESS OF THE INTEGRATIVE APPROACH FOR SPECIES DELINEATION

Species delineation, as part of an integrative taxonomy approach, relies on the combination of very different data (e.g. morphological, behavioural, ecological or genetic; Dayrat, 2005). These different types of data complement each other by solving ambiguities or by pointing out erroneous assumptions on the basis of any single character set. Consistency among the data strengthens the proposed species delineation, but, in most cases, conflicts among datasets can be expected (Padial & de la Riva, 2010). Such conflicts may indicate ongoing speciation processes that have not yet been finalized, e.g. on the basis of genetic data, allopatric populations are delineated into several lineages, but mechanisms for reproductive isolation are still missing. Padial & de la Riva (2010) argued that full congruence among data from various sources cannot be demanded or even expected. As a result of heterogeneous evolutionary forces, not all characters will be equally affected during speciation processes, implying that congruence among data is desirable, but not mandatory, for species delineation. Obviously, the extent to which these conflicts affect species delineation depends on the species concept applied.

Our analyses of COI resulted in eight distinct monophyletic lineages; all of these lineages are separated by COI distances of at least 6.8%. The results of the EF1α data showed no indication of gene flow among the lineages. Although not all of the lineages were monophyletic in the EF1α analyses, no sequence was shared among individuals of the different lineages. On the basis of these data, the eight lineages are qualified as distinct species based on the ESC, PSC *sensu* Mishler & Theriot and PSC *sensu* Wheeler & Platnick. Of these eight lineages, clasper scale morphology differs between the lineages *L. paradoxa*, *L. birchii*, *L.* cf. *tatei* 'Titanic', *L.* cf. *tatei* 'Carter's' and *L.* sp. 'Roskos', but the shape of the scales does not show a clear species-specific pattern (partly as a result of extensive intraspecific variation) between the lineages *L.* sp. 'Lagoon', *L.* cf. *parvispinus*

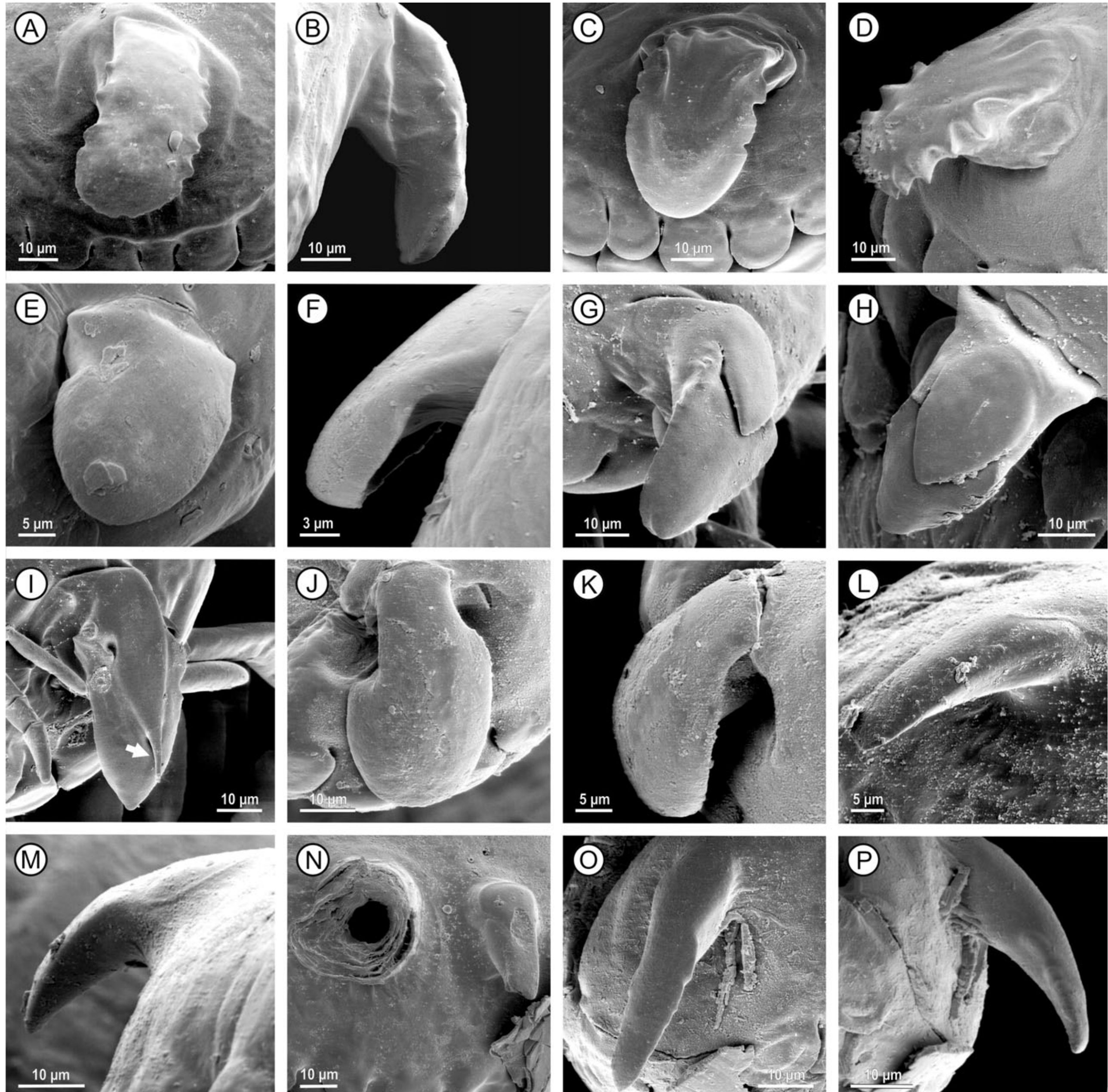

Figure 4. Scales at the tip of the movable finger of *Limnadopsis species*. A–D, *Limnadopsis paradoxa*: A, P.84167 first right clasper, medial view; B, P.84167, posterior view; C, P.84167 second right clasper, medial view; D, P.84170 second right clasper, anterior–medial view. E–F, *Limnadopsis* cf. *tatei* 'Titanic': E, P.84203 first right clasper, medial view; F, P.84203, anterior view. G–I, *Limnadopsis* cf. *tatei* 'Carter's': G, P.84209 second right clasper, posterior–medial view; H, P.84209 first right clasper, ventral view; I, P.84208 first right clasper, medial view (white arrow indicates palp-like structure). J–K, *Limnadopsis pilbarensis* P.84194 first right clasper: J, medial view; K, anterior view. L–N, *Limnadopsis multilineata* P.84154: L, first right clasper, medial view; M, frontal view; N, second right clasper, medial view. O–P, *Limnadopsis minuta* P.84153 first right clasper: O, medial view; P, posterior view.

'Buchanan' and *L.* cf. *parvispinus* 'Paroo' (the scales of both *L.* cf. *parvispinus* lineages are identical). For the first group, differences in clasper morphology add an argument for the species status based on the PSC *sensu* Wheeler & Platnick (diagnosability). As the scales of the movable finger potentially play an important role in the identification of suitable mating partners, species status is also indicated by the BSC, HSC

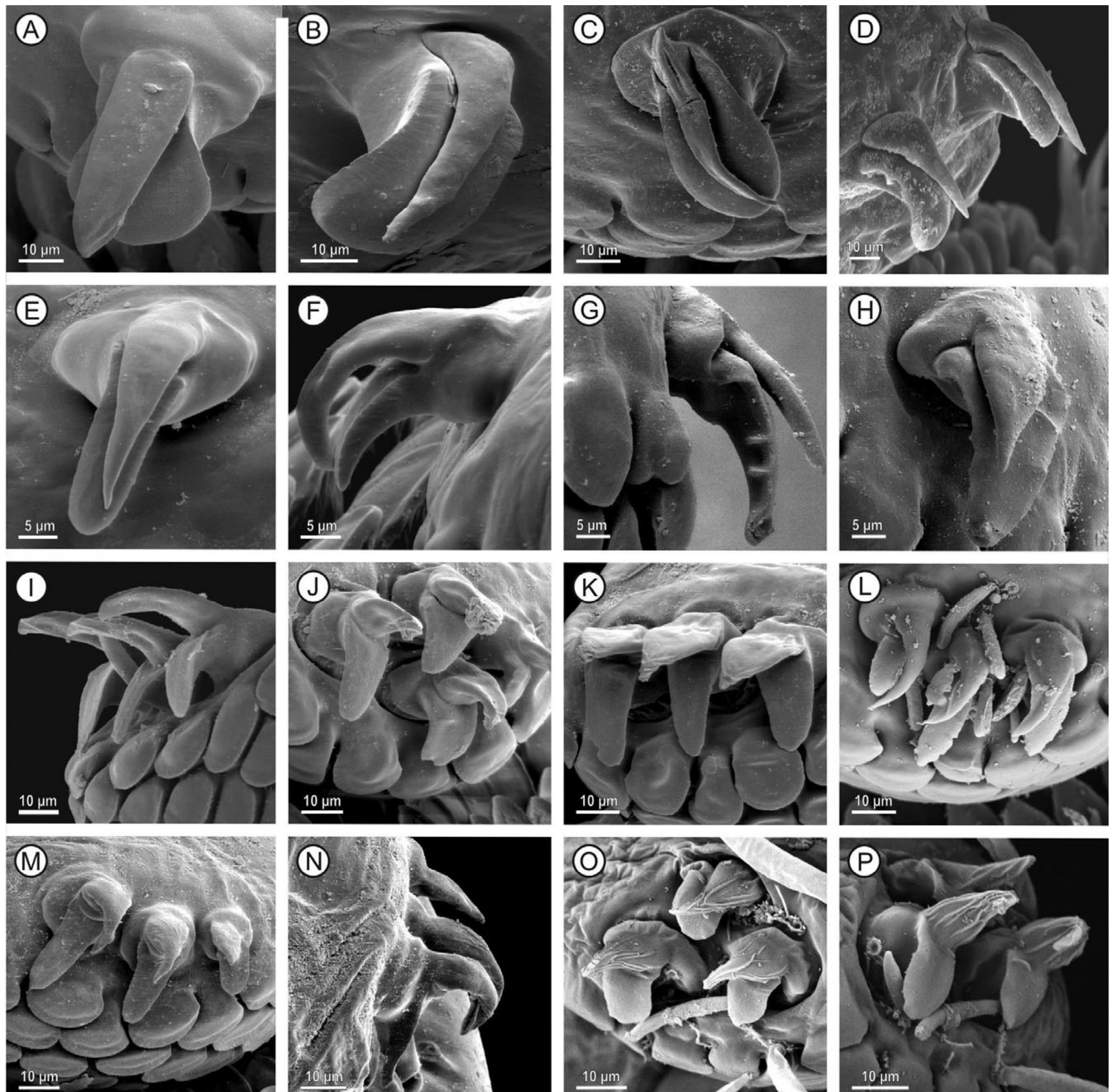

Figure 5. Scales at the tip of the movable finger of *Limnadopsis* species. A–D, *Limnadopsis birchii*: A, P.84144 first right clasper, medial view; B, P.84143 second right clasper, posterior–medial view; C, P.84152 first left clasper, medial view; D, ST5 first right clasper, posterior view. E–F, *Limnadopsis occidentalis*: E, P.84156 first right clasper, medial view; F, P.84156 frontal view. G–H, *Limnadopsis* sp. 'Roskos': G, P.85175 second right clasper, medial view; H, P.85175, posterior view. I–L, *Limnadopsis* cf. *parvispinus* 'Paroo': I, P.85022 first right clasper, anterior view; J, P.84181 first right clasper, posterior–medial view; K, P.84181 second right clasper, medial view; L, P.80898 first right clasper, medial view. M–N, *Limnadopsis* cf. *parvispinus* 'Buchanan' P.84186 second right clasper: M, medial view; N, posterior view. O–P, *Limnadopsis* sp. 'Lagoon' P.82580 first right clasper: O, medial view; P, posterior view.

and RSC. The sympatric (Timms & Richter, 2002) and partially syntopic (own observation) distribution of *L. birchii* with *L.* cf. *tatei* (*L.* cf. *tatei* 'Titanic' and/or *L.* cf. *tatei* 'Carter's') in the catchment area of the Paroo River strengthens the assumption that reproductive isolation mechanisms are effective between these species. This strengthens the species delineation based on BSC and HSC, in which sympatric

Table 4. Shape of the scales on the tip of the movable finger. The characteristics of the scales that are useful for the delineation of species are summed for each species and lineage of *Limnadopsis*, respectively. Species and lineages represent the initially identified species or the main lineages inferred from the cytochrome *c* oxidase subunit I (COI) dataset

	L. paradoxa	*L.* cf. *tatei* 'Titanic'	*L.* cf. *tatei* 'Carter's'	*L. pilbarensis*	*L. multilineata*	*L. minuta*	*L. birchii*	*L. occidentalis*	*L.* cf. *parvispinus* 'Paroo'	*L.* cf. *parvispinus* 'Buchanan'	*L.* sp. 'Lagoon'	*L.* sp. 'Roskos'
Number of scales	1	1	1	1	1–2*	1	1–2*	1	2–4*	2–4*	1–3*	1
Arrangement of scales (if > 1)	n.a.	n.a.	n.a.	n.a.	In a row	n.a.	In a row	n.a.	2–3 scales in a row, rarely in a rhombus, 4 scales in a rhombus	2–3 scales in a row, 4 scales in a rhombus	2 scales in a row, three scales triangular	n.a.
Sub-division	One pieced	One pieced	One pieced	One pieced	One pieced	One pieced	Bifid	Bifid	Bifid	Bifid	Bifid	Bifid
Form of scales (dorsal part of bifid scales)	Rounded, sometimes with triangular ledge ventrally	Rounded	Rounded with triangular ledge ventrally (ledge palp-like elongated in P.84208)	Rounded	Slender, pointed; second clasper with smaller hook-like scale	Slender, pointed, slightly flattened	Widely rounded, bent towards mf	Slender, not or weakly bent towards mf	Slender, not or weakly bent towards mf	Slender, not or weakly bent towards mf	Slender, not or weakly bent towards mf	Slender, not or weakly bent towards mf
Form of ventral part of bifid scales	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Slender (palp-like)	Mostly slender (palp-like); sometimes triangular	Slender (palp-like) or triangular†	Slender (palp-like) or triangular†	Triangular	Slender (palp-like)
Orientation of ventral to dorsal part of bifid scales	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Almost parallel	Almost parallel	Almost vertical	Almost vertical	Almost vertical	Almost parallel
Serration along the edges	Strong, several indentations	Minor	Minor	None	None	None	None	None	None	None	None	Minor
Length in µm (for bifid scales only dorsal part)	34–53	20–44	40–53	43	40 (hook-like scale: 30)	45	33–42	19–28	14–30†	14–30†	14–20†	29–35
Width in µm (for bifid scales only dorsal part)	22–34	19–36	20–23	25	9 (hook-like scale: 12)	9	23–31	8–9	8–16†	7–9†	7–9†	13–14

n.a., not applicable; mf, movable finger.
*Variable among the claspers of single individuals.
†Variable among the scales of a single claspers.

occurrence of closely related species is considered to be a test of the presence and effectiveness of the isolation mechanisms.

In analogy with the previous cases, differences in scale morphology among allopatric lineages may indicate separate species. This is important for the West Australian species *L. paradoxa*, *L. pilbarensis*, *L. multilineata*, *L. minuta* and *L. occidentalis*, for which only the scales were studied. The scales of all four species showed clear differences between these and all other *Limnadopsis* species, implying reproductive isolation (BSC, HSC) and mechanisms for the recognition of mates of the same species (RSC). The scales further represent a unique combination of character states (PSC *sensu* Wheeler & Platnick) and indicate the independent evolutionary fate of the species (ESC). The PSC *sensu* Mishler & Theriot (2000) cannot be applied, as this concept requires an a priori phylogenetic analysis. For an integrative approach, additional molecular data are lacking to corroborate the results of the scale morphology for these four species.

The delineation of *L.* cf. *parvispinus* 'Paroo', *L.* cf. *parvispinus* 'Buchanan' and *L.* sp. 'Lagoon' is the most complex and therefore most interesting case. The phylogenetic analyses of the COI dataset resulted in three reciprocal monophyletic lineages, which are also separated by large distances. The COI distances within each lineage are smaller (≤ 4.2%) than the distances among lineages (≥ 6.8% among the *L.* cf. *parvispinus* lineages, ≥ 12.4% for *L.* sp. 'Lagoon'). The COI distances between both *L.* cf. *parvispinus* lineages are, however, smaller than the distances between all other putative *Limnadopsis* species. In this context, also, *L.* sp. 'Roskos' should be considered. Its COI distances to the *L.* cf. *parvispinus* (10.4–13.8%) and *L.* sp. 'Lagoon' (12.3–12.8%) lineages, however, are similar or lower than the COI distance between the *L.* cf. *parvispinus* lineages and *L.* sp. 'Lagoon' (12.4–14.2%). This supports the delineation of *L.* sp. 'Lagoon' as a separate species from *L.* cf. *parvispinus*, as *L.* sp. 'Roskos' has already been delineated on the basis of all species concepts. The intralineage and interlineage EF1α distances of *L.* sp. 'Lagoon' and both *L.* cf. *parvispinus* lineages are of the same magnitude (up to 2.4%) and do not allow a separation of the lineages on the basis of genetic distances. However, *L.* cf. *parvispinus* is monophyletic in the phylogenetic analysis of the EF1α sequences. As mentioned above, the molecular data alone qualify to consider the lineages as distinct species under the ESC and the two PSCs. The shape of the scales does not show a clear species-specific pattern because of the extensive intraspecific variation in all three lineages. The scales of both *L.* cf. *parvispinus* lineages are too similar to be differentiated; thus, these two lineages, considering only the clasper morphology, would not be delineated into two species. *Limnadopsis* sp. 'Lagoon' differs slightly from the *L.* cf. *parvispinus* lineages (e.g. smaller number of scales, shorter scales and ventral part never palp-like shaped), but the differences in the shape of the scales are not as distinct as among the other studied species. The delineation of the *L.* cf. *parvispinus* lineages and *L.* sp. 'Lagoon' into different species is problematic under the BSC and HSC, because these three lineages seem to occur allopatrically with no known distribution overlap; therefore, the reproductive isolation has not yet been tested by sympatry. *Limnadopsis* cf. *parvispinus* 'Paroo' occurs in southern Queensland and large parts of New South Wales [as described for *L. parvispinus* by Timms (2009)]. *Limnadopsis* cf. *parvispinus* 'Buchanan' occurs in northern central Queensland [the occurrence of *L. parvispinus* in this area is not mentioned by Timms (2009)], at least 700 km apart from *L.* cf. *parvispinus* 'Paroo' and, for *L.* sp. 'Lagoon', only a single pool in south-eastern Queensland is known (geographically closer to *L.* cf. *parvispinus* 'Paroo' than to 'Buchanan'; no individuals of any of these lineages were found in the area in between these locations). When integrating all the data and the deduced assumptions, two species are congruently delineated: *L.* sp. 'Lagoon' and *L.* cf. *parvispinus*. The allopatric distribution of *L.* cf. *parvispinus* and *L.* sp. 'Lagoon' is problematic for the BSC and HSC, but the differences in scale morphology and the obvious lack of gene flow among these species also qualify for their delineation on the basis of these two species concepts.

The scales of both *L.* cf. *parvispinus* lineages are too alike to assume either reproductive isolation or effective mate recognition mechanisms between these lineages. As we favour a species concept that also considers reproductive isolation, we propose that *L.* cf. *parvispinus* 'Paroo' and *L.* cf. *parvispinus* 'Buchanan' represent two genetically divergent lineages of a single species. It is possible that the lineages will show an effective mechanism for reproductive isolation once they come into secondary contact, but this cannot be deduced from the data currently available.

The lineage *L.* sp. 'Roskos' has been delineated from all other lineages. Nevertheless, the overall morphology of the individuals and the shape of the scales are rather similar to *L. occidentalis*, which, however, has only been recorded from Western Australia (Timms, 2009) and has never been recorded from the area in which *L.* sp. 'Roskos' has been found (Timms & Richter, 2002). Therefore, these two might represent a single species. A final decision can only be made if genetic data for *L. occidentalis* become available.

In summary, all the *Limnadopsis* lineages revealed in the COI dataset could be treated as separate species on the basis of the PSC *sensu* Mishler & Theriot and the ESC without considering further data. To our understanding, however, this falls short

of the biological reality of these species and might artificially increase the number of recognized species (see also Ballard *et al*., 2002; Agapow *et al*., 2004). In most cases, species delineation was independent of the applied species concept (see also Laamanen *et al*., 2003 and Tan *et al*., 2010). Discrepancies arose when the various data types were incongruent (e.g. in the case of *L.* cf. *parvispinus*). Of course, these are the interesting cases for which the integration of the various species concepts is most important. If all types of data are not available for all individuals, this integrative approach still allows for well-founded assumptions regarding species delineation by direct comparison with other species for which more data are available. The following species could be delineated by the integrative approach: *L. birchii*, *L. minuta*, *L. multilineata*, *L. occidentalis*, *L. paradoxa*, *L.* cf. *parvispinus*, *L. pilbarensis*, *L.* cf. *tatei* 'Carter's', *L.* cf. *tatei* 'Titanic', *L.* sp. 'Lagoon' and *L.* sp. 'Roskos'. Overall, our study emphasizes the importance and value of an integrative approach to the resolution of taxonomic problems.

Genetic thresholds for species delineation?

One of the advantages of utilizing different datasets is the potential to determine the intra- and interspecific variation within each of these datasets. Between the eight main monophyletic lineages (including the two *L.* cf. *parvispinus* lineages), COI genetic distances were between 6.8 and 18.6% and, within these lineages, between 0.0 and 4.2%. This could imply a COI threshold value of about 5–6% to discriminate between intra- and interspecific genetic distances for *Limnadopsis* species. As argued above, however, the two *L.* cf. *parvispinus* lineages cannot be assigned to separate species unambiguously. If they indeed represent a single species (as deduced from the BSC, HSC and RSC) in an ongoing speciation process, the largest intraspecific genetic distance would be 9.9% and the lowest interspecific distance would be 9.6% (separating *L.* cf. *tatei* 'Titanic' and *L.* cf. *tatei* 'Carter's'). Such an overlap of intra- and interspecific distances is not uncommon and is one of the problems associated with DNA barcoding (Meyer & Paulay, 2005; Meier *et al*., 2006). A comparison with other branchiopod taxa reveals a wide range of intra- and interspecific distances for COI with no clear threshold. Maximum intraspecific genetic distances are up to 1.74% for *Daphnia magna* (De Gelas & De Meester, 2005), 2% for *Daphnia optusa* (Penton, Hebert & Crease, 2004), 0.3–4.3% for various *Daphnia* and *Ctenodaphnia* species (Adamowicz, Hebert & Marinone, 2004) and 5.7% for *Daphnia ambigua* (Hebert, Witt & Adamowicz, 2003b). Although Cladocera show rather large minimum interspecific COI distances, more than 16.2% for various *Daphnia* and *Ctenodaphnia* species (Adamowicz *et al*., 2004) and more than 15.4% for North American *Daphnia* species (Penton *et al*., 2004), in anostracans the minimum interspecific distances are often lower: 2.2% for *Chirocephalus* species (Ketmaier *et al*., 2003) and 2.1% for *Artemia* species (Muñoz *et al*., 2008). It should be noted that all but the last study used corrected distances (usually Kimura two-parameter corrected), rather than the uncorrected *p* distances used in this study, which increases their values slightly compared with the uncorrected *p* distances. Even considering this, the observed intraspecific distances within *Limnadopsis* species are in the upper range of the recorded intraspecific variations for branchiopods, even if both *L.* cf. *parvispinus* lineages are treated as two distinct species. The potential overlap of intra- and interspecific genetic variations emphasizes the limitations of a universal threshold for species delineation, and highlights the importance of the identification of threshold values for each taxonomic group independently.

Clasper characteristics as taxonomically valuable characters

The scales of the movable finger of the claspers have not been studied in detail previously; therefore, no a priori assumption regarding their variability was possible. The interspecific variation includes the differentiation into bifid or one-pieced scales, the presence/absence of serration along the edge, the presence/absence of a ledge, size differences, differences in the shape of the scale and different arrangements of the scales. The intraspecific variation generally accounts for minor differences in size and shape, e.g. differences in the number of indentations and whether or not the edges are serrated. Interestingly, the range of intra-individual variation among the four claspers is similar or identical to the variation observed within the whole species. The intraspecific variation in the number of scales is remarkable. In particular, the claspers of both *Limnadopsis* cf. *parvispinus* lineages and *L.* sp. 'Lagoon' feature varying numbers of scales, but also in *L. birchii* and *L. multilineata* single claspers bear two scales instead of one. Therefore, the number of scales is too variable within species to be a useful character for species delineation.

Our results suggest that the shape of the scales is species specific. This observation is congruent with the indications that parts of the clasper are part of SMRS (Paterson, 1993a) of these species. Components of the SMRS are expected to be under strong selection pressure, especially if closely related species occur in sympatry, leading to species-specific differentiations (character displacement; Brown & Wilson, 1956). All

closely related species that occur in sympatry feature scales which are clearly distinguishable: *L. parvispinus* and *L.* sp. 'Roskos' and *L. birchii*, *L.* cf. *tatei* 'Titanic' and *L.* cf. tatei 'Carter's'. However, closely related species which do not occur sympatrically (e.g. *L. parvispinus* and *L.* sp. 'Lagoon') are hardly differentiated in the shape of their scales. Further research focusing on the species-specific parts of the claspers, as well as their interactions with the females, might reveal important aspects of the mate recognition mechanisms of spinicaudatan species.

Our results highlight the usefulness of the scales at the tip of the movable finger for the delineation and identification of *Limnadopsis* species. At present, these scales seem to be the best single character to distinguish male specimens. For egg-bearing females, the shape and structure of the resting eggs carried under the carapace could be a similarly useful character (Timms, 2009). The scales might prove to be a useful character to delineate species of further spinicaudatan taxa.

Implications for *Limnadopsis* species diversity

Our results suggest that the species diversity of *Limnadopsis* is larger than anticipated in the last review by Timms (2009). In addition to the eight recognized species, three additional as yet undescribed species could be delineated: *L.* sp. 'Lagoon', *L.* sp. 'Roskos' and one of the two *L.* cf. *tatei* lineages. For the latter, we suggest that *L.* cf. *tatei* 'Titanic' represents *L. tatei* Spencer & Hall, 1896. The specimens collected from Marla (P.85864–P.85873) are closest to the type locality (Marla is about 200 km west of the poorly defined type locality area), and the specimens of this lineage resemble the original description of *L. tatei* much more closely than do specimens of the *L.* cf. *tatei* 'Carter's' lineage (this includes the number of body segments and telsonic spines; data not shown). Therefore, *L.* cf. *tatei* 'Carter's' is considered to represent an undescribed species.

Limnadopsis cf. *parvispinus* 'Paroo' most likely represents *Limnadopsis parvispinus* Henry, 1924 as the eggs 'Egg44' and 'Egg46' are from pools around Mossgiel, one of two type localities of *L. parvispinus*. If additional data support the delineation of the two *L.* cf. *parvispinus* lineages into two species, *L.* cf. *parvispinus* 'Buchanan' would have to be considered as a new species. So far, we consider all specimens identified in this study as *L.* cf. *parvispinus* to represent *L. parvispinus* Henry, 1924. Our study therefore increases the number of recognized *Limnadopsis* species to at least 11.

The study of Weeks *et al*. (2009), however, hinted at the presence of even further species. To integrate their results into this study, additional analyses were carried out (Bayesian analysis and Maximum Parsimony with specifications as above; trees not shown) including all *Limnadopsis* individuals, as well as selected *Limnadia* individuals and two *Eulimnadia* individuals as outgroups. As Weeks *et al*. (2009) incorporated a slightly different portion of the COI fragment, additional sequences were obtained using the primer pair MidCox1CrustForward/3′Cox1LimnReverse, as specified by Weeks *et al*. (2009), from a few chosen specimens for each main genetic lineage (these sequences are part of the sequences submitted to GenBank). It seems that what Weeks *et al*. referred to as '*Limnadopsis* sp. 2' is identical with *L. paradoxa* (identical COI sequence as P.84158) and '*Limnadopsis tatei* 2' is close to *L.* sp. 'Roskos' (2% genetic distance). Otherwise, none of their individuals could be assigned to any of the species included in this study. Their *L. tatei* specimens were not related to any of the two *L.* cf. *tatei* lineages of this study, and their *L. parvispinus* individuals were most closely related to *L.* sp. 'Lagoon'. '*Limnadopsis* sp. 3' is rather closely related to *L.* cf. *tatei* 'Carter's' (5.5% genetic distance). The discrepancy between these studies might be partially attributed to the fact that Weeks *et al*. (2009) did not incorporate the descriptions and redescriptions of Timms (2009), which may have led to identification problems. Therefore, some of the individuals of Weeks *et al*. (2009) may belong to one of the species described by Timms (e.g. *L. occidentalis*, *L. minuta*, *L. multilineata* or *L. pilbarensis*), but this cannot be clarified with the data currently available. Nevertheless, it becomes obvious that the actual diversity of *Limnadopsis* species is larger than previously thought, and it seems likely that even more species will be discovered.

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