Research Article

Morphological and Molecular Identification of Three *Ceriodaphnia* Species (Cladocera: Daphniidae) from Australia

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Australian *Ceriodaphnia* (Cladocera: Daphniidae) are examined using morphological attributes and two mitochondrial DNA (COI and 16s) and one nuclear DNA (28s) gene fragments to differentiate the species. The sequence data supports the existence of three species, that is, *C. dubia*, one reinstated species *C. spinata* Henry, 1919, and one new species *C.* sp. 1. Morphological characteristics were also able to accurately separate the three species. Furthermore, genetic analysis of COI sequences from *Ceriodaphnia* supported three clades. The high degree of correlation between morphological and molecular identification in this study indicates that mitochondrial markers, COI and 16s, are appropriate molecular markers for species discrimination and identification of *Ceriodaphnia*.

1. Introduction

Ceriodaphnia Dana, 1853 (Cladocera: Daphniidae), displays little diversification in terms of species richness and morphological disparity, with the genus currently comprising 14 "valid" species worldwide, predominantly based on morphology [1]. In addition, there are 21 *species inquirenda* and 24 species that are probably junior synonyms of previously described species [1]. There is limited morphological and genetic evidence to support this proliferation of the large number of proposed names. According to Smirnov and Timms (1983) [2], there are only five *Ceriodaphnia* species from Australia which includes one beaked species ('beak' = a rostral projection) i.e. *C. cornuta* Sars, 1885, and four non-beaked species *C. dubia* Richard, 1894, *C. laticaudata* Müller, 1867, *C. quadrangula* (Müller, 1785) and *C. rotunda* Sars, 1862. One further, non-beaked species, *C. pulchella* Sars, 1862, has been recorded since [3]. In addition to these, two more non-beaked species, *C. planifrons* Smith, 1909 and *C. spinata* Henry, 1919 were re-instated by Berner [4], thereby increasing the total number of recorded species from Australia to eight.

The literature on *Ceriodaphnia* sp. generally points towards the absence of divergent morphological characters for this group. Additionally, the historical taxonomic descriptions are incomplete and primarily focused on the head, antennule, antennae, postabdomen, carapace, reticulation, and rarely trunk appendages of *Ceriodaphnia*. Where morphological evidence is unclear, molecular techniques can be used to improve our understanding of taxonomic divergence and speciation. Barnett et al. [5] emphasised that genetics has become an increasingly important parameter in the classification and identification of organisms in comparison to more traditional morphological descriptors. The success of using Cytochrome Oxidase I (COI) gene region to distinguish species from a range of taxa and to reveal cryptic species has been remarkable. This method of matching unknown molecular sequences to species, however, is only effective for those which have been studied extensively using a variety of characters such as morphology, reproduction, ecology, and geographical distribution that have been well documented by researchers [6].

For example, the taxonomy of *Daphnia* has been the subject of intense investigations for over a century owing to its intraspecific variation. The gradual increase in the species discovery rate of *Daphnia* is due to the succession of molecular genetics from traditional taxonomy (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/258134).

The advancement in molecular techniques, especially the introduction of allozyme technique to study interspecific

hybridisation in *D. carinata* by Hebert [7], followed by DNA barcoding in 1994, saw a steady rise in species discovery, especially of cryptic species. Similarly, for other species of *Daphnia*, such as *D. pulex* where traditional taxonomy often resulted in grouping phenotypically similar species, genetic techniques have revealed a number of new species [8–15]. The study by Colbourne et al. [16] provided draft genomes of 200 mega bases for *D. pulex*, helping to understand environmental influences on gene function.

The objective of the present study was to consider all available data, morphological and molecular (mitochondrial and nuclear genes) of the valid eight *Ceriodaphnia* species, from Australia, and to clarify the taxonomical position of selected Australian *Ceriodaphnia* species.

2. Materials and Methods

Sixty female specimens of *Ceriodaphnia* (excluding *C. cornuta*, Sars, 1890, a beaked variety) preserved in 70% ethanol from 11 sampling sites in Australia (see Table 1) were used for morphological and molecular analyses.

2.1. Morphological Analysis. Eggs and young adults from a total of 60 ovigerous females were teased out from the brood chamber and stored in new 0.5 mL microtubes containing 90% denatured ethanol for genetic analysis. The rest of the body was transferred onto a slide containing Polyvinyl Alcohol (PVA) and left in a Greiner petri dish for 24 h. After 24 h equilibration, the specimen was dissected using tungsten needles and examined under a compound microscope for further detailed morphological analysis. Digital photographs were taken using Olympus BX51 microscope under high resolution using polarizing light and composite line drawings were made from these photographs for different parts of the specimen. Inbuilt imaging software Image J was used to calculate sizes of body parts. *Ceriodaphnia* species were identified morphologically using *taxonomic* keys of Shiel [3] and Kořínek [17]. Slide mounted *Ceriodaphnia* collections from Australian Museum, Sydney, Australia (AMS); Natural History Museum, University of Ohio, Norway (NHMUO); Naturkunde-Museum, Coburg, Germany (NMCL) museums were also examined and their morphological characteristics were compared with the Australian *Ceriodaphnia* specimens used during this study.

In addition to light microscopy, specimens were prepared for SEM as described in Berner and Rakhmatullaeva [18, 19]. The specimens were sputter-coated with gold and were examined under a Philips XL 20 SEM with an accelerating voltage of 10–20 KV, working distance between 9.6 and 14 mm and spot size at 3/4.

2.2. DNA Extraction, PCR, and Sequencing. DNA extraction was performed on eggs or young adults with DNeasy Tissue kit (Qiagen Inc.) according to the manufacturer's instructions.

Polymerase chain reaction was subsequently utilized to amplify the COI gene with Folmer primer pair (LCO 1490 and HCO 2198) [20]. Each 50 μL PCR reaction consisted of 5 μL of genomic DNA template, 3 μL of 50 mM MgCl$_2$, 5 μL of 10x Buffer, 1.5 μL of each primer, 1 μL of 10 μM dNTP's, 0.24 μL of Taq platinum polymerase, and 32.76 μL of DNA free Milli-Q water. PCR thermocycling was performed under the following conditions: 1 cycle of 2 min 30 sec at 94°C; 5 cycles each of 94°C for 35 sec, 48°C for 40 sec, and 72°C for 1 min; followed by 35 cycles each of 94°C for 30 sec, 56°C for 40 sec, and 72°C for 1 min; finishing with a step of 72°C for 10 min.

16s rDNA mitochondrial genes were amplified using 16s1 5$'$-CCGGAATTCCGCCTGTTTATCAAAAACA-3$'$ and 16s2 5$'$-CCCAAGCTTCTCCGGTTTGAACTCAGAT-3$'$ (modified Simon et al. 1994) [21] primers. PCR reaction as mentioned above. PCR thermocycling was performed under the following conditions: 1 cycle of 2 min 30 sec at 94°C; 5 cycles each of 94°C for 35 sec, 60°C for 40 sec, and 72°C for 1 min; followed by 35 cycles each of 94°C for 30 sec, 64°C for 40 sec, and 72°C for 1 min; finishing with a step of 72°C for 10 min. For 28s rRNA, the primers used for amplification were 28s1 5$'$-CCCGCTGAATTTAAGCATAT-3$'$ and 28s2 5$'$-TAGATGGTTCGATTAGTCTTTCGC-3$'$ [22]; PCR thermo cycling was performed under the following conditions: 1 cycle of 2 min 30 sec at 94°C; 5 cycles each of 94°C for 35 sec, 56°C for 40 sec, and 72°C for 1 min; followed by 35 cycles each of 94°C for 30 sec, 62°C for 40 sec, and 72°C for 1 min; finishing with a step of 72°C for 10 min.

The PCR products were run in 2% agarose gels containing 10 μL of SYBR Safe DNA gel stain (Invitrogen Inc.) for 2 to 4 h at 80 to 100 V and visualized using UV-transillumination. When amplified bands were sharp and clean, in few cases where there were double bands, the target band was cut and purified from the agarose gel using QIAquick Gel Extraction Kit to avoid contamination from any other nonspecific bands. PCR purified products were cycle-sequenced using BigDye Sequencing kit Terminator 3.1 (Applied Biosystems) for both forward and reverse directions. Cycle-sequencing reactions were carried out in 10 μL total volume, containing 1 to 3 μL of purified PCR product, 3.5 μL of sequencing buffer, 1 μL of Big Dye Terminator, and 0.5 μL of forward and reverse primer with total volume made up to 10 μL using DNA free Milli-Q water. The sequencing thermal cycle consisted of 1 cycle of 1 min at 95°C, followed by 25 cycles of 95°C for 15 sec, 50°C for 10 sec, and 60°C for 4 min, with final overnight incubation at 25°C. The sequencing product was then purified using Millipore TM384-SEQ Filter plates and 1x Tris Buffer. The purified PCR products along with primers (LCO1490 and HCO2198) were sent to the Australian Genome Research Facility Ltd., Australia, and Macrogen Inc., Republic of Korea, for sequencing on the Applied Biosystems 3730xl capillary sequencers.

2.3. Phylogenetic Analysis Based on cmtDNA. Both forward and reverse DNA sequences were analyzed and aligned using Clustal W application implemented in software Bioedit ver. 7.0.0. [23], with gap open penalty set to 100, so gaps become less frequent. The protein coding sequences of COI were translated into amino acids using MEGA ver. 5.05 [24], to check for stop codons. Phylogenetic analysis was conducted using Mega, PhyML, and Mr. Bayes on both

Table 1: Localities with coordinates and the GenBank accession numbers for *Ceriodaphnia* collected across Australia; —: not amplified.

Sites	Location	State	Sampling date	Location code	Water body type	Latitude	Longitude	GenBank accession numbers COI	16s	28s
1	Yanga National Park	New South Wales (NSW)	26/10/2010	NSYAN	Lake	34°42′19.04″S	143°35′30.73″E	KC020663–66	KC154331–34	—
2	Yanga National Park, Mercedes	New South Wales (NSW)	26/10/2010	NSMER	Lake	34°22′44.94″S	143°47′12.80″E	KC020667–71	KC154336–40	KC154371–72
3	Yanga National Park, Steam Engine	New South Wales (NSW)	26/10/2010	NSSTE	Lake	34°28′58.26″S	143°40′33.20″E	KC020662	KC154330	—
4	Parramatta	New South Wales (NSW)	Sample collected in 1986 by Moreno Julli. Since then, it was cultured and maintained in a laboratory	SYDCB	Lake	33°47′26.00″S	151°0′28.00″E	KC154279–83	KC154341–45	KC154358–62
5	Mannum, Mandina	South Australia (SA)	26/10/2009	SAMAN	Flood plain	36°16′29.99″S	139°54′40.41″E	KC154284–85	KC154326–29	KC154363–65
6	Snuggery	South Australia (SA)	20/10/2009	SASNU	Flood plain	36°33′45.00″S	140°0′7.88″E	KC020660	KC154322–25	KC154366–70
7	Myponga Reservoir	South Australia (SA)	2008–2010	SAMYP	Reservoir	35°24′10.06″S	138°25′43.14″E	KC154270–75	—	—
8	South Para Reservoir	South Australia (SA)	2008–2010	SASPA	Reservoir	34°41′47.28″S	138°51′41.63″E	KC154276–78	—	—
9	Chowilla	South Australia (SA)	10/02/2011	SACHOW	Flood plain	34°3′33.76″S	140°45′44.48″E	KC020655–59	—	—
10	Woorabinda	South Australia (SA)	15/10/2009	SAWOO	Flood plain	30°0′59.90″S	134°32′1.88″E	KC020661	—	—
11	Runaway Perth	Western Australia (WA)	10/03/2010	WARPER	Pond	31°56′11.10″S	115°58′31.78″E	KC020651–54	—	—

separate and concatenated mitochondrial (cmtDNA = 16s + COI, 1185 bp) datasets. Phylogenetic trees were inferred by Neighbour-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI). The best fit models of nucleotide substitution were selected using the Model Generator [25]; ML tree was constructed using PhyML ver. 3.0 [26]; branch support values were estimated using 100 bootstrap replicates. All other parameters were set to their default values. BI analysis was performed using Mr. Bayes ver. 3.1.2 [27]. NJ trees were constructed using MEGA ver. 5.05 and Kimura 2-parameter (K2P) distance with complete deletion of missing information. Four substitution rate categories were considered while gamma shape parameters, transition/transversion ratios, and nucleotide frequencies were estimated from the data. Proportions of invariable sites were set according to values given by models obtained from Model Generator with 100 bootstrap replications. Alignment gaps were treated as unknown characters. For Bayesian analysis the Markov Chain Monte Carlo chains were run for 10^6 generations and trees were sampled every 100 generations. Of the generated trees, the first 25% were eliminated as burn-in. Runs were checked for convergence and normal distribution in Tracer ver. 1.5 [28].

The sequences are deposited in GenBank under accession numbers KC154268–KC154287; KC020651–KC020671 for COI gene fragments; KC154322–KC154345 for 16s gene fragments; and KC154358–KC154372 for 28s gene fragments.

COI gene sequences for *Ceriodaphnia* used in this study are compared with published COI sequences available from GenBank (number of taxa = 124; number of haplotypes = 31; base pair length ≥ 500), most of which originated from North American taxa [29].

Abbreviations used for museum collections are as follows:

AMS = Australian Museum Sydney, Australia;

NHMUO = Natural History Museum, University of Oslo, Norway;

NMCL = Naturkunde-Museum, Coburg, Germany;

SAM = South Australian Museum, Australia.

3. Results

3.1. Descriptions of Species Based on Morphological Characteristics. Ten key morphological characteristics were used in the present study and are summarized in Table 2. Accordingly, of the Australian female specimens examined, the following three species were identified: *C. dubia*, *C.* sp. 1, and *C. spinata*.

3.1.1. Genus Ceriodaphnia Dana, 1853. Ceriodaphnia Dana, 1853: 1273; Müller, 1868: 125; Schoedler, 1877: 19; Winchell, 1883: 35; Stingelin, 1896: 211; Lilljeborg, 1900: 183; Lilljeborg, 1901: 675; Smith, 1909: 80; Sars, 1916: 315; Henry, 1922: 32.

Type Species. Ceriodaphnia quadrangular (O. F. Müller, 1785).

Diagnosis. Valves of the carapace ending in a posterior angle or a short spine. Head small, depressed and separated from body by a deep cervical groove. Carapace marked by a polygonal pattern. Antennules in female not freely movable. Ocellus always present. Ephippium triangular, containing more than one egg.

3.1.2. Ceriodaphnia dubia Richard, 1894 (Figures 6–8). Ceriodaphnia dubia Richard, 1894: 570; Delachaux, 1917: 80; Sars, 1916: 317; Berner, 1986: 16; Greenwood et al. 1991: 285.

Syn.: *Ceriodaphnia affinis* Lilljeborg, 1901: 675.
Syn.: *Ceriodaphnia limicola* Ekman, 1900: 70.
Syn.: *Ceriodaphnia acuminata* Ekman, 1900: 69.
Syn.: *Ceriodaphnia richardi* Sars, 1901: 21.

Type Series. Type locality: "lac Toba," N Sumatra, Indonesia.

Type Material. David G. Frey (DGF) collection was housed in Smithsonian Institution's Museum Support Center in Suitland, Maryland, USA. According to the hand written catalogue, sample DGF 723 was from "Balige (Lac Toba, Sumatra)," coll. in 24.xi.1891 by M. E. Modigliani. However, according to the label, this sample is from Lake Titicaca and collected in 1969. So the initial sample with number 723 is no longer present in the collection, and there are no other samples from Lake Toba in Richard's collection" [35].

Specimens Identified as Ceriodaphnia dubia Richard, 1894

Nontype Material. Australia: Victoria, no other data, NHMUO F19258 (in ethanol). South Africa: Cape of Good Hope, no other data, NHMUO F9266 (slide collection); Cape of Good Hope, no other data, NHMUO F9267 (slide collection). Central Africa: Lake Victoria, no other data, NHMUO F9309 (slide collection). Indonesia: Lake Toba, Sumatra, 1891-02-24, Frey, D. G. USNM 1120420 (100 specimens in ethanol).

Specimens Identified as Ceriodaphnia affinis Lilljeborg, 1901

Nontype Material. Sweden: Blekinge, Sturberg no other data, NHMUO F9583 (slide collection); Blekinge, Sturberg no other data, NHMUO F18489a (in ethanol); Blekinge, Sturberg no other data, NHMUO F18489b (in ethanol). Germany: Umgebung von Berlin, Hartwig, NMCL 11913.

Australia: slide of a dissected parthenogenetic female from Parramatta Lake near Sydney, NSW (33°47′26″S/ 151°00′28″E), deposited in the South Australia Museum (SAM). Dr. John Chapman (Office of Environment and Heritage, NSW, Sydney) collected this species in 1986 from Parramatta Lake in Sydney and since then it has been cultured and maintained in a laboratory (Moreno Jully, OEH, NSW, personal communication, 23 June 2014). Sample specimens of the species were provided by Dr. Tsuyoshi Kobayashi (OEH, NSW) on 26/10/2010. Genetic Reference number: COI + 16s = SYDCB001 − 05. Five ephippial females (undissected) stored in a vial containing 70% denatured alcohol deposited in the SAM, accession number SAM C7024.

Diagnosis. Parthenogenetic females: head small, 4.8 to 5.6 times of body length, moderately depressed with shallow

TABLE 2: Comparisons of selected morphological characters of eight *Ceriodaphnia* spp.; —: no information available.

Character	C. dubia (N = 5)	C. spinata (N = 5)	C. sp.1 (N = 5)	C. quadrangula (Müller, 1785 [30])	C. laticaudata Müller, 1867 [31]	C. rotunda Sars, 1862 [32, 33]	C. pulchella Sars, 1862 [32, 33]	C. dubia Richard, 1894
Size of adult female in mm	1–1.4	0.75–0.95	0.9–1.2	0.5–0.9	0.7–1	0.8–1	0.5–0.86	0.7–1
Posterior margin of carapace	Row of fine spinules punctuated partially or entirely with 10 to 12 spines	Approx. 1/3rd of the posterior end shows spines projecting from the edge of the carapace	Long flexible setules followed by a row of fine thin spines interrupted in between with fine spiniform setules rising parallel from the edge	acute and spine shaped	Long flexible setules followed by a row of fine thin spines interrupted in between with fine spiniform setules rising parallel from the edge	Is convex shaped with clear and coarse reticulation and serrated spines at the edge of the carapace. It ends on posterior side at a posterodorsal angle with blunt spine	Row of thin hairs continues till the rear edge, where it reaches its highest point near the junction of the posterodorsal angle. Posterodorsal angle shows presence of spine which is laid almost parallel to its edge	—
Reticulation and sculpture of carapace	Evenly sized polygonal in shape with heavy edges and dotted surfaces	Distinctly reticulated with relatively large raised nearly hexagonal meshes	Distinctly reticulated with relatively large flattened polygonal meshes	Coarser reticulation on the surface with nearly hexagonal structure	Is irregular in shape	Thick regular hexagonal reticulation	Strong polygonal reticulation	Cross-linking regular polygonal structure
Postabdomen	Moderately long and wide that tapers gradually in width towards the terminal claw	Narrow and half the length compared to the maximum body width	Broad and almost half in length compared to the maximum body width	Narrow and of medium size with rounded end	Broad with the securiform (ax) shape	Broad with two small obtuse and horn shaped angles or abdominal appendages at the posterior end	Postabdominal shape towards the end has been mentioned as wide (Lillieborg 1900b) [34], narrow, and elongated (Hellich 1877; Herrick 1884), approximately straight or slightly concave (Paggi 1986)	Moderately long and wide that tapers gradually in width towards the terminal claw
Anal denticles	7–10	8–10	9–10	8–10	9–11	7–9	7–14	8–10
Total number of setulated setae on III (E = endopodite; Ex = exopodite)	6 E1 = 2; Ex1 = 4	4-5 E1 = 1; Ex1 = 3/4	4 E1 = 0; Ex1 = 4	—	—	—	—	—
Number of setae on exopodite V	4	3	4	—	4	—	4	—
Middle pecten	21 long and stouter teeth	Presence of fine setules	With heavier and slightly longer spinules, compared to proximal pecten	—	Presence of larger spinules	—	Presence of larger spinules	Form 1 toothed pecten variety with a central pecten on the claw showing 18 to 24 finely setulated pectens

supraocular depression, elongated polygonal reticulations over frons, no fenestra on anterior surface of cervical notch. Eye moderately large in size, 2.1 to 2.6 times in head length, lenses prominent. Fornix smoothly rounded without lateral extensions and without a minute spine at broadest portion (Figure 1(a)). The rostral region with flat round pore (Figure 1(d)). Antennules short, anterior sensory hair arising from small peduncle, one-third the distance from apex, anterior sensory hair longer than either antennule body or aesthetascs. Ridge of cuticle encircles rostral pore. Coxal portion of antennae folded, with two sensory setae. Surface of basipod with nine irregular rows of fine spinules varying in length and thickness. Posterior ventral carapace margin with inner row of fine spinules punctuated partially or entirely with 12 spines and with one to three short, heavier, plumose spines dorsally at about 40% of distance below the posterior ventral margin. Surface of the carapace with polygonal reticulations (Figure 2(a)).

Trunk limb I with setulated setae on endites two to five (E2 to E5). Two ejector hooks situated on the anterior side of the limb. Trunk limb II with five endites E1 to E5 consisting of seven setulated setae and two soft setae; the gnathobase bears five setae and E2 bears one long and one short accessory spines. Trunk limb III consists of one exopodite and five endites. Trunk limb IV with eight setulated setae. Trunk limb V with a small sized exopodite bearing four setae of which two are long and two are small in size (Figure 2(d)).

Postabdomen slightly tapered and obliquely truncated distally. There are about eight anal denticles (Figure 3(a)). The postabdominal claw setules of proximal group short and slightly lighter in weight than those of the distal group. Middle pecten with 21 long and stouter teeth (Figure 3(d)).

3.1.3. C. sp. 1

Holotype. Slide of a parthenogenetic female, Yanga Lake, Sydney, NSW (34°42′19.04″S/143°35′30.73″E). Collected by Dr. T. Kobayashi (OEH, NSW) on 26-10-2010 (SAM C7551).

Material Examined. Five parthenogenetic females from Yanga Lake near Sydney, NSW (34°42′19.04″S/143°35′30.73″E), in a vial containing 70% denatured alcohol deposited in SAM, accession number SAM C7551. Barcode Reference: COI + 16s = NSYAN001 − 02, NSSTE002, NSMER003 − 05.

Diagnosis. Parthenogenetic females: head small, 3.9 to 4.33 times of body length and moderately depressed with shallow supraocular depression and irregular polygonal reticulations over frons. Fornix with extended hooks (Figure 1(b)). Cervical notch is not deep. Eye large in size, 1.9 to 2.25 times in head length, lenses prominent. Rostral region shows bulging vertical pore (Figure 1(e)). Antennules are long, anterior sensory hair rises from small peduncle 1/3rd distance from apex, anterior sensory hair longer than either the antennule body or aesthetascs. Coxal portion of antennae is folded and supplied with three sensory setae. Surface of basipod with 10 irregular rows of fine spinules varying in length and thickness. Posterior margin of carapace shows a line of fine spinules which are punctuated with spines. Surface of the carapace with hexagonal reticulations (Figure 2(b)).

Trunk limb I with setulated setae on endites 2 to 5 (E2 to E5), and two setae on E5. Trunk limb II comprises five endites E1 to E5, with four brush setae on gnathobase and two short accessory spines on E2. Trunk limb III exopodite consists of four setulated setae on the distal end and 2 small setulated setae on the lateral end. There are five endites on trunk limb III of which endites 1 to 4 are highly reduced and E5 bears a number of gnathobasic filtering setae. Trunk limb IV has five setulated setae at the distal end. Posterior end has two setae. Trunk limb V has a small sized exopodite bearing four setae of which two are long and two is smaller in size (Figure 2(e)).

Postabdomen is almost half in length compared to the maximum body width. There are eight recurved anal denticles (Figure 3(b)). The distal end of postabdominal claw is slightly concave and base is straight, with setules of proximal group being short and slightly lighter in weight than those of the distal group. Middle pecten with 31 long and thinner teeth (Figure 3(e)).

3.1.4. Ceriodaphnia spinata Henry, 1919 (pI. XL, Figures 1 and 2). Ceriodaphnia spinata Henry, 1919: 466.

Type Material. Holotype AMP 4327 (1 ovigerous female).

Type Series. Type locality: "Corowa," Australia.

Nontype Material. Australia: Victoria, Thornton, 1998-10-09, USNM 1121570 (5♀ in isopropyl alcohol); 1121571 (6♀, in isopropyl alcohol).

Material Examined. One female, Goulburn Billabong, Alexandria, Victoria, Australia, 06.08.1974 (AMP 27728); five ephippial females each from South Para Reservoir, Myponga Reservoir, Mannum and Snuggery Adelaide, SA; one parthenogenetic female from Mannum SAMAN001 (36°16′29.99″S/139°54′40.41″E), SA, sample collected by the author on 15/02/2009, deposited in SAM. Genetic Reference: COI + 16s = SAMAN001 − 02, SASNU001. Five ephippial females from Mandina (undissected) stored in a vial containing 70% denatured alcohol deposited in SAM, accession number: SAMC7572.

Diagnosis. The holotype (P: 4327) is a mounted slide of ovigerous female. The second slide-mounted specimen (P: 27728) of the same length, from Goulburn billabong, Alexandria, Victoria, Australia, is poorly preserved. Smirnov and Timms (1983) reported the total length of females in the range 0.8 to 1.2 mm.

Parthenogenetic Females. Head small, 5 to 5.5 times of body length, moderately depressed with absence of supraocular depression and presence of irregular polygonal and hexagonal reticulations, which are punctuated and stippled. Eye moderately large, 1.8 to 2.5 times in head length, lenses prominent. Edges or nodes of these reticulations are an acute V-shaped structure. Cervical notch not deep. Fornix with two small smooth denticles (Figure 1(c)). The rostral region shows flat round pore (Figure 1(f)). Eye moderately large size and lenses are prominent. Antennules are long, anterior sensory hair rises from small peduncle which is approximately half
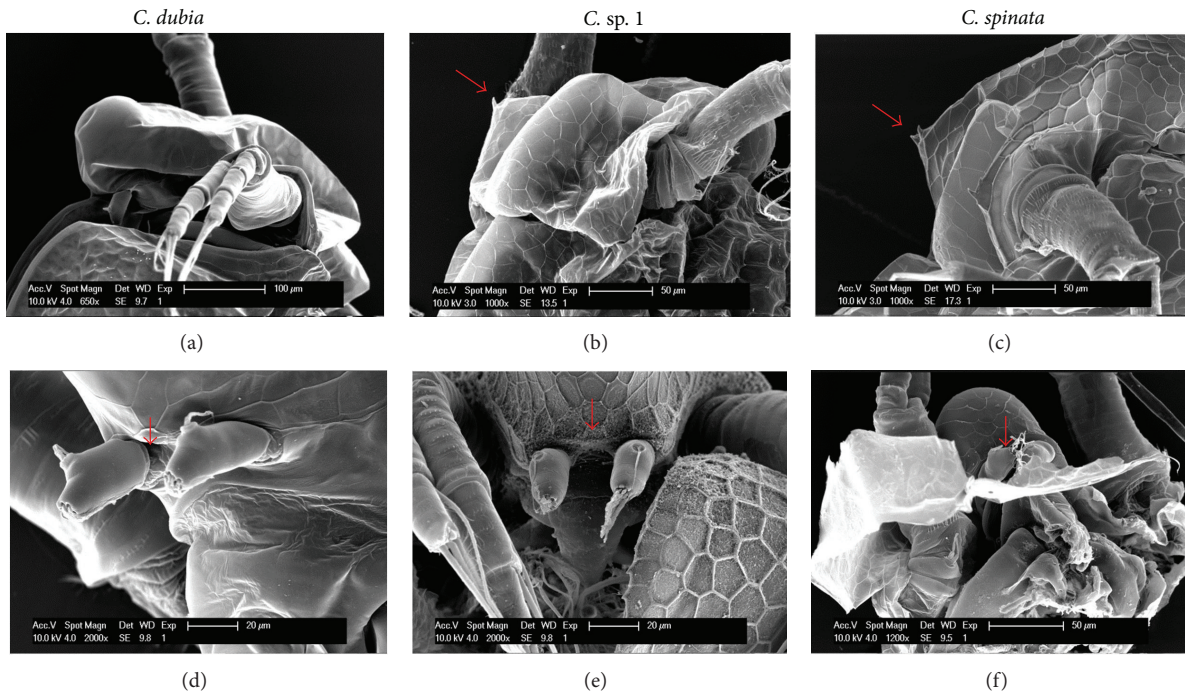
FIGURE 1: ((a)–(c)) SEMs of fornix. (a) Absence of denticles; (b) smooth extended hooks; (c) smooth small denticles. ((d)–(f)) SEMs of the rostral pore. (d) Flat; (e) bulging; (f) flat.
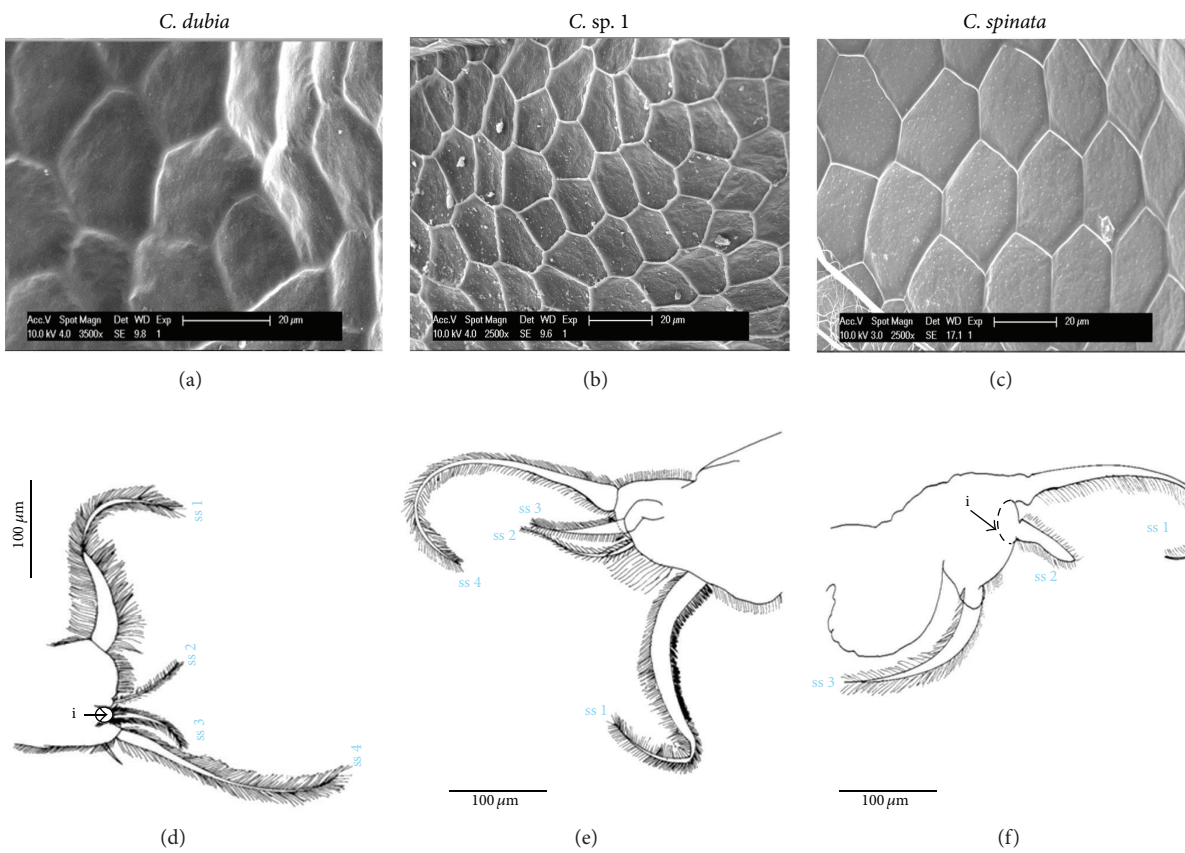

FIGURE 2: ((a)–(c)) SEMs of reticulation on the carapace. (a) Polygonal reticulation; ((b) and (c)) hexagonal reticulation. ((d)–(f)) Line drawing of Trunk Appendage V for the three species.

C. dubia

C. sp. 1

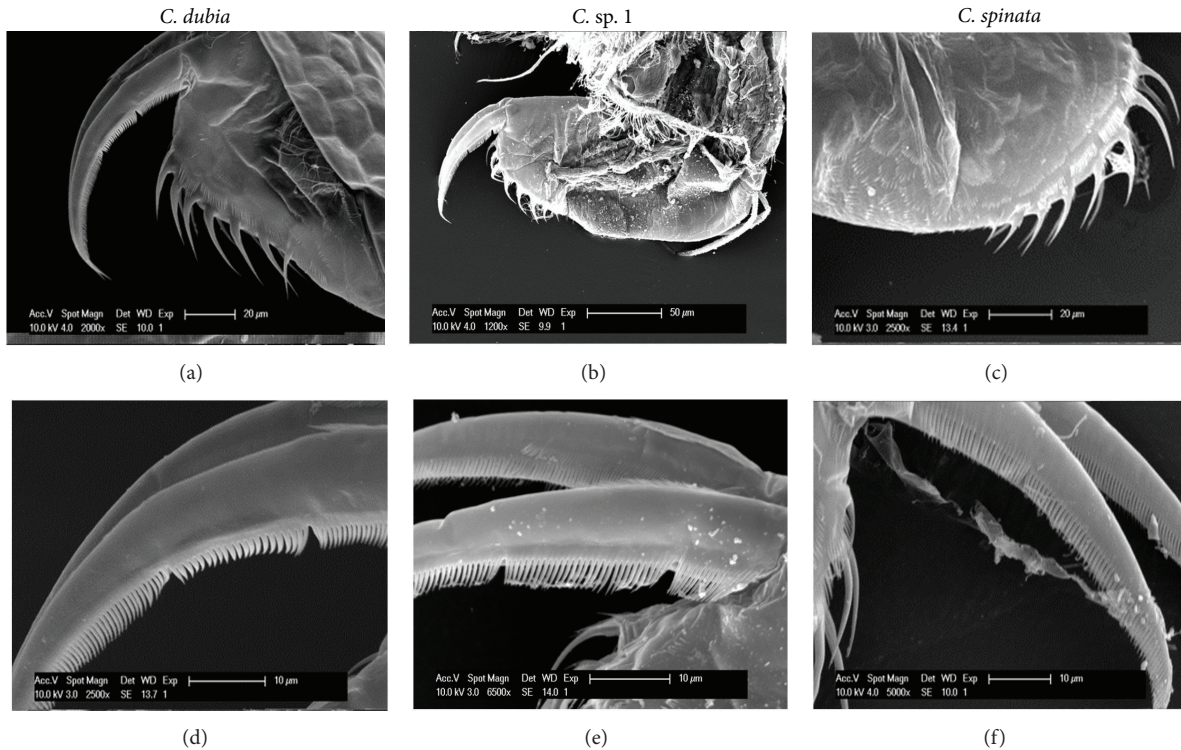C. spinata

(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 3: SEMs of postabdomen and denticles of the three species.

the distance from apex, anterior sensory hair longer than either the antennule body or aesthetascs. Coxal portion of antennae is folded and supplied with two sensory setae; lateral basal section of the basipod shows presence of ten plumose setae. Surface of basipod shows ten irregular rows of fine spinules varying in length and thickness. Posterior margin of the carapace shows no punctuating spine, unlike *C*. sp. 1 Posterodorsal angle is pointed and spiny. Surface of the carapace with hexagonal reticulations (Figure 2(c)).

Trunk limb I with setulated seta on endites 2 to 5 (E2 to E5); E5 shows the presence of three setae. Trunk limb II comprises of five endites E1 to E5: the gnathobase bears five brush setae and E2 bears two long accessory spines. Trunk limb III exopodite consists of three setulated setae on distal end and one small setulated seta on the lateral end. Trunk limb IV has four setulated setae in the distal end and the posterior end has a single seta. Trunk limb V has a small sized exopodite bearing three setae of which the distal and proximal one are the longest compared to middle setae (Figure 2(f)).

Postabdomen is almost half the length compared to maximum body width. There are nine recurved anal denticles (Figure 3(c)). The distal end of postabdominal claw is slightly concave and base is curved, with setules of proximal group shorter and thinner than those of the distal group (Figure 3(f)).

3.2. Phylogenetic Relationships Derived from cmtDNA. Basic statistics and selected substitution models for mtDNA sequences are shown in Table 3. The cmtDNA set yielded

a total of 14 unique sequences, consisting of 1185 aligned nucleotides, of which 220 sites were variable and 216 sites were parsimony informative. No internal stop codons and indels were detected. Trees based on individual gene fragments (not shown) and on cmtDNA (Figure 4) as well as nuclear marker 28s (Figure 5) yielded similar topologies in which:

(1) Clade 1 and Clade 2 comprising *C*. sp. 1 and *C. spinata* were supported by 100% bootstrap support and include populations from NSW and SA. Intraspecific pairwise differences for cmtDNA ranged between 0 and 1%, while minimal observed interspecific distance was 10% (ML corrected = 13%) (Table 4);

(2) Clade 3 comprising *C. dubia* was represented by laboratory cultured species from Parramatta, NSW, with bootstrap value of 100% and intraspecific divergence of 0% for cmtDNA (Table 4).

3.3. Comparison between Australian and North American Populations of Ceriodaphnia. Analysis of COI sequences between Australian *C*. sp. 1 and *C. spinata* and North American species of *Ceriodaphnia* (Figure 6) showed raw p-distance analysis between 2 and 21%, whereas corrected ML-distance analysis is 18 to 103% (Table 5). The exceptions to this are SYDCB001 and SYDCB003 specimens from Parramatta, NSW, which show genetic similarity with ZPLMX 452 and ZPLMX453 (*Ceriodaphnia* cf. *laticaudata*, Mexico); ZPLMX095 and ZPLMX859 (*Ceriodaphnia* cf. *acanthina*,
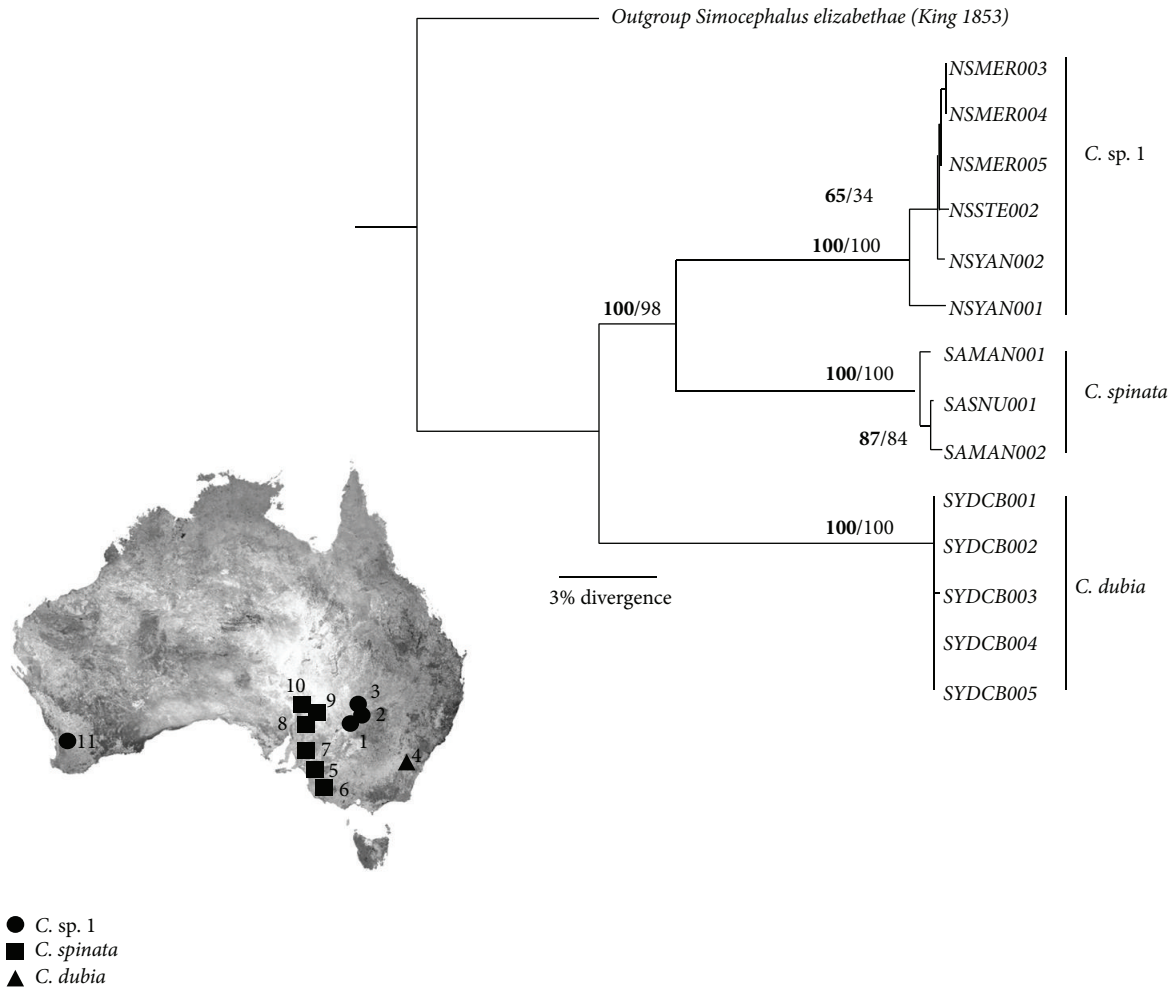
FIGURE 4: Phylogenetic trees inferred from concatenated mtDNA gene sequences for *Ceriodaphnia* within Australia. Numbers above branches are maximum likelihood (100 replicates) and numbers in bold are from Bayesian analysis. For location details refer to Table 1.

TABLE 3: Sequence information for the different gene fragments without the outgroup. Fragment length in base pair, number of variable sites (*V*), and number of gap positions or missing data (*G/M*).

Gene fragments	Length	*V*	*G/M*	Model test
mtDNA				
16s	540	75	—	K81uf + I
COI	645	148	—	K81uf + G
cmtDNA	1185	220	—	TVM + G
Nuclear				
28s	575	479	—	GTR + I + G

TABLE 4: Minimum and maximum cmt mtDNA (upper line in each cell) and 28s (bold bottom line in each cell) raw pairwise divergence within and between *Ceriodaphnia* species, with ML corrected divergence value inside the closed bracket (). The figures are in percentage (%) and 0 represents the sequence divergence value being <0.005%.

Species	*C.* sp. 1	*C. spinata*	*C. dubia*
C. sp. 1	0-1 (0-1) **0-1 (0-1)**		
C. spinata	10 (13–15) **8 (9-10)**	0-1 (0-1) **0 (0)**	
C. dubia	14 (24-25) **12–14 (17–19)**	15 (26-27) **9-10 (12–14)**	0 (0) **0–2 (0–2)**

Mexico) with 1–4% (raw p-distance) and ML distance of 1 to 6%.

4. Discussion

Molecular analysis of the published COI sequences available from GenBank including the sequences generated from this study (Figures 4 and 5) indicates the presence of three distinct *Ceriodaphnia* species. This is also well supported by morphology which showed consistent differences of ten key characters among and between *C. dubia*, *C.* sp. 1, and *C. spinata*.

Table 5: Minimum and maximum COI raw pairwise divergence within and between species with ML corrected divergence value inside the closed bracket () of the *Ceriodaphnia* complex. The figures are in percentage (%) and 0 represents the sequence divergence value being <0.005%. Asterisk represents species from Australia.

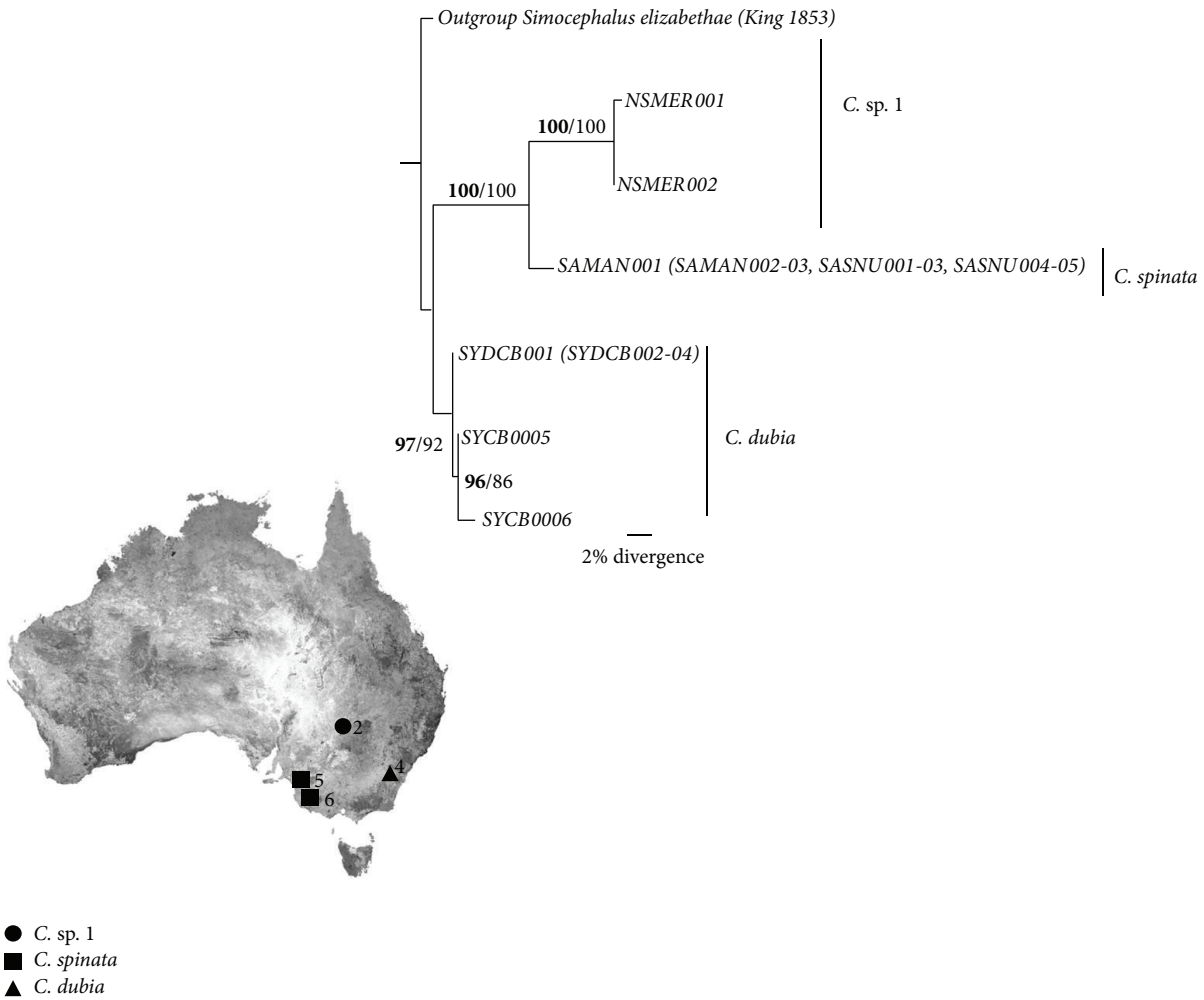|	*Ceriodaphnia* sp.	*C. laticaudata*	*Ceriodaphnia* sp.	*Ceriodaphnia* sp.	*Ceriodaphnia* sp.	*Ceriodaphnia* cf. *rigaudi*	*Ceriodaphnia* cf. *rigaudi* 2	*Ceriodaphnia* sp.	*Ceriodaphnia* *spinata**	*Ceriodaphnia* sp. 1*	*Ceriodaphnia* cf. *reticulata*	*Ceriodaphnia* sp.	*Ceriodaphnia* cf. *laticaudata*	*Ceriodaphnia* cf. *acanthina*	*C. dubia**	*C. dubia*
Ceriodaphnia sp.	0 (0)															
C. laticaudata	20 (80)	0 (0)														
Ceriodaphnia sp.	15-16 (64–66)	18-19 (64–67)	0–3 (0–4)													
Ceriodaphnia sp.	15 (58)	18 (73)	16-17 (57–60)	0 (0)												
Ceriodaphnia sp.	17 (68)	17 (83)	16-17 (67–70)	15 (51)	0 (0)											
Ceriodaphnia cf. *rigaudi*	17 (60-61)	18-19 (75–76)	15-17 (59–62)	15 (42-43)	6 (10-11)	0-1 (0-1)										
Ceriodaphnia cf. *rigaudi* 2	18 (64)	20 (79)	17-19 (63–65)	14 (46)	15 (43)	14 (35–36)	0-1 (0-1)									
Ceriodaphnia sp.	18 (64)	20 (79)	17-18 (63–66)	14 (46)	15 (43)	14 (35–36)	0-1 (0-1)	0 (0)								
Ceriodaphnia *spinata**	17-18 (64)	20 (89)	18-19 (73–76)	16 (67-68)	15-16 (78)	16 (69–70)	17 (73)	17 (73-74)	0 (0)							
Ceriodaphnia sp. 1*	17 (61-62)	19 (86)	16-17 (70–73)	17 (64-65)	18 (74-75)	17-19 (66-67)	19 (70-71)	19 (70-71)	13 (31-32)	0–2 (0–2)						
Ceriodaphnia cf. *reticulata*	19 (79)	21 (103)	18-21 (87–90)	17 (82)	16 (92)	16 (84)	15-16 (87)	16 (88)	16-17 (72)	19 (69)	0 (0)					
Ceriodaphnia sp.	20 (76-77)	21 (101)	18-21 (85–87)	17 (79–80)	16 (90)	16 (81-82)	16 (85–86)	16 (85-86)	16 (70)	19 (67-68)	3 (3)	1 (1)				
Ceriodaphnia cf. *laticaudata*	19 (65–67)	20 (90–92)	20-21 (73–77)	19 (68–70)	17 (78–80)	17-18 (70–72)	18-19 (74–76)	18-19 (74–76)	18 (59–61)	18 (55–58)	14-15 (37–39)	14-15 (35–37)	0–2 (0–2)			
Ceriodaphnia cf. *acanthina*	18 (71)	20 (96)	20-21 (79–82)	19 (74)	16 (84)	17-18 (76–77)	19 (80)	19 (80)	18-19 (64–65)	18-19 (61-62)	15 (43)	14 (41)	4-5 (5-6)	0 (0)		
C. dubia*	19 (68)	20 (92)	20-21 (76–79)	19 (71)	17 (81)	17 (73-74)	19-20 (76-77)	19-20 (77)	18 (61-62)	18 (58-59)	14 (40)	13-14 (37-38)	2-3 (2-3)	4-5 (5-6)	0 (0)	
C. dubia	17-18 (71–74)	19-20 (96–99)	17-20 (80–84)	17-18 (74–78)	15-16 (84–87)	16-17 (76–80)	18-19 (80–83)	18-19 (80-83)	17-18 (65–68)	18-19 (62–65)	14-15 (43–47)	14-15 (41–45)	11-12 (18–23)	11-12 (24–27)	10-11 (24–27)	0–3 (3)

FIGURE 5: Tree indicating the phylogenetic relationship inferred from 28s gene sequences for *Ceriodaphnia* within Australia. Numbers above branches are maximum likelihood (100 replicates) and numbers in bold are from Bayesian analysis. For location details refer to Table 1.

The specimens of *C. dubia* from Sydney, NSW (SYDCB), and specimens identified as *C. dubia* Richard, 1894, are morphologically the same. The *C. dubia* specimen from Sydney, NSW (SYDCB), appears to be morphologically the same as *C. dubia* Richard, 1894 in a "strict sense" (Berner, personal communication), hence *C. dubia* s.s. The variation in the number of setules on pecten has been highlighted by Berner (1986) [36] in US EPA report, were two form of toothed pecten variation of *C. dubia* was observed only under controlled environmental and nutritional conditions. However, the *C. dubia* description from New Zealand by Greenwood et al.(1991) [37] is similar to Berner (1986) [36] description of Form 1 toothed pecten variety with a central pecten on the claw showing 18 to 24 heavy finely setulated pecten, which are slightly longer than those of the adjacent setules, similar to *C. dubia* s.s studied here.

The lack of sufficient taxonomic detail for Australian *Ceriodaphnia* has resulted in incorrect identifications, leading to a great deal of confusion, with synonymization or clustering several distinct species like *C. planiformis* and *C. spinata* with *C. quadrangula* Lilljeborg (1900) [34], a holarctic species. *C. planiformis* and *C. spinata* are endemic Australian species

and therefore are not synonymous with *C. quadrangula*. The variations in size and shape of the postabdomen, number of setae on endopodite of limbs I, II, and V, presence or absence of punctuating spines along the posterior margin of the carapace, and variation in thickness of spinules of the middle pecten are stable characters that all point toward *C. planifrons* and *C. spinata* being distinctly separate species. These characters are also comprehensively documented by line drawings and SEM images by Berner [4].

Despite close morphological similarities of *C.* sp. 1 with *C. planifrons* reported by Berner [4], the loss of the holotype, and lack of well-preserved specimens of *C. planifrons*, nothing definitive can be said about this species.

There are a total of 11 unmounted specimens, approximately 30 years old, of *C. spinata* lodged in the Smithsonian Institution under accession codes 1121570 and 1121571 in Isopropyl alcohol. Isopropyl alcohol or isopropanol (IPA) has been a preferred choice for museums to store specimens for long term storage. Also, failure in extracting and amplifying DNA from >5 years preserved samples, in this study, available unmounted original material of *C. spinata* was not used for the genetic study. However, comparative light microscopical

Outgroup *Simocephalus elizabethae* (King 1853)

CERI0002 *Ceriodaphnia sp.* JRDW-2005   *C.* sp.

47/75

29/52

ZPLMX458 *C. laticaudata* Mexico: Coahuila
EU702084 *C. laticaudata* Mexico: Coahuila   *C. laticaudata*
100 /100

99/100
ZPLMX474 *C. sp.* Mexico: Chihuahua
ZPLMX476 *C. sp.* Mexico: Chihuahua
ZPLMX104 *C. sp.* Mexico: Durango
ZPLMX310 *C. sp.* Mexico: Durango
ZPLMX307 *C. sp.* Mexico: Durango
ZPLMX165 *C. sp.* Mexico: Durango   *C.* sp.
93/97

18/56

ZPLMX449 *C. sp.* Mexico: Quintana Roo   *C.* sp.
85/99
ZPLMX470 *C. sp.* Mexico: Veracruz   *C.* sp.
ZPLMX635 *C. cf. rigaudi 2* Guatemala: Alta Verapaz
ZPLMX303 *C. cf. rigaudi 2* Mexico: Tabasco
ZPLMX631 *C. cf. rigaudi 2* Guatemala: Alta Verapaz
ZPLMX629 *C. cf. rigaudi 2* Guatemala: Alta Verapaz   *C. cf. rigaudi*
94/83

28/98

22/57

100/100
ZPLMX467 *C. cf. rigaudi* Mexico   *C. cf. rigaudi*
ZPLMX464 *C. cf. rigaudi 1* Mexico: Durango
ZPLMX463 *C. sp.* Mexico: Chihuahua
ZPLM461 *C. sp.* Mexico: Chihuahua   *C.* sp.

20/40

98/100

100/100
SAMAN001 (SAMYP001-02; 05-07, SAWOO01, SACHOW01-04) Australia   *C. spinata* Australia
SASNU001 Australia

100/100
NSYAN001 *C. sp. 1* Australia
NSMER001 *C. sp. 1* Australia
NSYAN002 (NSSTE002) *C. sp. 1* Australia
NSMER002 *C. sp. 1* Australia   *C.* sp. 1 Australia
70/92

ZPLMX314 *C. cf. reticulata* Mexico: Durango   *C. cf. reticulata*
100/100
ZPLMX311 *C. sp.* Mexico: Durango
ZPLMX097 *C. sp.* Mexico: Campeche   *C.* sp.

98/100
ZPLMX452 *C. cf. laticaudata* Mexico: Coahuila
ZPLMX453 *C. cf. laticaudata* Mexico: Coahuila
ZPLMX095 *C. cf. laticaudata* Mexico   *C. cf. laticaudata*

84/100

79/87

75/97
ZPLMX859 *C. cf. acanthina* Mexico: Sonora   *C. cf. acanthina*
SYDCB001-02 *C. dubia s.s.* Australia
SYDCB003-05 *C. dubia s.s.* Australia   *C. dubia s.s.* Australia
88/98

ZPLCA029 *C. dubia* Mexico
100/100
EU702069 *C. dubia* Canada: Ontario
ZPLCA027 *C. dubia*
ZPLCA028 *C. dubia* Canada: Ontario
ZPLMX322 *C. dubia* Guatemala: Peten
ZPLMX170 *C. dubia* Guatemala: Peten
ZPLMX322 *C. dubia* Guatemala: Peten
ZPLMX323 *C. dubia* Guatemala: Peten
65/75
ZPLMX331 *C. dubia* Mexico
ZPLMX106 *C. dubia* Mexico
ZPLMX330 *C. dubia* Mexico
ZPLMX333 *C. dubia* Mexico
ZPLMX106 *C. dubia* Mexico   *C. dubia*
ZPLMX190 *C. dubia* Mexico
ZPLMX190 *C. dubia* Mexico
ZPLMX329 *C. dubia* Mexico
80/98
ZPLMX332 *C. dubia* Mexico
ZPLMX325C. *dubia* Mexico
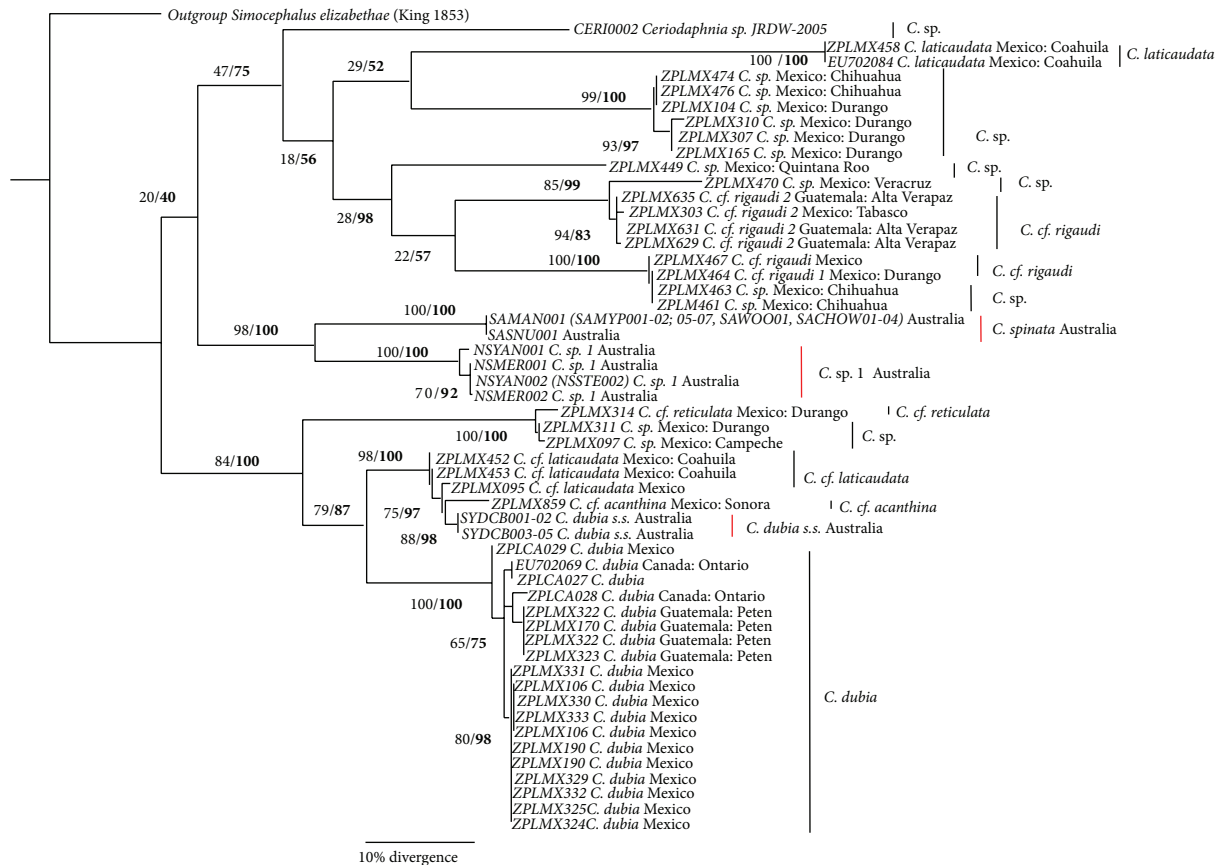ZPLMX324C. *dubia* Mexico

10% divergence

FIGURE 6: Maximum likelihood analysis of COI gene for *Ceriodaphnia* complex. Numbers above branches are maximum likelihood (100 replicates) and numbers in bold are from Bayesian analysis. Red bar represents species from Australia.

analysis of the type slide specimen and comparison with documented SEM and line drawings of Berner [4] helped narrow the third species to *C. spinata*.

4.1. Phylogenetic Analysis.

Phylogenetic relationships among species of *Ceriodaphnia* from Australia showed congruence between cmtDNA and nuclear gene tree topology with strong bootstrap support (Figures 4 and 5). The average bootstrap support values were moderate to high, suggesting that topologies estimated are reliable. Nevertheless, closely related species living in the same region formed a strongly supported monophyletic group *C.* sp. 1 and *C. spinata*. The low level of genetic divergence seen for *C. dubia* is probably due to the specimens being from a panmictic population from Parramatta River and laboratory cultured and maintained since 1986 (Julli, pers. comm.).

Phylogenetic analysis of mt COI sequences for *Ceriodaphnia* from different sites, many of which are from Mexico Elías-Gutiérrez [38], provided the first direct molecular evidence for genetic divergence between geographically isolated populations of *Ceriodaphnia*. Close genetic similarities between *C.* cf. *laticaudata* (raw p–distance of 2 to 3%, ML distance of 2 to 3%) and *C.* cf. *acanthina* (raw p-distance of 4 to 5%, ML distance of 5 to 6%) from Mexico to *C. dubia* and conversely large genetic differences with *C. dubia* (raw p-distance of 10 to 11, ML distance of 24 to 27%) from Guatemala

and Mexico indicate that the Australian population of *C. dubia* is genetically distinct and morphologically discrete.

In this work an integrated approach was used for achieving the goal of species identification and discrimination of *Ceriodaphnia* from Australia. The correlation between morphological and molecular identifications in this study indicates that both COI and 16s rRNA are appropriate molecular markers for species discrimination and identification of genus *Ceriodaphnia*. From this perspective the present study not only illustrates the usefulness of a combined morphogenetic approach for the relatively understudied genus *Ceriodaphnia*, but also provides the first DNA barcode reference for the three Australian *Ceriodaphnia* species studied.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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