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 Kofínek [17]. Slide mounted *Ceriodaphnia* collections from Australian Museum, Sydney, Australia (AMS); Natural History Museum, University of Ohio, Norway (NHMNU); 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 (LCO1490 and HCO2198) [20]. Each 50 μL PCR reaction consisted of 5 μL of genomic DNA template, 3 μL of 50 mM MgCl2, 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’ -CCCGAATTTCCGGCTTTATCAAAAAC-3’ and 16s2 5’ -CCCAGTTTCCGGGTTTGAACCTCAGAT-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 RNA, the primers used for amplification were 28s1 5’ -CCCGTGAATTTAAGCATAT-3’ and 28s2 5’ -TAGATGTTCCATTAGCCTTGGC-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 lx 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 |
|-------|----------|-------|---------------|---------------|----------------|----------------|----------------|----------------------------|
| 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°2'44.94"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 | Floodplain | 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 | Floodplain | 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 | Floodplain | 34°3'33.76"S 140°45'44.48"E | KC020655–59 — — |
| 10    | Woorabinda | South Australia (SA) | 15/10/2009 | SAWOO | Floodplain | 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; KC200651–KC200671 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: 21; Liljeborg, 1900: 183; Liljeborg, 1901: 675; Smith, 1909: 80; Sars, 1916: 315; Henry, 1922: 32.

Type Species. *Ceriodaphnia quadrangularis* (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). *Ceri-
daphnia dubia* Richard, 1894: 570; Delachaux, 1917: 80; Sars, 1916: 317; Berner, 1986: 16; Greenwood et al. 1991: 285.

Syn.: *Ceriodaphnia affinis* Liljeborg, 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 (DFG) collection was housed in Smithsonian Institution’s Museum Support Center in Suitland, Maryland, USA. According to the hand written catalogue, sample DFG 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 Titiaca 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 F9258 (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* Liljeborg, 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 I1913.

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

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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. laticeps* 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 spinules projecting from the edge of the carapace | Long flexible setules followed by a row of fine spines interrupted in between with fine spiny setules rising parallel from the edge | Long flexible setules followed by a row of fine spines interrupted in between with fine spiny 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 posteroventral angle with blunt spine | Row of thin hairs continues till the rear edge, where it reaches its highest point near the junction of the posteroventral angle. Posteroventral 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 secundiform (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 (Liljeborg 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 | 4 | 4 | 4 | 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 | Presence of larger spinules | Form I 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 spines varying in length and thickness. Posterior ventral carapace margin with inner row of fine spines 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 spines varying in length and thickness. Posterior margin of carapace shows a line of fine spines which are punctuated with spines. Surface of the carapace with polygonal 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 gnathobase 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 (pl. 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 Snuggerly 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.
Figure 3: SEMs of postabdomen and denticles of the three species.

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, Mexico).
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)| 0-1 (0-1)  |          |
| C. dubia    | 14 (24-25)| 15 (26-27)| 0 (0)    |

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*. 

Outgroup Simocephalus elizabethae (King 1853)
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.

| Species                        | Minimum | Maximum | COI corrected |
|--------------------------------|---------|---------|---------------|
| *Ceriodaphnia* sp.             |         |         |               |
| *C. laticaudata*               | 0 (0)   |         |               |
| *Ceriodaphnia* sp.             | 20 (80) | 0 (0)   |               |
| *Ceriodaphnia* sp.             | 15 (58) | 18 (73) | 0 (0)         |
| *Ceriodaphnia* sp.             | 17 (68) | 17 (83) | 15 (51)       |
| *Ceriodaphnia* cf. rigaudi     | 18 (64) | 20 (79) | H (46)        |
| *Ceriodaphnia* cf. rigaudi 2   | 17 (19) | 16 (63) | 14 (35)       |
| *Ceriodaphnia* sp.             | 18 (64) | 20 (79) | H (46)        |
| *Ceriodaphnia* sp.             | 17 (18) | 16 (63) | 14 (35)       |
| *Ceriodaphnia* spinata*        | 17-18 (64) | 20 (89) | 16 (67)       |
| *Ceriodaphnia* sp.             | 17 (61-62) | 19 (86) | 17 (64-65)    |
| *Ceriodaphnia* cf. reticulata | 19 (79) | 21 (103) | 17 (82)       |
| *Ceriodaphnia* sp.             | 20 (76-77) | 21 (101) | 17 (79-80)   |
| *Ceriodaphnia* sp.             | 19 (65-67) | 20 (90-92) | 19 (68-70) |
| *Ceriodaphnia* cf. acanthina  | 18 (71) | 20 (96) | 19 (74)      |
| *C. dubia*                     | 19 (68) | 20 (92) | 19 (71)      |
| *C. dubia*                     | 17-18 (71-74) | 19-20 (96-99) | 17-18 (74-78)  |
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) 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) is similar to Berner (1986) 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
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 Elias-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 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 morpho-genetic 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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**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.
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