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Phylogeny and Systematics of *Aponogeton* (Aponogetonaceae): The Australian Species

DONALD H. LES,[1,3] MICHAEL L. MOODY,[1] and SURREY W. L. JACOBS[2]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269-3043 USA;
[2]Royal Botanic Gardens, Sydney, New South Wales, 2000, Australia
[3]Author for correspondence (les@uconn.edu)

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ABSTRACT. *Aponogeton* is an important genus whose species are cultivated widely as ornamental aquatic plants. Although a fairly recent monograph has been published, the genus remains poorly studied systematically. We conducted a phylogenetic survey of *Aponogeton* that focused on relationships among the nine native Australian species as well as their relationship to other members of the genus. Our analyses included a phylogenetic assessment of morphological characters and molecular data obtained both from chloroplast (*trnK* 5′ intron, *matK*) and nuclear DNA (nrITS) loci. Molecular data provided evidence of hybridization and polyploidy as well as an informative overview of interspecific relationships in the genus. Two potentially new Australian species also were identified by the molecular data. Combined molecular data produced a well-resolved cladogram that enabled us to evaluate previous phylogenetic hypotheses based on non-explicit methods as well as the soundness of the existing classification of the genus. We conclude that Aponogetonaceae originated in Australia and subsequently radiated into Africa, Madagascar, and Asia, from which a secondary Australian diversification occurred resulting in a biphyletic origin of the native Australian species. A pattern of morphological distinctiveness coupled with low molecular divergence indicates relatively recent and rapid speciation of *Aponogeton* in Australia. Our results also demonstrate that in this group, morphological data are extremely unreliable taxonomically due to their extensive homoplasy. The phylogenetic relationships elucidated by this study provide evidence to support the establishment of two additional sections, ***Flavida*** and ***Viridis*,** which are described.

The freshwater monocotyledon genus *Aponogeton* L.f. (Aponogetonaceae) comprises approximately 50 species of obligate aquatic plants that are distributed mainly in tropical or subtropical regions of the Old World (Cook 1996; Hellquist and Jacobs 1998; Bruggen 1985). Analysis of *rbcL* sequence data indicates that the monotypic Aponogetonaceae occupy a relatively basal position near the families Juncaginaceae and Scheuchzeriaceae in one of two major clades that subdivide subclass Alismatidae (Les et al. 1997).

Although the genus is not a dominant element of any flora, *Aponogeton* is important economically as it long has been regarded as a valuable source of species suitable for use as aquarium plants. In particular, the unusual fenestrate-leaved *A. madagascariensis* (Mirb.) H. Bruggen (known as ''Madagascar lace plant'') has been in cultivation since 1855 (Tricker 1897) and continues to rank among the most popular ornamental freshwater aquarium plants. *Aponogeton distachyos* L.f. (''water hawthorne'') has been cultivated for more than two centuries, but mainly as an outdoor pond or water-garden ornamental (Bruggen 1985). It has been introduced to Victoria, Australia where it is regarded as an invasive weed (Gunasekera 2003). Another 15 species from Asia, Australia, and Madagascar are grown either widely or at least to a minor degree as ornamental aquarium plants (Kasselmann 1995). *Aponogeton distachyos* recently has become a popular food plant in South Africa where it is now cultivated intensively (Gunasekera 2003; Pemberton 2000). Several other species (*A. capuronii* H. Bruggen, *A. crispus* Thunb., *A. elongatus* F. Muell. ex Benth., *A. euryspermus* Hellq. & S. W. L. Jacobs, *A. madagascariensis*, *A. natans* (L.) Engl. & K. Krause, *A. queenslandicus* H. Bruggen, *A. vanbruggenii* Hellq. & S. W. L. Jacobs) have edible tubers, but are important only locally as food plants (Bruggen 1985; Cowie et al. 2000).

There are no Aponogetonaceae native to the New World. Over half of the species occur on the African continent (17 species) and in Madagascar (11 species) (Bruggen 1985; Kasselmann 1995). Ten *Aponogeton* species grow in India and Southeast Asia (Bruggen 1985; Cook 1996) with a subset of five in Sri Lanka (Thabrew and Thabrew 1983). Two species (both endemic) are known from New Guinea (Leach and Osborne 1985) and the remaining species are Australian. The South African *A. distachyos* has been introduced to Australia (New South Wales, Victoria, South Australia), Europe (England, France), New Zealand, North America (California), and South America (Argentina, Peru).

Aponogeton is poorly understood systematically. In *Aponogeton*, as in many aquatic plants, taxonomic study has been hampered by the similar, often convergent vegetative morphology of most species (sterile plants are notoriously difficult to identify), extensive phenotypic plasticity (Bruggen 1985; Hellquist and Jacobs 1998), and highly simplified reproductive structures. Consequently, there are few morphological characters that are useful for making taxonomic distinctions or that might serve as reliable phylogenetic markers (Bruggen 1985).

In the early 19th century, *Aponogeton* was subdivided

into two genera with *Ouvirandra* segregated to include plants with caducous tepals and large plumules. However, the latter genus was ill-defined and could not be maintained as circumscribed (Bruggen 1985). Several classifications of *Aponogeton* have emphasized the taxonomic importance of seed-coat (testa) number and inflorescence morphology but have treated infrageneric groups only informally. The most recent formal classification of *Aponogeton* was given by Camus (1923) who divided the genus into two sections: *Aponogeton* (flowers omnilateral) and *Pleuranthus* (flowers secund). Each section was divided further into two subsections that separated plants with simple versus forked inflorescences. The fact that this classification subdivides a group of nearly 50 species by incorporating only two characters attests to the difficulty in finding characters appropriate for systematic applications.

With admitted reluctance, Bruggen (1985) followed the classification of Camus (1923) in his monograph of 42 *Aponogeton* species even though he was not confident that the classification succeeded in depicting natural groups. Bruggen also believed that *Aponogeton* was unsuitable for cladistic analysis because of what he perceived as many reticulate relationships attributable to polyploidy. Nevertheless, within the broader classification, Bruggen identified several groups of species that he believed to be closely related. However, these proposed interspecific relationships essentially remain untested.

Thanikaimoni (1985, p. 11) evaluated phylogenetic relationships in *Aponogeton* by presenting a ''scheme depicting the morphological diversifications'' that he believed to indicate ''evolutionary trends'' in the genus (Fig. 1). Technically, this diagram is not a cladogram, but represents a phylogenetic hypothesis based upon Thanikaimoni's perception of interspecific relationships as indicated by transitional morphological series. By these phylogenetic relationships, the sections and subsections recognized by Camus (1923) all represent polyphyletic groups (Fig. 1) with the exception of *Aponogeton* sect. *Pleuranthus* subsect. *Monostachys*, which is monotypic. Thanikaimoni's phylogenetic perspective placed the Malagasy *A. longiplumulosus* H. Bruggen as basal in the genus, which led him to hypothesize that Aponogetonaceae originated in Madagascar. However, the soundness of the existing classification and these phylogenetic hypotheses cannot be ascertained until more empirical analyses have been undertaken.

Studies made during the past 35 years have provided evidence that Australia is an important center of diversity for *Aponogeton*. Although early workers recognized *Aponogeton elongatus* as the only native Australian species (Krause and Engler 1906), Bruggen's (1969) revision of Australian *Aponogeton* added three new species to yield a total of four native (*A. bullosus* H. Bruggen, *A. elongatus*, *A. hexatepalus* H. Bruggen, *A. queenslandicus*) and one nonindigenous species (*A. distachyos*) in the flora. Among these, *A. hexatepalus* was so distinctive by its forked inflorescence and flowers with six tepals that Bruggen (1969) doubted whether it shared a close relationship with any living *Aponogeton* species. Aston (1973) recognized the same five species in Australia, but also commented on an unnamed, ''proliferous'' taxon (i.e., producing vegetative plantlets in lieu of flowers in the inflorescence) in northern Queensland. Most recently, Hellquist and Jacobs (1998) have reevaluated the Australian Aponogetons thoroughly and described six new taxa including five new species: *A. kimberleyensis* Hellq. & S. W. L. Jacobs, *A. euryspermus*, *A. vanbruggenii*, *A. lancesmithii* Hellq. & S. W. L. Jacobs, and *A. proliferus* Hellq. & S. W. L. Jacobs (the latter corresponding to Aston's unnamed proliferous species).

In this study, we investigate relationships among Australian *Aponogeton* species in detail using an explicit, phylogenetic approach. Our work represents the first empirical phylogenetic analysis to be undertaken for any portion of *Aponogeton*. It provides the first test of various systematic hypotheses, including a phylogenetic appraisal of the classification developed by Camus (1923). We evaluate the use of several types of characters for phylogenetic analysis of *Aponogeton*, including both morphological features and molecular data.

In a taxonomically difficult genus such as *Aponogeton*, where reliable morphological characters are scarce, the analysis of molecular markers provides one alternative means of obtaining a relatively large number of characters suitable for phylogenetic analysis. However, even though the genetic basis of directly sequenced DNA regions might be perceived as unambiguous, the homology of molecular data also is subject to misinterpretations due to parallel substitutions and paralogous loci especially in polyploid species where gene duplications are prevalent (Page and Holmes 1998). This observation is pertinent because *Aponogeton* is highly polyploid. Reported counts indicate a chromosomal base number for the genus of $x = 8$, and an assortment of chromosome numbers ranging from $2n = 16$ to $2n = 100$ occurs among various species (Arends 1985). Indeed, *A. elongatus*, the only Australian *Aponogeton* for which a chromosome number has been reported, is polyploid ($2n = 40$).

Although Bruggen (1985) did not believe that *Aponogeton* formed natural hybrids, he observed several instances of apomixis (agamospermy), which often is associated with hybridization and polyploidy (Grant 1981); thus the potential for hybridization certainly exists in the genus. To safeguard against misleading results that might arise from analyses involving reticulate relationships such as those associated with hybridiza-

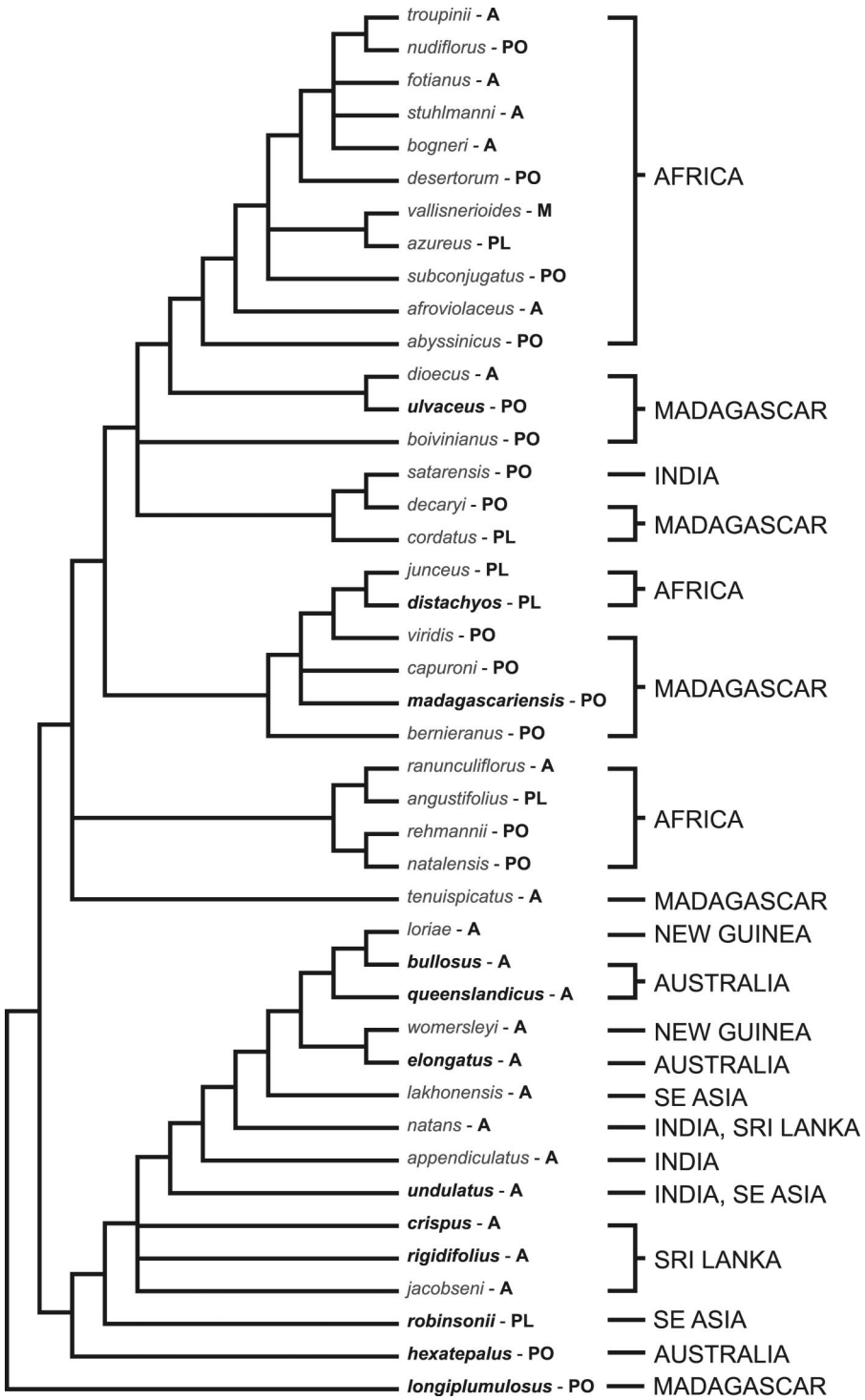

FIG. 1. Interspecific relationships in *Aponogeton* as indicated by morphological trends [redrawn in tree form from the diagram in Thanikaimoni (1985)]. Epithets of the species included in the present study are highlighted in bold. Native geographical distributions of the species are shown at right.

TABLE 1. List of taxa investigated, distributions, vouchers, specimens and Genbank accession numbers. Multiple accessions of a species are indicated by numbers in parentheses. The taxon *A.* ''indet.'' comprised vegetative material that could not be identified to species confidently. Two taxa (identified as *A.* ''species 1'' and *A.* ''species 2'') emerged as new species that remain unnamed at present. *Cycnogeton procerum* and *Tetroncium magellanicum* constituted the outgroup used in all molecular analyses. Specimens designed by ''cult.'' were obtained from sources of material grown in cultivation (see text). Citations with mutiple numbers (e.g., *Jacobs 8572 & Les 595*) reflect different personal numbers assigned to a single specimen gathered jointly by the collectors. The order of Genbank accession numbers for each taxon is: *matK, trnK* 5′ intron, nrITS (bracketed numbers [] = multiple cloned sequences recovered; NA = sequence not determined).

Aponogeton bullosus H. Bruggen, Australia (Queensland), *Jacobs 8572 & Les 595* (CONN, NSW); AY926279, AY926344, AY926318; *A. crispus* Thumb., SE Asia (cult.), *Jacobs 8537 & Les 563* (CONN, NSW); AY926263, AY926328, AY926288; *A. distachyos* L.f., S Africa (cult.), *Les s.n.* (CONN); AY926281, AY926346, AY926320; *A. elongatus* F.Muell. ex Bentham, (1), Australia (Queensland), *Jacobs 8525 & Les 551* (CONN, NSW); AY926266, AY926331, AY926296; *A. elongatus* (2), Australia (Queensland; cult.), *Les s.n.* (CONN); AY926267, AY926332, AY926297; *A. elongatus* (3), Australia (New South Wales), *Jacobs 9075* (NSW); AY926266, AY926331, AY926294; *A. elongatus* (4), Australia (New South Wales), *Jacobs 9074* (NSW); AY926266, AY926331, AY926295; *A. euryspermus* Hellq. & S.W.L.Jacobs (1), Australia (N Territory; cult.), *Jacobs 8532 & Les 558* (CONN, NSW); AY926273, AY926338, AY926308; *A. euryspermus* (2), Australia (W Australia), *Jacobs 8839* (NSW); AY926275, AY926340, AY926310; *A. hexatepalus* H.Bruggen, Australia (W Australia), *Sainty NSW434337* (NSW); AY926282, AY926347, AY926321; *A.* ''indet.'', Australia (Queensland), *Jacobs 8571 & Les 594* (CONN, NSW); AY926278, AY926343, AY926317; *A. kimberleyensis* Hellq. & S.W.L.Jacobs, Australia (W Australia), *Jacobs 8831* (NSW); AY926274, AY926339, AY926309; *A. lancesmithii* Hellq. & S.W.L.Jacobs, Australia (Queensland), *Jacobs 8567 & Les 590* (CONN, NSW); AY926277, AY926342, AY926316; *A. longiplumulosus* H.Bruggen, Madagascar (cult.), *Jacobs 8534 & Les 560* (CONN, NSW); AY926260, AY926325, AY926284; *A. madagascariensis* (Mirbel) H.Bruggen (1), Madagascar (cult.), *Jacobs 8535 & Les 561* (CONN, NSW); AY926261, AY926325, AY926286; *A. madagascariensis* (2), Madagascar (cult.), *Jacobs 8536 & Les 562* (CONN, NSW); AY926261, AY926325, AY926285; *A. proliferus* Hellq. & S.W.L.Jacobs, Australia (Queensland; cult.), *Jacobs 8523 & Les 549 (CONN, NSW); AY926276, AY926341, AY926315; A. queenslandicus* H.Bruggen (1), Australia (Queensland; cult.), *Jacobs 8524 & Les 550* (CONN, NSW); AY926265, AY926330, AY926293; *A. queenslandicus* (2), Australia (Queensland; cult.), *Jacobs 8526 & Les 552* (CONN, NSW); AY926265, AY926330, AY926289 [2.1], AY926290 [2.2], AY926292 [2.3]; *A. queenslandicus* (3), Australia (Queensland; cult.), *Jacobs 8541 & Les 567* (CONN, NSW); AY926265, AY926330, AY926298; *A. rigidifolius* H.Bruggen (1), Sri Lanka (cult.), *Jacobs 8529 & Les 555* (CONN, NSW); AY926262, AY926327, AY926287; *A. rigidifolius* (2), Sri Lanka (cult.), *Jacobs 8530 & Les 556* (CONN, NSW); AY926262, AY926327, AY926287; *A. robinsonii* A. Camus, Vietnam (cult.), *Jacobs 8806* (NSW); AY926280, AY926345, AY926319; *A. stachyosporus* de Wit, India (cult.), *Jacobs 8538 & Les 564* (CONN, NSW); AY926272, AY926337, AY926303 [1.1], AY926304 [1.2], AY926305 [1.3], AY926306 [1.4]; *A. "ulvaceus"* Baker (1), Madagascar (cult.), *Jacobs 8546 & Les 572* (CONN, NSW); AY926259, AY926324, AY926307 [1.1], AY926312 [1.2], AY926313 [1.3]; *A. ulvaceus* (2), Madagascar (cult.), *Jacobs 8543 & Les 569* (CONN, NSW); AY926259, AY926324, AY926283; *A. undulatus* Roxb. India (cult.), *Jacobs 8539 & Les 565* (CONN, NSW); AY926271, AY926336, AY926302; *A. vanbruggenii* Hellq. & S.W.L.Jacobs (1), Australia (N Territory; cult.), *Jacobs 8542 & Les 568* (CONN, NSW); AY926269, AY926334, AY926300 [1.1], AY926311 [1.2], AY926314 [1.3]; *A. vanbruggenii* (2), Australia (N Territory; cult.), *Jacobs 8533 & Les 559* (CONN, NSW); AY926268, AY926333, AY926299; *A.* ''species 1'', Australia (Queensland; cult.), *Jacobs 8528 & Les 554* (CONN, NSW); AY926264, AY926329, AY926291; *A.* ''species 2'', Australia (N Territory), *Jacobs 8801* (NSW); AY926270, AY926335, AY926301; *Cycnogeton procerum* Buchenau, Australia, *Beesley 449* (CBG); NA, AY926349, AY926323; *Tetroncium magellanicum* Willd., Chile, *Alvarez s.n.* (CONN); NA, AY926348, AY926322.

tion and polyploidy, we included both maternally-inherited (*trnK* 5′ intron; *matK*) and biparentally-inherited (nrITS) molecular markers in our study. In addition, we employed a molecular cloning strategy as a means of evaluating potentially paralogous loci. Using this approach we were able to make a preliminary assessment of the existing classification, achieve a reasonable phylogenetic assessment of Australian *Aponogeton*, and elucidate further details on the relationships of the Australian species to other species in the genus.

MATERIALS AND METHODS

Taxon Sampling. Thirty-three accessions of 23 taxa (21 Aponogetonaceae; 2 Juncaginaceae) were evaluated (Table 1). This sample included all nine species currently recognized as native to Australia, one African species (introduced to Australia), three Malagasy species and five Asian species. We examined multiple accessions for seven species (Table 1). Identification of all cultivated material was verified by the authors. The cultivated accessions of Australian species were collected originally from sites where species determinations had already been made (and prior vouchers collected) by coauthor SWLJ.

Morphological Analyses. To serve as an initial hypothesis of phylogenetic relationships in *Aponogeton*, we reconstructed, in tree format, the diagram presented by Thanikaimoni (1985) that purportedly shows interspecific relationships as inferred from the pattern of morphological diversification that he elucidated in the genus. For empirical analysis, a total of 19 morphological characters (5 vegetative, 14 reproductive) was scored for 17 *Aponogeton* species (Tables 2, 3). Following Bruggen (1985), we did not distinguish *A. stachyosporus* de Wit from *A. undulatus* Roxb. in the morphological analysis as it would have been scored with identical character states. Morphological characters were selected from those emphasized taxonomically by Bruggen (1969, 1985) and Hellquist and Jacobs (1998). We excluded characters that would have been autapomorphic (i.e., those varying in only one of the species). Several other characters (submersed leaf margin undulation; seed number and length; seed coat adherence; pericarp texture) were included in initial analyses, but were excluded when it became apparent that their high degree of homoplasy resulted in a nearly complete loss of resolution in resulting trees.

Morphological data were analyzed phylogenetically using unweighted maximum parsimony as implemented by the program PAUP* (Swofford 1998). Searches were conducted using the branch-and-bound algorithm (furthest addition sequence; MulTrees options) with all character states treated as unordered. We were unable to include members of the outgroup (Juncaginaceae, see below) in the morphological analyses due to our inability to score homologous states confidently between the two families. We relied on results from the molecular analyses (below), which clear-

TABLE 2. Morphological characters and character states used in phylogenetic analysis of *Aponogeton* (compiled from Bruggen, 1969; 1985; Hellquist & Jacobs, 1998).

Vegetative: 1. habit (0 = floating leaves only; 1 = leaves floating & submersed; 2 = leaves all submersed); **2.** maximum tuber length (0 = ≥4 cm; 1 = <3 cm); **3.** submersed leaf blade surface (0 = flat; 1 = bullate); **4.** maximum submersed leaf width (0 = >2.5 cm; 1 = ≤2.5 cm); **5.** floating leaf base morphology (0 = never cordate; 1 = commonly to rarely cordate).
Reproductive: 6. inflorescence habit (0 = emergent; 1 = emergent or floating; 2 = non-emergent); **7.** inflorescence morphology (0 = branched; 1 = unforked/rarely branched; 2 = unforked); **8.** peduncle (0 = not proliferous; 1 = proliferous); **9.** peduncle diameter (0 = equal to inflorescence rachis; 1 = > inflorescence rachis); **10.** spathe duration (0 = persistent; 1 = caducous); **11.** maximum spathe length (0 = long, > 1.5 cm; 1 = short, ≤1.5 cm); **12.** flower arrangement (0 = all around axis; 1 = secund in 2 rows); **13.** flower spacing (0 = loose; 1 = dense; 2 = dense or loose); **14.** tepal number (0 = 6; 1 = 2; 2 = 1); **15.** tepal color (0 = white/pink; 1 = green; 2 = yellow); **16.** number of tepal nerves (0 = 13; 1 = one); **17.** stamen number (0 = 8-16; 1 = 6; 2 = 4); **18.** testa number (0 = 1; 1 = 2); **19.** plumule (0 = absent; 1 = present).

ly indicated *Aponogeton hexatepalus* as sister to the rest of the genus, and used this species for ''ingroup'' rooting of the trees. Results were depicted by retrieving the strict consensus tree to which bootstrap values were added (1,000 replicates; same search options as described previously) to indicate the degree of internal support for each resolved branch. Missing or inapplicable data constituted 4.3% of morphological data cells and were treated as missing in all analyses. Character state distributions were examined for each morphological character using both ACCTRAN and DELTRAN optimizations on the tree derived from combined molecular data (see below).

Molecular Analyses. Specimens for molecular analysis were collected either in the field (12 accessions) or from material grown in cultivation (21 accessions). A large number of specimens (18 accessions) was obtained from Lance Smith (Kelso, Queensland, Australia) an aquatic plant propagator who maintains and preserves many *Aponogeton* species in pond culture. Voucher specimens were prepared for all specimens examined (Table1). Our selection of material provided for the analysis of all previously known Australian taxa (nine species) and other members of the genus to yield taxonomic coverage of both sections and three of four subsections as defined by Camus (1923). The monotypic subsection *Monostachys* (*A. vallisnerioides* Baker) was not included. We supplemented the material with multiple accessions for several species that possessed unusual morphologies (e.g., ''coarse-leaved'' and ''fine-leaved'' variants of *A. madagascariensis*, obtuse and acute leaf apex variants of *A. rigidifolius* H. Bruggen and several plants of Australian origin that we could not identify to species confidently using morphological characters). We included two genera of Juncaginaceae (*Cycnogeton*, *Tetroncium*) to function as outgroups in accordance with the *rbcL* survey by Les et al. (1997), which showed that family to be closely related to Aponogetonaceae.

Routine procedures as described in Moody and Les (2002) were followed for the extraction, amplification, and automated sequencing of ITS (ITS-1 and ITS-2 regions including the 5.8s rRNA gene) and cpDNA (*trnK* 5′ intron with an adjacent 5′ portion of the *matK* coding region). In addition it was necessary to develop two new sequencing primers: *ApotrnKR* (5′ATAATTTTGTTGATACAT) and *Apo340F* (5′ACGAGCTTATGTTCTTA). Nevertheless, we were unable to obtain complete *trnK* 5′ intron/*matK* sequences for *Cycnogeton* and *Tetroncium*. Sequencing was performed using an ABI 3100 automated sequencer. All sequences used in our analyses were newly generated and have been deposited in the GenBank database (Table 1).

Four Australian accessions showed numerous polymorphisms in their ITS sequence chromatograms, which indicated that they comprised mixed pools of similarly sized ITS fragments. To isolate these sequence variants we subcloned the polymorphic PCR amplification products into plasmids using TOPO TA cloning protocol (Invitrogen, Carlsbad, CA) as described in Les et al. (2004). All monomorphic sequences resulting from the cloned PCR products initially were added to the analyses as separate OTUs identified by their specimen of origin. We also observed several polymorphisms in the sequences derived from three Malagasy species (*A. longiplumulosus*, *A. madagascariensis*, *A. ulvaceus* Baker). Because subsequent phylogenetic analyses indicated that all sequences occurred within a single clade, and because this group was not our

TABLE 3. Matrix of morphological character states (from Table 2) used in a phylogenetic analysis of *Aponogeton*. (—) = data not applicable; (?) = data missing.

	Character number																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
A. bullosus	2	1	1	1	—	2	2	0	0	0	0	0	0	1	2	1	1	0	0
A. crispus	1	0	0	0	1	0	2	0	1	1	0	0	2	1	0	1	1	0	1
A. distachyos	0	0	—	—	0	0	0	0	1	1	0	1	0	2	0	0	0	0	1
A. elongatus	1	0	0	0	1	1	2	0	1	1	1	0	0	1	2	1	1	0	1
A. euryspermus	1	1	0	1	0	1	1	0	1	1	1	0	1	1	2	1	1	1	0
A. hexatepalus	0	0	—	—	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1
A. kimberleyensis	2	1	0	1	—	0	2	0	1	1	0	0	0	1	2	1	1	0	0
A. lancesmithii	2	0	1	0	—	1	1	0	1	1	1	0	1	1	2	1	1	1	0
A. longiplumulosus	2	1	0	0	—	0	0	0	1	1	0	0	1	1	0	1	1	0	1
A. madagascariensis	2	1	0	0	—	0	0	0	1	1	0	0	1	1	0	1	1	0	1
A. proliferus	2	1	0	1	—	0	1	1	1	0	1	0	0	1	2	1	2	?	?
A. queenslandicus	1	0	0	0	1	1	2	0	1	1	1	0	1	1	2	1	1	1	0
A. rigidifolius	2	0	0	0	—	0	2	0	0	1	0	0	1	1	0	1	1	0	1
A. robinsonii	1	1	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1	0	1
A. ulvaceus	2	1	0	0	—	0	0	0	1	1	1	0	1	1	0	1	1	1	0
A. undulatus	1	1	0	0	1	0	2	1	1	0	0	0	0	1	0	1	1	0	1
A. vanbruggenii	1	0	0	0	1	1	2	0	1	1	1	0	1	1	2	1	1	1	0

main group of interest, we did not subclone that material for further clarification. Cloning was unnecessary for the cpDNA data, which yielded monomorphic sequences for all species surveyed.

Sequences were aligned manually and analyzed for polymorphisms and/or variable sites using Sequencher (Gene Code Corp.) and MacClade 4 (Maddison and Maddison 2000). Phylogenetic analyses of molecular data were performed under maximum parsimony using PAUP* (Swofford 1998) (heuristic search; random taxon addition; TBR; characters unordered and weighted equally). Indels were treated as missing data, but five gaps in the *trnK* 5′ intron were included in the analysis as additional binary-coded characters (presence/absence of gap). The degree of internal support for recovered clades was indicated by the results of bootstrap values obtained from 500 replicates (same parameters as described for parsimony analysis). All results of analyses generating multiple, equally parsimonious trees were evaluated using strict consensus trees. The consensus trees were output as tree files to facilitate the representation of relative branch lengths. The nuclear (ITS) and cpDNA (*trnK* 5′ intron/*matK*) data initially were partitioned to enable their separate analysis. With these preliminary analyses yielding similar results, a combined analysis of all molecular data was carried out.

Molecular data were not combined with morphological data, because the latter could not be scored fully for the specimens used for molecular analyses, many having been collected in vegetative condition. However, ''species level'' morphological character state distributions were estimated by adding and mapping the morphological data to the combined molecular tree. This analysis was accomplished by providing the identical morphological character states for each specimen identified as conspecific in the molecular analysis.

Sequence homology for those OTUs characterized by multiple ITS alleles was evaluated by examining the distribution of clones on the cladogram resulting from phylogenetic analysis of the ITS data. In the case of *A. queenslandicus* and *A. vanbruggenii*, homologous alleles were determined by matching them to sequences obtained from monomorphic conspecific accessions. Matches of any other alleles to other taxa were interpreted as evidence of hybridization and they, along with other divergent alleles, were removed prior to performing the combined molecular data analysis. For *A. stachyosporus*, which showed several paralogous ITS alleles, we identified as homologs two identical clones that grouped with *A. undulatus*, a species with which it has been merged in past taxonomic treatments. The other clones formed an isolated cluster that did not associate closely with any species in the analysis and likely represent paralogous polyploid duplications (see discussion). We could not determine a homolog for one accession yielding several ITS alleles that did not associate with any taxon. We designated this accession as ''*A. ulvaceus*'' because its morphology resembled that species but also was uncharacteristic in several respects. These, as well as other anomalous sequences, were excluded from the combined molecular analysis.

All data used in phylogenetic analyses have been submitted to the TreeBASE database (study accession number: S1242; matrix accession number: M2166).

Results

Morphological Analyses. Maximum parsimony analysis of our morphological data set recovered six equal-length trees (48 steps) characterized by fairly high homoplasy (CI = 0.54, $CI_{(exc)}$ = 0.49, RI = 0.68). The strict consensus tree (Fig. 2) was poorly-resolved and characterized by low internal support (16–48% bootstrap values). Twelve of the characters (evaluated on the six maximum parsimony tree variants) had a consistency index (CI) less than or equal to 0.50. Higher consistency (0.67–1.00) was observed for seven characters (#1, 6, 13–17) (Table 2). The best-supported clade separated *A. hexatepalus* and *A. distachyos* from all other species. Other Australian species (excluding *A. hexatepalus*) formed two distinct subclades, but resolution was inadequate to establish their monophyly as a single clade. Although these results were supported only weakly, they agreed in some respects with Thanikaimoni (1985) who hypothesized the lack of a close relationship between *A. hexatepalus* and other Australian species. Although poorly supported, the monophyletic association of Malagasy species (Fig. 2) conflicted with Thanikaimoni's scheme, which showed all three species as distantly related (Fig. 1). Overall, the topology of the morphological cladogram showed higher compatibility than Thanikaimoni's hypothesis with respect to both the Camus classification and with geographical regions, although the low level of resolution rendered these assessments equivocal.

Molecular Analyses. Alignment of ITS data provided 930 aligned nucleotide sites for phylogenetic analysis (Fig. 3). Of these, 532 sites were constant and 214 were parsimony-informative. Under maximum parsimony we recovered 719 minimal-length trees (715 steps) characterized by moderate homoplasy (CI = 0.78; $CI_{(exc)}$ = 0.68; RI = 0.82). Resolved nodes were relatively well-supported (bootstrap values = 59–100%) with 14 of the nodes (56%) supported above 90%. The strict consensus tree (rooted by the two Juncaginaceae sequences) placed *A. hexatepalus* as sister to the rest of the genus (64% bootstrap support), succeeded in position by a metaphyletic group comprising the African *A. distachyos* and Asian *A. robinsonii* A. Camus (Fig. 3). The Malagasy species were resolved as monophyletic (bootstrap = 100%) as were the remaining Asian (bootstrap = 94%) and Australian species (bootstrap = 99%).

Because minor sequence variation occurs regularly in cloned DNA (Les et al. 2004), we identified as homologous those alleles differing only by a few base pairs from other accessions. The accession *A. queenslandicus* (2) yielded multiple cloned ITS alleles, including both an *A. queenslandicus* homolog and an *A. rigidifolius* homolog. This accession also produced a substantially divergent sequence clone that differed by 24 steps from the next closest sequence (*A. crispus*). These results indicate that the *A. queenslandicus* (2) accession represents a hybrid involving *A. queenslandicus* and *A. rigidifolius* whereas the divergent sequence probably indicates a paralog resulting from a polyploid duplication. Similarly, the accession *A. vanbruggenii* (1) yielded one cloned homolog [identical to *A. vanbruggenii* (2)], one homolog that allied with sequences of *A. bullosus*, *A. lancesmithii*, and *A. proliferus* (all identical), and a divergent sequence that differed from the nearest homolog by 14 steps, again probably a paralog due to polyploidy. Thus it appears that the accession *A. vanbruggenii* (1) represents a hybrid involving *A. van-*

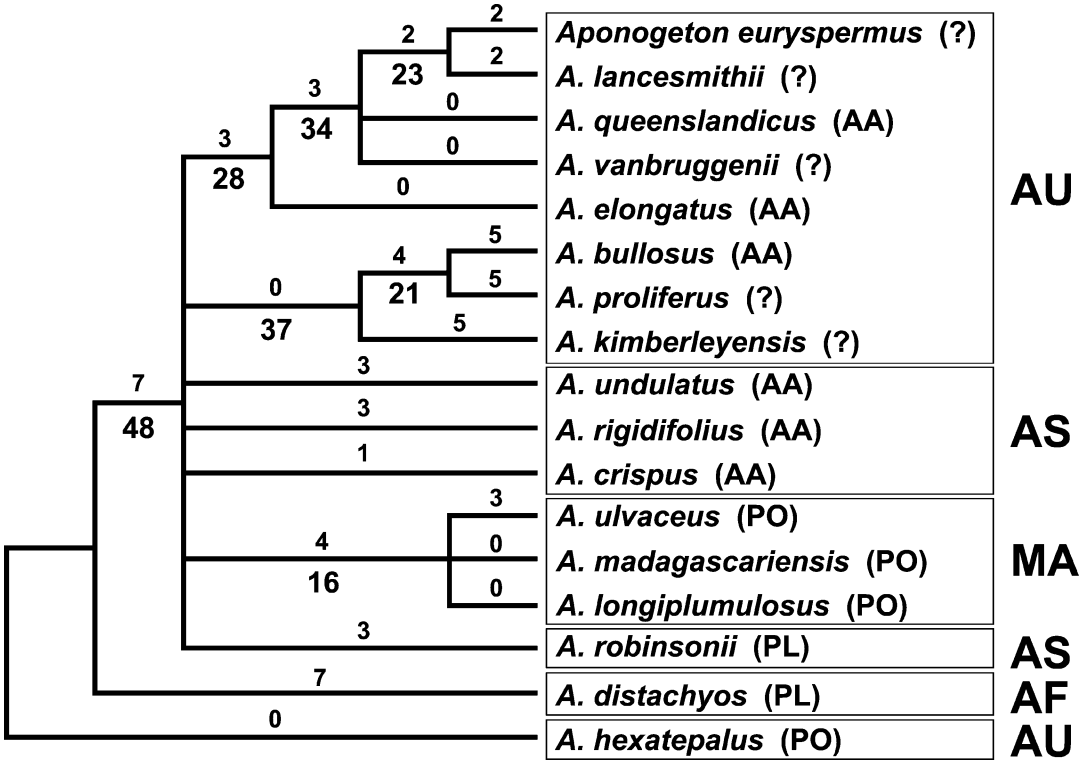

FIG. 2. Strict consensus of six maximum parsimony trees depicting the phylogenetic distribution of states for 19 morphological characters (Tables 2, 3). Branch lengths (tree steps) are indicated above nodes; bootstrap support (%) is indicated beneath nodes. Bracketed abbreviations after species names indicate their assignment to the classification proposed by Camus (1923): AA = sect. *Aponogeton* subsect. *Aponogeton*; PO = sect. *Aponogeton* subsect. *Polystachys*; PL = sect. *Pleuranthus* subsect. *Pleuranthus*; (?) designates newly described species not yet classified. Geographical distributions (for species grouped in boxes) are abbreviated as AF (continental Africa), AS (Asia), AU (Australia). Ingroup rooting using *A. hexatepalus* was performed in lieu of an outgroup (see text).

bruggenii as one parent and a member of the ''*A. bullosus*, *A. lancesmithii*, *A. proliferus* clade'' as the other. The cloned accession identified as ''*A. ulvaceus*'' yielded three divergent sequences that occurred within the Australian clade, but differed from the most similar sequences (*A. elongatus*) by 13–15 steps. No sequence clones were recovered that appeared to be homologous to any surveyed species, indicating probable hybridization with one of the Australian species. The relatively high degree of divergence of these ITS copies could indicate paralogous polyploid loci or chimeric sequences (produced subsequent to hybridization or as altered cloning artifacts) and our inability to recover alleles identical to either parent involved in the cross. The cpDNA of this accession (see below) matched that of *A. rigidifolius*, indicating that this unusual accession was not *A. ulvaceus* but a hybrid involving *A. rigidifolius*. *Aponogeton stachyosporus* produced two cloned identical homologs (differing from *A. undulatus* by seven steps) and two considerably divergent cloned sequences (presumably paralogs) differing from the presumed homologs by 60–70 steps and from each other by 13 steps. All cloned sequences from *A. stachyosporus* occurred within the well-supported Asian clade. There was no evidence of hybridization in this species, with the divergent sequences likely indicating paralogous polyploid duplications.

Two accessions of the distinctive *A. madagascariensis* differed by five steps and did not resolve as a clade (Fig. 3); however, the sequences contained a number of polymorphic sites and we did not subclone the PCR amplifications to isolate individual alleles. One accession [*A. madagascariensis* (1)] associated closely with *A. ulvaceus* (differing by 2 bps); whereas, the *A. madagascariensis* (2) accession was basal in the clade (Fig. 3). None of the Malagasy species surveyed differed by more than nine steps in the ITS tree. The ITS sequences of *A. stachyosporus* and *A. undulatus* were fairly distinct (differing by 7 bps). Both *A. crispus* and *A. rigidifolius* possessed distinct ITS sequences.

ITS data did not distinguish *A. bullosus*, *A. lancesmithii* or *A. proliferus*, despite their well-marked morphological differences as reported by Hellquist and Jacobs (1998). The ITS data also indicated that a speci-

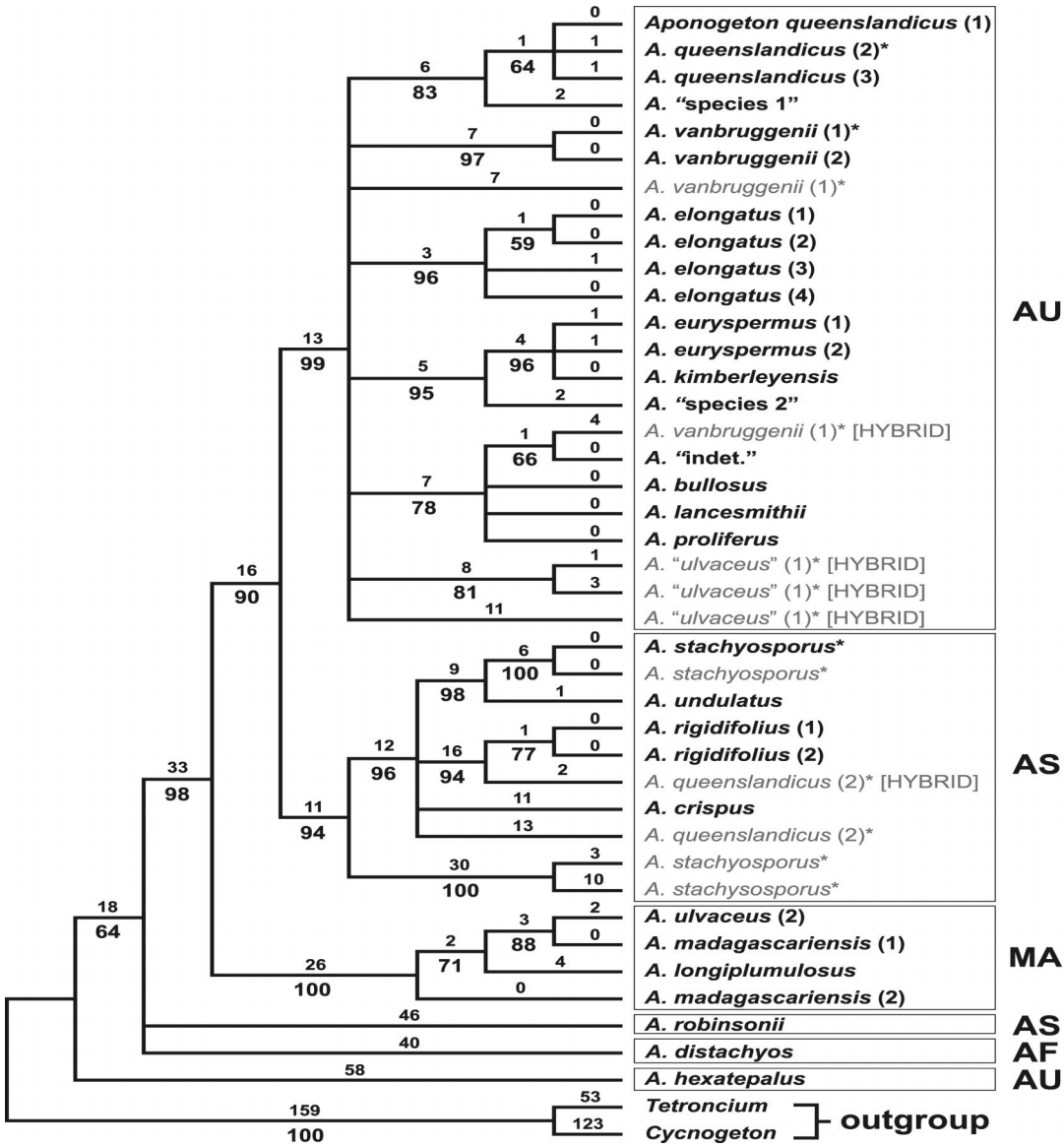

FIG. 3. Strict consensus of 719 maximum parsimony trees resulting from analysis of ITS data (40 *Aponogeton* sequences and two outgroup sequences). Branch lengths (tree steps) are indicated above nodes; bootstrap support (%) is indicated beneath nodes. Names followed by a bracketed number represent multiple accessions (see Table 1); those marked with an asterisk (*) are cloned sequences recovered from the same accession. Homologous sequences are indicated in bold type except where they occur in hybrids (so indicated and marked by light type). Paralogous sequences also are marked in light type (see text). Geographical distributions (for species grouped in boxes) are abbreviated as in Fig. 1.

men collected in vegetative condition that we found particularly difficult to identify (*A.* ''indet.''; suspected as being *A. elongatus*) either was *A. bullosus*, *A. lancesmithii*, or *A. proliferus* rather than *A. elongatus*, from which its sequence differed by 11–12 steps (Fig. 3). ITS data corroborated the distinctness of *A. elongatus*, *A. queenslandicus*, and *A. vanbruggenii* as well as a close relationship between *A. euryspermus* and *A. kimberleyensis*. However, further details of interspecific relationships among the Australian species could not be resolved by ITS data alone.

The possible existence of two new Australian *Aponogeton* species was indicated by the recovery of distinctive, monomorphic ITS sequences that did not associate closely with any of the species described previously. One taxon (*A.* ''species 1'') was similar to *A. queenslandicus* but differed by 3–4 steps from all three accessions of that species surveyed. The sequence of a

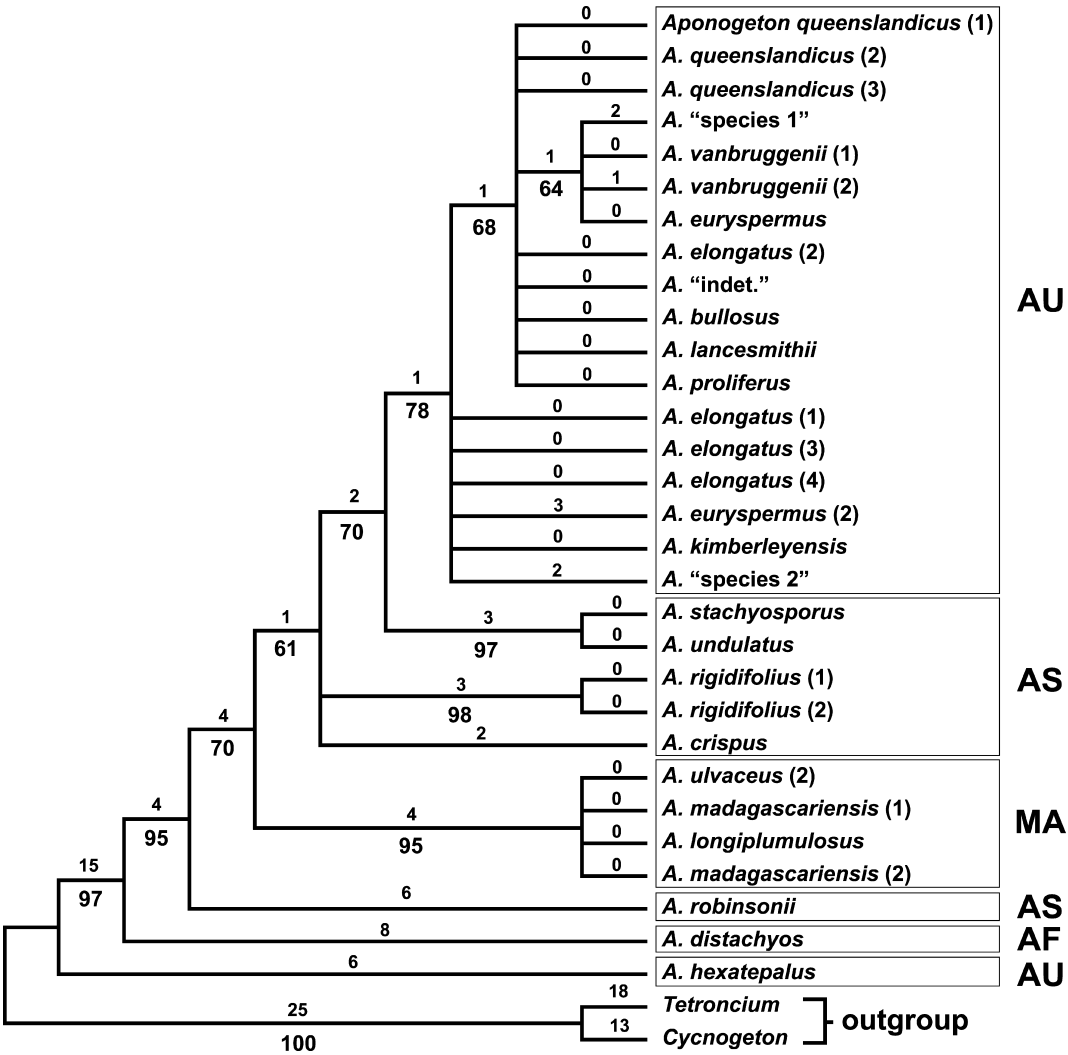

FIG. 4. Single most parsimonious tree resulting from analysis of cpDNA (*trnk* 5′ intron, *matK*) sequence data from 30 *Aponogeton* and two outgroup accessions. Branch lengths (tree steps) are indicated above nodes; bootstrap support (%) is indicated beneath nodes. Names followed by a bracketed number represent multiple accessions (see Table 1). Geographical distributions (for species grouped in boxes) are abbreviated as in Fig. 1.

second taxon (*A.* ''species 2'') was most similar to those of *A. euryspermus* and *A. kimberleyensis*, but differed from them by 6–7 steps.

Alignment of *trnK* 5′ intron/*matK* data provided 915 aligned nucleotide sites and five indel characters for phylogenetic analysis (Fig. 4). Of these, 803 sites were constant and 63 were parsimony-informative. Maximum parsimony analysis produced a single minimal-length tree (136 steps) characterized by low homoplasy (CI = 0.90; $CI_{(exc)}$ = 0.84; RI = 0.91). There was less resolution among closely-related species than we observed in the ITS analysis, but bootstrap support for nodes was similar, ranging from 61–100% with six nodes (50%) supported above 90% (Fig. 4). As with ITS data, analysis of cpDNA sequences also placed *A. hexatepalus* as basal but with even higher bootstrap support (97%). The cpDNA data further resolved the positions of *A. distachyos* (the sister group to the remaining *Aponogeton* species excluding *A. hexatepalus*) and *A. robinsonii* (the sister group to the remaining *Aponogeton* species excluding *A. distachyos* and *A. hexatepalus*) with high levels of support (Fig. 4). Likewise, analysis of cpDNA provided support for a clade comprising the three Malagasy species (95% support) although no finer resolution was achieved within that clade. The Asian species formed a polytomous, paraphyletic grade (in a basal position to a clade comprising all Australian species except *A. hexatepalus*) rather than a clade (Fig. 4). cpDNA provided moderate support (78% bootstrap) for an Australian clade (exclud-

ing *A. hexatepalus*), as congruent with the ITS data (Figs. 3, 4). Further details of relationships within this Australian clade could not be ascertained due to the virtual lack of resolution or support provided by the cpDNA sequences. However, both taxa identified as possible new species by ITS data also possessed distinct cpDNA sequences (Fig. 4).

The combined (ITS, cpDNA) molecular data set comprised 1845 aligned nucleotide sites and five indel characters of which 1335 (72%) were constant, 238 (13%) were variable but uninformative, and 277 (15%) were informative phylogenetically. Approximately 24% of the final molecular data matrix included missing data cells due mainly to the large number of gaps required for sequence alignment. Maximum parsimony analysis generated 18 minimal-length trees (769 steps) characterized by moderate homoplasy (CI = 0.83; $CI_{(exc)}$ = 0.73; RI = 0.84). The combined data strict consensus tree was more highly-resolved than either of those resulting from the independent analyses. Bootstrap support for nodes was similar, ranging from <50% (one node) to 100%; however, a slightly larger proportion (13 nodes; 57%) was supported above 90% (Fig. 5).

The combined molecular cladogram positioned *A. hexatepalus* as sister to the rest of the genus (97% bootstrap support) succeeded in position first by *A. distachyos* (82%), and then by *A. robinsonii* (100%; Fig. 5). Three major species groups were resolved as clades: the Malagasy species (100%), the ''Asian'' species (excluding *A. robinsonii*; 98%), and the ''Australian'' species (excluding *A. hexatepalus*; 100%). The arrangement of species within the Malagasy clade was identical to the ITS result given that cpDNA data provided no additional resolution within the group. Two subclades were resolved in the ''Asian'' clade; one comprising *A. undulatus* and *A. stachyosporus* (100%) and one comprising *A. rigidifolius* and *A. crispus* (60%). *Aponogeton stachyosporus* and *A. undulatus* were differentiated by seven steps in the tree. Within the ''Australian'' clade, two major subclades were resolved, but neither was particularly well-supported by bootstrap values. One subclade (72%) comprised *A. queenslandicus*, an undescribed taxon (*A.* ''species 1''), and *A. vanbruggenii* (Fig. 5). The other subclade (supported at less than 50%) was itself subdivided into three well-supported (88–100%) subclades that mirrored the ITS results. The second undescribed taxon (*A.* ''species 2'') occurred within the same subclade as *A. euryspermus* and *A. kimberleyensis*, but was distinguished from both species by 11–12 and 8 steps respectively, in the combined data tree (Fig. 5).

When mapped on the combined molecular data cladogram (ACCTRAN and DELTRAN reconstructions), the character states for many morphological characters showed extensive homoplasy. Only tepal color (character #15; Table 2) correlated unambiguously with the monophyly of the ''Australian'' clade whose representatives all uniquely possess yellow tepals (Fig. 5). Other morphological characters deemed to be highly informative taxonomically (e.g., testa number, character #15; Table 2) were homoplasious and showed numerous instances of multiple origins throughout the genus (Fig. 5). With one exception (*A. hexatepalus*), the classification developed by Camus (1923) was highly compatible with the cladogram topology generated using the combined molecular data (Fig. 5). The tree topology also depicted clades that, with the exception of *A. hexatepalus* and *A. robinsonii*, correlated well with distinct geographical regions.

Discussion

Our study of *Aponogeton* represents the first cladistic evaluation of these poorly-understood aquatic plants and also assesses the systematic utility of morphological and molecular data for phylogenetic reconstruction in the genus. Prior taxonomic studies have been based exclusively on morphology despite the confounding level of phenotypic variation encountered in many species (Bruggen 1985). Our incorporation of molecular characters provided a means of evaluating morphologically-based taxonomic hypotheses using an independent source of data. Such an approach is particularly important in *Aponogeton* where the influence of polyploidy and hybridization on morphology is yet to be determined. Our incorporation of molecular regions that are inherited both maternally (cpDNA) and biparentally (ITS) has been particularly useful as a means of providing genetic markers capable of identifying potential hybrids (Moody and Les 2002; Les et al. 2004).

Morphological Variation. An effective taxonomic assessment of *Aponogeton* based on morphology has proven to be notoriously difficult. Bruggen (1985) stressed the importance of studying plants extensively under cultivation in order to fully understand the range of phenotypic variation that can be encountered within a species. Leaf characters that appear to be distinctive, such as undulate margins or bullate laminas, can either appear or disappear when grown under different conditions. Similarly, some species vary widely in their ability to produce either submersed leaves (often absent from herbarium material) or floating leaves, making it particularly difficult to identify vegetative specimens that may lack one or the other leaf type, especially where such a character has been regarded as an important distinguishing feature. Understandably, there has been greater confidence in the taxonomic value of reproductive characters that, by virtue of their aerial disposition, are less influenced by the variable conditions of aquatic habitats. However, some species flower rarely in culture, making it difficult to

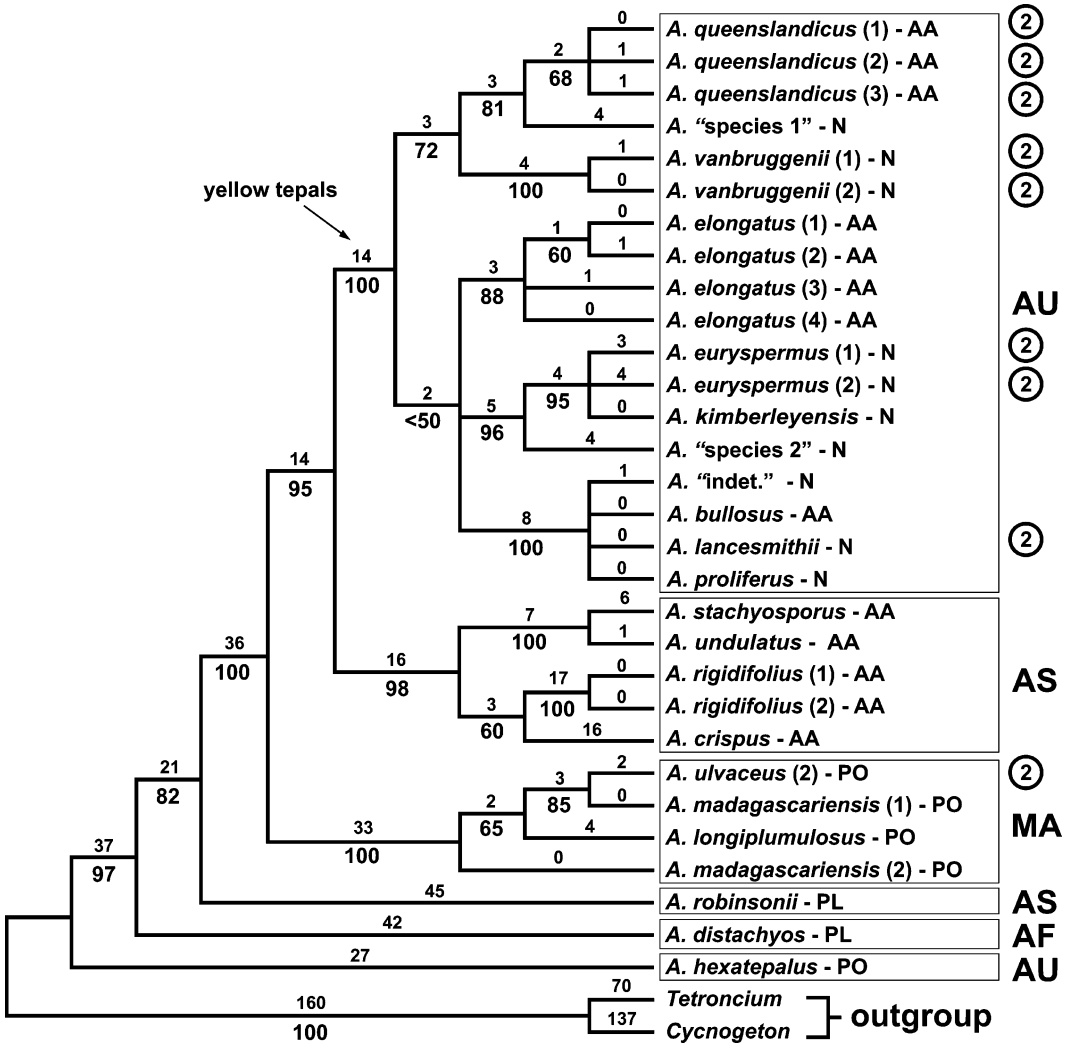

FIG. 5. Strict consensus of 18 maximum parsimony trees recovered from combined analysis of ITS and cpDNA sequence data obtained for 30 *Aponogeton* and two outgroup accessions. Branch lengths (tree steps) are indicated above nodes; bootstrap support (%) is indicated beneath nodes. Names followed by a bracketed number represent multiple accessions (see Table 1). Two-letter abbreviations after species names indicate their assignment to the classification proposed by Camus (1923): AA = sect. *Aponogeton* subsect. *Aponogeton*; PO = sect. *Aponogeton* subsect. *Polystachys*; PL = sect. *Pleuranthus* subsect. *Pleuranthus*; (N) designates accessions representing taxa that have not yet been classified. Geographical distributions (for species grouped in boxes) are abbreviated as in Fig. 1. The circled numbers outside the right edge of the boxes denote species having two testas (the unmarked accessions have one testa), a character state that is highly homoplasious. The character state of yellow tepals is unique to and occurs in all representatives of the Australian clade (designated by the arrow).

study the highly simplified reproductive characters and vegetative characters simultaneously. The relatively unstructured pattern of morphological variation in *Aponogeton* has resulted in a fairly small number of infrageneric classifications that mainly have defined only a few groups using few characters (e.g., Camus 1923).

The only phylogenetic assessment of *Aponogeton* made previously was by Thanikaimoni (1985) who elucidated relationships using a non-cladistic method that placed species in an inferred network based upon morphological trends observed in the genus (Fig. 1). By this approach, Thanikaimoni (1985) hypothesized a basal position for *A. longiplumulosus*, leading him to suggest a Malagasy origin of the genus (Fig. 1). However, the interspecific relationships suggested by Thanikaimoni are highly inconsistent with groups in the classification of Camus (1923) and show poor geographical correlation overall. Species assigned to section *Pleuranthus* are dispersed broadly across the genus and are embedded among different groups of species placed in section *Aponogeton*. Species of section *Apon-*

ogeton subsection *Polystachys* are not monophyletic but occur among different groups of species in subsection *Aponogeton*. The monotypic subsection *Monostachys* (section *Pleuranthus*) is not distinct but is embedded among representatives from subsection *Pleuranthus* as well as section *Aponogeton*. Geographically, the Malagasy species occur throughout the diagram and are interspersed among species from continental Africa and India (Fig. 1). The diagram depicts a biphyletic Australian element with *A. hexatepalus* in a near-basal position with the remainder of the Australian (and New Guinea) species forming a terminal clade derived from Asian species (Fig. 1). The Asian species are associated relatively closely and, with the exception of one species (*A. satarensis*), comprise a paraphyletic grade. From these examples, we perceive two fundamentally different perspectives of relationships in the genus: one (Thanikaimoni) inferring that relationships are best indicated by a plethora of morphological trends; and another (Camus) where only a few morphological characters define relatively large groups of related species. Geographical integrity among species is greater overall in the latter.

We assessed these hypotheses first by conducting a cladistic analysis of data (Tables 2, 3) comprising those morphological characters stressed most often in taxonomic treatments of *Aponogeton* (Bruggen 1969, 1985; Hellquist and Jacobs 1998). When analyzed phylogenetically (Fig. 2), we found that these data provided a poor assessment of clades within the genus by offering little resolution and poor support for all resolved nodes. Furthermore, some species believed to be closely related (e.g., *A. bullosus*, *A. lancesmithii*) were separated quite widely in the cladogram. However, the morphological data did indicate the distinctness of the Western Australian *A. hexatepalus* from all other Australian species, a result in agreement with Bruggen (1969; p. 136) who concluded that its ''. . . close relationship with any other species of *Aponogeton* does not seem probable.'' The clade of Malagasy species resolved by morphological data was supported weakly, but (with exception of *A. hexatepalus*) did unite species classified by Camus (1923) within sect. *Aponogeton* subsect. *Polystachys* (Fig. 2). Although the morphological cladogram was poorly resolved, the geographical groupings of species were consistent with results obtained by the molecular analyses (Figs. 3–5).

This analysis indicated that morphological data alone were insufficient for adequately resolving relationships in *Aponogeton*. The extensive homoplasy associated with the morphological character states also may explain why Thanikaimoni (1985) assumed numerous instances of parallel evolution and why Bruggen (1985) found it so frustrating to elucidate a satisfactory classification of the genus based on the bewildering, mosaic array of morphological features in the genus.

Molecular Anomalies. Overall, the interrelationships indicated by our phylogenetic analysis of molecular data were far more compelling than those indicated using morphological data. However, we initially had to clarify several anomalies that appeared in the molecular analyses, notably those inherent to the biparentally-inherited ITS sequences.

We first had to distinguish the homologous ITS sequences (Fig. 3) from a number of divergent sequences (see Materials and Methods) that were recovered from several species (*A. queenslandicus*, *A. stachyosporus*, *A. rigidifolius* [as *A.* ''*ulvaceus*''], *A. vanbruggenii*). We believe that these sequences are either paralogs resulting from polyploidy (probably the case in *A. stachyosporus* as the related *A. undulatus* is highly polyploid), result from incomplete gene conversion of divergent homologous loci subsequent to hybridization (possibly the case in *A. queenslandicus* (2), *A.* ''*ulvaceus*'' and *A. vanbruggenii* (1) which are hybrids but also could be polyploid) or are generated otherwise as cloning artifacts. Similarly divergent chimeric ITS sequences have been recovered from F_1 hybrids of other aquatic plants (Moody and Les 2002). Chromosome counts of these species would be helpful in evaluating these interpretations but as yet are unavailable.

Hybridization. The presence in a single specimen of homologous ITS sequences originating from two distinct species indicated that some accessions clearly were of hybrid origin. This approach revealed that Australian species are capable of hybridization with other Australian species [*A. vanbruggenii* (1) = *A. vanbruggenii* × *A. bullosus*, *A. lancesmithii* or *A. proliferus*] as well as Asian species [*A. queenslandicus* (2) = *A. queenslandicus* × *A. rigidifolius*]. Also compelling was the recovery of divergent sequences cloned from an accession of the Sri Lankan *A. rigidifolius* (that we erroneously had identified tentatively as *A.* ''*ulvaceus*'') from within the Australian clade (Fig. 3), which indicated the hybridization of *A. rigidifolius* with some Australian species (probably occurring while in cultivation). However, our failure to recover homologous clones from this accession precluded a more definitive assignment of parentage to this accession other than to *A. rigidifolius* with which it shared the maternally-inherited cpDNA markers. These examples indicate that intrinsic barriers to hybridization in *Aponogeton* may be weak even among species originating from distant geographical regions. Furthermore, we observed that hybrids generally resembled their maternal parent (as indicated by cpDNA sequences) to which the accessions were assigned taxonomically at the time of collection.

Bruggen (1985) believed that natural hybridization did not occur in *Aponogeton* despite the ability to cross

a number of species artificially. Unfortunately, we cannot evaluate this possibility further given that the hybrid accessions that we examined all originated from ponds in cultivation where the hybridization undoubtedly took place. Thus, we can demonstrate only that hybridization among these taxa is possible, but not that such hybrids could persist in nature. However, Bruggen (1985) may have overly relied on the ability of some morphological characters to estimate proclivity for hybridization. For example, he assumed that hybridization was ''highly improbable'' between species that differed by their testa number (Bruggen 1985). However, when mapped on the combined molecular cladogram, the distribution of species having single vs. double testa is highly homoplasious (Fig. 5). Moreover, there are a number of species in our analysis that group as sister species, yet differ by their testa number. Thus, hybridization between sister species of different testa number predictably would be much more likely than between distantly related species having the same testa number. Testa number is a good example of how homoplasious morphological data have provided misleading indications of phylogenetic relationships in *Aponogeton*.

***Major Phylogenetic Features of* Aponogeton.** Once we had accounted for the unusual paralogous, hybrid or otherwise divergent ITS sequences, the results from phylogenetic analyses of ITS and cpDNA yielded similar results. No data supported the recognition of a segregate genus *Ouviranda* as being distinct from *Aponogeton*. Several species proposed for inclusion within *Ouviranda* (e.g., *A. crispus*, *A. madagascariensis*, *A. undulatus*) did not form a clade but were embedded among other *Aponogeton* species (Figs. 2–5). Bruggen (1985) previously had dismissed the segregate *Ouviranda* because the type (*A. madagascarensis*) possesses characters that are discordant with those that allegedly define the genus.

Both molecular data sets (individually and combined) placed the W. Australian *A. hexatepalus* sister to the rest of the genus (Figs. 3–5) with moderate to strong internal support. This result differs from Thanikaimoni (1985) who regarded *A. longiplumulosus* as the ancestral *Aponogeton* species, but whose phylogenetic scheme placed *A. hexatepalus* as relatively closely related (Fig. 1). By specifically identifying *A. hexatepalus* as sister to the rest of *Aponogeton* (and *A. longiplumulosus* as comparatively derived), our results indicate that the genus is more likely to have originated in Australia and not in Madagascar as Thanikaimoni concluded.

The placement of the African *A. distachyos* in a position between *A. hexatepalus* and *A. robinsonii* also is well-supported by combined molecular data analysis (Fig. 5) and also varies considerably from Thanikaimoni (1985) whose phylogenetic scheme placed *A. distachyos* in a fairly derived position (Fig. 1). A survey of additional African species that includes both secund and omnilaterally-flowered species would be highly informative and would be necessary to estimate whether African species are monophyletic or reflect multiple colonizations. More in accord with Thanikaimoni's scheme was the phylogenetic placement of *A. robinsonii* in the combined molecular analysis (Fig. 5), which strongly supported an isolated position of the species between *A. distachyos* and the remaining species. Similarly, Thanikaimoni (1985) placed *A. robinsonii* in a relatively basal position near *A. hexatepalus* (Fig. 1). The Vietnamese *A. robinsonii* is unusual among other Asian *Aponogeton* by its secund flowers and paired spikes for which Camus assigned it to sect. *Pleuranthus* (otherwise comprising four African and one Malagasy species). Thus, it is not surprising that this species associated closely with the African *A. distachyos* (also sect. *Pleuranthus*) but distantly from the other Asian species surveyed (all sect. *Aponogeton*) in both molecular analyses.

Beyond these three relatively isolated, basal species, our combined molecular analysis resolved three major, highly-supported clades consisting of 1) Malagasy species, 2) remaining Asian species, and 3) remaining Australian species with the latter two clades constituting a sister group (Fig. 5). With one exception (*A. satarensis* Sundararagh., A. R. Kulk. & S. R. Yadav, which we did not survey), Thanikaimoni's (1985) scheme positioned the Asian species as a paraphyletic grade that gave rise ultimately to the Australian/New Guinea species (Fig. 1), a result not differing in essence from that depicted by our combined molecular cladogram. However, Thanikaimoni's (1985) placement of the Malagasy species that we surveyed differed substantially (see below).

Malagasy Species. All four accessions that we surveyed (*A. longiplumulosus*, *A. madagascariensis* (1, 2), *A. ulvaceus*) showed polymorphic ITS sequences, indicating that they might be of polyploid or hybrid origin. However, we did not clone any of these accessions for further clarification because they were not the focus of our study and because their sequences formed a single, well-supported clade (Fig. 3), thus providing adequate resolution of their phylogenetic position in the family. Notably, the two accessions of the distinctive ''lace plant'' (*A. madagascariensis*) did not associate together; the ITS sequence of one specimen with fine leaf fenestration [*A. madagascariensis* (1)] was more similar to that of *A. ulvaceus* than it was to a more coarsely fenestrate, conspecific accession [*A. madagascariensis* (2)]. Although the possibility that our accessions included a hybrid between *A. madagascariensis* and *A. ulvaceus* cannot be ruled out, further study and cloning would be necessary to clarify this question. Both species have been synthetically hybridized successfully (Bruggen 1985) and all three species appear to be closely related

by virtue of their identical cpDNA sequences (Fig. 4) and low level of ITS sequence divergence (Fig. 3).

The three Malagasy species included in our analysis formed a single clade, whether generated using morphological (Fig. 2), ITS (Fig. 3), or cpDNA (Fig. 4) data. Combined molecular data provided 100% bootstrap support for this clade (Fig. 5). This result is strongly at odds with Thanikaimoni's (1985) phylogenetic scheme, which depicted the three species as unrelated and dispersed widely across the genus (Fig. 1). Although we did not include a large sample of Malagasy species in our analysis, our results indicate that at least these species are much more closely related to each other than previously had been thought. Furthermore, our result is congruent with the classification of Camus (1923) who assigned all three species to subsect. *Polystachys*.

Asian Species. Bruggen (1985) treated *A. undulatus* and the narrower-leaved *A. stachyosporus* as conspecific, assuming that wide variability of leaf morphology existed in *A. undulatus*. This conclusion would be supported by our cpDNA sequences, which were identical in the two taxa (Fig. 4); however, we found their ITS sequences to differ by seven substitutions (Fig. 3), a level comparable to (or exceeding) the degree of ITS divergence that we observed between a number of other *Aponogeton* species. The ITS data would indicate that the distinctness of these two taxa should be reconsidered. We also compared two accessions of *A. rigidifolius* that differed conspicuously by their leaf-apex morphology (obtuse vs. acute). Despite this morphological difference, the ITS and cpDNA sequences of both accessions were identical, thus providing no evidence that these accessions might represent taxa worthy of nomenclatural distinction. Bruggen's (1985, p. 47) presumption that *A. rigidifolius*: ''. . . is, no doubt, closely related to *A. crispus*'' is corroborated by our combined molecular cladogram, which grouped the pair as sister species (Fig. 5). Thanikaimoni (1985) also depicted a close relationship between these species (Fig. 1). Considering the five species that we have now evaluated, the Asian Aponogetons appear to be biphyletic, with *A. robinsonii* clearly distantly related to the other species. A survey of additional Asian species (especially the secund-flowered *A. satarensis*) could readily determine whether additional clades exist among them.

Australian Species. Despite the opportunity that this study has given us to address other issues, our primary interest was to evaluate phylogenetic relationships among the Australian Aponogetons, a group in which many species have been described only recently. Because we had access to material of all the known Australian species, we believed that a comprehensive assessment was possible.

Our analyses of *Aponogeton* have provided conclusive evidence for the biphyletic origin of extant native Australian species with *A. hexatepalus* clearly not closely related to the other Australian taxa (see above). The sister position of *A. hexatepalus* to the rest of the genus not only points to a possible Australian origin for the family, but further indicates that Aponogetonaceae conceivably originated under temperate climatic conditions (both *A. hexatepalus* and *A. distachyos* presently inhabit temperate areas) and have radiated subsequently into the tropics. Camus (1923) classified *A. hexatepalus* within sect. *Aponogeton* (subsect. *Polystachys*) because of its branched, omnilaterally-flowered spikes. However, *A. hexatepalus* is distinct phylogenetically from other surveyed members of subsect. *Polystachys* that constituted a distinct clade (Fig. 5). The existing classification of *Aponogeton* would be improved by placing *A. hexatepalus* within a distinct section that better reflects its isolated position (Table 4). *Aponogeton hexatepalus* can be distinguished morphologically from other species in the genus by its six tepals that are green in color (Tables 2, 3).

All other Australian *Aponogeton* species occur within a clade (the ''Australian clade'') that is well-supported by ITS and cpDNA data (Figs. 3, 4). The combined molecular cladogram supports this group at 100%

TABLE 4. Refined classification of 18 *Aponogeton* species proposed as a result of phylogenetic analyses.

Aponogetonaceae J. Agardh
1. *Aponogeton* L.f.
  1. Section *Aponogeton*
    1. Subsection *Aponogeton*
      1. *A. crispus* Thunb.
      2. *A. rigidifolius* H.Bruggen
      3. *A. stachyosporus* de Wit
      4. *A. undulatus* Roxb.
    2. Subsection *Polystachys* A.Camus
      5. *A. longiplumulosus* H.Bruggen
      6. *A. madagascariensis* (Mirbel) H.Bruggen
      7. *A. ulvaceus* Baker
  2. Section *Flavida* Les, S.W.L.Jacobs & M.Moody
    3. Subsection *Flavida*
      8. *A. bullosus* H.Bruggen
      9. *A. elongatus* F.Muell. ex Bentham
      10. *A. eurystermus* Hellq. & S.W.L.Jacobs
      11. *A. kimberleyensis* Hellq. & S.W.L.Jacobs
      12. *A. lancesmithii* Hellq. & S.W.L.Jacobs
      13. *A. proliferus* Hellq. & S.W.L.Jacobs
      14. *A. queenslandicus* H.Bruggen
      15. *A. vanbruggenii* Hellq. & S.W.L.Jacobs
  3. Section *Pleuranthus* A.Camus
    4. Subsection *Pleuranthus*
      16. *A. distachyos* L.f.
      17. *A. robinsonii* A.Camus
  4. Section *Viridis* Les, S.W.L.Jacobs & M.Moody
    5. Subsection *Viridis*
      18. *A. hexatepalus* H.Bruggen

(Fig. 5). Thanikaimoni's (1985) phylogenetic scheme also portrayed the group as monophyletic with the inclusion of the two endemic New Guinea species (Fig. 1). Although our morphological analysis failed to resolve the Australian clade (Fig. 2), we found one character state (yellow tepal color) that uniquely defined the group (Tables 2, 3; Fig. 5). Bruggen (1969) commented that the yellow tepal color of the four native Australian species with which he was familiar was ''remarkable'' and uncommon elsewhere in the genus. Our studies confirm that all eight species within the Australian clade possess yellow tepals (Fig. 5); this morphological character state does indeed appear to represent a reliable taxonomic marker for the group. Of the New Guinea species (which we did not survey), *A. loriae* Martelli possesses yellow tepals; they are whitish in *A. womersleyi* H. Bruggen (Leach and Osborne 1985). It would be informative to include these species in subsequent analyses to determine whether they both are derived from the Australian clade as Thanikaimoni (1985) suggested.

All three species of the Australian clade that were known to Bruggen (1985) were placed by him within sect. *Aponogeton* subsect. *Aponogeton*, but Hellquist and Jacobs (1998) did not classify any of the five new species that they named in this group. From our results, it would be reasonable to classify all eight species of the Australian clade within sect. *Aponogeton* subsect. *Aponogeton*. However, the recognition of this clade as a distinct section of *Apongeton* also is justified and would further enhance the information content of the existing classification. Because the type species (*A. natans*) is not part of the Australian clade, the establishment of a new section name is necessary (Table 4).

Thanikaimoni (1985) concluded that species in the Australian clade were derived from a Malesian ''source,'' which itself was derived from Indian ''stock.'' Results from ITS and cpDNA analysis corroborate this hypothesis by placing the Australian and Asian species within a single clade (Figs. 3, 4). Internal support for this clade was high (95%) in the combined molecular analysis (Fig. 5). Although results of our morphological analysis were compatible (Fig. 2), the extremely poor resolution precluded a definitive assessment and provided no indication of morphological characters that might define this association.

Prior to this study, phylogenetic relationships among only four of the nine native Australian species had been hypothesized specifically, and these were estimated using non-cladistic methods (Fig. 1). Other authors (Aston 1973; Bruggen 1969, 1985; Hellquist and Jacobs 1998) had commented on possible relationships of the Australian species but again without the use of cladistic methods.

Bruggen (1985), who recognized only three species in what we have called the Australian clade, indicated that *A. bullosus*, *A. elongatus*, and *A. queenslandicus* were not particularly closely related, a result supported by our molecular analyses (Fig. 5). He remarked that the submersed leaves of *A. queenslandicus* sometimes resembled *A. bullosus* yet the species were ''easily distinguished'' by their reproductive characters. Although Bruggen (1985) observed that *A. elongatus* was ''impossible to distinguish'' vegetatively from some forms of *A. queenslandicus*, he believed that *A. queenslandicus* was related more closely to several Asian species (*A. lakhonensis*, *A. natans*) than to any of the Australian species (Bruggen 1985). Most likely, Bruggen based his conclusion on the presence of a double testa in these three species (cf. single in *A. elongatus* and *A. bullosus*), a character state he regarded as diagnostic taxonomically, but one that we have demonstrated as being highly homoplasious. With moderate support (72%), our combined molecular cladogram (Fig. 5) grouped *A. queenslandicus* as the sister species to *A. vanbruggenii*, a taxon not known to Bruggen but specimens of which he included in his concept of *A. elongatus*. Contrary to Bruggen's assessment, both species are fairly closely related to others in the Australian clade as indicated by the low level of molecular divergence among them (Fig. 5). Interestingly, Hellquist and Jacobs (1998) commented on a specimen of *A. vanbruggenii* from the Atherton Tablelands that greatly resembled *A. queenslandicus* vegetatively. Our phylogenetic analysis of morphological data (Fig. 2) also indicated a close relationship between the species. Within the *A. queenslandicus*/*A. vanbruggenii* clade was one accession (*A.* ''species 1'') that was difficult to identify conclusively. This taxon associates with, but is distinct from, *A. queenslandicus* at the molecular level (Fig. 5). We believe this taxon (for which we have not yet seen fruiting material) to represent an undescribed species and currently have initiated a more thorough study of it.

Bruggen (1969) presumed a close relationship between *A. bullosus* and *A. elongatus*, which Aston (1973) later remarked were ''not always easy to distinguish.'' Our results (Fig. 5) indicate that *A. bullosus* is related most closely to *A. lancesmithii* and *A. proliferus*, from which it cannot be distinguished by any of the molecular data that we evaluated. However, because the latter two species were not recognized by Bruggen at the time of his work (which included their material in *A. elongatus*), his hypothesized relationship between *A. bullosus* and *A. elongatus* is accurate given that the latter represents the next closest species phylogenetically (Fig. 5). Furthermore, because Bruggen actually had included material of what eventually was transferred to *A. lancesmithii* within *A. bullosus*, their close relationship as evidenced by molecular data is not surprising. *Aponogeton lancesmithii* resembles *A. bullosus* by its ability to produce bullate laminas (a trait unknown in other Australian species), but differs from *A.*

bullosus by possessing a double testa and extremely long-emergent inflorescences with chasmogamous flowers (*A. bullosus* has short usually submerged inflorescences with cleistogamous flowers). The species are identical for the molecular data that we surveyed. Here is yet another example of the potentially misleading homoplasy associated with testa number, which varies even between what appear to be very closely related species. Hellquist and Jacobs (1998) believed initially that *A. lancesmithii* was a hybrid between *A. bullosus* and *A. elongatus* but ultimately concluded that it was a distinct species. Our molecular analyses provided no evidence of hybridization between these species.

Aponogeton proliferus is one of only two proliferous species in the genus, the other being the Asian *A. undulatus* (Bruggen 1985). Despite the uniqueness of this feature, there is no question that proliferous shoots arose independently in the genus as evidenced by the vastly different placement of these two species phylogenetically (Figs. 3–5). Morphological data indicate a close relationship between *A. proliferus* and *A. bullosus* but not with *A. lancesmithii*, which is placed among a group of species possessing a double testa (Fig. 2). However, the close relationship of *A. proliferus* to both *A. bullosus* and *A. lancesmithii* is supported strongly by molecular data (Figs. 3, 5) and again calls into question the phylogenetic utility of testa number. Hellquist and Jacobs (1998) surmised that *A. proliferus* was most closely related to *A. elongatus*; however, the present results show these species to be considerably more distantly related.

Hellquist and Jacobs (1998) suggested a close relationship between *A. kimberleyensis* and *A. euryspermus* and also between the latter and *A. elongatus*. Morphological data separate these species phylogenetically (Fig. 2), but mainly by virtue of their different testa numbers. Molecular data (Figs. 3, 5) group *A. kimberleyensis* and *A. euryspermus* as sister species with a high degree of bootstrap support (95%), a result that raises further doubt on the taxonomic reliability of testa number. The association of these two sister species with *A. elongatus* (as proposed by Hellquist and Jacobs) is legitimate given their proximity to the latter species phylogenetically (Fig. 5). However, there is a confounding issue regarding one accession (*A.* ''species 2'') that we could not identify confidently to species, and which groups as a sister to the clade comprising *A. kimberleyensis* and *A. euryspermus* (Fig. 5). Tentatively, we consider this material to represent a second undescribed species whose taxonomic status we have deferred pending further study.

Lastly we consider the relationships of *A. elongatus*, a species that has been allied variously to *A. bullosus* (Bruggen 1969), *A. euryspermus*, *A. lancesmithii*, and *A. proliferus* (Hellquist and Jacobs 1998). Indeed, *A. elongatus* is closely related to all of these species (Fig. 5) and groups centrally in the Australian clade by its morphology (Fig. 2). Unfortunately, the limited resolution of our morphological and molecular cladograms precludes a more precise estimation of relationship for *A. elongatus*. Hellquist and Jacobs (1998) recognized two distinct subspecies of *A. elongatus* whose evaluation would require the use of genetic markers having finer resolution than those that we surveyed. However, our observation of minor DNA sequence divergence among the four accessions of *A. elongatus* that we surveyed indicates that this species possesses at least a moderate degree of interpopulational genetic variation.

Overall, the elucidation of phylogenetic relationships on the basis of perceived morphological trends (e.g., Thanikaimoni 1985) has depicted species relationships successfully in some instances but has failed badly in others. On the other hand, the classification developed by Camus (1923) incorporated only a few characters but appears to be reasonably compatible with our phylogenetic assessment of the genus, requiring only slight modifications (Table 4).

Molecular data have proven to be far more reliable than morphology for elucidating phylogenetic relationships in *Aponogeton*, yielding cladograms with relatively high resolution and nodal support that greatly facilitate study of the genus. The comparison of biparentally-inherited ITS sequences has provided evidence of hybridization in several instances and detected paralogous loci that reflect the extensive polyploidy that pervades the genus. Certainly, a more comprehensive assessment of chromosome numbers for *Aponogeton* would provide a useful adjunct to phylogenetic studies of the group. Intrinsic barriers to hybridization do not appear to be developed strongly in *Aponogeton* despite the apparent lack of natural hybrids, which may be due to the appropriate species rarely growing sympatrically. Molecular data also have indicated the possible existence of two undescribed *Aponogeton* species from Australia, the distinctness of which, had gone unnoticed previously from material that had been evaluated only morphologically, and indicates that there may be further benefit from studying populations still referred to *A. elongatus*.

Our analyses indicate that Aponogetonaceae conceivably originated in Australia and experienced early radiations into Africa and Asia. Subsequent diversification in *Aponogeton* yielded relatively discrete groups that radiated in Madagascar, Asia and ultimately back to Australia where considerable speciation has occurred since. Australian *Aponogeton* species represent an actively evolving group in which new species continue to be discovered. The recent origin of several species (e.g., *A. bullosus*, *A. lancesmithii*, *A. proliferus*) is evidenced by their distinct morphology coupled with a virtual lack of detectable molecular divergence in the genes sequenced.

The study of additional species in a similar fashion will be necessary before a comprehensive, phylogenetically defensible classification of *Aponogeton* can be achieved. However, this study has made some progress towards this objective by providing evidence in support of establishing two new sections (Table 4) that we describe below.

Taxonomic Treatment

1. **Aponogeton** L.f. sect. **Flavida** Les, M. Moody & S. W. L. Jacobs, sect. nov.—Type Species: *A. queenslandicus* H. Bruggen

Tepalis tribus, inflorescentia omnino sulphurea a congeneribus differt.

Differing from other related sections in having three tepals and a completely sulphur-yellow inflorescence. The section contains 8–10 species distributed throughout tropical regions of Australia.

2. **Aponogeton** L.f. sect. **Viridis** Les, M. Moody & S. W. L. Jacobs, sect. nov.—Type Species: *A. hexatepalus* H. Bruggen

Tepalis sex vice tribus, semper viridibus, floribus circum axem patentibus, inflorescentia ramosa viridique, a congeneribus differt. Hieme florens; in terram hieme inundatam.

Differing from other related sections in having six always green tepals instead of three, flowers spread around the axis, and the inflorescence branched and green. Flowering in winter; growing in winter-flooded habitats. The section comprises one species (*A. hexatepalus*), which is distributed in temperate regions of Western Australia.

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