



# Roxburgh was right: *Aponogeton microphyllus* and *Aponogeton undulatus* are distinct species

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*Aponogeton microphyllus*, previously placed under the synonymy of *A. undulatus*, is recognized here as a distinct species based on morphology, chromosome number, and molecular phylogenetics (nuclear ribosomal internal transcribed (ITS) spacer region). Observations on the type and live specimens revealed morphological differences between the two species. *Aponogeton microphyllus* flowered regularly and set seeds. *Aponogeton undulatus* flowered rarely, did not set seeds, but showed formation of young plantlets on the inflorescence axis. Similarly, different chromosome numbers were recorded in *Aponogeton microphyllus* and the two forms of *A. undulatus*, viz., AF1 and AF2, which occur in distinct populations. *Aponogeton microphyllus* exhibited polysomaty with root-tip cells showing  $2n=40, 42,$  and  $44$  chromosomes. The two forms of *A. undulatus*, AF1 and AF2, showed  $2n=84$  and  $86$  chromosomes, respectively. Based on the ITS data, both species occupied two separate clades. Plastid *trnK* intron region indicated a close relationship between both species. Our study suggests the need for comprehensive phylogenetic analyses of *A. undulatus* across its distribution range based on more advanced techniques such as high-throughput sequencing data to understand the *A. undulatus* species complex and to detect natural hybrids of this species.

**Keywords.** Aquarium plant; cpDNA; cytogenetics; hybridization; ITS; phylogeny; polysomaty

## 1. Introduction

The Cape pondweed family (Aponogetonaceae Planch.) is a monogeneric family comprising 60 species. These tuberous hydrogeophytes occur mainly in the tropical and subtropical regions of the Old World (POWO 2023). The species of this genus usually bear a simple or two-branched spike (fragrant biforked inflorescence in *A. distachyos* L.f.). Some species are also known for their unique leaves (fenestrate leaves in *A. madagascariensis* (Mirb.) H. Bruggen). The most important utility of the genus is its use as an ornamental freshwater aquarium plant (van Bruggen 1985).

The species of this genus are mostly concentrated in Africa, Madagascar, Asia, and Australia. In India, Yadav and Gaikwad (2003) carried out a taxonomic revision of the genus, describing seven species. Since then two new species, viz. *A. nateshii* S.R. Yadav (Yadav *et al.* 2015) and *A. wolfgangianus* S.R. Yadav (Yadav 2017), have been described. Very recently, Dey *et al.* (2021) re-collected *A. lakhonensis* A. Camus from the Poba Reserve Forest, Dhemaji District, Assam, after a gap of 123 years. At present, nine species occur in India, of which five are endemic (Yadav *et al.* 2015; Yadav 2017). Taxonomic delimitation has always been tricky in this genus on account of polymorphism and frequent hybridization (van

Bruggen 1985). Consequently, species complexes are usually observed in nature. Hybridization has been described as ‘common’ in this genus, which includes mainly self-compatible species, several of which are also interfertile (Les and Philbrick 1993). Both natural and artificial hybrids have been reported. The natural hybrids are *A. appendiculatus* x *A. undulatus*, *A. crispus* x *A. undulatus*, and *A. natans* x *A. undulatus* (Les and Philbrick 1993; Gaikwad et al. 1998). Gaikwad et al. (1998) reported natural hybrids (*A. appendiculatus* x *A. undulatus*) from two places, Padubidri, Udipi Taluk, Karnataka, and Khodungullor, Thrissur District, Kerala. Both populations show characters intermediate between *A. undulatus* and *A. appendiculatus*. The natural hybrids from Padubidri produce many inflorescences, but fail to develop fruit and seeds because of the absence of stamens in flowers, and the plants propagate vegetatively by forming young plants on inflorescence-like axes as in *A. undulatus* and have submerged leaves as in *A. appendiculatus* (Gaikwad et al. 1998). The hybrids from Khodungullor also produce many inflorescences with normal flowers, but this population does not set seeds because of high pollen sterility and, hence, propagate vegetatively by formation of propagules on inflorescence-like axes (Gaikwad et al. 1998). Cytogenetical studies of the hybrids revealed diploid numbers of  $2n=74$  and  $84$  for the Khodungullor and Padubidri populations, respectively. Artificial hybrids (*A. decaryi* x *A. satarensis*, *A. bovinianus* x *A. ulvaceus*, and *A. madagascariensis* x *A. ulvaceus*) have also been generated and are found to be vigorous and flowering abundantly (van Bruggen 1985; Yadav 1995).

As a part of taxonomic studies on Indian *Aponogeton*, we collected some specimens from Bankati, on the way to Dudhwa Wildlife Sanctuary, Lakhimpur, Uttar Pradesh. After critical scrutiny of the relevant literature and examination of fresh material, we found that the specimens tallied with Roxburgh’s species *A. microphyllus* Roxb. van Bruggen (1985) reduced this species to a synonym of *A. undulatus*. Therefore, the aims of the present study were (1) to determine whether *A. undulatus* and *A. microphyllus* are distinct species, using morphology, karyology, and molecular phylogenetics and (2) to determine whether *A. undulatus*/*A. microphyllus* are part of a species complex.

## 2. Materials and methods

### 2.1 Taxonomy

*Aponogeton microphyllus* was collected from Bankati, near Dudhwa Wildlife Sanctuary, Lakhimpur, Uttar

Pradesh. *Aponogeton undulatus* Form 1 (AF1) was collected from Kittur, Belgaum District, Karnataka, and *A. undulatus* Form 2 (AF2) from Thrissur District, Kerala. Flowering was not observed in AF1, whereas AF2 flowered regularly. Both the forms propagated vegetatively by formation of young plants on an inflorescence-like axis. Morphological analysis and description are based on observations made in the field and examination of live specimens. The identity of the specimen was confirmed by consulting protologs and types. Descriptions were made following the terminology of Hickey and King (2001).

### 2.2 Cytogenetics and pollen viability

Materials for cytogenetical studies were taken from the plants maintained in the Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India. The voucher specimens of the cytogenetically examined plants were deposited in the Herbarium of the Department of Botany, Shivaji University, Kolhapur, India (table 1).

For mitotic studies, the root tips obtained from tubers were used after pre-treatment with an aqueous saturated solution of para-dichlorobenzene at 8–10°C for 4 to 5 h. Root tips were fixed in freshly prepared modified Carnoy’s fluid (3:1 of ethanol and propionic acid, respectively) for 24 h. Fixed roots were washed thoroughly in distilled water, hydrolyzed in 1 N HCl, and stained and squashed with 2% propionic-orcein. For meiotic studies, floral buds of appropriate size were fixed in Carnoy’s fluid. Anther smears were prepared in 2% propionic-orcein. Meiotic and pollen viability studies for AF1 could not be carried out as these plants do not flower. Suitable somatic and meiotic plates from freshly prepared slides were photographed with a LEICA DM 750 microscope.

Pollen viability was estimated through stainability using glycerolacetocarmine (1:1). A total of 2500 pollen were analyzed. Stained pollen were taken as fertile or viable, while shriveled and unstained pollen were scored as sterile or non-viable. Slides were photographed with a LEICA DM 750 microscope.

### 2.3 Molecular phylogeny

A total of eight *Aponogeton* species were collected from different regions in India. The plant material was identified, and voucher specimens deposited in the herbarium, Department of Botany, Shivaji University, Kolhapur, India (table 1).

**Table 1.** Taxa included in the study, their locality, sample code, voucher details and GenBank accession number of the gene regions used

| Sr. No. | Species  | Locality                           | Sample code | Voucher  | ITS accession number   | <i>trnK</i> intron accession number |
|---------|--|------------------------------------|-------------|----------|--|-------------------------------------|
| 1.      | <i>A. appendiculatus</i>                             | Mananthavady, Kerala               | A7          | RNC-231  | -  | OR398472                            |
| 2.      | <i>A. crispus</i>                                    | Sira, Tumkur, Karnataka            | 8           | SRY-1019 | -  | OR398468                            |
| 3.      | <i>A. lakhonensis</i>                                | Poba reserve forest, Demaji, Assam | A2          | RNC-251  | OR381831   | -                                   |
| 4.      | <i>A. microphyllus</i>                               | Bankati, Lucknow, Uttar Pradesh    | 3           | RNC-225  | OR381828   | -                                   |
| 5.      | <i>A. microphyllus</i>                               | Bankati, Lucknow, Uttar Pradesh    | 4           | RNC-226  | OR381830   | OR398464                            |
| 6.      | <i>A. microphyllus</i>                               | Bankati, Lucknow, Uttar Pradesh    | A5          | RNC-255  | OR381829   | OR398465                            |
| 7.      | <i>A. microphyllus</i>                               | Bankati, Lucknow, Uttar Pradesh    | Am          | RNC-263  | -  | OR398466                            |
| 8.      | <i>A. natans</i>                                     | Badami, Bagalkot, Karnataka        | A10         | RNC-264  | -  | OR398467                            |
| 9.      | <i>A. nateshii</i>                                   | Jaitapur, Ratnagiri, Maharashtra   | A12         | RNC-244  | OR381824   | -                                   |
| 10.     | <i>A. undulatus</i> (form 1) (AF1)                   | Kittur, Belgaum, Karnataka         | Ka          | RNC-267  | OR381826   |                                     |
| 11.     | <i>A. undulatus</i> (form 2) (AF2)                   | Thrissur, Kerala                   | A3          | RNC-269  | OR381487, OR381488, OR381489, OR381490, OR381491, OR381492, OR381493, OR381494, OR381495 | OR398461, OR398462                  |
| 12.     | <i>A. undulatus</i> (probably hybrid) (form 3) (AF3) | Ponnani, Kalady, Kerala            | 12          | -        | -  | OR398460                            |
| 13.     | <i>A. undulatus</i> (form 4) (AF4)                   | Wayanad, Kerala                    | 10          | RNC-265  | -  | OR398469                            |
| 14.     | <i>A. undulatus</i> (form 6) (AF6)                   | Amgaon, Bhandara, Maharashtra      | 9           | RNC-268  | OR381827   | -                                   |
| 15.     | <i>A. wolfgangianus</i>                              | Panamaram, Wayanad, Kerala         | A6          | SRY-402  | OR381823   | -                                   |
| 16.     | <i>A. wolfgangianus</i>                              | Panamaram, Wayanad, Kerala         | A9          | RNC-256  | OR381822   | OR398471                            |

**Table 2.** Taxa included in this study and GenBank accession numbers of the gene regions used

| Sr. No. | Species   | ITS  | <i>trnk</i>                            | Reference  |
|---------|---|--|--|--|
| 1.      | <i>Aponogeton abyssinicus</i> Hochst. ex A.Rich.  | KJ922026, KJ922027, KJ922028, KJ922029                     | KJ939682, KJ939684, KJ939683           | Chen et al. 2015   |
| 2.      | <i>A. angustifolius</i> Aiton                     | KJ922030, KJ922031   | KJ939688, KJ939689                     | Chen et al. 2015   |
| 3.      | <i>A. azureus</i> H. Bruggen                      | KJ922035   | KJ939679, KJ939680, KJ939681           | Chen et al. 2015   |
| 4.      | <i>A. boivinianus</i> Baill. ex Jum.              | HQ456424, HQ456425, KJ922063, KJ922066, KJ922065, KJ922068 | HQ456474, HQ456475, KJ939640, KJ939642 | Les and Tippery 2013; Chen et al. 2015                                   |
| 5.      | <i>A. bruggenii</i> S.R.Yadav & Govekar           | HQ456426, KJ922090, KJ922091                               | HQ456476, KJ939662, KJ939663, KJ939664 | Les and Tippery 2013; Chen et al. 2015                                   |
| 6.      | <i>A. bullosus</i> H. Bruggen                     | AY926318   | AY926344                               | Les et al. 2005  |
| 7.      | <i>A. capuronii</i> H. Bruggen                    | HQ456427   | HQ456477                               | Les and Tippery 2013   |
| 8.      | <i>A. crispus</i> Thunb.                          | AY335958, AY926288, KJ922092, KJ922093, HQ456429,          | KJ939654, KJ939656, HQ456478           | Les et al. 2003; Les et al. 2005; Chen et al. 2015; Les and Tippery 2013 |
| 9.      | <i>A. crispus</i> x <i>A. rigidifolius</i>        | HQ456432, HQ456433, HQ456434, HQ456435                     | HQ456479, HQ456480, HQ456481           | Les and Tippery 2013   |
| 10.     | <i>A. decaryi</i> Jum. ex Humbert                 | KJ922071, KJ922072   | KJ939645, KJ939646                     | Chen et al. 2015   |
| 11.     | <i>A. distachyus</i> L.f.                         | AY926320, HQ456436   | AY926346, HQ456482                     | Les et al. 2005; Les and Tippery 2013                                    |
| 12.     | <i>A. eggersii</i> Bogner & H. Bruggen            | HQ456437, HQ456438   | HQ456483, HQ456484                     | Les and Tippery 2013   |
| 13.     | <i>A. elongatus</i> F.Muell. ex Benth.            | AY926294, AY926295, AY926296                               | AY926331                               | Les et al. 2005  |
| 14.     | <i>A. euryspermus</i> Hellq. & S.W.L. Jacobs      | AY926308, HQ456440   | AY926338, HQ456485                     | Les et al. 2005; Les and Tippery 2013                                    |
| 15.     | <i>A. fugax</i> J.C.Manning & Goldblatt           | KJ922109   | KJ939685                               | Chen et al. 2015   |
| 16.     | <i>A. gottlebei</i> Kasselm. & Bogner             | KJ922078, KJ922079   | KJ939667, KJ939668                     | Chen et al. 2015   |
| 17.     | <i>A. hexatepalus</i> H. Bruggen                  | AY926321   | AY926347                               | Les et al. 2005  |
| 18.     | <i>A. jacobsenii</i> de Wit                       | KJ922096, KJ922097   | KJ939674, KJ939675                     | Chen et al. 2015   |
| 19.     | <i>A. kimberleyensis</i> Hellq. & S.W.L. Jacobs   | AY926309   | AY926339                               | Les et al. 2005  |
| 20.     | <i>A. lakhonensis</i> A.Camus                     | KJ922110   | KJ939650                               | Chen et al. 2015   |
| 21.     | <i>A. lancesmithii</i> Hellq. & S.W.L. Jacobs     | AY926316   | AY926342                               | Les et al. 2005  |
| 22.     | <i>A. longiplumulosus</i> H. Bruggen              | AY335956, AY926284, HQ456428                               | AY335991, AY926325                     | Les et al. 2003; Les et al. 2005; Les and Tippery 2013                   |
| 23.     | <i>A. madagascariensis</i> (Mirb.) H. Bruggen     | AY926285, AY926286   | -                                      | Les et al. 2005  |
| 24.     | <i>A. madagascariensis</i> var. <i>major</i> Baum | HQ456444   | HQ456488                               | Les and Tippery 2013   |
| 25.     | <i>A. masoalaensis</i> Bogner                     | HQ456445   | HQ456489, KJ939638, KJ939639           | Les and Tippery 2013; Chen et al. 2015                                   |
| 26.     | <i>A. natans</i> (L.) Engl. & K. Krause           | KJ922101.1, KJ922102.1, KJ922104                           | KJ939651, KJ939652, KJ939653           | Chen et al. 2015   |
| 27.     | <i>A. proliferus</i> Hellq. & S.W.L. Jacobs       | AY926315   | AY926341                               | Les et al. 2005  |

**Table 2** (continued)

| Sr. No. | Species   | ITS  | <i>trnk</i>                                      | Reference   |
|---------|---|--|--|---|
| 28.     | <i>A. queenslandicus</i> H. Bruggen                       | AY926290, AY926293, AY926298                     | AY926330   | Les <i>et al.</i> 2005  |
| 29.     | <i>A. ranunculiflorus</i> Jacot Guill. & Marais           | KJ922044, KJ922045, KJ922046                     | KJ939676, KJ939677, KJ939678                     | Chen <i>et al.</i> 2015   |
| 30.     | <i>A. rehmannii</i> Oliv                                  | KJ922049, KJ922050, KJ922052                     | KJ939686, KJ939687                               | Chen <i>et al.</i> 2015   |
| 31.     | <i>A. rigidifolius</i> H. Bruggen                         | AY335957, AY926287                               | KJ939670, KJ939671                               | Les <i>et al.</i> 2003; Les <i>et al.</i> 2005; Chen <i>et al.</i> 2015 |
| 32.     | <i>A. robinsonii</i> A.Camus                              | AY926319, HQ456448                               | AY926345, HQ456490, KJ939635, KJ939636, KJ939637 | Les <i>et al.</i> 2005; Les and Tippery 2013; Chen <i>et al.</i> 2015   |
| 33.     | <i>A. satarensis</i> Sundararagh., A.R.Kulk. & S.R. Yadav | KJ922041, KJ922042, KJ922043                     | KJ939644   | Chen <i>et al.</i> 2015   |
| 34.     | <i>A. stachyosporus</i> de Wit                            | AY926304, AY926305                               | AY926337   | Les <i>et al.</i> 2005  |
| 35.     | <i>A. stuhlmannii</i> Engl.                               | KJ922057, KJ922058, KJ922056                     | KJ939657, KJ939658, KJ939659                     | Chen <i>et al.</i> 2015   |
| 36.     | <i>A. subconjugatus</i> Schumach. & Thonn.                | KJ922060, KJ922061, KJ922062                     | KJ939672, KJ939673                               | Chen <i>et al.</i> 2015   |
| 37.     | <i>A. tenuispicatus</i> H. Bruggen                        | KJ922082, KJ922084, KJ922088, KJ922089           | KJ939665, KJ939666                               | Chen <i>et al.</i> 2015   |
| 38.     | <i>A. ulvaceus</i> Baker                                  | AY926307, AY926312, AY926313, AY926283, HQ456450 | AY926324, HQ456491                               | Les <i>et al.</i> 2005; Les and Tippery 2013                            |
| 39.     | <i>A. undulatus</i> Roxb.                                 | AY926302   | AY926336, HQ456492                               | Les <i>et al.</i> 2005; Les and Tippery 2013                            |
| 40.     | <i>A. vanbruggenii</i> Hellq. & S.W.L. Jacobs             | AY926299, AY926300                               | AY926333, AY926334                               | Les <i>et al.</i> 2005  |
| 41.     | <i>A. womersleyi</i> H. Bruggen                           | HQ456452   | HQ456493   | Les and Tippery 2013  |
| 42.     | <i>Wiesneria triandra</i> (Dalzell) Micheli               | AY335953   | HQ535983   | Les <i>et al.</i> 2003; Les and Tippery 2013                            |

DNA was extracted from either live plants or silica gel-dried plants by the CTAB method (Doyle and Doyle 1987). The nuclear ribosomal DNA internal transcribed spacers (ITS) and the plastid tRNA-Lys (*trnK*) intron along with partial maturase K (*matK*) gene were used for molecular phylogenetics analysis as in Chen *et al.* (2015). The ITS region was amplified using ITS4 and ITS5 primers (White *et al.* 1990) and the plastid (cp) *trnK* intron region was amplified using M-apo-f1 and M-apo-r1 primers (Chen *et al.* 2015).

PCR amplification was done using a 2X PCR master mix (APS Labs, Pune, India). Each 25  $\mu$ L reaction consisted of 12.5  $\mu$ L 2X master mix, 1  $\mu$ L each of the forward and reverse primers (10 pmol/ $\mu$ L), and 1  $\mu$ L of

template DNA extracted by the CTAB method which was approximately 100 ng. The PCR cycle was as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, followed by a final extension for 5 min. The PCR products were visualized in 1% agarose gels stained with 0.5  $\mu$ g/mL ethidium bromide. PCR product purification and Sanger sequencing were performed by Barcode Biosciences, Bengaluru, India. For one specimen of *A. undulatus*, the ITS PCR products were cloned into a TA-cloning vector and plasmids were isolated from 10 colonies and sequenced from both forward and reverse directions. PCR product cloning and sequencing of the cloned plasmid DNA was outsourced to Barcode Biosciences, Bengaluru, India.

Contigs from both forward and reverse strands were assembled using pregap4 and gap4 modules of the STADEN package ver. 2.0.0b11 (Bonfield and Whitwham 2010). The assembled contigs were separately aligned with sequences of *Aponogeton* available in the NCBI GenBank database (table 2) and aligned using the MUSCLE algorithm in Aliview ver. 1.28 (Larsson 2014). Alignments were checked and refined manually in Aliview.

The congruence of the two datasets was tested using the partition homogeneity test as implemented in PAUP ver. 4.0b10 (Swofford 2003), and the null hypothesis that the datasets are congruent was rejected ( $p=0.01$ ). As  $p=0.01$ , we analyzed the two genes separately. The best model of sequence evolution was checked using jModelTest ver. 2.1.10 (Darriba et al. 2012). Bayesian phylogenetics analysis was performed using MrBayes ver. 3.2.7a (Ronquist et al. 2012) using the CIPRES portal ([www.phylo.org](http://www.phylo.org)) (Miller et al. 2010). The Bayesian phylogenetic (BP) analysis consisted of two independent runs of 4 chains of Metropolis-coupled Monte Carlo simulations run for 2 million generations sampling trees every 1000 generations. The convergence of the runs was monitored with effective sample sizes (ESS) which were greater than 200. The first 20% of the sampled trees were discarded as the burn-in and the remaining trees were summarized. Trees were visualized in FigTree ver. 1.4.4. Maximum likelihood (ML) phylogenetic analysis was performed using

**Table 3.** Comparative morphological features of *A. microphyllus* and *A. undulatus* Form 2 (AF2)

| Attributes   | <i>Aponogeton microphyllus</i>  | <i>Aponogeton undulatus</i> Form 2 (AF2)                         |
|--------------|---|--|
| Spathe       | Persistent  | Caducous   |
| Tepal colour | White   | Pinkish white  |
| Tepal        | Spathulate, oblong ovate, caducous, 4–7 × 2 mm  | Obovate, caducous, 3–5 × 2 mm                                    |
| Stamens      | Filaments 1.5–1.7 mm long, anthers yellow coloured  | Filaments 1.4–1.5 mm long, anthers dark bluish to black coloured |
| Carpel       | 3, 0.8–1.5 × 0.5–0.8 mm   | 3, 1.2 × 0.8 mm  |
| Follicle     | Beaked  | Not developed  |
| Seed         | 5 × 2 mm, testa simple  | Not developed  |
| Embryo       | 4–5 × 1.5–2 mm, plumule with two young foliage leaves, plumule attached to the embryo at base | Not developed  |

**Figure 1.** Karyotypes in two species of *Aponogeton* (a, b, c) Mitotic metaphases showing  $2n=40$ , 42 and 44 chromosomes, respectively (*A. microphyllus*), note secondary constrictions (arrowheads); (d) pollen mother cell (PMC) at diakinesis showing  $n=20$  bivalents (*A. microphyllus*); (e) Mitotic metaphase showing  $2n=84$  (*A. undulatus* AF1); (f) Mitotic metaphase showing  $2n=86$  chromosomes (*A. undulatus* AF2); meiotic stages in *A. undulatus* AF2 (g–l); (g) PMC at diakinesis showing  $n=43$  bivalents; (h, i) PMCs at metaphase I showing precocious separation (arrowheads); (j) PMC at anaphase I showing a bridge (arrowhead); (k) PMC at metaphase II showing precocious separations (arrowheads); (l) PMC at telophase II showing bridge and laggards (arrowheads). Scale bars: 5  $\mu$ m.

RAxML ver. 8.2.12 (Stamatakis 2014). ML bootstrap analysis was performed using the GTRGAMMA model under the non-parametric bootstrapping method with 100 replicates in RAxML.

### 3. Results

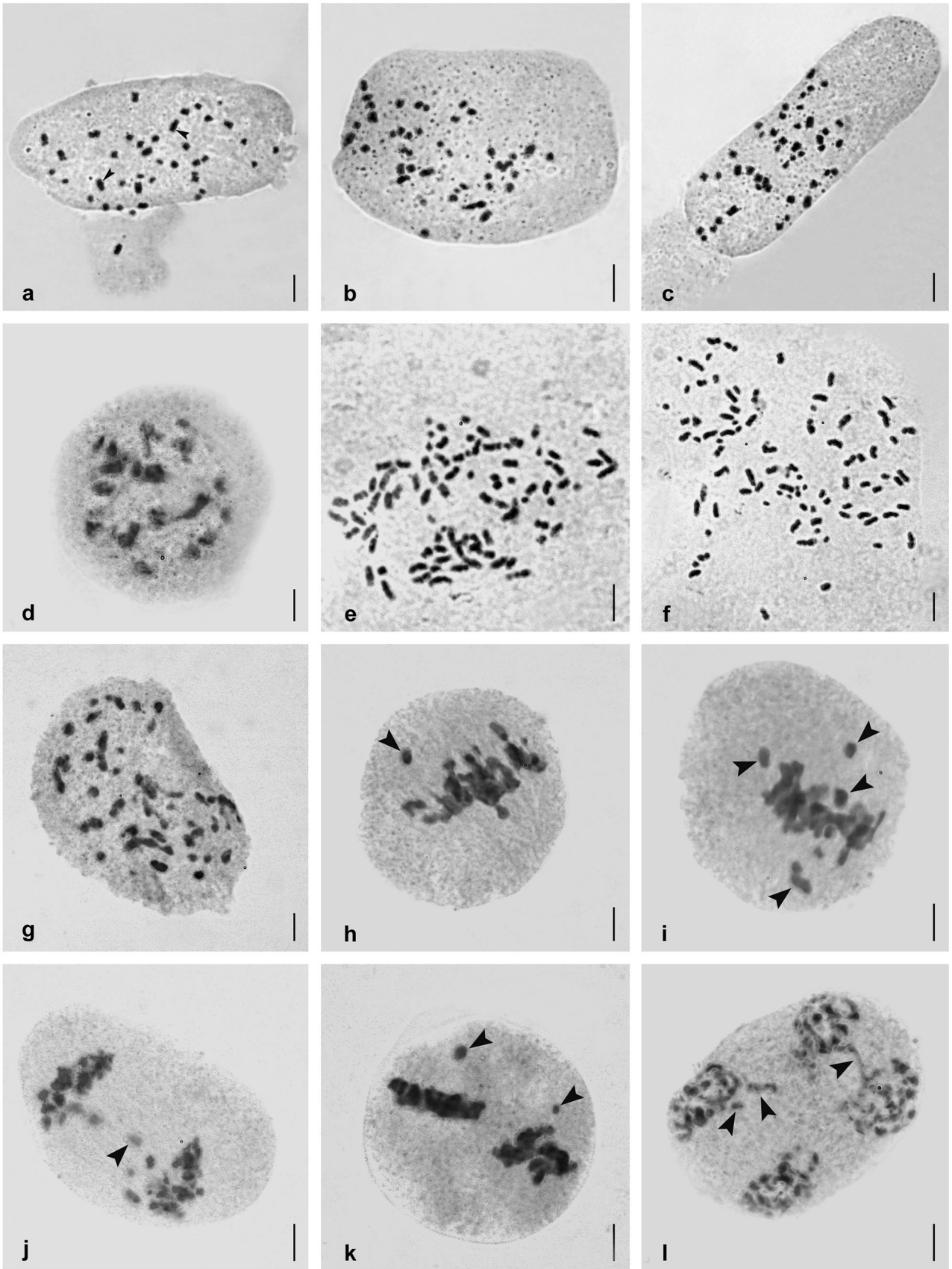
#### 3.1 Cytogenetics and pollen viability

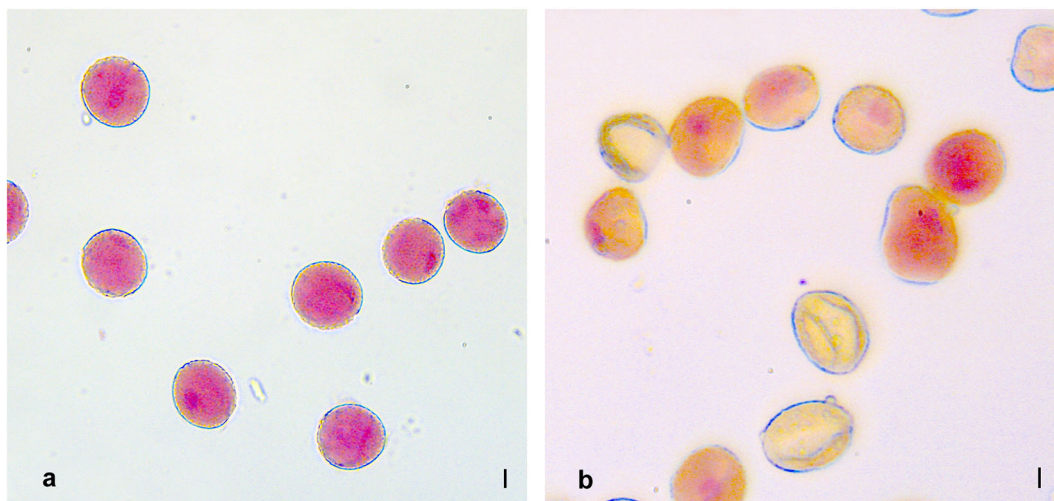
*Aponogeton microphyllus* exhibited polysomaty. Three counts with  $2n=40$ , 42, and 44 chromosomes were observed (figure 1a–c). The chromosome number  $2n=40$  was the most frequent and observed in 47.62% of the nuclei, whereas  $2n=42$  and 44 was recorded in 30.16% and 22.22% of the nuclei, respectively. In addition to numerical alterations, structural alterations in chromosomes were observed. In one of them, i.e.,  $2n=40$ , two chromosomes with secondary constrictions were noted (figure 1a). *Aponogeton undulatus* AF1 and AF2 showed  $2n=84$  and  $2n=86$  chromosomes, respectively (figure 1e, f). Meiosis was normal in *A. microphyllus* and  $n=20$  bivalents were observed at diakinesis (figure 1d). *Aponogeton undulatus* AF2 showed  $n=43$  bivalents at diakinesis (figure 1g) with meiotic anomalies such as bridge formation, precocious separation, and laggards (figure 1h–l).

*Aponogeton microphyllus* exhibited high (98%) pollen viability (figure 2a) and seed set (figure 3g). AF2 had very low (30%) pollen viability (figure 2b) with no seed set. These plants propagated vegetatively by the formation of propagules (figure 3m, n).

#### 3.2 Molecular phylogeny

The aligned ITS dataset consisted of 111 accessions and 837 nucleotides. There were 564 variable sites, of which





**Figure 2.** Pollen viability. **(a)** *A. microphyllus*; **(b)** *A. undulatus* (AF2). Note the stained pollen (viable) and unstained pollen (non-viable). Scale bars: 5  $\mu\text{m}$ .

462 were parsimony-informative. The cp *trnK* intron dataset consisted of 86 accessions and 1010 nucleotides of which 233 were variable and 176 were parsimony-informative. The BP and ML trees were similar in topology (ML tree not shown). The ITS tree (figure 4) was better resolved than the plastid *trnK* intron tree (figure 5). The best-fit model of sequence evolution for the ITS dataset and cpDNA datasets was GTR+G and SYM+G, respectively.

In the ITS dataset tree, the three different accessions of *A. microphyllus* formed a well-supported clade. This clade also contained samples named *A. stachyosporus* de Wit which were reported in other studies (AY926306 and AY926303 from Les et al. 2005) (table 3). In the same clade, one sequence from a cloned ITS region obtained from *A. undulatus* (A3 specimen, clone no. 3) collected from Thrissur, Kerala, India, was recovered.

The other clones of the same specimen, A3 (RNC-269), collected as *A. undulatus* from Kerala, and whose chromosome number indicates high ploidy formed a clade along with *A. stachyosporus* (AY926305, AY926304) and *A. undulatus* (AY926302) from Les et al. (2005). Another specimen, 10, collected as *A. undulatus* from Wayanad, Kerala, formed a close relationship with *A. crispus* Thunb. specimens although not strongly statistically supported. The occurrence of one isoform of ITS from A3 specimen of *A. undulatus* Roxb. in the *A. microphyllus* clade indicates the hybrid nature of this specimen.

The plastid *trnK* dataset was not well resolved, and the posterior probability (PP) as well as ML bootstrap (BS) supports of the majority of the branches were not significantly high. This might be due to fewer

parsimony-informative characters (figure 5). The samples of *A. microphyllus* nested within *A. undulatus* with shallow branch lengths but with good branch supports. *Aponogeton wolfgangianus* was also found closely associated with *A. undulatus* in the *trnK* data. This clade was the closest in relation to another clade consisting of *A. womersleyi* H. Bruggen and *A. euryspermus* Hellq. & S.W.L. Jacobs and *A. lakhonensis* A. Camus. This sister relationship between these two clades was not well supported. The sample A3 used for ITS cloning and sequencing was in the *A. undulatus* clade, proving the maternal lineage of the hybrid.

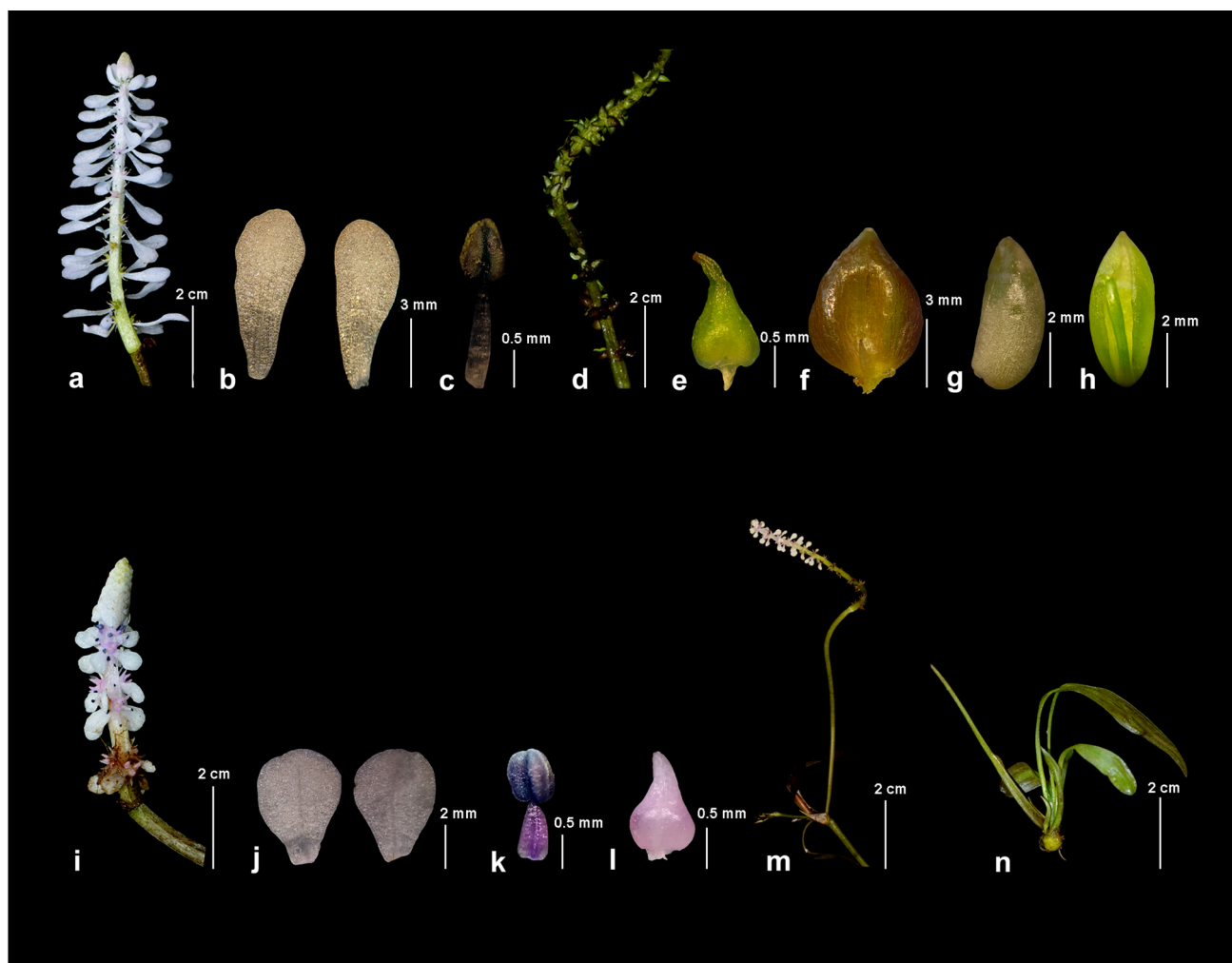
### 3.3 Morphological study

***Aponogeton microphyllus* Roxb.**, Fl. Ind. Ed. Carey (1832) 2:211 (figures 3a–h and 6)

**Type** Roxb. Icon No.1232

**Description** Tuberos, monoecious, perennial, aquatic herbs. *Tubers* globose to obovoid, 1–2 cm, roots fibrous. *Leaves* both submerged and floating; submerged leaves petiolate, petiole ca. 15 cm long, lamina ca. 10×4 cm, elliptic lanceolate, obtuse or rounded at apex, base rounded or rarely cordate, leaf margin undulate, main nerve with 6–7 parallel nerves; floating leaves petiolate, petiole ca. 20–25 cm long, lamina ca. 8×3 cm, elliptic lanceolate, obtuse apex, base rounded, main nerve with 5–6 parallel nerves. *Inflorescence* a single spike, spatheate, spathe persistent, peduncle ca. 22–25 cm long, cylindrical; spike simple, 4–8 cm long, flowers turned in all directions on axis. *Flowers* bisexual, tepals 2, 4–7×2 mm, oblong-





**Figure 3.** Comparison of morphological features of *A. microphyllus* and *A. undulatus* (AF2). (a–h) *A. microphyllus*. (a) Inflorescence; (b) tepals; (c) stamen; (d) infructescence; (e) gynoeceium; (f) fruit; (g) seed; (h) embryo; (i–n) AF2; (i) inflorescence; (j) tepals; (k) stamen; (l) gynoeceium; (m) inflorescence with a young plantlet (propagule); (n) the young plantlet (propagule) forming a new individual.

obovate, obtuse, 1-nerved, caducous, white coloured. *Stamens* 6, exserted, filamentous; filaments  $1.5 \times 1.7$  mm long, narrow above and widened towards base; anthers 2-celled, globose, basifixed, yellow coloured. *Carpels* 3,  $0.8-1.5 \times 0.5-0.8$  mm, ovules 2, basal. *Follicles*  $5-7 \times 4$  mm with short, terminal, curved beak. *Seeds* ca.  $5 \times 2$  mm with simple testa, embryo ca.  $4-5 \times 1.5-2$  mm, plumule attached at the base with young foliage leaves.

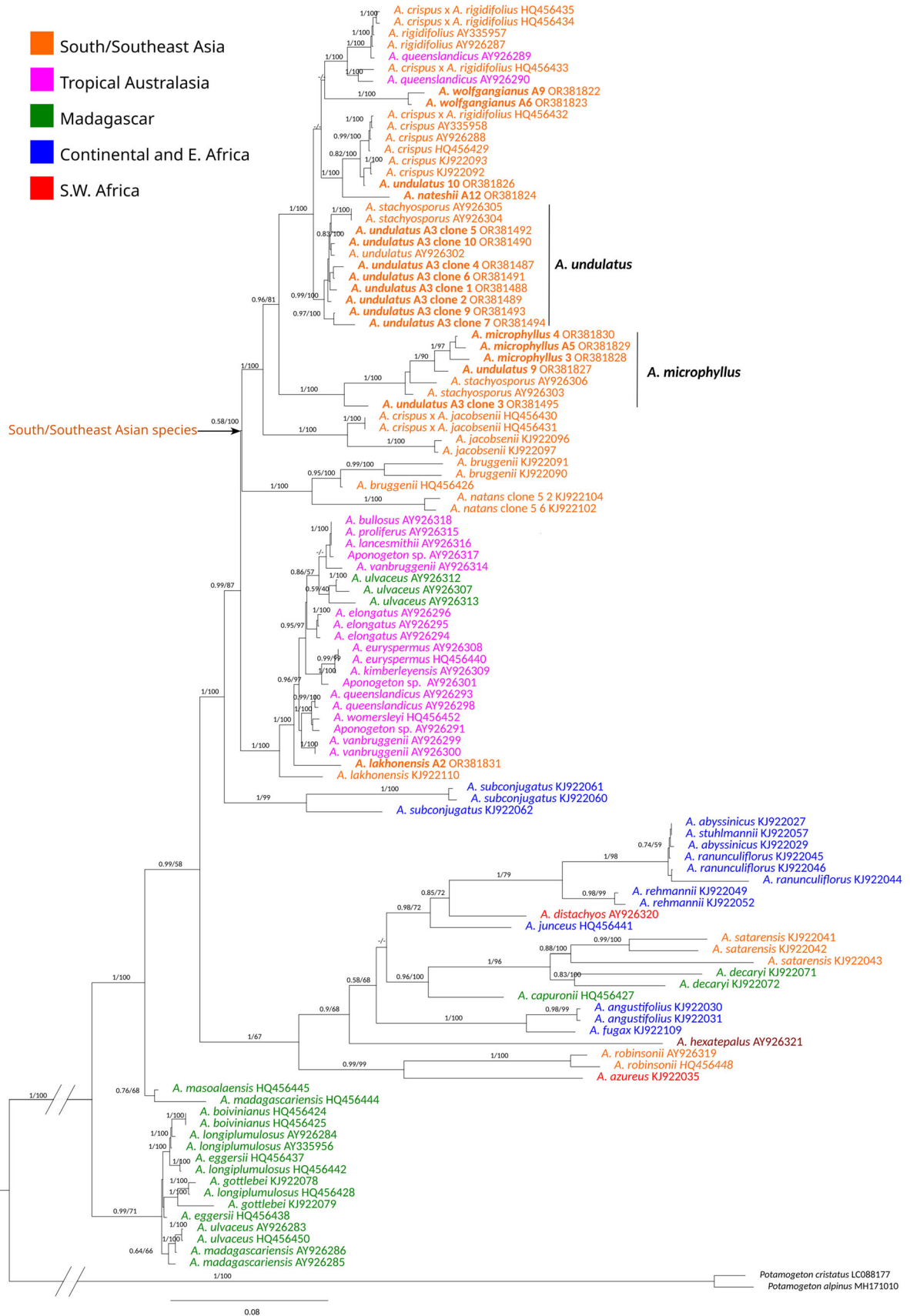
**Phenology** flowering and fruiting from May to September

*Aponogeton undulatus* Roxb., Fl. Ind. Ed. Carey (1832) 2:211 (figures 3i–n, 7)

**Type** Roxb. Icon No. 936

**Description** Tuberos, monoecious, perennial, aquatic herbs. *Tubers* globose to obovoid  $1-4 \times 1-2$  cm, roots fibrous. *Leaves* both submerged and

floating; submerged leaves petiolate, petiole ca. 15–30 cm long, lamina ca.  $9-15 \times 1.5-2.5$  cm, linear-lanceolate, acute apex, base cuneate, leaf margin undulate, main nerve with 5–7 parallel nerves; *floating leaves* petiolate, petiole ca. 25–30 cm long, lamina ca.  $10 \times 4$  cm, elliptic lanceolate, obtuse apex, base rounded, main nerve with 5–6 parallel nerves. *Inflorescence* a single spike, spatheate, spathe caducous, peduncle ca. 25–30 cm long, cylindrical; spike simple, 4–6 cm long, flowers turned in all directions on axis. *Flowers* bisexual, tepals 2,  $2.5-3 \times 2$  mm, oblong-obovate, rounded, 1-nerved, caducous, pinkish-white coloured. *Stamens* 6, exserted, filamentous; filaments  $1.4 \times 1.5$  mm long, narrow above and widened towards base; anthers 2-celled, globose, basifixed, dark bluish to black coloured. *Carpels* 3,  $1.2 \times 0.8$  mm, free, unilocular.



◀ **Figure 4.** Bayesian phylogenetic tree based on the ITS region. Numbers above branches are posterior probabilities and maximum-likelihood bootstrap values, respectively. Sequences generated for this study are indicated in bold text. GenBank accession numbers are indicated beside taxon names. Scale indicates the number of substitutions along the branch lengths. Branches are colour-coded according to their biogeographic distribution. (Orange: S/S.E. Asia, Pink: Tropical Australasia, Green: Madagascar, Blue: Continental and E. Africa, Red: S.W. Africa.)

**Phenology** flowering from May to September, fruiting absent.

The differences between *A. microphyllum* and *A. undulatus* are listed in table 3.

## 4. Discussion

### 4.1 Cytogenetics and pollen viability

The chromosome count in *Aponogeton* varies from  $2n=16$  (*A. distachyos* L.f. and *A. madagascariensis* (Mirb.) H. Bruggen) to  $2n \sim 126$  (*A. junceus*) (van Bruggen 1985). Other chromosome counts recorded in this genus are  $2n=24, 26, 32, 38, 40, 48, 50, 52, 56, 60, 68, 70, 74, 76, 78, 80, 92$ , and  $\sim 100$  (van Bruggen 1985; Gaikwad *et al.* 2014).

*Aponogeton undulatus* shows tremendous variation in chromosome number with  $2n \sim 70, \sim 74$ , and 86 (van Bruggen 1985; Gaikwad 2000; Rice *et al.* 2014). The count of  $2n=32$  cannot be verified as it lacks voucher details (see Rice *et al.* 2014) and this could be a wrong identification. Non-flowering populations of *A. undulatus* collected from Bhandara District, Maharashtra, and Dastikoppa, Dharwad District, Karnataka, had  $2n=70$  chromosomes, whereas flowering populations of *A. undulatus* from Kittur, Belgaum District, Karnataka, and Ghadbadya Lake, near Amgaon, Bhandara District, Maharashtra, had  $2n=74$  and  $2n=86$  chromosomes, respectively (Gaikwad 2000). In our studies, we observed  $2n=84$  chromosomes in AF1 and  $2n=86$  chromosomes in AF2. Meiotic anomalies such as chromosome clumping and laggards have been reported in *A. undulatus* flowering individuals ( $2n=86$ ) (Gaikwad 2000). We also observed meiotic anomalies such as bridge formation, precocious separation, and laggards in AF2. Consequently, AF2 showed very low (38%) pollen viability.

Polysomy, the occurrence of different chromosome numbers in the cells of the same root tip, has been

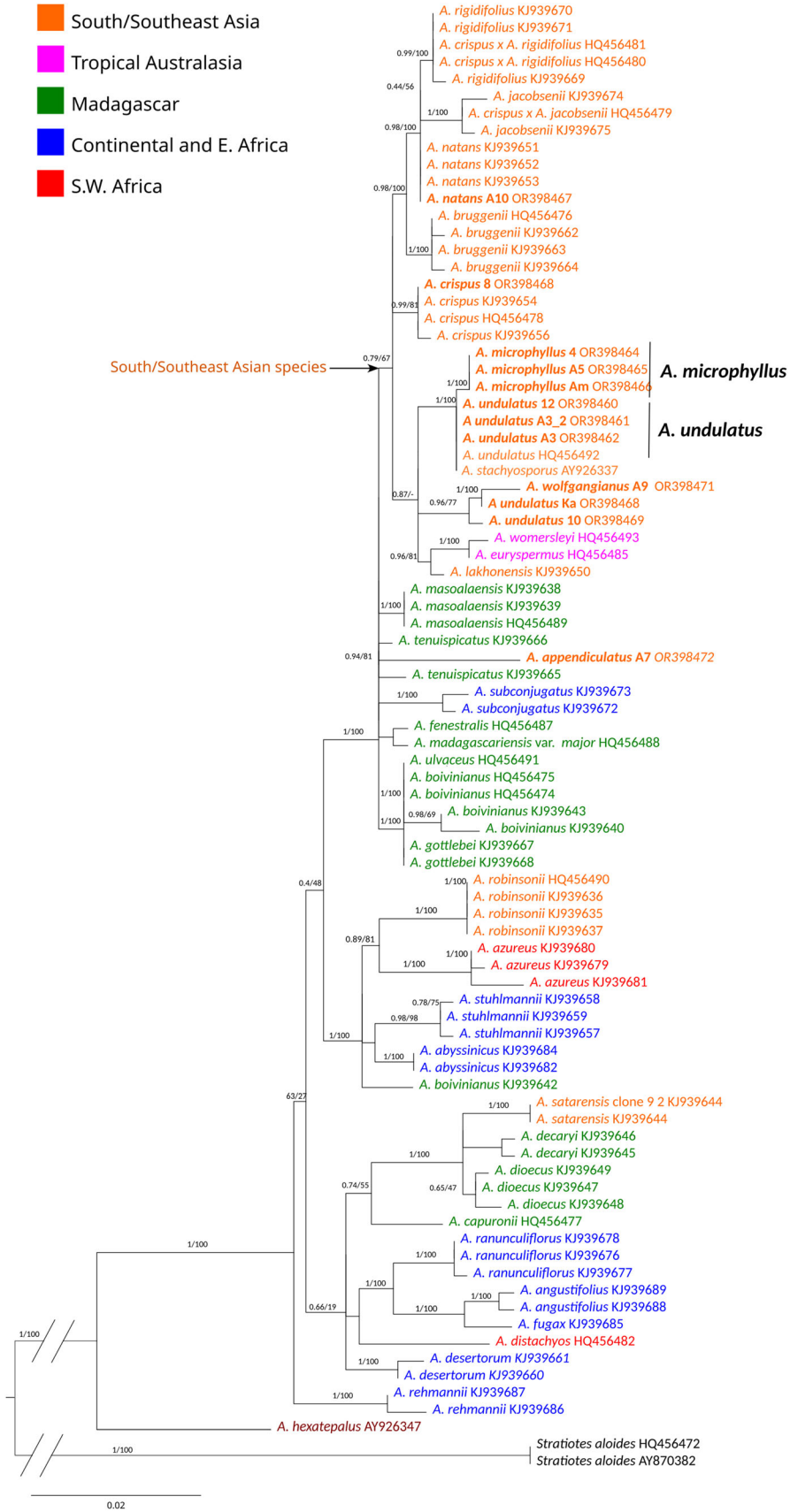
reported in many monocots such as *Lilium* Tourn. ex L., *Drimiopsis* Lindl. & Paxton, *Ornithogalum* L., *Urginea* Steinh., *Allium* L., *Cymbidium* Sw., *Bulbophyllum auricomum* Lindl., *Cymodocea nodosa* (Ucria) Asch., and *Dendrobium pierardii* R.Br. In the majority of cases, aneuploidy was observed frequently (Sen 1973; Fukai *et al.* 2002; Than *et al.* 2011; Gargiulo *et al.* 2020). Recently, Gargiulo *et al.* (2020) reported aneusomy and polysomy in *Cymodocea nodosa* (Cymodoceaceae). Similarly, polysomy and chromosome number variation has been observed in the freshwater plant *Ceratophyllum demersum* L. by Gargiulo *et al.* (2022), who reported euploid and aneuploid ( $2n=24, 48, 52, 62, 72, 78, 82, 86, 92, 98$ ) chromosome numbers as well as nuclei with different amounts of DNA (2C, 4C, 6C, 8C, 10C) in a single plant. Gargiulo *et al.* (2020, 2022) suggested that both endoreduplication and regular or anomalous mitoses together were responsible for the formation of nuclei with diverse chromosome numbers and ploidy levels in somatic cells. In the present investigation, we observed polysomy in the root-tip cells of *A. microphyllum* ( $2n=40, 42$ , and 44) along with structural changes involving secondary constrictions. It has been suggested that the nucleocytoplasmic ratio in such polysomy cases is maintained in the tissue taken as a whole, that is, between the entire cytoplasm and the nuclear complement of the tissue, rather than of the individual cells (Sen 1973). The exact cause of polysomy in *A. microphyllum* is not known as of now. The data on cytogenetics and pollen viability suggest that both species are genetically different. *Aponogeton microphyllum* has low chromosome number and high pollen viability and reproduces sexually. On the other hand, *A. undulatus* has high chromosome number, polymorphism, and low pollen viability, and propagates vegetatively, indicating its hybrid origin.

### 4.2 Molecular phylogeny

The molecular phylogeny of the genus was constructed based on one nuclear marker and one plastid DNA marker, ITS and *trnK* intron, respectively. A total of 45 species and 36 species was included in the nuclear ITS and cp *trnK* intron data, respectively.

According to the ITS dataset, *A. undulatus* and *A. microphyllum* fall into two separate clades. However, in cpDNA data, *A. undulatus* and *A. microphyllum* show a close sister relationship. The close affinity of *A. undulatus* and *A. microphyllum* might have led to natural hybridization, and some specimens collected for

- South/Southeast Asia
- Tropical Australasia
- Madagascar
- Continental and E. Africa
- S.W. Africa



◀ **Figure 5.** Bayesian phylogenetic tree based on the plastid *trnK* intron. Numbers above branches are posterior probabilities and maximum-likelihood bootstrap values, respectively. Sequences generated for this study are indicated in bold text. GenBank accession numbers are indicated beside taxon names. Scale indicates number of substitutions along the branch lengths. Branches are colour-coded according to their biogeography. (Orange: S/S.E. Asia, Pink: Tropical Australasia, Green: Madagascar, Blue: Continental and E. Africa, Red: S.W. Africa.)

this study might be those hybrids. Specimen A3, collected as *A. undulatus*, shows at least two isoforms of the ITS gene sequence. Among the nine sequences obtained by cloning the PCR product, one (A3) was found nested in the *A. microphyllus* clade (figure 4). Similar natural hybrids have been reported in previous works but without molecular sequence data (Les *et al.* 2005). Hybridization followed by polyploidization as a source of speciation has been reported in several plants (Soltis and Kuzoff 1995; Ackerman and Wen 2003; Kim and Donoghue 2008; Surveswaran *et al.* 2018).

Although *A. undulatus* and *A. microphyllus* have been synonymized as *A. undulatus* by van Bruggen (1985), several morphological features, karyology, and molecular phylogenetics using nuclear ITS provide evidence that they are distinct species. However, due to their close evolutionary history, as seen in the maternally inherited *trnK* intron data, the species might be able to hybridize naturally.

Some specimens collected as *A. stachyosporus* (Les *et al.* 2005) fall under the *A. undulatus* clade as well as the *A. microphyllus* clade in our study (figure 4). However, in the cpDNA phylogeny (figure 5), *A. stachyosporus* is placed along with *A. undulatus*. Les *et al.* (2005) have similar findings. They found that cpDNA sequences were identical in *A. undulatus* and *A. stachyosporus*; however, ITS sequences of the species differed by seven substitutions, indicating their distinctness. This might be the reason why Chen *et al.* (2015) used the name *A. stachyosporus* in their phylogeny (based on the combined nuclear and plastid data) but indicated that the species is synonymous with *A. undulatus*. A perusal of the protolog of *A. stachyosporus* revealed that the species was collected from Johore (now Johor, a state in Malaysia) from the Malay Peninsula and exhibited vivipary (de Wit 1958). The plants produced a digitately branched inflorescence, giving rise to reduced sterile inflorescences and young plants (de Wit 1958). The morphology of *A. stachyosporus* matches completely with the AF2, A3,

and Ma specimens in our study. Therefore, the results of the present study provide clear evidence that *A. stachyosporus* is nothing but *A. undulatus*, which is a hybrid species.

In the ITS phylogeny, the widespread *A. crispus*, *A. rigidifolius*, and *A. wolfgangianus* were found in a close relationship, and one specimen of *A. nateshii* was also found inside this clade. The relationships between these taxa are well resolved by both nuclear and plastid markers. *Aponogeton natans* was found closely related to *A. brugennii* in the ITS data. Another widespread species, *A. lakhonensis*, was found in a clade consisting of Australian and Asian species with good branch support. There are some incongruencies between the cpDNA and ITS datasets, as expected (Reisberg and Soltis 1991; Doyle 1992). It was also found that in the ITS tree, one of the forms, i.e., *A. undulatus* (form 4), is sister to *A. crispus*, which indicates that these species may interbreed to produce hybrids.

The South/Southeast Asian species form a clade in both nuclear ITS and cpDNA datasets which is weakly supported in the cpDNA dataset (0.79/67) and only well supported by Bayesian posterior probability in the ITS dataset. In the cpDNA data, Australian *A. eurysperumus* and *A. womersleyi* are included in the South/Southeast Asian clade and this clade has relatively low support (only ML bootstrap support). The paraphyletic *A. undulatus* nested with *A. microphyllus* and sometimes in *A. wolfgangianus*. The ITS/plastid lineages of the *A. crispus* hybrids are more closely related to *A. natans* than to other *A. crispus* accessions themselves.

The close association of the *A. undulatus* dataset with *A. wolfgangianus* in the cpDNA dataset indicates a phylogenetic close relationship of these species. To further understand reticulation events in these closely related species, much more sequence data are required. To cite an example, in *Lachemilla* (Focke) Rydb. (Rosaceae), to resolve the incongruence between nuclear and cpDNA markers, a dense sampling of 396 nuclear loci by target capture approach and nearly complete plastome sequences from 27 species were used to resolve the relationships among the major groups. The data also helped understand the multiple sources of conflict between gene trees and species trees inferred with a multitude of approaches (Morales-Briónes *et al.* 2018). Such an approach should be applied in *Aponogeton*, after sampling the genus from all over the world. In any case, the specimens of *A. undulatus* and *A. microphyllus* presented a major challenge in obtaining the sequences for our study. This might be due to their hybrid nature and varying ploidy levels.



**Figure 6.** *Aponogeton microphyllum* Roxb. (Roxburgh drawing No. 1232) [Reproduced with the permission of the Board of Trustees, Royal Botanic Gardens, Kew].

Therefore, a complete sampling of the specimens in various locations as well as reduced representation genome sequencing followed by phylogeny analysis are very important for a future study.

#### 4.3 Taxonomy

Roxburgh (1832) described two species, *A. microphyllum* (figure 6) and *A. undulatus* (figure 7), but the former was synonymized under *A. undulatus* by van Bruggen (1985). He could not find any specimen of *A. microphyllum* and concluded that *A. microphyllum* represents a poor specimen of *A. undulatus*. Subsequent

workers (Yadav and Gaikwad 2003) also considered *A. microphyllum* conspecific to *A. undulatus*. Roxburgh (1832) described the flowers as blue and the spathe as caducous, but his drawing (figure 6) depicts white flowers and a persistent spathe. This was also pointed out by van Bruggen (1985). Our observations revealed that *A. microphyllum* has white flowers and a persistent spathe, whereas *A. undulatus* has pinkish-white flowers and a caducous spathe. van Bruggen (1985) observed that *A. undulatus* rarely flowers in cultivation. He also observed the formation of a forked inflorescence, proliferation, and malformations of the inflorescence in the *A. undulatus*. de Wit (1958) also recorded branched inflorescence and propagules in *A. undulatus*. Yadav



**Figure 7.** *Aponogeton undulatus* Roxb. (Roxburgh drawing No. 936) [Reproduced with the permission of the Board of Trustees, Royal Botanic Gardens, Kew].

and Gaikwad (2003), based on their collections of *A. undulatus* from different localities in India, made similar observations. They found that some of these populations produced 1–3 inflorescences on the same axis on which propagules had developed and flowered

regularly; however, these plants failed to set seeds, whereas in some populations flowers were not observed (Yadav and Gaikwad 2003). Our observations are consistent with those of van Bruggen (1985) and Yadav and Gaikwad (2003). *Aponogeton undulatus*

AF1 flowers rarely, and therefore, the species has adapted vegetative propagation by forming a propagule, whereas *A. undulatus* AF2 flowers regularly but fails to set seeds. *Aponogeton undulatus* AF2 sometimes produces branched inflorescences that develop propagules which, when detached, form a new plant (figure 3m, n). *Aponogeton microphyllus* flowers regularly and sets seeds (figure 3g).

In conclusion, based on morphological studies, chromosome numbers, and molecular phylogenetic analysis of the ITS region, our study confirms that *A. microphyllus* is not conspecific to *A. undulatus*. Our studies also suggest that *A. undulatus* has a hybrid origin. There is a possibility that four species, viz., *A. appendiculatus*, *A. crispus*, *A. microphyllus*, and *A. undulatus*, hybridize whenever their distribution ranges overlap. Overall, it can be said that the Indian specimens of *Aponogeton* would provide an interesting model to study hybrid speciation and karyology by employing tools of molecular biology and techniques such as flow cytometry.

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## Declarations

**Conflict of interest** Authors have no conflict of interest to declare.

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