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Multilocus phylogenetics of smooth clam shrimps (Branchiopoda, Laevicaudata)

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Abstract

Laevicaudatan branchiopods, also called 'smooth clam shrimps' or 'pea shrimps', are rare crustaceans found exclusively in temporary, small freshwater bodies, which stay dry most of the year. Only 42 laevicaudatan species have been described so far, 90% of which belong to the genus *Lynceus*. The first multilocus phylogeny of the group is provided here, based on 15 *Lynceus* species from North and South America, Europe, Africa, Asia, Australia and New Caledonia and using nine molecular markers (two mitochondrial and seven nuclear genes, including newly designed primers). Genetic data suggest populations of *Lynceus brachyurus* from Europe and North America to represent a complex of cryptic species and sister group to all other laevicaudatans. Species from Thailand, Japan, Mongolia and China formed a distinct East Asian clade. A Southern Hemisphere (Gondwanaland) clade, composed of Chilean, Australian and New Caledonian taxa, was found weakly clustering with an African *Lynceus* species. Relaxed molecular clock analyses indicate a Pangean origin of Laevicaudata, with further diversification due to vicariance and the continued splitting of continents. Rostrum characters, which are particularly relevant for laevicaudatan systematics, were re-evaluated and provide morphological evidence supporting molecular clades. Our worldwide overview of Laevicaudata evolution highlights that

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recent sampling from Africa and South America is scarce, and that further DNA efforts should focus on *Paralimnetis* and *Lynceiopsis* species.

KEYWORDS

Gondwana, historical biogeography, Laevicaudata, molecular clock, Southern Hemisphere

1 | INTRODUCTION

Smooth clam shrimps (Branchiopoda: Laevicaudata) are among the most rare, bizarre-looking and understudied Crustacea (Martin & Belk, 1988; Rogers & Olesen, 2016). Several unique structures (=synapomorphies) distinguish these globular and small (1.5–7 mm) freshwater crustaceans from other clam shrimps (Spinicaudata and Cyclestherida), such as their disproportionate head, mandibles with transverse ridges, multilobed flaps (laminae abdominalis) placed dorsolaterally for holding eggs, dorsal hinge between the carapace valves, and peculiar ‘UFO-shaped’ larvae with the first antennae modified as large horns (Fryer & Boxshall, 2009; Olesen, 2005; Richter, 2004; Rogers & Olesen, 2016) (see Figure 1). Smooth clam shrimps are essentially benthic animals, often making slow-swimming excursions into the water column using their antennae (Fryer & Boxshall, 2009; Patton, 2014; Sigvardt & Olesen, 2014). Laevicaudatans can be found in ephemeral freshwater habitats like vernal pools, seasonal wetlands, woodland pools, river flood pools, rock pools, clay pans, playa lakes, salt-pans, permafrost wetlands, tundra pools and alpine pools that remain dry most of the time and appear either seasonally or episodically throughout the year (Brendonck et al., 2008; Rogers, 2009, 2014). The instability of these habitats makes laevicaudatans challenging to sample and successful collecting is more a matter of timing than technique (Martin et al., 2016).

All Laevicaudata species ($n = 42$) currently recognized (Rogers & Olesen, 2016; Sigvardt et al., 2019) are grouped into a single family (Lynceidae Stebbing, 1902) and three genera, namely *Lynceus* Müller, 1776, *Lynceiopsis* Daday, 1912, and *Paralimnetis* Gurney, 1931 (Rogers & Olesen, 2016). *Lynceus* is the largest genus by far, comprising about 90% ($n = 37$) of the total species diversity, and is present on every continent except Antarctica. *Lynceiopsis* and *Paralimnetis* include just two species from Africa and three from the Americas, respectively (Martin & Belk, 1988). The first laevicaudatan described (i.e. *Lynceus brachyurus* O.F. Müller, 1776), was collected from a now destroyed pool north of Copenhagen, Denmark (Rogers & Olesen, 2016). Species from other continents have been gradually added since then, with Daday's monograph (1927) being the first major contribution to Laevicaudata taxonomy. Significant studies include the taxonomic revisions from the Americas by Martin and

Belk (1988) and Australia by Timms (2013). Minor revisions and species descriptions from Argentina, Canada, Chile, New Caledonia, Thailand, South and North China, and Mongolia have recently been published (Olesen et al., 2016; Pessacq et al., 2011; Rogers et al., 2015, 2016; Shu et al., 2019; Sigvardt et al., 2019, 2020) and most of those are summarized in the Laevicaudata catalogue by Rogers and Olesen (2016). African Laevicaudata are in need of revision, with descriptions mostly scattered in a number of older publications (Barnard, 1924; Barnard, 1929; Daday, 1927; Gauthier, 1936; Martin & Belk, 1988; Thiele, 1907; Thiery, 1986). Laevicaudatans are poorly represented in the fossil record, typically recognized by the presence of a roundish carapace devoid of growth lines and possessing the impression of a maxillary gland; the oldest putative laevicaudatan being from the Permian with possible soft part preservation from the Jurassic (Hegna & Astrop, 2020).

Laevicaudatan species can be distinguished based on the form of the head/rostrum, male claspers, female lamina abdominalis etc. (e.g., Martin & Belk, 1988; Rogers & Olesen, 2016; Timms, 2013), but virtually no attempts have been made to address evolutionary relationships within Laevicaudata using morphological or molecular data. Previous molecular studies included few species and/or genes (e.g. deWard et al., 2006; Stenderup et al., 2006; Schwentner et al., 2018). The most comprehensive molecular study so far only presents a preliminary laevicaudatan phylogeny based on a single gene (Sigvardt et al., 2019). This is not surprising considering that fresh material is very difficult to obtain due to the ephemeral nature and threatened status of their habitats (Martin et al., 2016). Phylogenetic and biogeographic distribution patterns of Spinicaudata and other branchiopods have been under debate during the last decades (e.g. Olesen, 2009; Richter et al., 2007; Schwentner et al., 2009, 2020; Xu et al., 2011), but laevicaudatan relationships remain practically unexplored. Most smooth clam shrimp species and their largest morphological diversity are found in the Southern Hemisphere ($n = 27$) (e.g. Barnard, 1929; Daday, 1927; Timms, 2013), so these continents are crucial to any study on evolution and biodiversity of Laevicaudata. The origin of Australian laevicaudatan clam shrimps or how they relate to remote populations from New Caledonia are not known. Similarly, the unusually large distribution of some taxa like *Lynceus brachyurus* (with a Holarctic distribution) is unexpected and may suggest cryptic speciation.

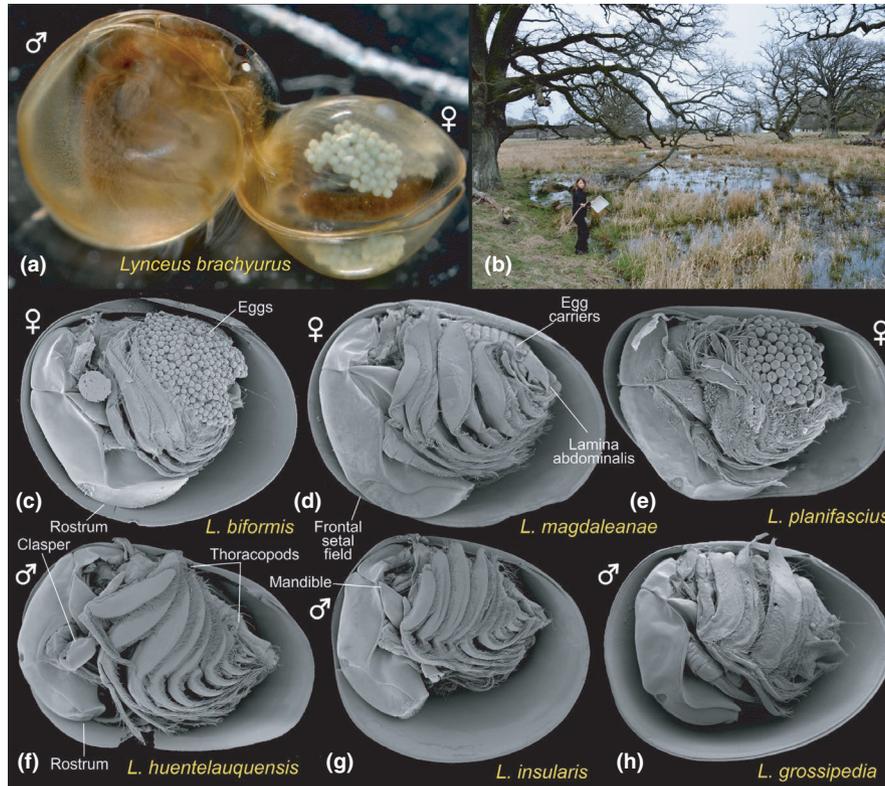


FIGURE 1 Laevicaudata (Crustacea: Branchiopoda) diversity and Northern European habitat. All illustrated species have been included in the study.—a. *Lynceus brachyurus*, mating couple from locality in ‘b’.—b. *Lynceus brachyurus* locality in the Deer Garden north of Copenhagen (Denmark) less than 10 km from the now destroyed type locality for *L. brachyurus* (type species for *Lynceus*) (see Rogers & Olesen, 2015).—c. *L. biformis* from Yangminshan National Park, Taipei (Taiwan) (NHMD-615844).—d. *L. magdaleanae* from Yanneymooning Rock pit gnamma, Mukinbuden, Western Australia (Australia) (NHMD-615928).—e. *L. planifascius* from Khon Kaen (Thailand) (NHMD-615849, topotype).—f. *L. huentelauquensis* from pool in road bed, Huentelauquén Plains, Coquimbo Region (Chile) (NHMD-265550, paratype).—g. *L. insularis* from doline (ultramafic sink hole), Le Mont-Dore, South Province (New Caledonia) (NHMD-82632, paratype).—h. *L. grossipedia* (paratype) from Tuv Province, Uguu nuur (Mongolia) (NHMD-616086)

To address these questions, a multilocus molecular phylogenetic study is carried out here, based on two mitochondrial and seven nuclear markers and a worldwide collection of *Lynceus* species. Significant efforts were made to include specimens from Europe, Africa, Asia, Australia, and North and South America. This allowed us both to analyse the molecular evolution of Laevicaudata and its connection with observed biogeographic patterns and to assess the status of *L. brachyurus*. Laevicaudata systematics is discussed considering the new phylogenetic results and selected morphological data (rostrum morphology).

2 | MATERIAL AND METHODS

2.1 | Molecular marker selection and primer design

A small set of four universal primers pairs was initially tested with positive results, including primers for both mitochondrial (COXI and 16S) and nuclear (18S and H3)

markers (Table S1). To design a set of primers amplifying other nuclear genes, next-generation (NGS) sequencing was carried out on several *Lynceus* species and *Cyclestheria hislopi* (Baird, 1859) (Cyclestherida). Prior to library building, small aliquots of each extract were analysed with Qubit and on Bioanalyzer for fragment size estimation and concentration. TruSeq Nano kit Illumina libraries were paired-end sequenced (2×150 bp) using the Illumina HiSeq3000 chemistry. Genomic library preparation and Illumina HiSeq sequencing were carried out at GenoToul (INRA, France) and the Modern Lab, Natural History Museum of Denmark (University of Copenhagen, Denmark). After a first round of revision of this manuscript, sequences from a South African species (*Lynceus triangularis*) were included. DNA library preps for this sample were performed with a Nextera XT kit (Illumina) and sequenced on an Illumina Miseq at Plateforme iGenSeq, Institut du Cerveau—ICM (Hôpital Pitié Salpêtrière, France).

Paired-end reads were subjected to quality inspection using FastQC software (Andrews, 2010), cleaned using Trimmomatic 0.36 (Bolger et al., 2014) and used

for de novo genome assembly with Velvet (Zerbino & Birney, 2008). The OrthoDB comprehensive catalogue of orthologs (i.e. descendants from a single gene) was used to annotate conserved genes within genome assemblies. The following five genes were selected to design a new set of primers with PRIMER3 (<http://bioinfo.ut.ee/prime-r3-0.4.0/>) using default parameters: pre-mRNA-splicing factor 18 (EOG090X03WO), Golgi phosphoprotein three homolog sauron (EOG090X0A2Q), Queuosine salvage protein (EOG090X0A16), NADH-cytochrome b5 reductase (EOG090X0BKI), and BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3 (EOG090X090D) (Table S1). Loci were selected to represent a diverse range of functional categories, from replication, recombination and repair to coenzyme transport and metabolism (Kriventseva et al., 2019).

2.2 | DNA extraction and PCR analyses

Although DNA extraction was carried out on many laevicaudatan samples (see Table S2), difficulties with old museum material were a limiting factor for molecular analyses and attempts on several taxa were failed. A total of 24 samples, representing 15 *Lynceus* species obtained from multiple collections and sampling trips worldwide, were finally included in the analyses (together with four outgroups) (Table 1). The preferred DNA extraction method was DNeasy Kit (QIAGEN) because it always gave positive results on fresh/ethanol-fixed material, but alternative methods were also tested for recalcitrant samples, involving digestion with: (A) Proteinase K following Gilbert et al. (2007) (B) KOH, and (C) AE buffer +Proteinase K following Palero et al. (2010). The amount of tissue used for DNA extraction varied from 2–3 legs (rare specimens) to whole specimens (1–7 mm, carapace valves often removed). Prior to library building or PCR amplification, small aliquots of each extract were analysed for DNA content, either by running a 1% agarose gel or by using Qubit and Bioanalyzer. Extracted samples showing no or little DNA (and excluded from the analyses) are shown in the Supplemental Material (Table S2). PCR amplification was carried out using 2 µl DNA in a total reaction volume of 15 µl, consisting of 4.3 µl milliQ water, 7.5 µl of Tmix, and 0.6 µl of each primer (forward and reverse) using either universal or newly designed primers (Table S1). The PCR thermal profile used was: 95°C for 15 min for initial denaturation, followed by 35–40 cycles of denaturation at 95°C for 30s, hybridization at 50°C or 54°C for 30s, elongation at 72°C for 30s, and a final extension at 72°C for 15 min. Amplified PCR products were sent out to MACROGEN (Madrid, Spain) for Sanger sequencing. Obtained gene sequences have been deposited in GenBank under accession numbers as indicated in Table 1.

2.3 | Phylogenetic analyses

Each set of gene sequences was aligned separately using MAFFT and alignments for all genes were then concatenated. To improve reliability, conserved (ungapped) blocks of sequence were extracted from each alignment by using Gblocks server with default settings (Castresana, 2000). The best-fit substitution model was tested for each gene individually using MrAIC 1.4.6 (Nylander, 2004) and selected according to the Akaike information criterion with a correction for small sample sizes (AICc). The maximum-likelihood (ML) phylogenetic tree construction method was applied as implemented in Phyml v.3.0 (Guindon et al., 2010). Aligned and concatenated sequences were also used to estimate phylogenetic relationships with the Bayesian inference approach implemented in BEAST v.2.4.7 (Bouckaert et al., 2014). We used each selected model of DNA sequence evolution with an estimated proportion of invariable sites and a Gamma distribution of rates across five classes as suggested by MrAIC (see Results). We estimated a rooted phylogeny with BEAST, using a random starting tree and applying an uncorrelated lognormal relaxed clock and a Yule model as tree prior. Four independent Markov chains were run in BEAST for 100 million generations, sampling every 10,000th generation. We summarized the chains using Tracer v.1.6 (Rambaut & Drummond, 2013) and visually inspected the trace plots (all showed good mixing and convergence). The effective sample sizes (ESS) for the runs were above 200 for most parameters reported in Tracer. We converted the posterior tree distributions into a maximum clade credibility (MCC) tree using TreeAnnotator v.1.4.8 (from the BEAST package). The tree was graphically edited to include species morphological data and geographical distribution using CorelDRAW (Corel Corporation) (Figure 2).

2.4 | Divergence time estimation

A relaxed-clock analysis was used to infer divergence times using information from the fossil record to calibrate the molecular phylogeny. It has been shown that the ‘uncorrelated relaxed-clock’ models, in which the mutation rates in each branch can vary within particular constraints, perform better than a strict molecular clock or the correlated models (Drummond et al., 2006). We used the Bayesian relaxed-clock uncorrelated lognormal approach as implemented in BEAST v1.4.7 (Drummond & Rambaut, 2007) with the corresponding model of sequence evolution previously inferred for each gene partition and a Yule process as tree prior. It is well established that Branchiopoda is monophyletic based on both morphological and molecular data (Lozano-Fernandez et al., 2019; Olesen, 2007; Schwentner et al., 2018). We used *Cyclestherioides* and *Leaia* fossils for calibrating the Cladocera (Cladocera + Cyclestherida) and the

TABLE 1 List of species included in this study

Species	Locality, collector and year	Museum & ID	A2Q	A16	16S	CO1	18S	Morphology
<i>Lynceus baylyi</i> (Timms, 2013)	Australia, WA: 12 km North of Trayning Pit Gnamma Nr. 3; B. V. Timms; 19 May 2016	NHMD-615846/ZS 002	MZ463681	MZ463695	MZ539585	MNS15424	MZ539570	
<i>Lynceus baylyi</i>	Australia, WA: Yellari Pit via Beacon; B. V. Timms; 19 May 2016	NHMD-615847/ZS 075	-	-	-	-	-	X
<i>Lynceus biformis</i> (Ishikawa, 1895)	Japan, Shiga, Livsatsu, rice paddies of Kataoka-Cho; M.J. Grygier; 21 May 2001	NHMD-81868/ ZMUC-CRU-4020/ ZS 017	MZ463682	MZ463696	MZ539586	MNS15425	MZ539571	
<i>Lynceus biformis</i>	Japan, Tano-Hanakuma, Takatsuki City, Osaka Prefecture, irrigated paddy field. 34°57'18.4"N, 135°35'23.7"E; So Ishida; 31 May 2018	NHMD-615843/ZS 089	-	-	-	-	-	X
<i>Lynceus biformis</i>	Japan, Okamoto, Gamou, Higashiohmi-shi, Shiga-ken; 35.037721, 136.189629; Souichirou Okoda; 24 May 2018	NHMD-615871	-	-	-	-	-	
<i>Lynceus biformis</i>	Taiwan: Taipei, Yangmingshan National Park; C. C. Wang, 2015	NHMD-615844	-	-	-	-	-	X
<i>Lynceus brachyurus</i> (Müller, 1776)	Denmark, Pond close to 'Trepilelægen', Deer Garden, North of Copenhagen; J. Olesen; 28 April 2017	NHMD-232312/ZS 031/CS88	MZ463683	MZ463697	MZ539588	MNS15427	MZ539572	
<i>Lynceus brachyurus</i>	USA, California: Sacramento County, NE of junction of Grant Line Road and Kiefer Boulevard, Kiefer Landfill Wetland Preserve. 38°31.469"N, 121°11.631"W; C.W. Witham; 31 Marts 2018	NHMD-615853/ZS 068	MZ463684	MZ463698	MIK356563	MNS15426	MZ539573	X

(Continues)

TABLE 1 (Continued)

Species	Locality, collector and year	Museum & ID	A2Q	A16	16S	CO1	18S	Morphology
<i>Lynceus brachyurus</i>	USA, California: Sacramento County, Sloughhouse; D.C. Rogers; 1 April 2008	NHMD-265530/ DCR-696/ZS 006	-	-	-	-	-	
<i>Lynceus brachyurus</i>	USA, Ohio: Delaware, Stratford Ecological Center (north Pool); W. Patton; 31 May 2018	NHMD-615865/ZS 066	-	-	MZ539587	-	-	
<i>Lynceus brevifrons</i> (Packard, 1877)	USA, Colorado: Weld County: Pawnee National Grasslands, 40°50'29.58"N, 104°30'19.27"W, 1589 masl.; Z.M.S. Sigvardt, J. Olesen & D.C. Rogers; 13 September 2016	NHMD-265534/ BRP3/CS89	MZ463685	MZ463700	MK356564	MZ539936	MZ539574	X
<i>Lynceus brevifrons</i>	USA, New Mexico: Harding County, Kiowa National Grassland, Rock Lake Playa; D. Garcia de la Cadena; 31 July 2004	NHMD-265536/ DCR-618/ZS 085	-	MZ463699	-	-	-	
<i>Lynceus gracilicornis</i> (Packard, 1871)	USA, Georgia: Liberty County: St Catherine's Island, Epheneral Wetland in former Zebra Pasture; J. Jensen; 8 June 2005	NHMD-265538/ DCR-611/ZS 005	-	-	MZ539589	MZ539937	-	
<i>Lynceus gracilicornis</i>	USA, Florida, Leon County, Leon City, Lake Manson area, past intersection of Road 305 and 303; T. Spears; 29 August 2001	NHMD-265539/ DCR-393/ZS 084	-	-	-	-	AF144215	
<i>Lynceus gracilicornis</i>	USA, Georgia: Early County, TNC Shackleford-William's Bluff Preserve, The Nature Conservatory (TNC); J. Jensen & T. Floyd; 21 Marts 2003	NHMD-265540/ DCR-516	-	-	-	-	-	X

(Continues)

TABLE 1 (Continued)

Species	Locality, collector and year	Museum & ID	A2Q	A16	16S	COI	18S	Morphology
<i>Lynceus gracilicornis</i>	USA, Georgia: Baker County, George Sand Pond, Ichauway Plantation; J. Jensen; 20 Marts 2003	NHMD-265541/ DCR-521	-	-	-	-	-	X
<i>Lynceus grossipedia</i> (Sigvardt et al., 2020)	Mongolia: Tuv Province, Uguu nuur (Lake), 47.673917°N, 108.356333°E, altitude 1,307 m a.s.l.; M. Alonso; 24 August 2017	NHMD-616086/ZS 056	MZ463686	MZ463701	MZ539590	MNS15429	MZ539575	X
<i>Lynceus grossipedia</i>	China: Jilin Province, Qianguo County, Songyuan City, Chaghanhua Town, DongPao Lake; 124° 15.636', 44° 36.177'; S. Shu; 14 September 2017	NHMD-615880/ZS 061	-	-	-	-	-	X
<i>Lynceus huentelauquensis</i> (Sigvardt et al., 2019)	Chile: Coquimbo province, pool on the Huentelauquén Plains near Huentelauquén City; 31°35'19.9"S, 71°30'59.6"W; J. Pizarro-Araya; 16 October 2015	NHMD-265547/ZS 021	MZ463687	MZ463702	MK356565	MZ539938	MZ539576	X
<i>Lynceus huentelauquensis</i>	Chile: Coquimbo province, pool on the Huentelauquén Plains near Huentelauquén City; J. Pizarro-Araya; 6–8 January 2016	NHMD-265550	-	-	-	-	-	X
<i>Lynceus insularis</i> (Olesen et al., 2016)	New Caledonia: Yate, temporary watershed, 22.263238, 166.952401; C. Pöllbauer; 1 May 2018	NHMD-615886/ZS 060	-	-	-	-	-	X
<i>Lynceus insularis</i>	New Caledonia: South Province, Mont-Dore, doline (limestone sinkhole); 22°19'32.38"S, 166°54'07.26"E; C. Pöllbauer; 14 May 2010	NHMD-82633/ ZMUC-CRU-4787	MZ463688	-	MZ539591	MZ539939	MZ539577	

(Continues)

TABLE 1 (Continued)

Species	Locality, collector and year	Museum & ID	A2Q	A16	16S	CO1	18S	Morphology
<i>Lynceus insularis</i>	New Caledonia: South Province, Mont-Dore, doline (limestone sinkhole); 22°19'32.38"S, 166°54'7.26"E; C. Pöllbauer; 14 May 2010	NHMD-82632/ ZMUC-CRU-4786	-	-	-	-	-	X
<i>Lynceus macleayanus</i> (King, 1855)	Australia, NSW: Bloodwood, Sue's Pan; D.C. Rogers; 26 July 2015	NHMD-232861/ DCR-916/ZS 008	MZ463689	MZ463703	MK356566	MNS15430	MZ539578	X
<i>Lynceus macleayanus</i>	Australia: NSW: northwest, Marsilea Pan, Bloodwood Station, Paroo; B.V. Timms; 13 May 2016	NHMD-615887/ZS 080	-	-	MZ539592	-	-	-
<i>Lynceus magdaleanae</i> (Timms, 2013)	Australia, SA: Peela Rock, Pit 1 via Wudinna; B.V. Timms; 13 May 2016	NHMD-232860/ BRP4/CS90	MZ463690	MZ463704	MK356567	MNS15431	MZ539579	-
<i>Lynceus magdaleanae</i>	Australia, WA: Yanneymooning Rock pit gnamma via Mukinbuden WA; B.V. Timms; 18 May 2016	NHMD-615928	-	-	-	-	-	X
<i>Lynceus planifasciatus</i> (Rogers et al., 2016)	Thailand: Khon Kaen, Mueang Khon Kaen District, Don Han; 16°18'45.88"N, 102°52'31.37"E; D.C. Rogers & L. Sanoamuang; 19 June 2015	NHMD-615849/ DCR-889/ZS 004	-	-	MZ539594	-	-	-
<i>Lynceus susanneae</i> (Timms, 2013)	Australia, WA: Cocklebidy gnamma Nullarbor Plain; B.V. Timms; 16 May 2016	NHMD-615888/ZS 003	MZ463692	-	MZ539595	MNS15432	MZ539581	X
<i>Lynceus tatei</i> (Brady, 1886)	Australia, WA: large central pool, Wanarra Rock, via Perenjori, 50 km ESE of 29°31'23.5"S, 116°47'38.1"E; B.V. Timms; 20 August 2011	WAM C51608/ZS 010	MZ463693	MZ463706	MK356568	MNS15433	MZ539582	-

(Continues)

TABLE 1 (Continued)

Species	Locality, collector and year	Museum & ID	A2Q	A16	16S	COI	18S	Morphology
<i>Lynceus tatei</i>	Australia, WA: Ngopitchup Swamp via Kojonup; MZ?; 19 October 2010	WAM C51610	-	-	-	-	-	X
<i>Lynceus trinagularis</i> (Barnard, 1929)*	South Africa, Eastern Cape Province, 6 km East of King William's Town; 32°53.71'15" S 27°18.873'E; Musa Mlambo; 8 November 2017	NHMD-XXXX	MZ463691	MZ463705	MZ539593	MZ539940	MZ539580	X
<i>Lynceus</i> sp.	Australia, Queensland: big flat temporary wetland ca. 140 km N of Hughenden; 19°51'03.4"S, 144°16'08.5"E; B.V. Timms; 12 April 2018	NHMD-615894/ZS 074	-	MZ463707	MZ539596	MZ539941	MZ539583	X
<i>Cyclohextheria hislopi</i> (Baird, 1859)	Thailand: Pond outside the University building, Mahasarakham University, Talat, Mueang Maha Sarakham District; J. Olesen, Z.M.S. Sigvardt & S. Savatnalinton; 2 November 2016	NHMD-615897/ BRP1/CS87	MZ463680	MZ463694	MZ539584	MZ539935	MZ539569	
<i>Daphnia pulex</i> (Leydig, 1860)	BioSample: SAMN02744063	GenBank	GCA_000187875	GCA_000187875	AF117817	AF117817	AF014011	
<i>Eulimnadia texana</i> (Packard, 1871)	BioSample: SAMN05965515	GenBank	GCA_002872375	GCA_002872375	GCA_002872375	GCA_002872375	GCA_002872375	
<i>Triops cancrivormis</i> (Bosc, 1801)	Japan	GenBank	GCA_000981345	GCA_000981345	AB084514	AB084514	EF189638	

Note: A dash (-), indicates missing sequence

BMNH, British Museum (Natural History), London, United Kingdom
 NHMD, Natural History Museum of Denmark, University of Copenhagen
 WAM, Western Australian Museum, Australia
 ZS/BRP/CS, molecular extraction numbers.

GenBank Accession numbers are given for each individual and molecular locus. Sequences in bold are generated for this study. Samples used for scanning electron microscopy (rostrum morphology and for Figure 1) are indicated in the last column (always another specimen than used for DNA extraction, in some cases another population).

*Specimens similar to both *L. lobatsianus* Barnard, 1929 and *L. trinagularis* Daday, 1927, but sampling site closest to the locality of compared *L. cf. lobatsianus* (BMNH 1972.1.27.9-28). However, *L. lobatsianus* is poorly described and African *Laevicauda* data are in general in need of revision.

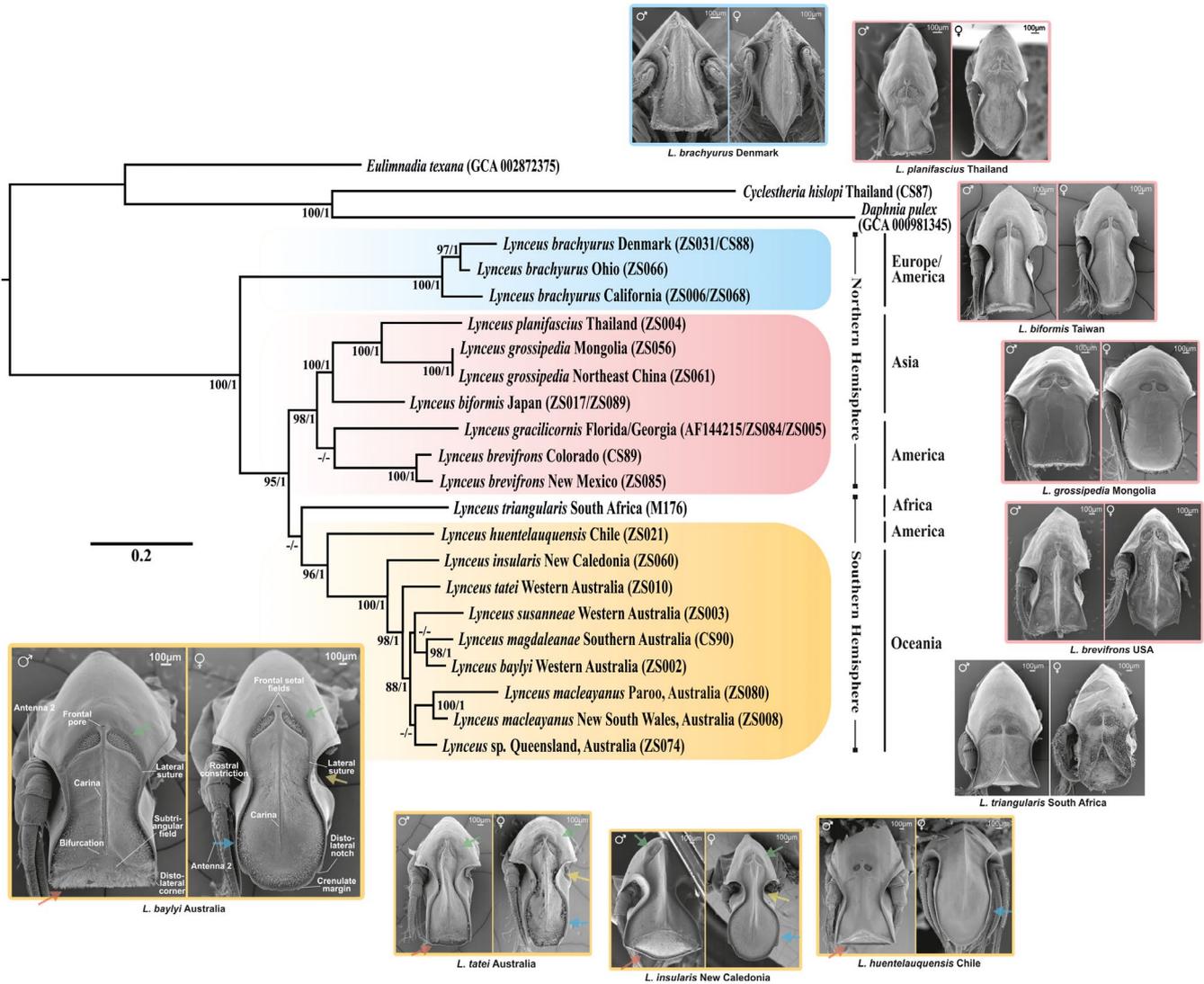


FIGURE 2 Maximum Likelihood phylogeny of laevicaudatan species. Bootstrap support values above 70% (before slash) and Bayesian Posterior Probabilities above 95% (after slash) are shown for each node. Geographical occurrence as well as information (SEM images) on male and female rostrum morphology are shown (see *L. baylyi* for applied terminology). Coloured arrows point at clade supporting rostral morphology (see Discussion)

Onychocaudata (=non-Laevicaudata diplostracans) clades, following Raymond (1946) and Wolfe et al. (2016), respectively. Lognormal prior distributions (mean in real time \pm standard deviation) were used to time-calibrate the crown-group Cladocera (250 \pm 50 Mya) and Onychocaudata (390 \pm 50 Mya). Four independent Markov chains were run in BEAST for 100 million generations, sampling every 10,000th generation. The lower and upper bounds of the 95% highest posterior density (HPD) interval were obtained for every node using the software TreeAnnotator and FigTree (Figure 3).

2.5 | Morphological analyses

For most species included in the molecular analyses, key characters of male and female rostrum were examined with

scanning electron microscopy (SEM). Specimens were dissected to expose relevant structures (left antennae often dissected off). Samples were dehydrated in a graded ethanol series, critical point dried, mounted on stubs, coated with metal and examined in a JEOL JSM-6335-F (FE). SEM images of *Lynceus baylyi* are presented at larger size with applied terms of rostrum morphology (Figure 2).

3 | RESULTS

3.1 | DNA extraction and PCR analyses

A significant amount of museum material (e.g. from Africa, India and America) was tested to cover underrepresented regions as well as taxa for which fresh material could not

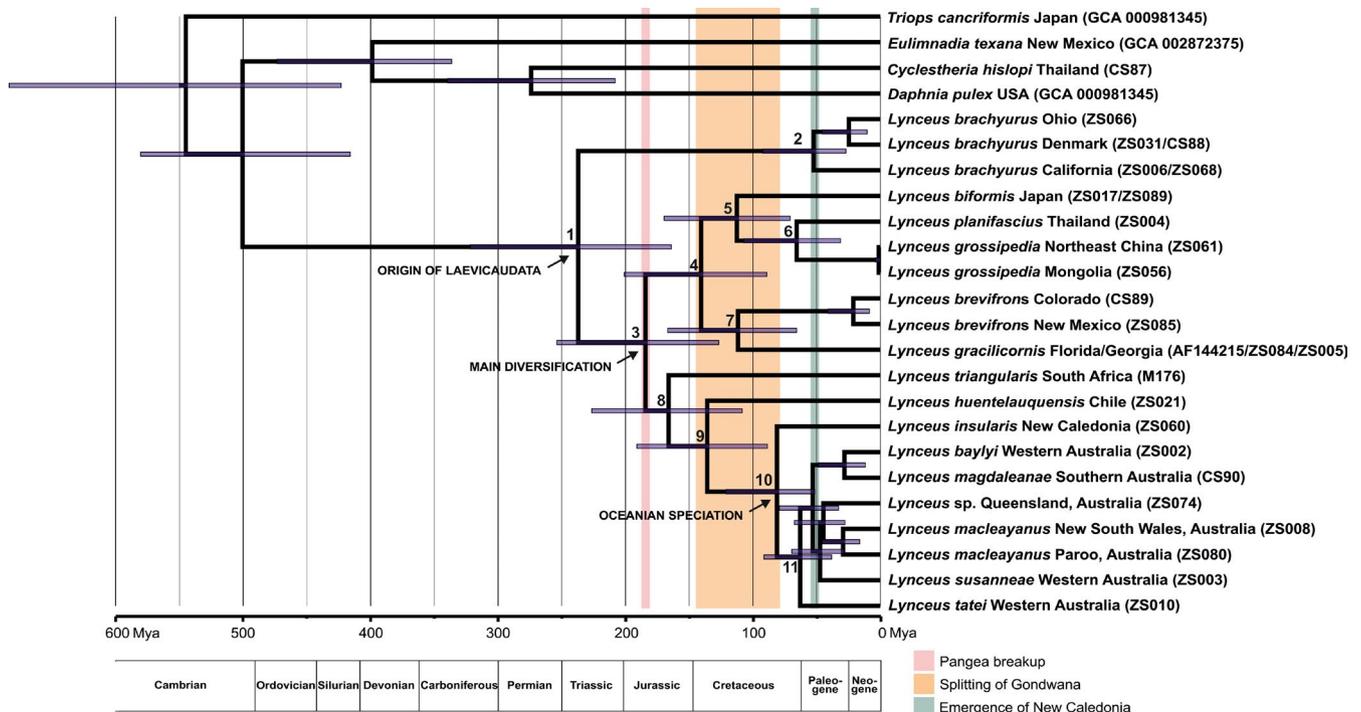


FIGURE 3 Divergence time estimates based on relaxed molecular clock dating. The nodes corresponding to the origin of the Onychocaudata (=non-Laevicaudata diplostracans) and the Cladoceromorpha (Cladocera + Cyclestherida) were time-calibrated following Raymond (1946) and Wolfe et al. (2016)

be obtained (especially *Paralimnetis* and *Lynceiopsis*). Several attempts were carried out to sequence ancient material both using NGS and Sanger sequencing without success and, to avoid future exploitation of unique museum specimens, details on tested vouchers are included in Table S2. Museum material showed very low DNA content after extraction (as checked with agarose gel and/or Qubit), despite the use of different protocols (see Materials and Methods). Samples being more than 100 years old were processed in the ancient DNA lab in Copenhagen but gave no positive results (see Table S2). As could be expected, the highest success rate was obtained with fresh material (collected after 2001) stored in high concentration ethanol ($\geq 96\%$) and cold (fridge or freezer). Proteinase K following Gilbert et al. (2007) and the DNeasy kit (QIAGEN) had the highest success among the different extraction methods (see Materials and Methods).

3.2 | Phylogenetic analyses

Final length for each gene alignment after running Gblocks was: 360 bp (89% of 401 bp) for 16S, 1726 bp (75% of 2287 bp) for 18S, 656 bp (87% of 750 bp) for EOG090X03WO, 625 bp (64% of 973 bp) for EOG090X090D, 532 bp (23% of 2217 bp) for EOG090X0A16, 561 bp (53% of 1046 bp) for EOG090X0A2Q, 669 bp (70% of 945 bp) for EOG090X0BKI, 1532 bp (97% of 1565 bp) for COXI, 263 bp (100% of

263 bp) for H3. Therefore, the concatenated alignment including all 9 molecular markers comprised a total of 6,924 bp (66% of 10,447 bp). The strong reduction in alignment length observed for EOG090X0A16 was due to the presence of a unique and long (~1 kB) intron sequence in the *Triops cancriformis* assembly from Genbank. Model selection results using AICc were TVM + I + G (lnL = -2680.33), TrN + I (lnL = -3902.57), GTR + I + G (lnL = -4937.32), GTR + G (lnL = -5746.41), GTR + G (lnL = -4932.63), TVM + I + G (lnL = -3681.01), TIM + I + G (lnL = -4963.90), TIM + I + G (lnL = -10680.76) and HKY + G (lnL = -1463.80), respectively.

Every *Lynceus* species for which more than one population was included in the analyses (i.e. *Lynceus brachyurus*, *L. brevifrons* and *L. macleayanus*) formed a well-supported monophyletic clade in both maximum likelihood and Bayesian analyses (Figure 2). A clade including three *L. brachyurus* populations from Europe and North America (USA) was strongly supported and sister to all the remaining Laevicaudata samples. Within this *L. brachyurus* clade, the population from Ohio (Eastern USA) seemed to be closer to the European (Denmark) than to the Californian (Western USA) population, but bootstrap support was only marginally significant (71%) and the clade was not recovered using Bayesian inference. Genetic divergence levels among different *L. brachyurus* populations suggest that they might in fact represent a complex of cryptic species. Sequences of *L. brachyurus* from all three localities revealed high

divergences when using the conservative 16S rDNA gene (K2P values ranging between 0.059 and 0.077); and the COXI divergence observed between samples from Denmark and California ($K2P = 0.181 \pm 0.019$) is well above the inter-species genetic threshold observed in clam shrimps and other crustaceans. The remaining *Lynceus* were divided in a Southern Hemisphere clade (including representatives from Africa, Chile, Australia, and New Caledonia) and a Northern Hemisphere clade that comprised a North American subclade (*L. brevifrons* and *L. gracilicornis*) with low bootstrap support and a well-supported Asian clade that included species from Japan (*L. biformis*), Thailand (*L. planifascius*), and a recently described species from Mongolia/North China (*L. grossipedia*) (Figure 2). Within the Asian clade, the Japanese lineage (*L. biformis*) seems to have diverged first, whereas the material from Thailand (*L. planifascius*) grouped together with the Mongolian/Chinese species. In the Southern Hemisphere clade, *L. triangularis* (Africa) diverged first but with low bootstrap support, *L. huentelauquensis* from South America (Chile) grouped with a clade including *L. insularis* (New Caledonia) and all the Australian species. Further well-supported groups were found within the Australian clade, with *L. tatei* splitting early from all other Australian taxa (bootstrap 82%). *Lynceus macleayanus* appeared as sister to a putative new *Lynceus* species from North Queensland (see Discussion), while the well-supported *L. magdaleanae/L. baylyi* clade (bootstrap 100%) grouped with *L. susanneae*, but with low bootstrap support (Figure 2).

3.2.1 | Divergence time estimation

The results obtained from the relaxed molecular clock analysis (Figure 3) support an early divergence within *Lynceus* of the lineage leading to Northern Hemisphere *L. brachyurus*, which separated from the rest as early as ~237 Mya (95% HPD = 164–321 Mya) (node 1). The split between the second Northern clade (North American and East Asian species) (node 4) and the Southern Hemisphere clade (node 8) would have occurred around 184 Mya (95% HPD = 126–254 Mya) (node 3). Within this Northern Hemisphere clade, with a most-recent common ancestor (MRCA) placed about 140 Mya (95% HPD = 89–200 Mya) (node 4), the *L. biformis* lineage would have split from other Asian taxa about 112 Mya (95% HPD = 71–169 Mya) (node 5), while the Thailand and the Mongolia/China species would have diverged about 65 Mya (95% HPD = 31–107 Mya) (node 6). The earliest divergence within the Southern Hemisphere clade, giving rise to the South African *L. triangularis* is dated to 166 Mya (95% HPD = 108–226 Mya) (node 8), while the split of the South American *L. huentelauquensis* is dated 135 Mya (95% HPD = 88–191 Mya) (node 9). The Australia and New Caledonia species split 81 Mya (95% HPD = 51–121 Mya)

(node 10), and the MRCA of all the Australian species included in the analyses would be placed around 62 Mya (95% HPD = 38–91 Mya) (node 11).

3.3 | Rostral morphology of included species

The rostrum morphology of most species/populations included in the molecular analyses was studied (not all depicted in this work) and briefly described below (Figure 2). Most studied specimens had their left second antenna dissected off for detailed studies.

AUSTRALIA. *Lynceus baylyi* (Figure 2). Male: rostrum apex truncate; rostral carina bifurcating distally towards distolateral corners, apically forming a broad, subtriangular field covered with dense setation. Female: rostrum broadly rounded with distolateral notches; margin between notches crenulate; fine setation concentrated along margin. Male and female: frontal setal fields characteristically ovate, broadest medially tapering laterally extending into lateral sutures which are directed obliquely towards rostral apex. *Lynceus tatei* (Figure 2). Rostrum generally similar to *Lynceus baylyi* but elongate and strongly constricted at point of second antennal insertion; carina very prominent; lateral sutures oriented vertically. Female: rostrum rounded but more quadratic of shape; margin between distolateral notches subcrenulate. Morphology of other Australian species can be summarized as follows: Rostrum in *Lynceus magdaleanae* in general very similar to *Lynceus baylyi* but carina often broader (variable); Rostrum in *Lynceus macleayanus* generally similar to *Lynceus baylyi* except female distal part more narrowly rounded and margin between distolateral notches subcrenulate; Rostrum in *Lynceus susanneae* generally similar to *Lynceus baylyi* but rostral carina only weakly developed (females) or even absent (males), in female distal margin subcrenulate with distolateral notches lacking; Rostrum in *Lynceus* sp. (North Queensland) very similar to *L. magdaleanae* but differs in female rostrum being less broadly rounded, with margin strongly crenulate.

NEW CALEDONIA. *Lynceus insularis* (Figure 2). Rostrum generally similar to *Lynceus baylyi* but in both male and female markedly constricted in the region of second antennal insertion, making apex appear very broad; setal fields smaller and less ovate.

AFRICA. *Lynceus triangularis* (Figure 2). Male: rostral apex strongly truncate and abbreviated; carina short extending from frontal pore and bifurcating approx. halfway towards distal margin, forming deep invaginated subtriangular setose field apically; distolateral corners prominent. Female: carina bifurcating forming a broad field similar to the male but distal margin broadly rounded; distolateral notches and crenulation/denticulation absent. Male and female: frontal setal fields subcircular to slightly ovate laterally; lateral sutures obliquely oriented.

CHILE. *Lynceus huentelauquensis* (Figure 2). Rostrum similar to *Lynceus baylyi* but male with more 'triangular' rostral shape and rostral fornice extending obliquely in straight line to distolateral corners (in anterior view); carina partly weakly developed and distal margin subcrenulate. Female distal surface without setation. Male and female frontal setal fields markedly small with straight upper margins; lateral sutures placed below setal fields (not in contact), horizontally oriented.

ASIA. *Lynceus bififormis* (Japan) (Figure 2). Male: rostral apex truncate; rostral carina bifurcating towards distolateral corners forming relatively narrow subtriangular field distally; bifurcating extensions of carina and upper 2/3 of subtriangular field densely setose. Female: rostral apex rounded and greatly denticulate, distolateral notches absent. Male and female: frontal setal fields subcircular to slightly ovate; lateral sutures extending vertically from lateral part of setal fields (in contact). *Lynceus planifascius* (Thailand) (Figure 2). Male: rostrum generally like *L. bififormis* but subtriangular field narrower, setation restricted to rim on bifurcating extensions of carina. Female: rostrum narrowly rounded, distally with folding margins making it appear subtriangular (SEM artefact?), narrow distal part subdenticulate. Male and female: frontal setal fields subcircular; lateral sutures extending obliquely below setal fields (not in contact); rostrum in lateral view with characteristically flattened anterior margin (Rogers et al., 2016). *Lynceus grossipedia* (Mongolia/China) (Figure 2). Description based on Mongolian material. Male: rostrum terminating abruptly distally (no subtriangular field), with setal rim along margin. Female: rostrum broadly rounded, distal margin denticulate. Male and female: rostrum markedly broad (anterior view), with indistinct marginal constriction where second antenna insert; double carina bifurcating between frontal setal fields, running in parallel 2/3 of rostrum length, diverging in distal 1/3 of rostrum; frontal setal fields subcircular; lateral sutures extending almost horizontally below setal fields (not in contact).

EUROPE. *Lynceus brachyurus* (Denmark) (Figure 2). Male: rostrum truncate with carina non-bifurcating along entire midline; distal margin with setal row. Female: apex with three prominent protrusions, two sharp distolateral notches and one long medial spine. Male and female: rostrum strongly constricted in region of second antennae, with pronounced carina extending from proximal (cervical suture) to distal (apex); frontal setal fields distinctly oval (four-five times longer than broad), oriented vertically; rim of fine setae laterally on each side of rostrum.

USA. *Lynceus brevifrons* (Figure 2). Male: rostral apex with very narrow subtriangular field, setal row on branches of bifurcation of carina. Female: trilobed rostral apex, medial spine less elongate than in *L. brachyurus*. Male and female: frontal setal fields subcircular, lateral sutures extending obliquely from near setal fields. *Lynceus gracilicornis* (not

depicted). Male: rostrum truncate with carina non-bifurcating along entire midline, no setation present. Female: apex narrowly rounded with folding margins, apparently subtriangular. Male and female: frontal setal fields broadly oval; lateral sutures extending vertically from lateral part of setal fields (in contact).

4 | DISCUSSION

4.1 | Pangea, extreme climate and the origin of Laevicaudata

The first comprehensive molecular phylogeny for laevicaudatan clam shrimps, based on nine loci and including 15 *Lynceus* species sampled worldwide, is presented here, and assessed in the light of new rostrum morphology data. The absence of a well-resolved phylogeny has prevented scientists from addressing key evolutionary and biogeographic questions for laevicaudatans until now. Our results show that the main Laevicaudata clades are strongly correlated with geography (i.e. continents) and suggest vicariance due to continental drift as the dominant explanatory factor for the macroevolution of smooth clam shrimps. Relaxed molecular clock estimates indicate that current Laevicaudata lineages appeared about 237 Mya (95% HPD = 164–321 Mya), which is congruent with the existence time of Pangea, a mostly dry supercontinent with seasonal rainfalls (Parrish, 1993) probably favourable for branchiopods with diapausing eggs as part of their lifecycle (Gueriau et al., 2016). Furthermore, the MRCA of present-day Laevicaudata is estimated to have occurred after the Permian–Triassic mass extinction (about 250 Mya). This dating is congruent with the known fossil record of laevicaudatans, which, according to Hegna and Astrop (2020) possibly reaches from the Middle Permian to the Jurassic and Cretaceous. Interestingly, the timing of laevicaudatan origin appears to be correlated with a fast increase in aridity and global temperature (Sahney & Benton, 2008; Smith & Botha-Brink, 2014), which may have led to an increase in niche availability and a reduction in number of predators and competitors allowing laevicaudatan populations to expand.

4.2 | Main Laevicaudata clades: Vicariance and allopatry

The northernmost clade (*L. brachyurus*) diverged early from the remaining *Lynceus*, which further split into three main lineages: one clade with species from North America and Asia, another including the South African *L. triangularis*, and the last one formed by all the remaining species. The splitting of those three lineages is estimated to have occurred 184 Mya (95% HPD = 126–254 Mya), which aligns with

the onset of Pangea breakup. The first lineage comprises an Asian clade (including species from Japan, Thailand, China and Mongolia) and a weakly supported North American clade (including *L. brevifrons* and *L. gracilicornis*). High support for a Southern Hemisphere clade, including species from South America and Oceania, and our molecular clock estimates suggest a Gondwanaland origin of the clade. This biogeographic pattern agrees with those previously observed in freshwater crayfish or plants and could be explained by an Antarctic land bridge connecting Australia and South America (Sanmartin & Ronquist, 2004; Toon et al., 2010).

The Australian species clusters with the New Caledonian species (*L. insularis*) and the splitting into two separate lineages is estimated to have occurred about 81 Mya (95% HPD = 51–121 Mya). Interestingly, the dating of this split coincides with the timing for a separation between Australia and the ancient microcontinent Zealandia (e.g. New Caledonia and New Zealand) (Hedges, 2018). However, the geological history of Zealandia is controversial. There is evidence for marine flooding of New Caledonia in the Late Cretaceous (65 Mya) to late Eocene (~45 Mya) (Hedges, 2018; Pelletier, 2007), which would favour later dispersal (e.g. by resting eggs) as the explanation for the occurrence of *Lynceus* at these remote islands. On the contrary, flora composition suggest that some parts of New Caledonia have remained exposed (He et al., 2016), thereby serving as a terrestrial refugium. Discordances on the complete Eocene drowning of New Caledonia are not negligible (Giribet & Baker, 2019), and vicariance could explain the disjunct distribution of the Australian/New Caledonian *Lynceus* clade.

4.3 | Concordance between rostral morphology and molecular phylogeny

Rostral morphology characters are congruent with some of the clades outlined above. The early divergence of *L. brachyurus* from the remaining laevicaudatans agrees with its unique carina, which extends all the way from the cervical suture to the rostral apex (most pronounced in females), like that of *Cyclestheria hislopi*, and is possibly plesiomorphic.

The Southern Hemisphere species, which are strongly supported by molecular data (except *L. triangularis*), are also supported by rostrum morphology. In practically all males, the rostral apex is adapted for a tight fit to the female carapace during mating (Sigvardt & Olesen, 2014), and in the Southern Hemisphere species, this distal rostral area is modified further into a large subtriangular field, probably providing even firmer support (orange arrows in Figure 2). The male of the African *L. triangularis* also has a modified rostral apex, but different from the other Southern Hemisphere species, which is in accordance with only weak molecular support for the clustering of these species. Also, the Southern

Hemisphere species (again except *L. triangularis*) are supported by the female rostrum being long and broadly rounded (blue arrows in Figure 2).

The Oceania clade (Australia and New Caledonia) received strong molecular support which is congruent with rostrum morphology. The rostral similarities for this clade include a strong constriction at the point where the second antennae insert (yellow arrows in Figure 2), and markedly obliquely orientated frontal setal fields (green arrows in Figure 2), in contrast to the circular or oval shape of these in all other examined species. Despite partly holding a phylogenetic pattern, rostral morphology shows much evolutionary plasticity, and clearly needs to be evaluated in a larger morphological context including for example male claspers and other characters.

4.4 | Paleogene diversification and alternatives to continental drift

Laevicaudata speciation events occurring after the onset of the Paleogene (65 Mya) cannot be simply attributed to vicariance, mainly because Pangea had already completed its breakup into the present-day continents by the end of the Mesozoic. Recent studies point out that dispersal through bird- or wind-facilitated transport of resting/diapause eggs could determine branchiopod distribution (Fryer, 1996; Rogers, 2014), particularly at shorter geographic distances as indicated for spinicaudatan clam shrimps (Schwentner et al., 2012). Dispersal of resting eggs by birds could have played an important role for laevicaudatan distribution and speciation patterns and, interestingly, radiation of modern birds also occurred after the onset of the Paleogene (James, 2005). Phylogeography of Eurasian species is probably related to more complicated processes occurring on the Northern Hemisphere, such as temporary connections between landmasses (Brikiatis, 2016) possibly transported by large land mammals (Rogers et al., 2021). Genetic divergence levels between North American and European *L. brachyurus* are like those observed among well-established *Lynceus* species from Australia, which suggests the presence of cryptic species and agrees with the remarkable wide distribution and large intraspecific variation observed in *L. brachyurus* (Daday, 1927). However, a detailed morphological revision of further *L. brachyurus* samples, supplemented by population genetic analyses, should be conducted to determine the origin and validity of these putative cryptic taxa.

5 | CONCLUSIONS AND OUTLOOK

Our phylogenetic results have a direct impact on Laevicaudata evolutionary biology and systematics, but further DNA

efforts should focus on the American *Paralimnetis* and the African *Lynceiopsis*. This will allow for testing the reciprocal monophyly of the laevicaudatan genera and the relation of these rare genera to the more diverse and widespread *Lynceus*. Likewise, more samples from a wider geographic region will be needed to test the robustness of the North American and Asian clade, particularly samples from India, Taiwan and South China. Finally, future research on laevicaudatan systematics will clearly benefit from using non-rostral characters (e.g. male claspers, female laminae abdominalis), which are likely to hold important phylogenetic information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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