

## *Nymphaea kakaduensis* (Nymphaeaceae), a new species from the northern portion of the Northern Territory, Australia

C. Barre Hellquist<sup>1,2</sup>, Andre Leu<sup>3</sup> and Michael L. Moody<sup>4</sup> 

<sup>1</sup>Department of Biology, Emeritus, Massachusetts College of Liberal Arts, North Adams, MA 01220, USA

<sup>2</sup>Botanical Research Institute of Texas (BRIT), Fort Worth, Texas, 76107, USA

<sup>3</sup>PO. Box 800, Mossman, Queensland 4873, Australia

<sup>4</sup>Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968, USA

Author for correspondence: [c.barre.hellquist@mcla.edu](mailto:c.barre.hellquist@mcla.edu)

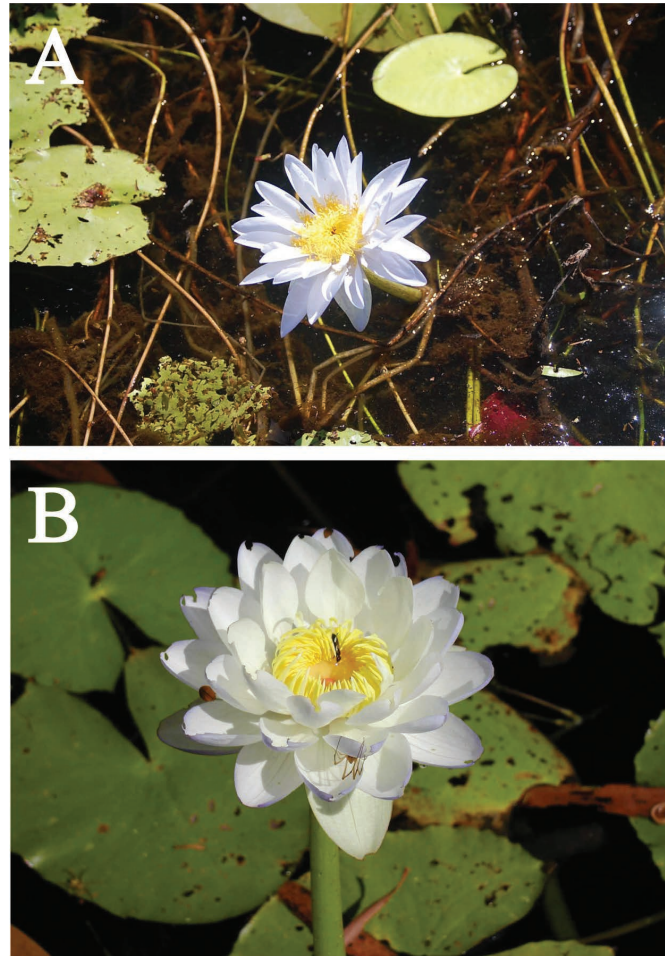
### Abstract

*Nymphaea kakaduensis* C.B.Hellq., A.Leu & M.L.Moody (Nymphaeaceae) is described from Kakadu National Park, Northern Territory. This new species is endemic to the “Top End” of the Northern Territory and was formerly included in *N. violacea* Lehm. The distinct floral form of *N. kakaduensis* of having generally smaller flowers with blunt-tipped petals and different coloration than *N. violacea* instigated a genetic study of taxa from the region. The cpDNA *trnL* (UAA) - *trnF* (GAA) intergenic spacer region was sequenced from samples from across northern Australia and a haplotype network analysis was conducted. Plants from populations that had the distinct floral form of *N. kakaduensis* are found to be genetically distinct from *N. violacea* in northern Australia sharing 4 SNPs and a notable 23 bp indel in the cpDNA intergenic spacer region to support the morphological attributes and species designation.

### Introduction

The number of *Nymphaea* L. species recognised in Australia has expanded considerably since Conard's (1905) treatment of the genus determined all *Nymphaea* in Australia to be *N. gigantea* Hook., with *N. violacea* Lehm. designated as a variety. Extensive collections and studies by numerous investigators over the last century have now revealed a diversity of Australian *Nymphaea* species (e.g. Aston 1970; Conard 1905; Borsch *et al.* 2007; Jacobs 1992, 1994; Cowie *et al.* 2000; Jacobs and Hellquist 2006, 2011; Jacobs and Porter 2007; and Löhne *et al.* 2008). Today, there are 18 native species of Australian *Nymphaea* described (Jacobs and Hellquist 2011).

Conard's (1905) treatment of *Nymphaea* considered *N. gigantea* var. *violacea* (Lehm) Conard as a smaller form within *N. gigantea* and Aston (1970) reiterated this interpretation. *Nymphaea violacea* was again formally recognised with lectotypes designated by Jacobs (1992) and it is now accepted as a distinct species (Short 2000; Jacobs and Porter 2007). *Nymphaea violacea* is abundant throughout the Australian tropical regions of Queensland, Northern Territory and Western Australia and it occurs in New Guinea (Jacobs and Porter 2007). It is highly variable with flowers that may be blue, mauve, pink or white. The petals have been described as acute-tipped, blunt or intermediate between the two characteristics. The sepals and petals are frequently ascendant giving the flower an acute floral profile (Fig. 1A).

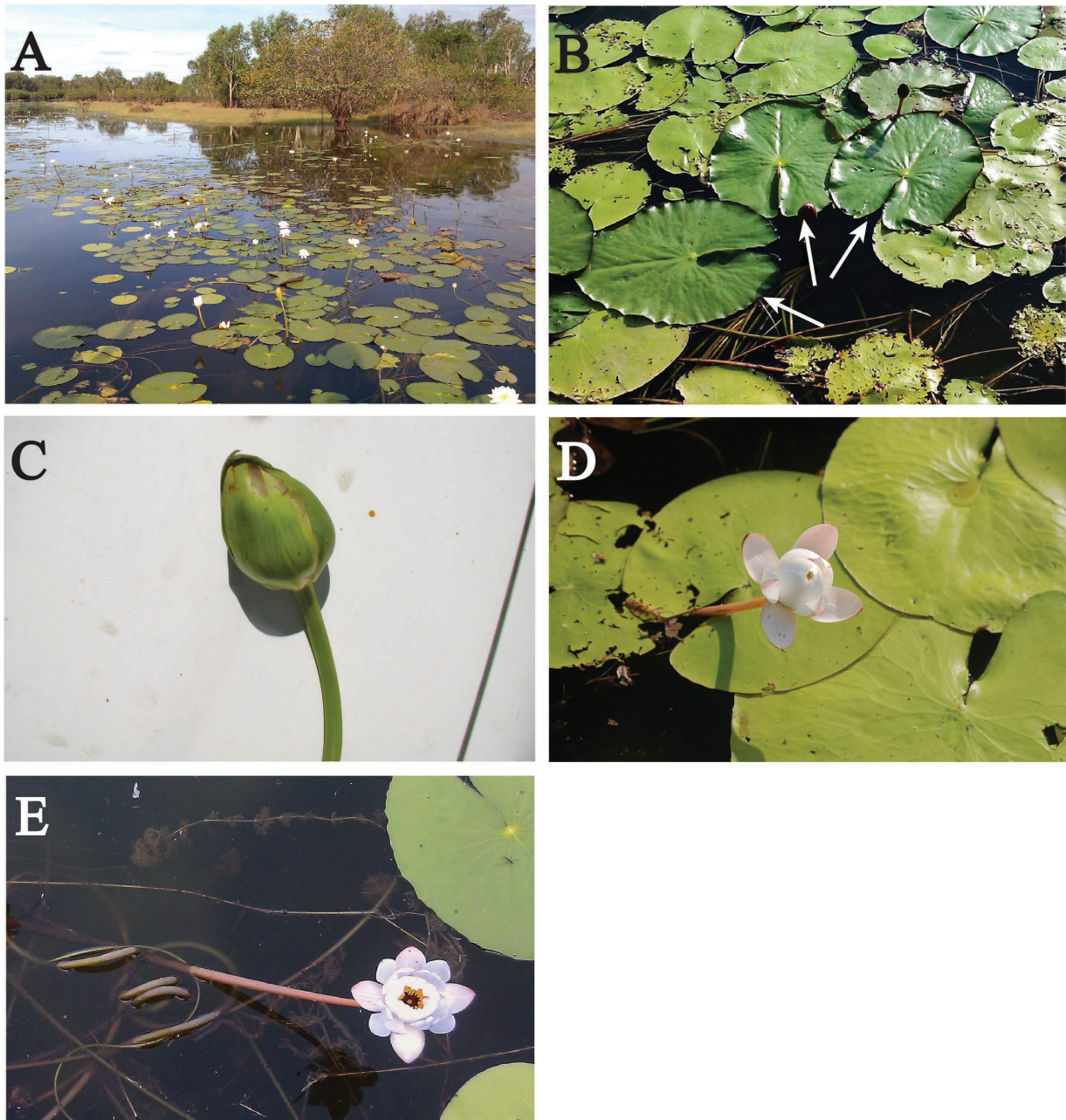


**Fig. 1.** Floral characteristics of *Nymphaea violacea* and the closely related *N. kakaduensis*. A. *N. violacea* with characteristic acute oblanceolate petals with tinges of bluish coloration (Kakadu population), B. *N. kakaduensis* with white coloured, blunt ovate–elliptic petals tinged with pale blue at the apex and along the margins (Lake Jabiru population). Photos: C.B. Hellquist.

Kakadu National Park is located in the “Top End” or northernmost region of the Northern Territory, east of Darwin and west of the Arnhem Land Aboriginal Reserve. The region has many wetlands, rivers, streams and billabongs that are well known for their waterlily populations. The senior author first visited Kakadu in 1997, with subsequent collecting trips to the area in 2002, 2005, and 2011. One of the largest wetland complexes within Kakadu National Park is the floodplain billabong of the South Alligator River at Yellow Waters (Cowie *et al.* 2000). *Nymphaea* taxa found in this region include the night blooming species *N. pubescens* Willd. and the day-blooming *N. macrosperma*, Merr. & L.M. Perry, *N. hastifolia* Domin., *N. nouchali* Burm. f. and *N. violacea* (see key below). Morphologically distinctive hybrid *N. violacea* × *N. macrosperma* have also been identified in the region and have been confirmed using molecular data (Löhne *et al.* 2008).

*Nymphaea violacea* is the most widespread and abundant species in Kakadu National Park and is common in Yellow Waters with many of the open water plants having characteristic large, blue flowers with acute petals (Fig. 1A). In Jim Jim Creek, its billabong system and surrounding wetlands, we found multiple sites inhabited by populations initially believed to be *N. violacea* but which, upon closer observation, appeared to be morphologically distinct (Fig. 2A). Initially, the darker green upper leaf surface drew our attention to plants now described as *N. kakaduensis*, as they were mixed among the lighter green leaves of *N. violacea* (Fig. 2B). The plants had flower buds that were blunt and wider (Fig. 2C) compared to the typically shorter angular buds of *N. violacea*. In addition, the flowers of *N. kakaduensis* were smaller with blunt-tipped petals and often a slight pink hue (Fig. 2D–E), but could be infused with more blue coloration (Fig. 2B) like those found in Jim Jim Billabong and Lake Jabiru. The fruiting peduncles were also coiled at the type locality (Fig. 2E); however, more field observations will be required to determine if this characteristic is consistent across populations. Here, we describe and provide genetic support for plants with this form as a new species of *Nymphaea* from the Northern Territory designated as *N. kakaduensis*.





**Fig. 2.** Habitat and characteristics of *Nymphaea kakaduensis*. A. Plants of the type location, Jim Jim Creek, Kakadu National Park. B. Dark green leaves of *N. kakaduensis* (see arrows) surrounded by leaves of *N. violacea*. C. Blunt flower bud. D. White flower infused with pink. E. Coiled peduncles. Photos: C.B. Hellquist and A. Leu.

## Methods

DNA was extracted from dried leaf material of 25 samples of *Nymphaea* from 24 populations in northern Australia (Table 1). Five samples are recognised as having morphological features of *N. kakaduensis* and 14 of *N. violacea*. *Nymphaea macrosperma* (6 accessions) was also included to address if there was evidence that *N. kakaduensis* was a result of recent hybridisation between *N. macrosperma* and *N. violacea* as these species are known to hybridise in the region. DNA extractions were performed using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. The polymerase chain reaction (PCR) was used to amplify the cpDNA *trnL* (UAA) – *trnF* (GAA) intragenic spacer region using the primers TabF and TabC (Taberlet *et al.* 1991) following the protocol of Shaw *et al.* (2005). The PCR products were treated with ExoSAP-IT (Affymetrix) to eliminate unincorporated primers and dNTPs before sequencing. The PCR products were sequenced using the same primers used for PCR and BigDye terminator chemistry (Applied Biosystems), on an ABI 3130xl automated sequencer (Applied Biosystems) at the UTEP Genomics Analysis Core in the Border Biomedical Research Center.

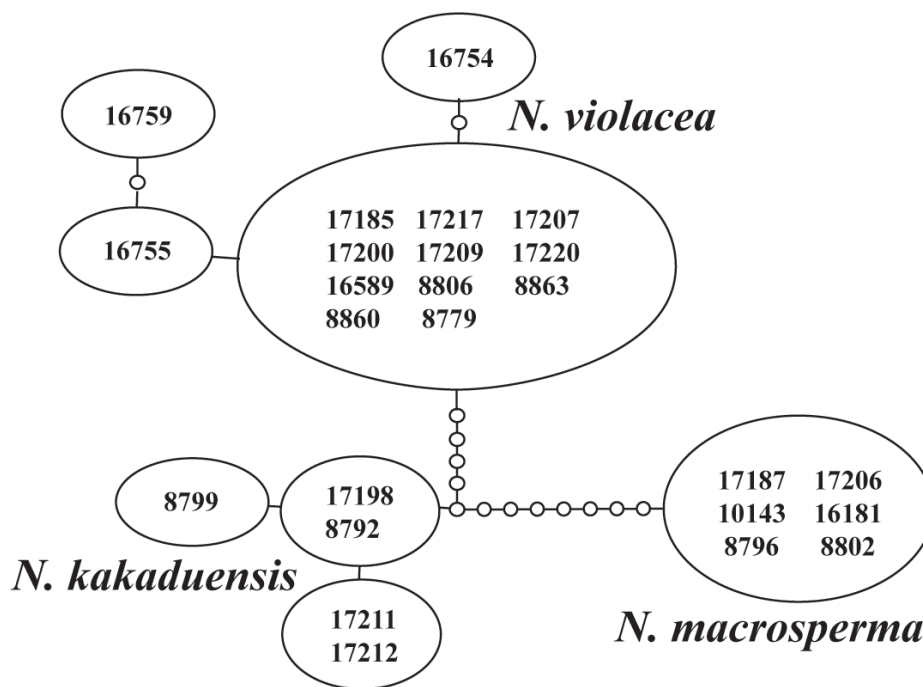
**Table 1. Specimens used for network analysis. Included are the herbarium voucher, coordinates, location description and GenBank accession numbers.**

Taxon	Voucher	Location & Coordinates	GenBank #
<i>N. kakaduensis</i>	Jacobs & Hellq. 8792, DNA	Lake Jabiru, Jabiru, Kakadu N.P., N.T. 12°40.429'S, 137°09.546'E	MW427905
<i>N. kakaduensis</i>	Jacobs & Hellq. 8799, B, BRIT, PERTH	Jim Billabong, Kakadu N.P., N.T. 12°56.538'S, 132°33.076'E	MW427903
<i>N. kakaduensis</i>	Hellq., Leu, F. Baird & D. Miller 17198, CNS, DNA, GH, NSW	Billabong, Yellow Waters, Kakadu N.P., N.T., 12°54.523'S, 132°31.629'E	MW427904
<i>N. kakaduensis</i>	Hellq., Leu & F. Baird 17211, CNS, BRIT, DNA, GH, NSW	Billabong, upper end of Jim Billabong Creek, Kakadu, N.P., N.T., 12°57.676'S, 132°34.516'E	MW427902
<i>N. kakaduensis</i>	Hellq., Leu & F. Baird 17212, BRI, DNA, NSW	Billabong, upper end of Jim Billabong Creek, Kakadu, N.P., N.T., 12°57.676'S, 132°34.516'E	MW427901
<i>N. macrosperma</i>	Jacobs & Hellq. 8796, B, DNA, GH, K, NSW	Four Mile Hole, Wildman River, Kakadu N.P., N.T., 12°54'S, 132°31'E	MZ069075
<i>N. macrosperma</i>	Jacobs & Hellq. 8802, CNS, DNA	Top Swamo, Yellow Waters, Kakadu N.P., N.T., 12°54'S, 132°31'E	MZ069076
<i>N. macrosperma</i>	K. Wilson 10143, Jacobs & Hellq.	Parry's Lagoon N.R., SE. of Wyndham, W.A., 15°32.969S, 128°15.592	MZ069077
<i>N. macrosperma</i>	Hellq., Wiersema & Lahriing 16181, DNA, NSW	Island Billabong, Kakadu N.P., N.T., 12°33'31" S, 132°52'30"	MZ069078
<i>N. macrosperma</i>	Hellq. & Leu 17187, CNS, DNA	Elsy River crossing of Roper R., N.T., 15°00'42" S, 133°14'22" E	MZ069074
<i>N. macrosperma</i>	Hellq., 17206, Leu, F. Baird & D. Miller, CNS, DNA	Yellow Waters, Kakadu N.P., N.T., 12°53'59" S, 132°30'45" E	MZ069073
<i>N. violacea</i>	Jacobs & Hellq. 8779, B, CNS, DNA, NSW	Kangaroo Creek, Borroloola Rd., N.T., 16°49.841'S, 137°09.546'E	MW427899
<i>N. violacea</i>	Jacobs & Hellq. 8806, NSW	Fogg Dam, Middle Point, N.T., 12°33.631'S, 131°17.989'E	MW427898
<i>N. violacea</i>	Jacobs & Hellq. 8860, B, DNA, NSW, PERTH	Hidden Valley, Kununurra, W.A., 15°46.334'S, 128°45.051'E	MW427895
<i>N. violacea</i>	Jacobs & Hellq. 8863, B, DNA, GH, NSW	Little Roper River, Mataranka, N.T., 15°46.334'S, 128°45.051'E	MW427900
<i>N. violacea</i>	Hellq. & Leu 16589, CNS	Lagoon N. of Mt. Molloy cemetery, Qld., 16°41.12.4'S, 145°19.34'E	MW427892
<i>N. violacea</i>	Hellq. & Leu 16755, GH	Stockpond 8 km S. of Coen, Qld., 14°00.288'S, 143°11.436'E	MW427894
<i>N. violacea</i>	Hellq. & Leu 16754, DNA	Palmer River Roadhouse lagoon, Qld., 16°06.443'S, 144°46.630'E	MW427893
<i>N. violacea</i>	Hellq. & Leu 16759, CNS	Langi Lagoon, Mungkan Kaanju N.P., Qld., 13°27.052'S, 142°42.49'E	MW427897
<i>N. violacea</i>	Hellq. & Leu 17185, CNS, DNA	Mataranka, swamp, N.T., 14°55.091'S, 138°04.041'E	MW427886
<i>N. violacea</i>	Hellq., Leu, F. Baird & D. Miller, 17200, CNS, DNA	Flooded roadside, Cooina, Kakadu N.P., N.T., 12°53.817'S, 132°31.125'E	MW427889
<i>N. violacea</i>	Hellq., Leu, F. Baird & D. Miller 17207, CNS, DNA	Yellow Waters, Kakadu N.P., N.T., 12°53.988'S, 132°30.758'E	MW427888
<i>N. violacea</i>	Hellq., Leu, F. Baird & D. Miller 17209, CNS, DNA, NSW	Yellow Waters, Kakadu N.P., N.T., 12°53.707'S, 132°30.317'E	MW427891
<i>N. violacea</i>	Hellq. & Leu 17217, BRI	Toomba Station, south of Lolworth Creek, Qld., 19°59.297'S, 145°33.858'E	MW427887
<i>N. violacea</i>	Hellq. & Leu 17220, CNS	Toomba Station; flooded grassland, Qld., 18°58.898'S, 145°35.197'E	MW427890

Sequence chromatograms were assembled and edited in Sequencher 4.2 (GeneCodes Corp.). The *trnL* (UAA) – *trnF* (GAA) region was then manually aligned using MacClade 4.08 (Madison and Madison 2000) and indels were determined following Moody and Les (2010). The indels were expressed as binary characters (T/A) at the end of the sequence following Moody *et al.* (2016) to be used for haplotype network analyses. Haplotype networks were constructed from the cpDNA region sequences using statistical parsimony as implemented in TCS 1.21 (Clement *et al.* 2000), with a 95% connection limit and with gaps treated as missing data.

### Molecular Results

The final cpDNA *trnL* (UAA) – *trnF* (GAA) region alignment for the haplotype network included 25 accessions and had a length of 902 base pairs. There were 8 haplotypes identified as a result of the network analysis (Fig. 3). All *N. kakaduensis* accessions were found to belong to three haplotypes that varied by 1 or 2 single nucleotide polymorphisms (SNPs) and were divergent from all *N. violacea* accessions by at least 4 SNPs and 2 indels (23 bp and 2 bp). *Nymphaea kakaduensis* accessions were divergent from *N. macrosperma* accessions by 5 SNPs and 3 indels (27 bp, 9 bp and 7 bp). Most *N. violacea* sampled shared a common haplotype, but there was some variation based on SNPs.



**Fig. 3.** Haplotype network based on cpDNA *trnL* (UAA) – *trnF* (GAA) using statistical parsimony for *Nymphaea kakaduensis*, *N. macrosperma* and *N. violacea* accessions from Northern Australia. Ovals represent haplotypes, and size is proportional to number of accessions sharing a given haplotype. Lines between small circles indicate mutational steps between haplotypes (SNPs or Indels). Each number represents accessions presented in Table 1.

### Discussion

Floral characteristics were consistent among sampled population of *N. kakaduensis*. Flowers of *N. kakaduensis* had most petals with a blunt apex and ovate to elliptic shape; whereas, *N. violacea* has oblanceolate petals with a commonly acute apex. Sepals are also generally shorter in *N. kakaduensis* (up to 6 cm) compared to *N. violacea* (up to 11.5 cm). Seed size has been useful to distinguish Australian *Nymphaea* (Jacobs and Porter 2007; Jacobs and Hellquist 2011). There was consistent differences in seed size between *N. kakaduensis* (on average 2.0 × 1.3 mm from five populations) and *N. violacea* (on average 1.5 × 0.9 mm from 17 “Top End” populations). Seed size measured from 25 Queensland populations of *N. violacea* were similar to those from the “Top End” of the Northern Territory at an average of 1.5 × 1.0 mm. The seeds of *N. kakaduensis* also have a latitudinal ridge that is lacking in *N. violacea*.

Given the floral variation and seed differences between *N. kakaduensis* and *N. violacea*, molecular data provides further evidence for the new species designation. The cpDNA haplotype network indicated that samples with the aforementioned morphological variation from four populations in the Kakadu region were genetically distinct from *N. violacea* and *N. macrosperma* (Fig. 3), which are commonly found in the same waterbodies as *N. kakaduensis*. The *trnL* (UAA) – *trnF* (GAA) intragenic spacer has been considered a useful species level DNA barcode (Hollingsworth *et al.* 2011) and has been used for this purpose in other groups for species identification (e.g. Bączkiewicz *et al.* 2017; Liu *et al.* 2012). In this case, the molecular data support the morphological evidence for *N. kakaduensis*.



Many smaller billabongs and lagoons within Kakadu National Park have populations with apparent *N. kakaduensis* characteristics, including Island Billabong, Patonga Billabong, and Coonjimba Billabong (within the former Ranger Mineral leases) that were not sampled for molecular analyses. Additional occurrences of *Nymphaea* populations outside of Kakadu National Park from the “Top End” including Girraween Lagoon at Howard Springs, Manton Dam, and the wetlands at Humpty Doo deserve further analysis as possible occurrences of *N. kakaduensis*. A much broader survey of the northern wetlands of the Northern Territory along with the generation of molecular data for all taxa present at each location will provide a better understanding of geographical extent of *N. kakaduensis* and other species.

### Taxonomic Treatment

*Nymphaea kakaduensis* C.B.Hellq., A.Leu & M.L.Moody, *sp. nov.*

*Type:* Australia: Northern Territory: Kakadu National Park, billabong at upper end of Jim Creek, SE of Rt. 21, west of Patonga Community, 12°57.676'S, 132°34.516'E, 29 April 2011, C.B. Hellquist 17211, A. Leu, and F. Baird (holotype: DNA; isotypes: BRIT, CNS, GH, NSW).

Perennial with a globose rhizome. Leaf blade oval–oval-elliptic, dark green above, dark purple below, 15.6–17.4 cm long, 11.8–16.6 cm wide; margins entire to sinuate-crenate to slightly undulate; petiole, green–brownish green, some streaked; stipules fused for c. 2 cm. Flower raised above the surface, fragrant. Sepals 3.0–6.0(–8.0) cm long, 1.0–2.6 cm wide, white, pink/brown or green above mostly flecked or streaked, apex acute-blunt or blunt. Petals usually white, occasionally blue, becoming pinkish or blue at apex. Petals 14–27(–36), 2–5 cm long, 0.6–2.0 cm wide, ovate–elliptic, some narrowing at base; apex blunt, obtuse, rounded, occasionally blunt-acute, flower shape is generally cupped, 5.5–10.5 cm wide (avg. 8.3 cm). No gap between petals and stamens. Stamens yellow, 80–200; anther length 3–10 mm; filament length 2–24 mm; carpels 14–30. Fruit globose, c. 2.0–4.0 cm diam; green at apex, sepals attached, seeds dark brown–dark olive, ovoid, with 1 longitudinal ridge; seed size avg. 2.0 (1.8–2.1) × 1.3 (1.2–1.3) mm, beak lacking or to 1.0 mm. (Figure 4)

**Diagnostic characters:** Differs from *N. violacea* by presence of smaller, mostly white flowers with upturned, blunt ovate–elliptic petals. Sepals and petals are often faintly pink-tipped and buds are blunt at the apex. Seeds are larger on average, 2.0 × 1.3 mm. The adaxial leaf surface is a darker green than in *N. violacea*.

**Distribution:** Currently only known from Kakadu National Park, in Jim Jim Creek and Billabong, Home Billabong, and Lake Jabiru. Probably in Island Billabong, Patonga Billabong, and Coonjimba Billabong, and the species may be more widespread in the Top End.

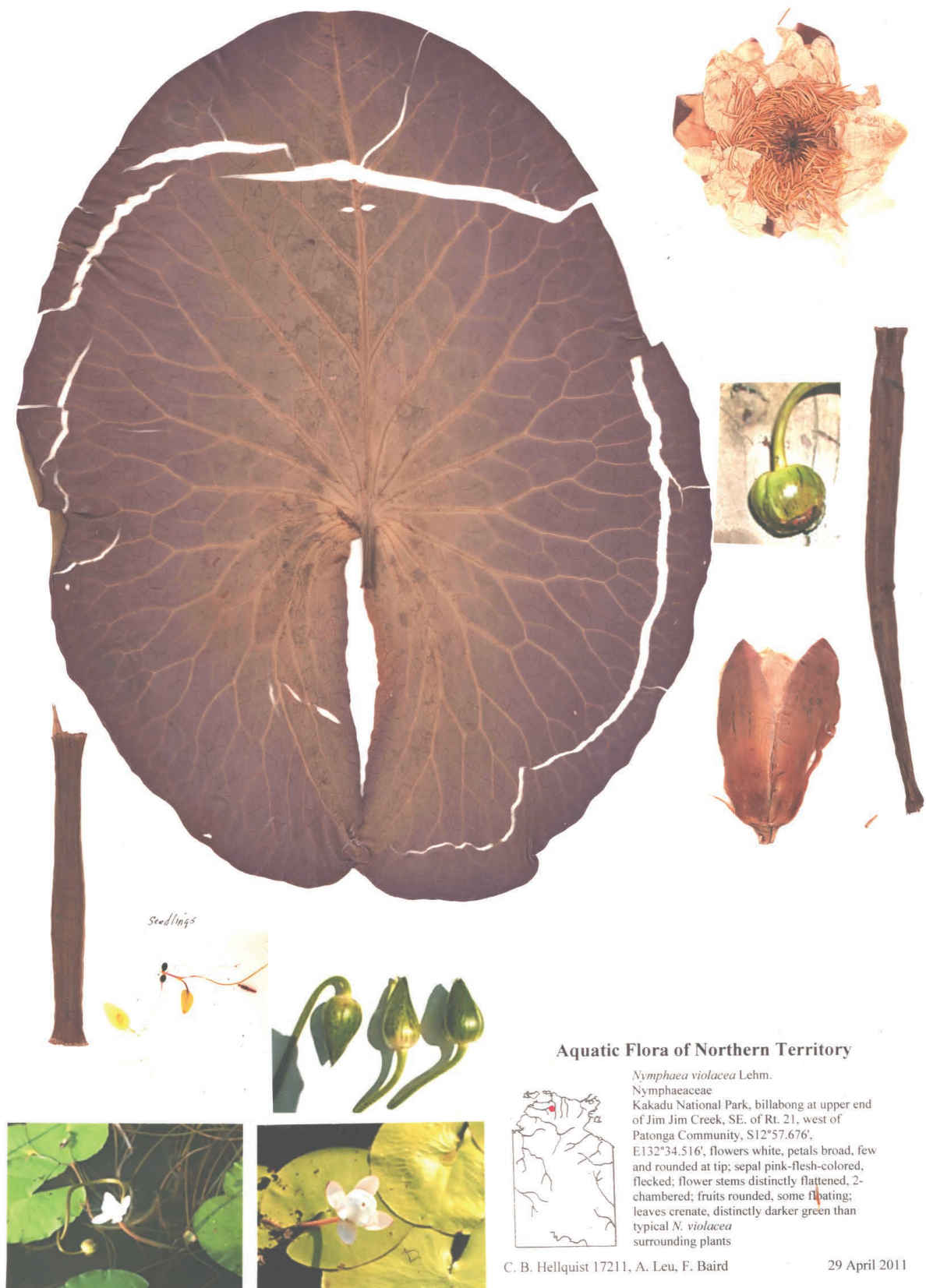
**Ecology:** The species grows in usually permanent billabongs. Recorded in association with *Nymphaea macrosperma*, *N. pubescens* and *N. violacea*.

**Phenology:** Flowering and fruiting recorded for April to June.

**Specimens examined:** NORTHERN TERRITORY: Kakadu National Park, billabong southwest of Home Billabong near Yellow Waters shore, 12°33.6918'S, 131°18.0747'E, 28 April 2011. C.B. Hellquist 17198, A. Leu, F. Baird & D. Miller (BRIT, CAS, DNA, GH); Kakadu National Park, Jim Jim Billabong, 12°56'32"S, 132° 73.4743', 28 April 2011, C.B. Hellquist 17202 & A. Leu, F. Baird & D. Miller (CNS, DNA); Kakadu National Park, billabong at upper end of Jim Jim Creek, SE of Rt. 21, west of Patonga Community, 12°57.676'S, 132°34.516'E, 29 April 2011, C.B. Hellquist 17212, A. Leu & F. Baird (DNA, NSW); Kakadu National Park, Lake Jabiru, Jabiru, 12°40'26"S, 132°50'32"E, 15 June 2002, S.W.L. Jacobs 8792 & C.B. Hellquist (CNS, DNA, NSW); Kakadu National Park, Jim Jim Billabong, 12°56'32"S, 132°33'05"E, 17 June 2002, S.W.L. Jacobs 8799 & C.B. Hellquist (B, BRIT, NSW, PERTH); Kakadu National Park, Jim Jim Billabong, 12°56'32"S, 132°33'05", 17 June 2002, S.W.L. Jacobs 8800 & C.B. Hellquist (CNS, DNA, NSW).

**Conservation status:** Locally restricted on current knowledge, but protected within Kakadu National Park and not under immediate threat.

**Etymology:** The epithet refers to Kakadu National Park where the species occurs.



**Fig. 4.** Holotype: Australia: Northern Territory: Kakadu National Park, billabong at upper end of Jim Jim Creek, SE of Rt. 21, west of Patonga Community, 12°57.676'S, 132°34.516'E, 29 April 2011, C.B. Hellquist 17211, A. Leu, and F. Baird, (holotype: DNA)

### Key to *Nymphaea* of Kakadu National Park and “Top End” coastal floodplains

1. Plants with distinct gap between petals and sepals ..... 2
- 1: Plants with petals grading into stamens ..... 3
2. Seeds 3–4.5 mm long, 2–3 mm wide; leaf margin toothed; flowers blue ..... *N. macrosperma*
- 2: Seeds c. 0.75 mm diam; leaf margin entire to slightly sinuate; flowers white ..... *N. nouchali*
3. Leaf margin dentate; leaf underside pubescent; flowers bloom at night until noon ..... *N. pubescens*
- 3: Leaf margin entire or sinuate; leaf undersurface glabrous; flowers bloom during day ..... 4
4. Seeds c. 1 mm diam., stamens 50–100; plants of seasonal shallow waters during wet season ..... *N. hastifolia*
- 4: Seeds greater than 1 mm diam.; stamens 80–200; plants mostly of permanent waters ..... 5
5. Flower petals oblanceolate with usually acute apex; flower buds acute at apex; seeds averaging 1.5 (1.4–2.0) × 1.0 (0.9–1.6) mm ..... *N. violacea*
- 5: Flower petals ovate–elliptic with usually blunt apex; flower buds blunt at apex; seeds averaging 2.0 (1.8–2.1) × 1.3 (1.2–1.3) mm ..... *N. kakaduensis*<sup>1</sup>

<sup>1</sup>All measurements obtained from specimens collected by S.W.L. Jacobs and/or C.B. Hellquist.

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