Zoological Studies

Molecular Evaluation of the Fairy Shrimp Family Branchinectidae (Crustacea: Anostraca) Supports Peripatric Speciation and Complex Divergence Patterns

D. Christopher Rogers[1],* and Andres Aguilar[2]

[1]*Kansas Biological Survey, Kansas University, Higuchi Hall, 2101 Constant Avenue, Lawrence, KS 66047-3759 USA.*
  *Correspondence: E-mail: branchiopod@gmail.com (Rogers)
[2]*Department of Biological Sciences, California State University, 5151 State University Drive Los Angeles, CA 90032 USA.*
  E-mail: aguil67@calstatela.edu (Aguilar)

The Branchinectidae is a diverse and widely distributed group of anostracans. The majority of work on the group has focused on the morphological delineation of taxa and biogeography. Here we present a molecular phylogeny for select members of the family to better understand the distribution of morphological variation among species, and test biogeographic models of speciation for the group. Although we conducted both molecular and morphological phylogenies for the Branchinectidae, the morphological analysis did not support our molecular phylogeny and it did not support previous species group concepts based on geography. Our molecular phylogenetic analysis suggests that the family may have originated in Eurasia and found support for numerous species groups. These phylogenetic groups assisted in delineating species groups that are all definable morphologically and/or ecologically. The peripatric speciation model was supported from our analysis, offering credence to previously published speciation models in anostracans. This suggests that these processes may be important in other Branchiopoda and should be rigorously evaluated when delineating species.

Key words: *Branchinecta*, *Archaebranchinecta*, Species groups, Phylogeny, Branchiopoda.

BACKGROUND

Anostracan crustaceans are among the least known of all faunal elements in seasonally astatic aquatic habitats, yet they are used as indicators of ephemeral wetland habitat health and functionality in the United States (Rogers 1998; Lang and Rogers 2002), Brazil (Rogers and Ferreira 2007) and Australia (M. Coleman & B. Datsun personal communication; B. Timms personal communication). For example, of the 26 anostracan species reported from California, only two were known prior to 1980, and eight were not described until after 1990. Large branchiopods have become flagship animals for ephemeral wetland habitat imperilment and are the subject of increasing conservation attention (Federal Register 1994; Eriksen and Belk 1999; Brendonck et al. 2008). Currently, five large branchiopod crustacean species are afforded protection under the federal Endangered Species Act, and other species are proposed for protective status. Documentation of the diversity of anostracan crustaceans and their associated habitats can identify trends in habitat and species loss relative to land use practices. Effective conservation management of anostracan crustaceans requires knowledge of their phylogeny, taxonomy, distribution and habitat requirements.

The Branchinectidae is comprised of two extant genera: *Archaebranchinecta*, with three species, and *Branchinecta*, with 51 species (Rogers 2013; Cohen et al 2019). The species of *Archaebranchinecta* are limited to the Americas (one fossil in North America, two extant in South America) whereas *Branchinecta* is distributed through the Holarctic and the Neotropic regions, with one species occurring in the northernmost Antarctic (Rogers 2013; Rogers and Coronel 2011). *Branchinecta* has two primary centres of diversity; one in western North America (29 species) and one in southern South America (16 species) (Rogers 2006), with the remaining six species in Eurasia. Of these 51 species, 31 species are narrow range endemics, 12 species are known from two or less localities each, and four species are protected under the USA Endangered Species Act (Eng et al. 1990; Fugate 1993; Belk and Fugate 2000; Rogers and Fugate 2001; Belk and Rogers 2002; Rogers 2006; Rogers et al. 2006 2011; Rogers and Lorenz 2015). The reason for the high western North American endemism is the complex geochemical and recent geological history of this region (Rogers 2014a 2015). Colonization of newly available habitats (*e.g.*, exposure of 'new' terrestrial environments via uplift or erosion) and/or novel environments (*e.g.*, saline versus alkaline pools) may be important for driving speciation in the Branchinectidae and allied anostracans (Rogers 2015). Additionally, *Branchinecta* is the most diverse anostracan genus in South America, further suggesting that this group is the prime system to study not only in situ continental divergence, but South American adaptive radiations.

The monophyly of Branchinectidae has been strongly supported by recent phylogenetic analyses (*e.g.*, Remigio and Hebert 2000; Weekers et al. 2002), but relationships within the family have not been clearly elucidated. Morphological diagnosis of anostracan species is generally straightforward as there is strong sexual selection by the females for large and showy male second antennae and associated structures. The most reliable characters used to delineate anostracan species are the form of the male second antennae (Lynch 1972). Other characters, such as the form and/or ornamentation of the female head, dorsum, and brood pouch, the structure and shape of the male gonopods, and the external morphology of the eggs are of limited use at the species level (Lynch 1972; Rogers and Fugate 2001; Belk and Rogers 2002; Rogers 2002 2006; Rogers et al. 2006). These features may display a large amount of homoplasy and be inappropriate for determining phylogenetic relationships among anostracan groups.

In recent molecular phylogenetic studies of the Australian *Branchinella* (Remigio et al. 2003; Pinceel et al. 2013), it was found that only one of the morphologically defined groups described by Geddes (1981) was monophyletic, and all others were polyphyletic. Similarly, the morphological features described by Hamer et al. (1994a b) and Maeda-Martinez et al. (1995) proved to be homoplastic based on the molecular phylogeny of *Streptocephalus* (Daniels et al. 2004). A detailed analysis of a single species complex (*Branchinella longirostris* Wolf, 1911) found no relationship between frontal appendage morphology and molecular relatedness (Zofkova and Timms 2009). These studies indicate that the morphological features commonly used to differentiate the various anostracan species may not be that reliable for determining evolutionary relationships, although other characters may exist that will provide more information than previously thought. Therefore, independent molecular phylogenetic analysis is warranted to establish closely related subgeneric groups and serve as a scaffold by which to better understand anostracan species level morphological differentiation.

We initiated this study to gain a better understanding of the evolutionary relationships among members of the Branchinectidae. Our initial goal was to evaluate if morphologically similar species within the family are in fact closely related from a molecular phylogenetic perspective. We simultaneously investigated the phylogenetic utility of a suite of morphological features, alone and in combination with molecular data. The phylogenetic hypotheses generated from molecular and morphological data were then used to assess biogeographic hypotheses for the group and assess the systematics of the family.

MATERIALS AND METHODS

Specimen Collection

We examined the morphology of all 54 branchinectid species. Character states of seven species were only available via the literature; however, the remainder were examined directly. We examined 1,727 male and 1,801 female branchinectid fairy shrimp from both described genera (3,528 individuals total). The specific collecting data of the material used in this study are presented in appendix 1. Thirty specimens from 29 species were sequenced for the molecular study.

Specimens were either adults preserved in 95% ethyl alcohol or reared from eggs in the laboratory. For each population that was reared from eggs, we collected soil from natural, dried field sites. We made soil collections by sampling at many locations across each dried habitat and then homogenizing the soil in plastic bags. Approximately 500 mL (volume) of

this field-collected soil was placed in the bottom of a 37 L aquarium and hydrated with deionized water. The aquarium was maintained at 25–28°C, with gentle aeration, 12 hours light/dark cycle, and fed a mixture of brewer's yeast and ground vegetable flake fish food.

Laboratory Analysis

Preserved specimens were examined using a Wild M8 dissection stereomicroscope. Some preserved specimens have been in storage over 10 years, making amplification of large fragments via the polymerase chain reaction (PCR) difficult. Members from the Chirocephalidae (*Eubranchipus oregonus* and *Linderiella occidentalis* – Table 1) were used as outgroup taxa, following previous phylogenetic analyses (Remigio and Hebert 2000; Weekers et al. 2002). DNA was extracted from individual samples with a modified CTAB extraction protocol for anostracans (Aguilar 2011). Samples were macerated in 2x CTAB buffer (Teknova) and 10 μL of Proteinase K (10 mg/mL) and allowed to incubate overnight at 60°C. Following incubation samples were extracted once with Phenol-Chloroform-Isoamyl alcohol (25:24:1) and once with Chloroform-Isoamyl Alcohol (24:1). DNA was then precipitated overnight in ethanol (with 3 μL of 3 M NaOH) at -20°C. DNA was pelleted via centrifugation, washed once with 70% ethanol, and allowed to air dry. The DNA pellet was resuspended in 100 μL on sterile ddH$_2$O. All DNA extractions were quantified on a Nanodrop 1000 spectrophotometer.

We targeted three genes for phylogenetic evaluation: two mitochondrial (16S and 12S) and a single nuclear locus (28S). Primer sequences and sources are listed in table 2. All reactions were conducted in 30 μL volumes with 1x PCR Buffer II

Table 1. Specimens used in phylogenetic analysis and corresponding GenBank Accession Numbers

Species	Sample location	16S	12S	28S
Ingroup taxa				
B. campestris	Carrizo Plains, CA, USA	MT010634	MT010665	MT010695
B. coloradensis	Ada, Idaho, USA	MT010635	MT010666	MT010696
B. conservatio	Solano, CA, USA	MT010635	MT010667	MT010697
B. constricta	Fremont, WY, USA	MT010637	MT010668	MT010698
B. cornigera	Grant, WA, USA	MT010638	MT010669	MT010699
B. dissimilis	Modoc, CA, USA	MT010638	MT010670	MT010700
B. ferox	Jordan	MT010640	MT010671	MT010701
B. gaini	Antarctic Peninsula	MT010641	MT010672	MT010702
B. gigas	Washoe, NV, USA	MT010642	MT010673	MT010703
B. granulosa	Chile	MT010643	MT010674	MT010704
B. hiberna	Humboldt, NV, USA	MT010644	MT010675	MT010705
B. lateralis	Wyoming, USA	MT010647	MT010678	MT010708
B. lindahli-1	Mineral, NV, USA	MT010648	MT010679	MT010709
B. lindahli-2	Lancaster, CA, USA	MT010649	MT010680	MT010710
B. longiantenna	Carrizo Plains, CA, USA	MT010650	MT010681	MT010711
B. lutulenta	Grant, WA, USA	MT010646	MT010677	MT010707
B. lynchi	Merced, CA, USA	MT010651	MT010682	MT010712
B. mackini	Washoe, NV, CA	MT010652	MT010683	MT010713
B. mediospinosa	Kansas, USA	MT010645	MT010676	MT010706
B. mesovallensis	San Joaquin, CA, USA	MT010653	MT010684	MT010714
B. oriena	Douglas, NV, USA	MT010654	MT010685	MT010715
B. orientalis	Turkey	MT010655	MT010686	MT010716
B. oterosanvicentei	Baja California, MX	MT010656	MT010687	MT010717
B. packardi	Cibola, NM, USA	MT010657	MT010688	MT010718
B. paludosa	Manitoba, CAN	MT010658	MT010689	MT010719
B. potassa	Nebraska, USA	MT010659	MT010690	MT010720
B. raptor	Elmore, ID, USA	MT010660	MT010691	MT010721
B. sandiegonensis	San Diego, CA, USA	MT010661	MT010692	MT010722
B. serrata	Fremont, WY, USA	MT010662	MT010693	MT010723
Outgroup taxa				
Eubranchipus oregonus	Siskiyou Co., CA, USA	MT010664	-	MT010725
Linderiella occidentalis	Merced, CA, USA	MT010663	MT010694	MT010724

(ABI), 2.0 mM MgCl$_2$, 0.08 mM of each dNTP, 0.4 μM of each primer and 1 unit of Amplitaq (ABI). Thermal cycling was performed on an ABI3700 thermocycler with the following temperature profile: initial denaturation for 5 min at 94°C followed by 30–35 cycles at 94°C for 30 s, primer specific annealing temperature (Table 2) for 30 s and an extension at 72°C for 45 s, followed by a 5 min extension at 72°C. PCR products were run out on a 2% agarose gel to check for amplification prior to sequencing. All PCR products were cleaned with the EXO-SAPIT kit (USB) and sequenced in both directions on an Applied Biosystems 3730 automated sequencer.

We were only able to acquire genetic samples from just over half (55%) of the extant species, as sampling in South America was limited.

Phylogenetic analysis

Sequence trace files were imported into SEQUENCHER (Genecodes Inc.), and inspected by eye for read quality. Consensus reads were constructed for each individual/locus combination and exported to MUSCLE (Edgar 2004) for alignment using default alignment parameters. Sequence evolution models were evaluated for each gene fragment and for the concatenated dataset with the Bayesian Information Criterion (Schwartz 1978) in jMODELTEST v1.10 (Posada 2008). A total of 51 morphological characters (section 4.1.) were coded for all species that we were able to obtain molecular data for, including outgroup taxa (matrix in Table S1).

MrBayes v3 (Ronquist and Huelsenbeck 2003) was used to reconstruct phylogenies in a Bayesian framework, and the dataset was partitioned based on the three gene fragments (12S, 16S, 28S) or the three gene fragments and the morphological data. We used the standard discrete model (Lewis 2001) with gamma rate variation for the morphological partition in the Bayesian analysis. Four simultaneous runs of six MCMC chains were run for a total of 5×10^6 generations, sampling trees every 100 generations for a total of 50,000 trees. The first 25% of trees (12,500) were discarded as burn in. A 50% consensus tree was estimated from the remaining sampled trees to better show the conflicts. Lastly, we performed a phylogenetic analysis of just the 51 coded morphological features. This analysis was done in PAUP4 (Swofford 1997) using a heuristic search and no character weighting. We ran 1,000 bootstrap replicates to assess node confidence.

List of morphological characters

1. Female second antenna (AII) medial surface [0] smooth; [1] medial spine; [2] lateral bulge; [3] medially chitinized.
2. Female AII length: [0] normal; [1] elongate.
3. Male AII extending to: [0] thoracic segment (TH) IV; [1] Th V; [2] Th VI; [3] Th VIII; [4] Th XII.
4. AII proximal vs distal antennomere length: [0] prox < dist; [1] prox = dist; [2] prox > dist.
5. Antennal appendages: [0] none; [1] anteriobasal.
6. Pulvilli: [0] absent; [1] present.
7. Apophyses: [0] flat; [1] cylindrical; [2] transverse; [3] conical.
8. Medial AII spines: [0] microdenticles only; [1] small scattered; [2] small, in rows; [3] generally distributed; [4] large, in discrete row(s); [5] on medial projection.
9. Anterior AII spines: [0] absent; [1] present.
10. Medial AII surface: [0] smooth; [1] convex; [2] ridged; [3] hooked.
11. Medial AII denticles: [0] microdenticles only; [1] generally distributed; [2] systematic field.
12. AII distal antennomere: [0] subcylindrical; [1] anteriorly flattened; [2] narrowly laterally flattened; [3] broadly laterally flattened; [4] triangular in cross section, smooth.
13. AII distal antennomere torsion: [0] none; [1] anteriorly.
14. AII Distal antennomere arc: [0] none; [1] present.
15. AII Distal antennomere arc: [0] proximal; [1] medial; [2] even; [3] distal.
16. AII apex: [0] subacute; [1] rounded; [2] anteriorly produced; [3] truncated.
17. AII subapical constriction: [0] absent; [1] present.

Table 2. PCR primers used in this study

Primer	Sequence (5' to 3')	Annealing temperature	Reference
16Sbr-3'	CCG GTT TGA ACT CAG ATC A	56°C	Palumbi 1996
16Sar-5'	CGC CTG TTT ATC AAA AAC AT	-	Palumbi 1996
Branch 12S-F1	AAG GAT TTG GCG GTY CTT AAA	52°C	This study
Branch 12S-R1	GAG CTT AAT TCA AAT TCT YAN TAT TTT	-	This study
Branch 28SF1	GGG TTA AAC GGA TGG ACC TT	56°C	This study
Branch 28SR1	CGC CTT CGG TCT TTA TCA AC	-	This study

18. AII apex form: [0] entire; [1] bilobed, anterior lobe small; [2] bilobed, posterior lobe small; [3] trilobed.
19. AII apex direction: [0] distally; [1] medially; [2] laterally; [3] posteriolaterally.
20. AII rasp: [0] absent; [1] present.
21. Body sensory papillae: [0] absent; [1] present.
22. Praepipodites: [0] two; [1] one.
23. Endopodites: [0] filtering; [1] female filtering, male scraping; [2] "basket" type; [3] raptorial.
24. Female thoracic dorsolateral projection surface: [0] smooth; [1] papillose; [2] denticulate.
25. Female TH I dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
26. Female TH II dorsal surface: [0] smooth; [1] Medial convexity; [2] Lateral convexity [3] Medial and lateral convexities.
27. Female TH III dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
28. Female TH IV dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
29. Female TH V dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
30. Female TH VI dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
31. Female TH VII dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
32. Female TH VIII dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
33. Female TH IX dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
34. Female TH X dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
35. Female TH XI dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
36. Female TH XII dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
37. Female TH XIII dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
38. Female TH XIV [genital segment] dorsal surface: [0] smooth; [1] medial convexity; [2] lateral convexity [3] medial and lateral convexities.
39. Female abdominal (AB) IV dorsolateral projections: [0] absent; [1] present.
40. Brood pouch: [0] fusiform; [1] pyriform; [2] penduculate; [3] cylindrical; [4] spherical
41. Brood pouch lateral projections: [0] absent; [1] present.
42. Brood pouch extends to: [0] AB I; [1] AB III; [2] AB IV; [3] AB V; [4] AB VI; [5] AB VII
43. Ovaries in thorax: [0] TH 12/13; [1] TH 11; [2] TH 10; [3] TH 9; [4] TH 8; [5] TH 7; [6] TH 6
44. Ovaries in abdomen: [0] none; [1] AB 1; [2] AB 2; [3] AB 3; [4] AB 4; [5] AB 5; [6] AB 6
45. Gonopod ventral projections: [0] none; [1] present.
46. Gonopod distal spined projections: [0] none; [1] one; [2] two.
47. Gonopods extend to: [0] AB I; [1] AB II; [2] AB III.
48. Testicular rami in: [0] abdomen; [1] abdomen and thorax.
49. Testis in abdomen extending to: [0] AB 2; [1] AB 3; [2] AB 4; [3] AB 6.
50. Egg surface: [0] rough; [1] small polygons; [2] medium polygons; [3] large polygons; [4] denticulate.
51. Polygon ridges on egg surface: [0] absent; [1] normal; [2] flanged; [3] spined; [4] obscure.

Test for peripatric speciation

We tested for peripatric speciation using the approach of Barraclough and Vogler (2000). Briefly species ranges were estimated using museum collection records and published accounts. We concentrated on North American species as our dataset is biased towards these taxa and the occurrence records are better for these species. They were imported into Google Maps to estimate areas. A pruned ultrametric tree was generated from the DNA only dataset using the penalized likelihood algorithm (Sanderson 2002; Kim and Sanderson 2008) implemented in the R package app (Paradise et al. 2004) and clade sequence divergence was estimated using tip to node distances from the ultrametric tree for each clade of interest. Range symmetry, estimated as the range size of the clade with the smaller size divided by the sum of the range sizes of each clade (Barraclough and Vogler 2000), was transformed (arcsin of double the value) and plotted against sequence divergence for each clade. Higher range symmetry values indicate more similar geographic size distributions (*e.g.*, the total area of the ranges is similar between taxa), while lower symmetry values indicate greater differences in geographic size distributions (*e.g.*, the total area of the ranges is dissimilar between taxa—small versus large ranges). Spearman's rank correlation was used to assess the significance of each relationship.

RESULTS

Phylogenetic analysis

DNA sequences for the three gene fragments were obtained for 28 *Branchinecta* species and the two outgroup species. The sequence evolution model estimation was based on the Bayesian Information Criterion (BIC) in jMODELTEST (Posada 2008). Analysis of each partition indicated the following models were appropriate for each gene fragment: 16S – HKY + I (p = 0.391); 12S – GTR + G (α = 0.296); 28S – GTR + G (α = 0.266).

The results of the molecular and molecular/ morphological data analysis gave highly congruent results that supported numerous groups within *Branchinecta* (Figs. 1, 2). Only posterior probabilities > 0.90 are reported as evidence for strong support. There was limited support for monophyletic groups of species: '*coloradensis*' group, '*cornigera*' subgroup,

'*gigas*' group, 'southern' group, '*lindahli*' group, '*paludosa*' group and '*sandiegonensis*' subgroup (Fig. 1). The '*lindahli*' group had low support in the molecules only analysis (0.82) and the '*packardi*' group had low support in the combined analysis (0.87). The current phylogenetic analyses could not resolve many of the interior branches, though the addition of the morphological data did lead to increased interior branch resolution with low statistical support (Figs. 1, 2). The analyses that included molecules indicated that a trio of species, *B. ferox*, *B. orientalis* and *B. raptor*, were always basal to all other branchinectids analysed here; the two Eurasian species basal to all other branchinectids had low statistical support (Figs. 1, 2). We found 15 equally parsimonious trees with just analyzing morphological data. A 50% majority consensus of these 15 most parsimonious trees had many discrepancies with the trees that included molecular data (Fig. 3). However, there was little to no bootstrap support for any groupings we observed in the molecular datasets.
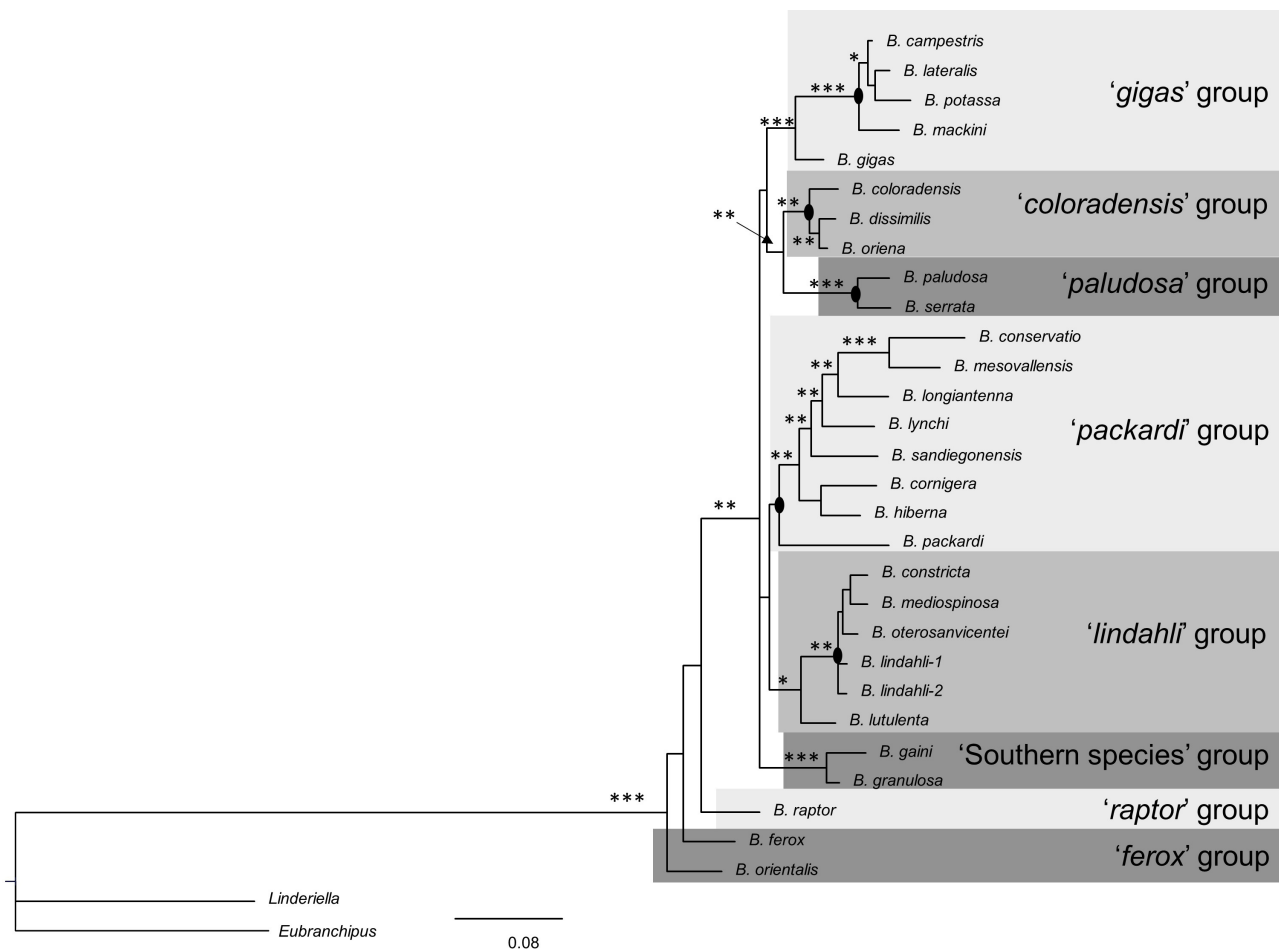

Fig. 1. Bayesian inference phylogeny based on 16S, 12S, 28S and 50 morphological characters. Nodes with filled circles indicate sister group taxon relationships composed of basal broad and narrow range sister taxa. Posterior probabilities: *p = 0.90–0.94; **p = 0.95–0.99; ***p = 1.0.

We found a significant positive relationship between pairwise clade distance and transformed range symmetry (Fig. 4). This relationship indicates the species which are more closely related have less symmetric (more asymmetric) ranges.

SYSTEMATICS

Branchinectidae Daday, 1910 (*sensu* Rogers & Coronel, 2011)

Branchipodidae pro partim: Packard 1883
Branchinectidae Daday, 1910; Linder 1941; Tasch 1969; Belk 1982; Brtek & Mura 2000; Belk & Schram 2001; Maeda-Martinez et al. 2002; Rogers & Coronel 2011; Rogers 2013)

Diagnosis: Gonopods free and separated, extending ventrolaterally, with rigid base bearing a medial projection. Vas deferens not dorsally looped, lacking clearly defined seminal vesicles. Head lacking a frontal appendage. Second antennae never medially fused, widely separated by labrum, antennal appendages lacking. Male proximal antennomere often spinose, dentate and/ or tuberculate medially, and/or posteriorly, pulvilli and apophyses present or not. Second maxilla robust, elongated, well developed, with 5–30 apical rigid setae, and 3–5 anteriorly directed soft setae on the anteriomedial surface. Eleven pairs of thoracopods with each thoracopod bearing one prae-epipodite. Female thoracic segments may have rows or paired rows of lateral and/or dorsal projections. Body length ranging from 6 to 100+ mm. Two genera.

Archaebranchinecta Rogers & Coronel, in Rogers 2019

Branchinecta Daday, 1910: Harding 1940; Belk & Brtek 1995; Brtek & Mura 2000; Belk & Schram 2001
Archaebranchinecta Rogers & Coronel, 2011; Rogers 2013; Rogers 2019; Cohen et al. 2019

Diagnosis: Genital segments expanded, gonopod rigid bases extending to base of abdominal segment II,
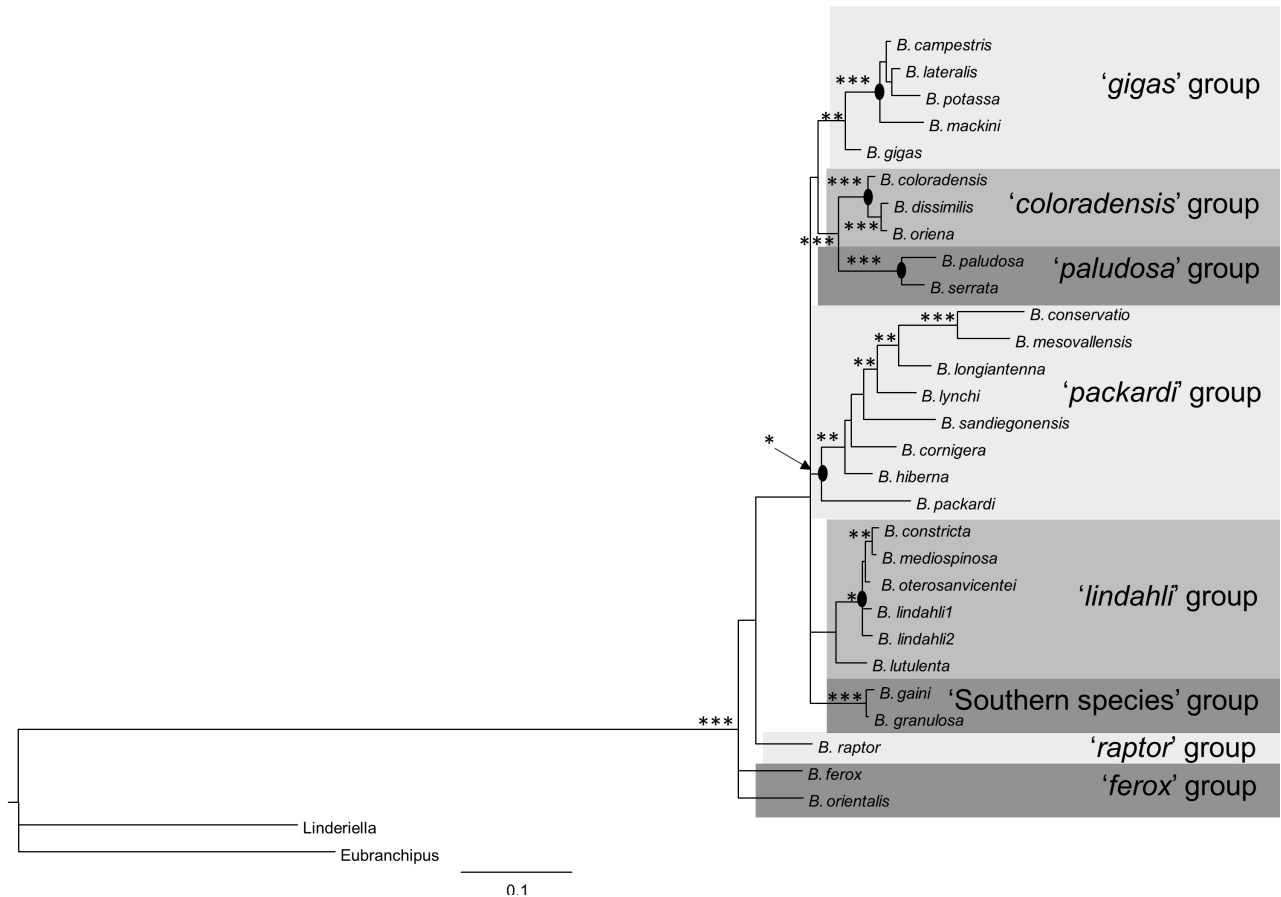

Fig. 2. Bayesian inference phylogeny based on 16S, 12S, and 28S. Nodes with filled circles indicate sister taxon relationships composed of basal broad and narrow range sister taxa. Posterior probabilities: *p = 0.90–0.94; **p = 0.95–0.99; ***p = 1.0.

and bearing a single proximomedial fleshy lobe and chitinized medial projection, terminating in biramal structure. Biramal structure proximal ramus conical and subacute. Distal ramus arcuate, spiniform, directed medially, connected to proximal ramus by a ridge. Gonopod eversible portion with one anterior and one lateral longitudinal denticle row. Apical most portion hemispherical, situated in recess with ten to twelve anterioapically scattered acute spinules. Abdominal segment I bearing a ventral chitinous plate covered in denticles. Three species are included in this genus, and *Archaebranchinecta pollicifera* (Harding 1940) is the type by designation (Rogers 2019).

Species attributed

Archaebranchinecta aimara Cohen et al. 2019
†*Archaebranchinecta barstowensis* (Belk & Schram, 2001)
    = †*Branchinecta barstowensis* Belk & Schram, 2001
Archaebranchinecta pollicifera (Harding, 1940) (*sensu* Rogers &
Coronel, 2011)
    = *Branchinecta pollicifera* Harding, 1940

Branchinecta Müller, 1788 (*sensu* Rogers & Coronel, 2011)

Branchinecta Müller, 1788; Packard 1874, 1883; Verrill 1869; Shantz 1905; Daday 1910; Linder 1941; Belk & Brtek 1995; Brtek & Mura 2000; Belk & Schram 2001; Maeda-Martinez et al. 2002; Rogers & Coronel 2011; Rogers 2013
Artemis Thompson, 1834
Branchiopsyllus Sars, 1897; Linder 1941; Belk 1982 (fide Vekhov 1989)
Artemiella Daday, (fide Linder 1932, 1941)

Diagnosis: Genital segments not expanded, each gonopod extending ventrolaterally, visible in dorsal view, with rigid base bearing a spiniform medial projection. Gonopod eversible portion with one or two denticulate tubercles apically or subapically. Apex truncated. Abdominal segment I lacking a ventral
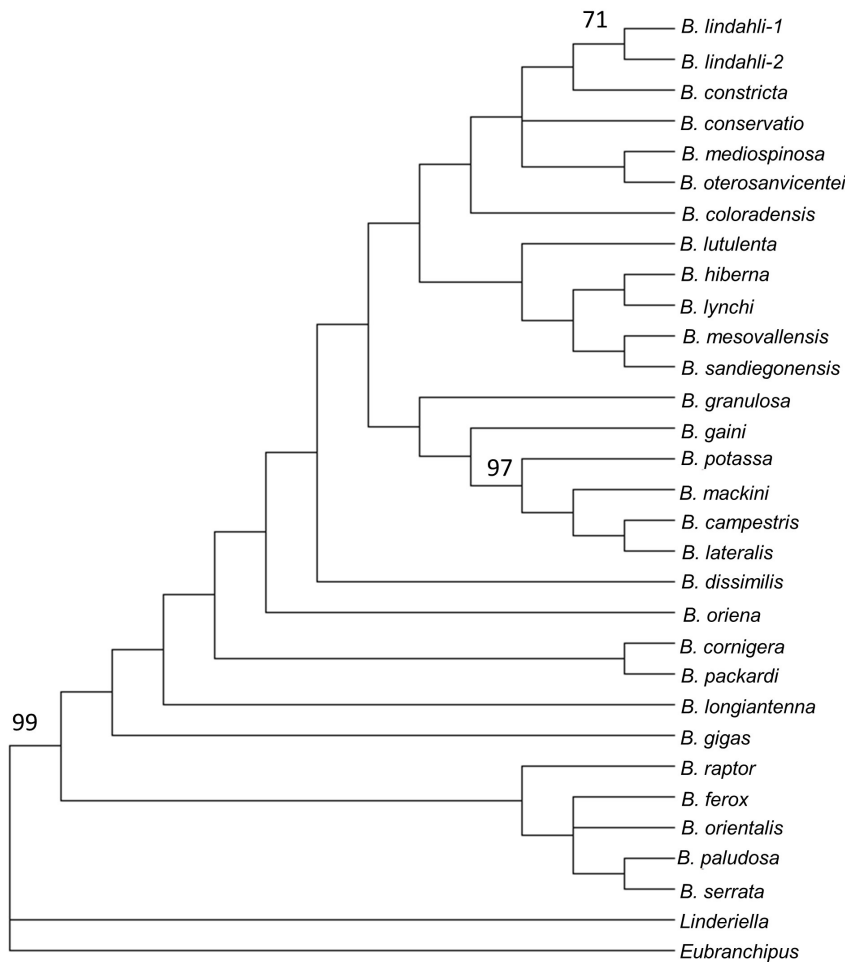

Fig. 3. 50% Majority consensus tree from the 15 most parsimonious trees produced from a heuristic search of 51 morphological characters in PAUP. The proportion of 1,000 bootstrap replicates above 70% are shown.

chitinized plate. Brood pouch variable, may be elongate, pedunculate, conic or pyriform. Females may have corneous or papillose cephalic projections. Type by monotypy *Branchinecta paludosa* (Müller, 1788).

Comments: There are 52 species presently recognized (Rogers 2013; Rogers and Lorenz 2015) in seven species groups. Due to the lack diagnosis or definition in the work of Brtek and Mura (2000), we have made no effort to associate our species groups with theirs; any attempt would be pure speculation.

Subdivision: All the following species groups and incertae sedis taxa are part of *Branchinecta*. The shared characters between the *ferox* and the *raptor* groups, and the fact that these groups were consistently basal in our analyses, suggest that *Branchinecta* may have had a Eurasian origin, and via a stochastic dispersal event, invaded North America and rapidly differentiated across that continent in a manner similar to *Streptocephalus* (Daniels et al. 2004).

"*ferox*" species group

Diagnosis: Eyes normal; male second antennae arcuate and subacute; female second antennae lacking a medial spine; body lacking sensory papillae; female thoracic segments lacking any dorsolateral projections; first and last limb pairs shortest; ovaries uniramal limited to the abdomen (except *B. ferox*, which is biramal, extending anteriorly to thoracic segment X), and posteriorly in the abdomen to abdominal segment IV, V, or VI; brood pouch fusiform, extending to abdominal segment III, IV, V or VI; testes always uniramal extending posteriorly from genital segments into abdominal segment IV, V, or VI; cercopods
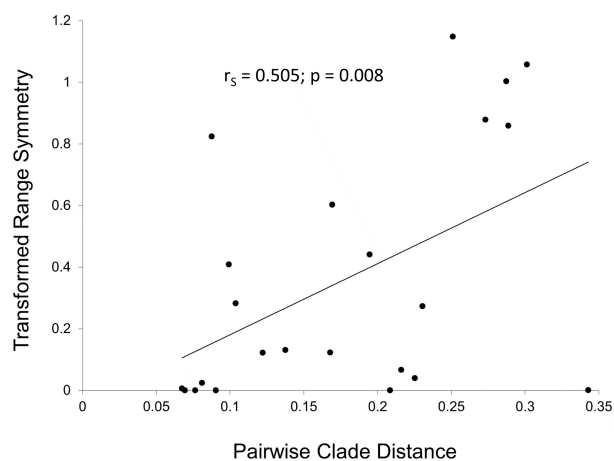

Fig. 4. Relationship between pairwise clade distance and transformed range symmetry. The Spearman's rank correlation ($r_s = 0.505$) is significant ($p = 0.008$).

cylindrical, tapering, shorter than the last two abdominal segments, medial surface with setae, lateral surface with or without setae. No geochemical association data is available.

Comments: Three species, morphologically similar to *B. raptor* in the overall general form of the male second antennae and the cylindrical cercopods. However, in *B. raptor* the cercopods are glabrous, whereas in the *ferox* group, marginal setae are always present medially in all three species, but on the lateral side may only be present distally or not at all in *B. ferox* and *B. orientalis*.

Species attributed

Branchinecta ferox (Milne-Edwards, 1840) (*sensu* Petkovski, 1991)
    = *Branchipus ferox* Milne-Edwards, 1840
    = *Branchipus eximius* Baird, 1861
    = *Branchipus* (*Branchinecta*) *ferus* Brauer, 1877
    = *Branchipus ferox* f. *aestivalis* Daday, 1890
    = *Branchipus ferox* f. *hibernalis* Daday, 1890
    = *Branchipus ferox* f. *vernalis* Daday, 1890
Branchinecta minuta Smirnov, 1948
Branchinecta orientalis Sars, 1901 (*sensu* Petkovski, 1991)
    = *Branchinecta cervantesi* Margalef, 1947
    = *Branchinecta ferox orientalis* Sars, 1901

"*raptor*" species group

Diagnosis: Eyes reduced; male second antennae elongate, arcuate and subacute; female second antennae lacking a medial spine; body covered in sensory papillae; female thoracic segments lacking any dorsolateral projections; anterior most limbs longest; ovaries uniramal limited to the abdomen; brood pouch pyriform, extending to abdominal segment III; testes uniramal extending posteriorly from genital segments into abdominal segment VI; cercopods elongate, nearly as long as the abdomen, cylindrical, tapering, and glabrous. All other anostracans have the first and last limb pairs smaller than the middle pairs. This species is only known from habitats with a substrate salinity of 5 mS/cm and containing 25% calcium carbonate (Rogers 2014a).

Comments: *Branchinecta raptor* shares several important character states with the *ferox* group (see comments under the *ferox* group below) and with *Branchinecta gigas* in the *gigas* group. Both *B. raptor* and *B. gigas* are specialized predators. Both species are super giants among anostracans (growing over 100 mm), have dramatically reduced eyes, bodies covered in sensory papillae that are used to detect movement in water by potential prey items, and elongated, whip like cercopods (Rogers et al. 2006). The significance of these characters must needs be examined, especially

as they do not appear in any other anostracan. These characters may suggest a stronger relationship than was elucidated by our genetic analyses.

Species attributed

Branchinecta raptor Rogers, Quinney, Weaver & Olesen, 2006

"Southern" species group

Diagnosis: (Limited) Eyes normal; male second antennae subcylindrical and highly variable; female second antennae lacking a medial spine; body lacking sensory papillae; female thoracic segments variously ornamented; first and last limb pairs shortest; ovaries biramal or uniramal; brood pouch variable; testes uniramal, limited to the abdomen; cercopods, fringed with setae, flattened and shorter than last two abdominal segments.

Comments: This group is not defined at this time, and is poorly resolved genetically and morphologically. Only two species from the southern hemisphere were available for molecular study and eleven were studied morphologically. The two species we analysed genetically grouped separately from the remaining members of the genus, regardless of their great morphological variety. While it is possible that all southern species belong to a single clade, we expect that once more taxa are available for study, other monophyletic groups will be revealed. The limited resolution we have now suggests that it is possible that the southern hemisphere species are the result of radical differentiation from a single, stochastic colonization event from North America. The species examined morphologically all share the basic form of the teste (uniramal, limited to the abdomen), and the cercopods. However, with as few taxa as we have for molecular analysis at this time, it is only considered a possibility.

Species attributed

Branchinecta achalensis César, 1985 (*sensu* Cohen, 1987)
   = *Branchinecta uruguayensis* Rogers and Lorenz, 2015
Branchinecta brushi Hegna & Lazo-Wasem, 2010
Branchinecta ferrolimneta Rogers & Ferreira, 2007
Branchinecta fueguina Cohen, 2008
Branchinecta gaini Daday, 1910
Branchinecta granulosa Daday, 1902 (*sensu* Cohen, 1992, 1995)
   = *Branchinecta santacrucensis* César, 1987a (fide Cohen, 1992)
Branchinecta iheringi Lilljeborg, 1889 (*sensu* Cohen, 1993, 1995b)
Branchinecta leonensis César, 1987b
Branchinecta palustris Biraben, 1946 (*sensu* Cohen, 1981)
Branchinecta papillata Rogers, de los Rios, & Zuniga, 2008 (*sensu* Cohen, 2012)
Branchinecta papillosa Biraben, 1946
Branchinecta prima Cohen, 1983

Branchinecta roacensis Cohen, 1982
Branchinecta somuncurensis Cohen, 1983
Branchinecta tarensis Birabén, 1946
Branchinecta tolli (Sars, 1897) (*sensu* Vekhoff, 1989)
   = *Branchiopsyllus tolli* Sars, 1897
   = *Artemiella skorikowi* Daday, 1910
   = *Branchinecta skorikowi* (Daday, 1910)
Branchinecta valchetana Cohen, 1981
Branchinecta vuriloche Cohen, 1985

"*lindahli*" species group

Diagnosis: Eyes normal; male second antennae subcylindrical, generally with the proximal antennomere undifferentiated (except *B. constricta*) and the distal antennomere laterally flattened with apex bent medially; female second antennae lacking a medial spine; body lacking sensory papillae; female thoracic segments IV–X (except *B. lutalenta*, with segments III–VII) always with a dorsolateral subconical or hemispherical projection, segments I and II always lacking projections; first and last limb pairs shortest; ovaries biramal, extending anteriorly from the genital segments into the thorax as far as thoracic segment VI; brood pouch fusiform or cylindrical, extending to abdominal segment IV, V or VI; testes always uniramal extending posteriorly from genital segments into abdominal segment II, III, or IV; cercopods, fringed with setae, flattened and shorter than last two abdominal segments. Species in this group are associated with substrates that have 0–11% gypsum, 3–30% calcium carbonate, and salinity of 0–24 mS/cm (Rogers 2014a). They are strongly associated with sodium and calcium salts and one species (*B. potassa*) only occurs where potassium salts are present.

Comments: Female *B. coloradensis* ("*coloradensis*" species group) also share the same dorsolateral thoracic projections, but the ovary only extends anteriorly to thoracic segment IX. Females in other defined species groups either have no projections, only some of thoracic segments IV–VII with projections but not all, or have the projections as transverse ridges or paired (*i.e.*; two per side).

Species attributed

Branchinecta constricta Rogers, 2006
Branchinecta lindahli Packard, 1883 (*sensu* Lynch 1964)
Branchinecta lutulenta Rogers & Hill, 2013
Branchinecta mediospinosa Rogers, Dasis & Murrow, 2011
Branchinecta oterosanvicentei Obregón-Barboza, Maeda-Martínez, García-Velazco & Dumont, 2001

"*packardi*" species group

Diagnosis: Eyes normal; male second antennae

with proximal antennomere subcylindrical, and variously ornamented; body lacking sensory papillae; female thoracic segments II–VII with some combination of a dorsolateral projections either paired or single; first and last limb pairs shortest; brood pouch fusiform, pyriform, or cylindrical; cercopods, fringed with setae, flattened and shorter than last two abdominal segments.

Species attributed

Branchinecta belki Maeda-Martínez, Obergón-Barboza & Dumont, 1992
    = *Branchinecta* sp. A in Maeda-Martinez 1991
Branchinecta conservatio Eng, Belk & Eriksen, 1990
Branchinecta cornigera Lynch, 1958
Branchinecta hiberna Rogers & Fugate, 2001
Branchinecta longiantenna Eng, Belk & Eriksen, 1990
Branchinecta lynchi Eng, Belk & Eriksen, 1990
Branchinecta mesovallensis Belk & Fugate, 2000
Branchinecta mexicana Maeda-Martínez, Obergón-Barboza & Dumont, 1993
    = *Branchinecta* sp. B in Maeda-Martínez 1991
Branchinecta packardi Pearse, 1912 (*sensu* Lynch 1964)
Branchinecta sandiegonensis Fugate, 1993

"*paludosa*" species group

Diagnosis: Eyes normal; male second antennae subcylindrical, pulvilli lacking (except in *B. kaibabensis*, which has each pulvillus borne on a subconical mound) with the proximal antennomere medial surface with a single, longitudinal row of spines, and the distal antennomere not flattened, tapering, with apex directed distally; female second antennae lacking a medial spine; body lacking sensory papillae; female thoracic segments smooth (except *B. serrata* which has a dorsolateral conical protrusion on segments III–VII; first and last limb pairs shortest; ovaries biramal extending anteriorly from the genital segments into the thorax as far as thoracic segment VII or VI; brood pouch fusiform or pedunculate, extending to abdominal segment VII; testes uniramal, extending posteriorly from genital segments into abdominal segment III or IV; cercopods, fringed with setae, flattened and shorter than last two abdominal segments. This species group is associated with substrates lacking gypsum, 0–10% calcium carbonate, and 0–2 (rarely) mS/cm substrate salinity (Rogers 2014a).

Comments: *Branchinecta longiantenna* in the "*packardi*" species group has the second antenna proximal antennomere bearing spines in several longitudinal rows, not just one. *Branchinecta cornigera*, also of the "*packardi*" species group, has the second antennae distal antennomere not flattened and tapering, but lacks a longitudinal spine row on the proximal antennomere. The "*raptor*" and "*ferox*" species groups

also have the second antenna distal antennomere tapering, but are separated from this group by the form of the cercopod.

Species attributed

Branchinecta kaibabensis Belk & Fugate, 2000
Branchinecta paludosa (Müller, 1788)
    = *Cancer stagnalis* Fabricius, 1780
    = *Cancer paludosus* Müller, 1788
    = *Branchipus paludosus* Kröyer, 1838
    = *Artemis paludosus* Thompson, 1834
    = *Branchipus middendorfianus* Fisher, 1851
    = *Branchipus groenlandicus* Verrill, 1869
    = *Branchipus groenlandica* Verrill, 1869
    = *Branchipus arctica* Verrill, 1869
    = *Branchipus arcticus* Verrill, 1869
    = *Branchinecta groenlandica* Packard, 1874
    = *Branchinecta arctica* Packard, 1874
    = *Branchipus verrilli* Miers, 1877
    = *Branchipus grubei* Gerstäcker, 1879
    = *Branchinecta polonica* Gajl, 1934
Branchinecta paludosa tjanshanica Akatova, 1987
Branchinecta serrata Rogers, 2006

"*coloradensis*" species group

Diagnosis: Eyes normal; male second antennae subcylindrical, generally with the proximal antennomere medial surface covered in scattered, cylindrical spines, and the distal antennomere laterally flattened with apex directed medially; female second antennae lacking a medial spine; body lacking sensory papillae; female thoracic segments without dorsal projections, (except *B. coloradensis*, which has dorsolateral hemispherical protrusions on thoracic segments I–VIII); first and last limb pairs shortest; ovaries biramal, extending anteriorly from the genital segments into the thorax as far as thoracic segment IX or VIII; brood pouch fusiform or cylindrical, extending to abdominal segment V or VI; testes uniramal (biramal in *B. dissimilis*, extending anteriorly to thoracic segment VIII) extending posteriorly from genital segments into abdominal segment III; cercopods, fringed with setae, flattened and shorter than last two abdominal segments. This species group is associated with substrates containing 0–5% gypsum, 0–30% calcium carbonate, a substrate salinity of 0–8 mS/cm, and is strongly associated with low levels of calcium salts (Rogers 2014a).

Species attributed

Branchinecta coloradensis Packard, 1874 (*sensu* Lynch, 1964)
    = *Branchinecta shantzi* Mackin, 1952
Branchinecta dissimilis Lynch, 1972
Branchinecta oriena Belk & Rogers, 2002

"*gigas*" species group

Diagnosis: Eyes normal or reduced; male second antennae elongate, extending posteriorly to thoracic segment VIII (except *B. gigas*, which extends to thoracic segment VI), pulvillus absent or greatly obscured (except *B. potassa*), apex turned laterally, or flattened and bent medially (*B. gigas*); female second antennae lacking a medial spine; body lacking sensory papillae, except *B. gigas*, which is covered in sensory papillae; female thoracic segments I–XI always with a dorsomedial ridge (except *B. gigas*, which lacks protrusions); first and last limb pairs shortest; ovaries biramal or uniramal and limited to thorax, extending anteriorly from the genital segments into the thorax as far as thoracic segment VI or VII; brood pouch is pedunculate, and extends to abdominal segment VII (except in *B. gigas*, which is pyriform and extends to abdominal segment III); testes always uniramal extending posteriorly from genital segments into abdominal segment III; cercopods flattened and shorter than last two thoracic segments and fringed in setae, except in *B. gigas* (see comments under *raptor* species group), which are cylindrical, nearly as long as the abdomen, and with a ventrolateral plumose setal fringe and a distomedial dorsal setal fringe. Species in this group are strongly associated with substrate calcium salts, and occur on substrates containing 0–20% gypsum, 0–80% calcium carbonate, and substrate salinities of 5–32 mS/cm (Rogers 2014a).

Comments: No one morphological character unites all these species, as *B. gigas* is particularly specialized as a predator. Only the posterior extension of the testes (to abdominal segment III) is shared by all members of this group. However, this character is not exclusive to the *gigas* species group; it appears in the "*coloradensis*" group as well. Similarly, *B. potassa* seems to share characters with other groups (pulvillus, as well as female dorsal ornamentation characters) that are otherwise not found in this species group. However, all species have the same geochemical associations.

Species attributed

Branchinecta campestris Lynch, 1960
Branchinecta gigas Lynch, 1937
Branchinecta lateralis Rogers, 2006
Branchinecta mackini Dexter, 1956 (*sensu* Belk, 2000)
Branchinecta potassa Belk, 1979
Branchinecta readingi Belk, 2000

Incertae sedis

Comments: This species is unattributed at this time. Material was not available for molecular or morphological examination and the original description and subsequent redescriptions (Daday 1910; Vekhov 1989) were insufficient to determine morphological affinities.

Species attributed

Branchinecta tolli (Sars, 1897) (*sensu* Vekhov, 1989)
   = *Branchiopsyllus tolli* Sars, 1897
   = *Artemiella skorikowi* Daday, 1910
   = *Branchinecta skorikowi* (Daday, 1910)

DISCUSSION

Phylogeny of Branchinectidae

We present here the first molecular phylogenetic evaluation of the Branchinectidae. The fact that the Eurasian taxa arise from several successive basal dichotomies (Fig. 1) suggests that the family may have originated in Eurasia. Our initial phylogenetic appraisal was unable to resolve many of the interior branches within the family. However, we were able to recover a number of statistically supported species groups that also contained highly resolved relationships among more closely related species. The "*paludosa*" and "*lindahli*" groups contain species that can be classified as morphologically similar based on characteristics of the male's second antenna. The "*gigas*" and "*packardi*" groups are highly diverse with regard to male antennal characteristics and female brood pouch morphology. However, each of these groups possesses interesting physiological and biogeographical features.

The two species that are considered gigantics are not closely related (*B. gigas* and *B. raptor*). This suggests that gigantism has evolved more than once within this family. These two species both require longer ponding durations and both are highly predatory. Early work suggested that these species were closely related (Rogers et al. 2006); however, our molecular phylogenetic analysis clearly supports multiple origins for gigantism.

We only have two South American species in this analysis and they are closely related (*B. gaini* and *B. granulosa*). We could not definitively resolve the relationship between these and the North American species, as they are part of a polytomy. Studies on morphology suggest that a single species (*B. packardi*) may be the ancestral species for most South American *Branchinecta* (Fugate 1992). However, as the two branchinectid genera are found on both North and South America, we suggest that, while North America has served as the ancestral location for South American branchinectids, there may have been multiple invasions

from North America into South America. This cannot however be substantiated with our data and requires much more rigorous analysis of the South American branchinectid fauna.

Also of interest are the federally protected *Branchinecta* species in the western United States (*B. sandiegonensis*, *B. lynchi*, *B. conservatio*, *B. longiantenna*), which belong to a single monophyletic clade, the '*packardi*' group, with four other species. Six species in this group are endemic to westernmost North America (Washington, Oregon, California and adjacent coastal Mexico), with *B. packardi* ranging throughout the Great Plains and deserts of Canada, USA and Mexico, and *B. hiberna* occurring across the Great Basin Desert in California, Oregon, Idaho and Nevada.

Brtek and Mura (2000), in their larger review of the Anostraca, provide a very confused diagnosis of the Branchinectidae (Rogers 2006 2013) dividing the species into groups. Brtek and Mura (2000) justify their scheme in one sentence: "One genus only: *Branchinecta* Verrill, 1869 which can be divided (according to the morphology of the male antenna, among other features) into five distinct species groups at least…". However, they never explain or describe their analyses or the specific characters used, nor define which species belong in each group (Rogers 2006). Brtek and Mura (2000) then describe these groups based on geography (quoted directly from Brtek and Mura 2000:1043):

(1) "*paludosa*-group" circumpolar and glacial relicts;
(2) "*ferox*-group", roughly between 55° and 30°N;
(3) "*coloradensis*-group" (the whole of the Americas, Antarctica, Arctic regions of Asia and most of eastern Europe);
(4) "*pollicifera*-group" (area of Lake Titicaca);
(5) "*cornigera*-group" (U.S.A.).

As it is not possible to exactly ascertain which species were meant for which groups, some inferences can be drawn. We cannot be sure which taxa they considered glacial relicts. It appears that the "*ferox*-group" of Brtek and Mura (2000) roughly corresponds to our own group of the same name, and the "*pollicifera*-group" is the genus *Archaebranchinecta*. Our molecular results do not support the remaining groups of Brtek and Mura (2000), and our morphological analyses support neither our molecular results nor this previous study's species groups.

The level of resolution in our analyses is insufficient to treat our species groups as formal taxonomic units (*e.g.*, subgenera). Our species groups demonstrate specific evolutionary relationships with various levels of support, and additional analyses, with more genes and more species, is necessary before greater taxonomic resolution can be acheived.

Peripatric speciation

An interesting finding of this work is that a peripatric model of speciation may be applied to the Branchinectidae of North America. In many instances we find evidence for well supported sister species relationships between a widespread species and a narrow range species and in some instances the narrow range species is at the periphery of wide range species distribution. This is expected as seasonally astatic aquatic habitats drive speciation through isolation (Rogers 2014a b c 2015). Rogers (2014a) predicted that new species must evolve allopatrically or peripatrically, in peripheral unoccupied habitats, via small genetically isolated founder populations. This is also supported by examination of anostracan biogeographic patterns: of the described anostracan species worldwide, 56.2% are reported from ten or fewer localities each and 28.7% from only the type locality (Rogers 2013 2015). In the Branchinectidae, we have a similar situation with 37.0% reported from ten or fewer localities each and 24.1% from only the type locality (Rogers 2013; Rogers and Lorenz 2015). Furthermore, the Anostracan Biogeographic model proposed by Rogers (2015) predicts that anostracan genera would consist of small clades, each consisting of a basal, widespread species, with peripheral, isolated, insular sister species (Rogers 2015).

The sister species relationships between wide and narrow range species in question that support the peripatric model in our phylogenetic analysis include *B. gaini* and *B. granulosa*, *B. lateralis* and *B. potassa*, *B. lindahli* and *B. oterosanvicentei*, *B. cornigera* and *B. hiberna*, and *B. paludosa* and *B. serrata*. The pattern presented here is intriguing and supports the peripatric model in some but not all instances of diversification for this group. Additional data that could bolster this conclusion include taxa that may be related to to the widely distributed *B. packardi* (*B. belki*, *B. mexicana*), a more detailed delineation of species limits and geographic boundaries, and the inclusion of environmental data.

Interestingly, our analysis indicated little to no phylogenetic information with our coded morphological characters. High support was found for the genus *Branchinecta* and a group that contained *B. potassa*, *B. mackini*, *B. campestris* and *B. lateralis*. This indicates that the morphological features studied here, while useful in diagnosing taxonomic units within the Branchinectidae, are not useful for phylogenetic purposes, or the useful characters were either not treated or were lost in the background noise of our larger data set. We were able to develop limited morphological and/or ecological definitions for all our

species groups. Future studies should attempt to identify phylogenetically informative morphological characters within the Branchinectidae and other Anostracans.

Hybridization has been recently reported in the Branchinectidae (*B. lindahli* and *B. sandiegonensis*) (Simovich et al. 2013) and been observed under laboratory conditions (Maeda-Martinez et al. 1992). Interestingly there are a number of *Branchinecta* species that are known to co-occur in the same pools (*e.g.*; *B. lindahli* and *B. longiantenna*, *B. longiantenna* and *B. lynchi*, *B. conservatio* and *B. lynchi*, *B. mackini* and *B. cornigera*), yet no morphological intermediates (potential hybrids) have been reported. While we did not examine any specimens that showed intermediate morphology, further research will undoubtedly uncover additional instances of hybridization in the Branchinectidae, but other factors (amplexial morphology and life history characteristics) should also be examined to determine the conditions under which hybridization may occur. A more detailed analysis of hybridization, or a lack thereof, in this group will be useful for establishing the robustness of our proposed phylogenetic relationships.

CONCLUSIONS

The phylogenetic hypothesis presented here shows that distinct morphological and molecular based groupings exist in the Branchinectidae. While additional data are needed to provide stronger support to interior and basal nodes, we were able to provide support for peripatric speciation in this group. We also identified a monophyletic clade ('*packardi*' group) that contains all the federally protected *Branchinecta* species, among others.

Authors' contributions: DCR conducted the morphological character analyses and the species group morphological definitions. AA conducted all the molecular analyses and developed the cladograms and phylogenies. Both authors worked equally on the manuscript.

Competing interests: DCR and AA declare that they have no conflict of interest.

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Ethics approval consent to participate: Ethics approval is not necessary.

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Supplementary Materials

Appendix 1. Material examined. (download)

Table S1. Morphological Character. (download)