# *NYMPHOIDES HUMBOLDTIANA* (MENYANTHACEAE) IN FLORIDA (U.S.A.) VERIFIED BY DNA DATA[1]

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ABSTRACT

Certain *Nymphoides* populations in Florida, U.S.A., previously identified as the non-native *N. indica* (L.) Kuntze, are actually *N. humboldtiana* (Kunth) Kuntze, as verified using nuclear and plastid DNA data. These new records of *N. humboldtiana* in Florida are the only known localities in the U.S.A. outside of Uvalde County, Texas. *Nymphoides humboldtiana* is native to Texas, México, the Caribbean, and Central and South America. The newly identified Florida populations found near the city of Tampa in Manatee County (Ward Lake and Braden River) and Fort Walton Beach in Okaloosa County (Bass Lake) are presumed to be native. *Nymphoides humboldtiana* and *N. indica* share many superficial similarities, most notably white petals with ciliate hairs. Our findings suggest that certain morphologically-determined populations of *N. indica* in the U.S.A. may be incorrectly identified. Such populations might be re-examined using DNA methods to avoid any management actions against *N. humboldtiana*.

RESUMEN

Las poblaciones de algunas *Nymphoides* en Florida, U.S.A., previamente identificadas como la no nativa *N. indica* (L.) Kuntze, son actualmente *N. humboldtiana* (Kunth) Kuntze, tal como se ha verificado usando datos del ADN nuclear y plastidial. Estas nuevas citas de *N. humboldtiana* en Florida son las únicas localidades conocidas en U.S.A. Fuera del condado de Uvalde, Texas. *Nymphoides humboldtiana* es nativa de Texas, México, el Caribe, y América Central y del Sur. Las poblaciones identificadas de Florida encontradas cerca de la ciudad de Tampa en el condado de Manatee (lago Ward y río Braden) y Fort Walton Beach en Okaloosa County (lago Bass) se asume que son nativas. *Nymphoides humboldtiana* y *N. indica* comparten muchas similitudes superficiales, la más notable pétalos blancos con pelos ciliados. Nuestros hallazgos sugieren que ciertas poblaciones determinadas morfológicamente de *N. indica* en U.S.A. pueden estar identificadas incorrectamente. Tales poblaciones deberían ser re-examinadas usando métodos de ADN para evitar cualesquiera acciones de manejo contra *N. humboldtiana*.

Key Words: floating hearts, invasive species, nuclear and chloroplast DNA, taxonomic classification, range extension, rare and endangered species

## INTRODUCTION

*Nymphoides humboldtiana* (Kunth) Kuntze is an aquatic floating-leaved herb native to the Americas, which is nearly identical morphologically to the paleotropical *N. indica* (L.) Kuntze (Ornduff 1969). *Nymphoides* species have many morphological similarities, including floating leaves that support small clusters of insect-pollinated flowers (Tippery & Les 2011). There are four species native to the U.S.A., including *N. aquatica* (J.F. Gmel.) Kuntze, *N. cordata* (Elliott) Fernald, *N. grayana* (Griseb.) Kuntze, and *N. humboldtiana* (Tippery et al. 2011, 2015), as well as three exotic species: *N. cristata* (Roxb.) Kuntze, *N. peltata* (S.G. Gmel.) Kuntze, and *N. indica* (Burks 2002; Tippery et al. 2015). *Nymphoides humboldtiana* and *N. indica* are difficult to distinguish because they both have flowers in umbellate clusters and white petals that are densely covered with ciliate hairs (Ornduff 1969; Tippery et al. 2011). Owing to their morphological similarity, Ornduff (1969)

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synonymized the tetraploid *N. humboldtiana* with the diploid *N. indica*, and subsequent authors and collectors used the latter name for their descriptions and collections. More recently, however, molecular evidence has supported the retention of *N. humboldtiana* as a separate species (Tippery & Les 2011) and elucidated its allopolyploid evolutionary origin (Tippery et al. 2018).

Whereas *N. humboldtiana* is native to Central and South America (Tippery et al. 2015), *N. indica* is native to tropical latitudes in Africa, Asia, and Australia (eFloras 2017), where it grows in temporary monsoonal wetlands and slow-flowing rivers (Middleton 1998). *Nymphoides indica* is known to occur in Florida, particularly as a horticultural species in water gardens (Tippery et al. 2015). Although not officially listed on federal or state noxious weed lists, *N. indica* is actively targeted for control and removal (Willey & Langeland 2011). Prior to its discovery in Uvalde County, Texas, *N. humboldtiana* was unknown in the U.S.A. (Saunders 2005; Tippery et al. 2015). *Nymphoides humboldtiana* and *N. indica* can be distinguished from the morphologically similar *N. grayana*, also native to Central and South America, because the latter has entirely yellow petals with ciliate hairs (Ornduff 1969; Jacono 2002). In the U.S.A., *N. grayana* is presumed to be native but grows only in Florida (Tippery et al. 2015). Two other native species, *N. aquatica* and *N. cordata*, are dioecious with predominantly glabrous white petals, and their largely overlapping ranges extend from the Gulf Coast north into Canada (Tippery & Les 2013). The introduced Eurasian species *N. peltata*, with glabrous yellow petals, has a scattered distribution throughout North America (including Florida), where it likely has escaped from cultivation multiple times (Stuckey 1973; Pfingsten et al. 2017). *Nymphoides cristata*, native to tropical Asia, has mostly glabrous white petals, each with a distinctive median wing; this species occurs in Florida waterways as an introduced aquatic weed (Burks 2002; Jacono 2002; Tippery et al. 2015), and more recently it has spread into neighboring states (Thayer & Pfingsten 2017a).

Owing to the rather recent arrival of non-native species and the recent verification of *N. grayana*, many *Nymphoides* species in Florida are unfamiliar to botanists and land managers. Moreover, many diagnostic floral features preserve poorly and are difficult to observe on dried herbarium specimens. However, molecular identification tools are becoming increasingly useful for identifying species and distinguishing ecologically and evolutionarily distinct entities. Only recently, molecular evidence verified the existence of *N. grayana* in Florida, where it likely arrived via natural dispersal (Tippery et al. 2015). The purpose of the present work is to determine whether or not certain Florida populations are genetically identified as either *N. humboldtiana* or *N. indica* and to ascertain if these populations are native or exotic, respectively. These species determinations are important and ultimately affect invasive species management of *Nymphoides* in Florida (Willey & Langeland 2011).

## MATERIALS AND METHODS

Plant material was collected from three populations in Florida, including one accessed from a low bridge across Bass Lake near Fort Walton Beach (Okaloosa County: 30°27'12.384"N, 86°36'51.0834"W) and two others sampled by boat near Tampa (Manatee County): Ward Lake (27°25'53.45"N, 82°28'56.74"W) and Braden River (27°25'36.58"N, 82°28'41.13"W). Additional material was obtained from two herbarium specimens: a prior collection from Bass Lake (*Butera s.n.*, 09 Aug 2016, USF291968) and a yellow-petaled *Nymphoides* from Bonita Springs, Lee County, Florida (*Shagall s.n.*, 9 May 2002, USF231612). Seeds were obtained from plants growing at the Ward Lake locality and imaged using a Leica M80 dissecting microscope, with focus-stacking via the Leica Application Suite ver. 4.3.0 (Leica Biosystems Inc , Buffalo Grove, Illinois).

Genomic DNA was extracted and sequenced for one individual from each of the three populations (i.e., Bass Lake, Ward Lake, and Braden River) and from herbarium specimens, for one nuclear (internal transcribed spacer, ITS) and one plastid (*trnK* 5' intron) region, following Tippery and Les (2011). DNA regions were amplified and sequenced using the ITS primers designed by Blattner (1999) and the *trnK* 5' intron primers DL19 and 1011R (Tippery et al. 2008). Newly obtained sequences were aligned to those previously deposited in GenBank and compared using the program Mesquite ver. 3.31 (Maddison & Maddison 2017).

## RESULTS

We examined populations of *Nymphoides* from Florida to determine if these were *N. indica* or *N. humboldtiana* based on DNA evidence from both the nuclear and plastid gene regions (Tippery & Les 2011). Morphologically, both species have flowers with a yellow corolla throat and white petals with a ciliate upper surface (Fig. 1). Seeds of the Ward Lake plants matured to a black color (Fig. 2), with seeds being shades of tan at earlier stages of maturity. Individual seeds were slightly compressed and nearly orbicular in outline, approximately 0.81–0.84 × 0.90–0.91 mm. The seed surface was smooth to rugulose, with no tuberculate projections, and the indistinct hilum occupied a modest depression in the seed outline.

With respect to both nuclear and plastid DNA data, the plants collected from three Florida populations were identical or nearly identical to previous collections of *N. humboldtiana*. The two Manatee County collections (Ward Lake and Braden River) were genetically identical to each other and to plants from Brazil (ITS: JF926326 / *trnK*: JF926410), Ecuador (JF926376 / KR080307), México (JF926327 / JF926412), and Texas (JF930150 / JF930151). The Okaloosa County collection (Bass Lake) had an identical ITS sequence (not including ambiguous sites) to a *N. humboldtiana* plant previously collected from Brazil (JF926325). The *trnK* sequence of this specimen, however, matched sequences that were obtained from *N. grayana* (KR080306). (It should be noted that the *trnK* region is poorly diagnostic for this group of species, with previously reported sequences showing only one nucleotide difference between *N. grayana* and *N. humboldtiana* accessions.) The herbarium specimen collected from Bass Lake in 2016 produced an identical ITS sequence to our 2017 specimen from that lake, and the Lee County specimen with yellow petals had an ITS sequence identical to *N. grayana* plants collected in Florida (KR080304) and Cuba (KR080301 / KR080302).

## DISCUSSION

Molecular tools for distinguishing closely related species can aid managers in decisions related to invasive species control, particularly to differentiate between invasive species introductions versus range expansions of native species. Our finding that both *N. humboldtiana* and *N. indica* exist in Florida suggests that these populations should be evaluated using DNA techniques before plant control procedures are undertaken.

Although *N. humboldtiana* and *N. indica* share many similarities, it appears that seed characteristics may be useful for telling them apart. Seeds of Florida *N. humboldtiana* plants lack tubercles and are nearly identical in size and surface ornamentation to seeds of other conspecific collections (Fig. 2; Chuang & Ornduff 1992). Seed descriptions for the wide-ranging and genetically variable *N. indica*, by contrast, nearly always include mention of surface tubercles (Raynal 1974; Sivarajan et al. 1989; Aston 2003; Tippery & Les 2011). The Florida *N. indica* plants that were verified previously using DNA data (Tippery et al. 2015) were genetically identical to plants from India, making that region a likely candidate for the source of cultivated plants. Seeds of *N. indica* in India are distinctly tuberculate, making the surface ornamentation a potentially useful character for distinguishing *N. humboldtiana* from *N. indica* in Florida. Even the presence of seeds on Florida *N. humboldtiana* plants may be informative because this species (as well as many other heterostylous *Nymphoides* species) possesses a self-incompatibility system that should preclude seed production in genetically uniform populations (Barrett 1980). Several *Nymphoides* species including *N. indica* are capable of clonal vegetative reproduction, which would enable a small number of introduced plants to overtake a water body and have a severely reduced ability to produce seeds due to their self-incompatibility (Shibayama & Kadono 2007; Wang et al. 2005). *Nymphoides humboldtiana* populations that produce abundant seeds thus presumably include both floral morphs of the heterostyly syndrome, or their self-incompatibility has broken down (Ganders 1979); further study will be required to elucidate the floral morph diversity and the extent to which self-compatibility functions in Florida *N. humboldtiana* populations.

As to the question of how *N. humboldtiana* may have arrived in Florida, useful clues are given by its current distribution, seed and propagule dispersal mechanisms, and dispersal vectors. *Nymphoides humboldtiana* grows in North and South America, from Uruguay north to southern Tamaulipas in México, the Caribbean (Tropicos.org 2011), and Uvalde County, Texas (Tippery et al. 2011). The Texas population is about 1350 km

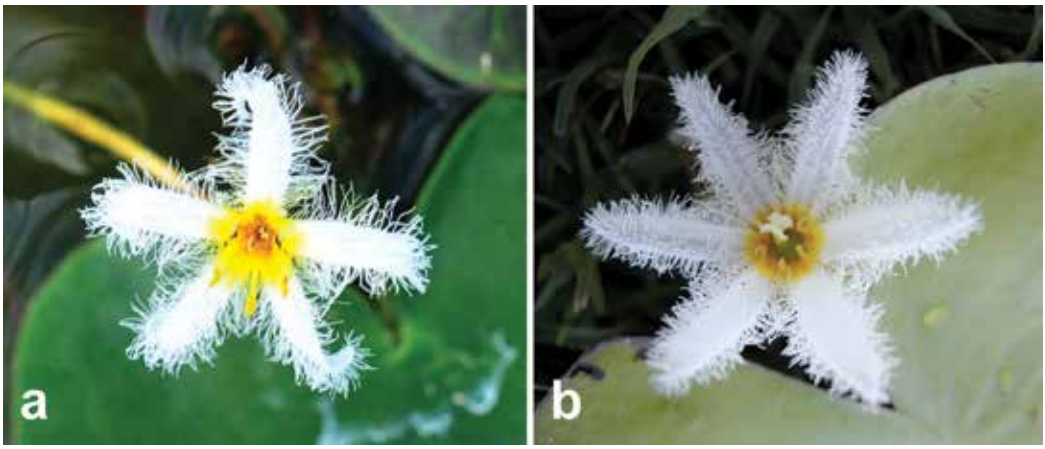


Fig. 1. (**a**) *Nymphoides humboldtiana* collected in Bass Lake, Florida (photo by Larry Allain, U.S. Geological Survey), and (**b**) *N. indica* Lily Creek Lagoon in Kununurra, Western Australia (photo by Nicholas P. Tippery, UW Whitewater). Note that both species have ciliate white petals and a yellow corolla throat.


Fig. 2. Seeds of *Nymphoides humboldtiana* collected (**a**) in Ward Lake, Manatee County, Florida (this study), and (**b**) near Paramaribo, Suriname (*Kramer & Hekking 2544*, UC1328696), alongside seed of *N. indica* (**c**) collected in Indonesia (*Vogel 5964*, L.2700818). Scale bar = 250 µm.

west of the nearest known population in Florida (Bass Lake), but Caribbean populations of *N. humboldtiana* are nearer (Ornduff 1969). *Nymphoides* species variously disperse as seeds, seedlings, or rhizome fragments via water and animals (Middleton 1999). Waterfowl flyways may provide a conduit for seed dispersal between the Caribbean and Central and South American populations to Florida (Figuerola & Green 2002). A similar route of introduction has been suggested for *N. grayana*, which has also been recently identified in Florida (Tippery et al. 2015).

We were able to confirm the identity of a previous collection from Bass Lake (*Butera s.n.*, 09 Aug 2016, USF291968) using molecular methods, and this evidence verifies that the *N. humboldtiana* population there has existed for at least two years. The Braden River population, with a much earlier collection record (*Shuey 2585*, 02 Dec 1983, FLAS167731 / FSU000064662 / USF176793), is provisionally identified here as *N. humboldtiana*. If verified, this collection would represent the earliest documented occurrence of *N. humboldtiana* in Florida. Besides the populations that were verified in this study using molecular data, several other localities have been reported for *N. indica* in Florida. A specimen of *N. indica* that was confirmed previously using DNA (Tippery et al. 2015) was found growing in cultivation near Lake Livingston, Avon Park, Polk County, Florida (*Watts s.n.*, CONN00111881). Other reported *N. indica* localities in Florida, which merit further investigation

using molecular methods, include Highland Oaks Park, Miami, Dade County (Thayer & Pfingsten 2017b); Mary Holland Park, Bartow, Polk County (Center for Invasive Species and Ecosystem Health 2015); and Lake Fairview and Lake Orlando, Orlando, Orange County (Thayer & Pfingsten 2017b). Two specimens that were collected from Cypress Lake, Kissimmee, Osceola County, either directly (*Harris s.n.*, 20 Apr 1996, FSU000064664) or from later cultivation in Orange County (*Burks 1168*, 11 Nov 1999, FSU000064663), describe flowers with yellow corollas and thus likely represent *N. grayana* (Tippery et al. 2015). The identity of one additional *N. grayana* population in Bonita Springs, Lee County, Florida (*Shagall s.n.*, 9 May 2002, USF231612), collected from a locality similar to material that was examined previously (*Watts s.n.*, 19 Nov 2010, CONN00111882; Tippery et al. 2015), was confirmed during this study.

There are no *N. humboldtiana* herbarium specimens collected from Florida prior to 1983, so presumably the species arrived fairly recently via natural or anthropogenic means. The verified distribution of *N. humboldtiana* in Florida (in Manatee County and Okaloosa County) comprises two water bodies separated by a large distance (approximately 500 km), and the source of introduction to these counties could have been from nearby or more distant populations. The rather extreme genetic distance between the two water bodies argues for two independent introductions: the ITS genetic distance between plants from these two counties is equal to the maximum recorded distance between any two *N. humboldtiana* collections to date, and the *trnK* genetic distance equals that found among three closely related *Nymphoides* species (*N. fallax*, *N. grayana*, and *N. humboldtiana*). The two Manatee County localities, which were collected from contiguous water bodies, likely represent descendants from a single introduction event.

Clearly, *N. humboldtiana* might be considered a native component of the aquatic flora of Florida, while *N. indica* cannot. Because *N. humboldtiana* easily may be confused with *N. indica*, there is a question whether either species should be controlled until it can be ascertained how aggressive *N. indica* may be in Florida. At least in India, *N. indica* dies quickly after cutting underwater, in contrast to *N. cristata*, which regrows quickly after cutting (Middleton 1990; Middleton et al. 2015). Especially because aquatic plant control methods might harm *N. humboldtiana*, a useful precaution would be to genetically analyze populations bearing a morphological resemblance to either *N. indica* or *N. humboldtiana* before any further management procedures are undertaken.

Voucher specimens (GenBank accession numbers for ITS / *trnK* 5' intron follow the herbarium code for each specimen; —: sequence not obtained): **NYMPHOIDES GRAYANA—U.S.A. Florida. Lee Co.:** Bonita Springs, 9 May 2002, *Shagall s.n.* (USF; MG845505/—). **NYMPHOIDES HUMBOLDTIANA—U.S.A. Florida. Manatee Co.:** Ward Lake, Bradenton, 27°25'53.45"N, 82°28'56.74"W, 26 Oct 2017, *Sowinski s.n.* (USF; MG845508 / MG845511); Braden River, Bradenton, 27°25'36.58"N, 82°28'41.13"W, 26 Oct 2017, *Sowinski s.n.* (USF; MG84550/ MG845512). **Okaloosa Co.:** Bass Lake, Fort Walton Beach, 30°27'12.384"N, 86°36'51.0834"W, 09 Aug 2016, *Butera s.n.* (USF; MG845507 —), 17 Mar 2017, *Anemaet s.n.* (USF; MG845506/MG845510).

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