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Delimiting invasive *Myriophyllum aquaticum* in Kashmir Himalaya using a molecular phylogenetic approach

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ABSTRACT. *Myriophyllum aquaticum* (Vell.) Verdc. (family Haloragaceae) is one of the most invasive and destructive South American aquatic plant species and is present in a wide range of geographic regions, including the Kashmir Himalaya. Confusion regarding the taxonomic delimitation of *M. aquaticum* in the Himalayan region impedes effective and targeted management. Hence, our goal was improve the identification of *M. aquaticum* for exclusive delimitation from other related species in the study region using a molecular phylogenetic approach. A maximum parsimony tree recovered from phylogenetic analyses of the internal transcribed spacer sequences of nuclear ribosomal DNA was used to authenticate the identification of *M. aquaticum*. The results of this study can be used for targeted management of this tropical invader into the temperate Kashmir Himalaya.

Key words: *Myriophyllum aquaticum*; Kashmir Himalaya; India; Molecular authentication; Internal transcribed spacer; Invasion management

INTRODUCTION

While the family Haloragaceae has been of special interest to botanists since the beginning of the 19th century, it remains poorly understood (Moody and Les, 2010). Several instances of errors in taxonomic classification in the family Haloragaceae have recently been overcome using data obtained from molecular phylogeny approaches (Moody and Les, 2007; Thum and Lennon, 2010; Thum et al., 2011). *Myriophyllum* L. (family Haloragaceae), commonly known as "water milfoil" is a cosmopolitan genus consisting of aquatic or semi-aquatic herbs. With approximately 68 species, the genus has 3 main centers of distribution, including Australia, India/Indochina, and North America. In the Kashmir Himalayan region, the genus *Myriophyllum* is represented by 4 species. These species include *M. aquaticum* (Vell.) Verdc, *M. spicatum* L., *M. tuberculatum* Roxb., and *M. verticillatum* L. (Arshid et al., 2011). *M. spicatum* is a destructive invasive species present in a wide range of geographical regions (Moody and Les, 2007). *M. aquaticum* (commonly referred to as parrot-feather) is another rapidly spreading congeneric species that has taken over marshy and aquatic habitats at different spatial scales in different temperate and tropical regions.

M. aquaticum has recently emerged as an aggressive invader with alarming impacts on aquatic habitats, particularly in the lakes and wetlands of the Kashmir Himalayan region (Shah and Reshi, 2012, 2014). Taxonomic inaccuracies and identification problems significantly limit targeted control measures and precise management strategies of this aquatic invasive species in the Himalayan valley of Kashmir. Although an attempt has recently been made to identify *M. aquaticum* based on morphological features (Arshid et al., 2011), correctly identifying such species using precise molecular approaches is very important. The internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) has gained much attention during the last two decades not only due to its efficacy in carrying out phylogeny of the plants at lower taxonomic level but also for its reorganization as the most trusted markers available for the DNA barcoding of the plants (Ali et al., 2013). Hence, the goal of the present study was to assess nrDNA ITS sequence-based molecular authentication and identification of *M. aquaticum,* and its exclusive delimitation from related or morphologically similar species.

MATERIAL AND METHODS

During the present study, 5 lakes (Anchar, Dal, Manasabal, Nigeen, and Wular) and 5 wetlands (Haigam, Hokersar, Rakhi-Arath, Shallabugh, and Mirgund) varying in depth, trophic status, and level of disturbance were surveyed for collection of *M. aquaticum*. At least five individuals were collected from each water body surveyed. Total genomic DNA was extracted using the DNeasy Plant Mini Kit from Qiagen. The nrDNA ITS regions were amplified using the primers ITS1 and ITS4 as described by White et al. (1990). Double-stranded polymerase chain reaction (PCR) products were produced via 35 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min, with a 10-min final extension cycle at 72°C. PCR products were purified using a SolGent PCR Purification kit-Ultra (SolGent, Daejeon, South Korea) and sequenced using the same primers using 2 µL BigDye, 1 µL primer (20 pM), and template DNA and purified water to reach a 10-µL reaction volume. Cycle sequencing used 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing products were visualized on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences were edited using the ABI Sequence Navigator (Applied Biosystems). Each

sequence generated was searched using the Basic Local Alignment Search Tool (BLAST) to identify sequence similarity to other Haloragaceae sequences available in GenBank. The sequences generated from the samples, including MAS1, MAS5, MAS6, and MAS9 (GenBank accession Nos. KC012919-KC012922) as well as MAS2, MAS3, MAS4, and MAS8 (GenBank accession Nos. KC012915-KC012918) showed similarity to *Ceratophyllum demersum* L. and *Myriophyllum* species, respectively; thus, only the second set of sequences were included in the data matrix for phylogenetic analyses. Sequences of *Myriophyllum* species available in the GenBank were retrieved (Table 1) and analyzed together with the sequences generated from the target species of the present study. Outgroup sequences were also retrieved from NCBI GenBank and included in the analysis. The sequences of *Laurembergia repens* (L.) P.J. Bergius, *Gonocarpus montanus* (Hook. f.) Orchard, *Haloragis odontocarpa* F. Muell. were selected as the outgroup (Table 1) because of their close phylogenetic relationship with *Myriophyllum* and based on the results of previous studies (Moody and Les, 2010). Sequence alignment of the final data set was performed using CLUSTAL_X version 1.81 (Thompson et al., 1997). Aligned sequences were then subsequently manually adjusted using BioEdit (Hall, 1999). The data matrix as a nexus file were exported to MEGA5 (Tamura et al., 2011) and analyzed using maximum parsimony (MP) methods. Insertion-deletions (indels) were scored as single characters when we had confidence in positional homology. The MP tree was obtained using the close-neighbor-interchange algorithm (Nei and Kumar, 2000) using search level 3 (Felsenstein, 1985; Nei and Kumar, 2000), in which initial trees were obtained using a random addition of sequences (10 replicates).

Table 1. Taxon and GenBank accession numbers included in the molecular phylogenetic analysis.

Taxon	GenBank accession No.
Myriophyllum alpinum Orchard	EF178720
Myriophyllum aquaticum (Vell.) Verdc.	EF526367
Myriophyllum aquaticum (Vell.) Verdc. (MAS2)*	KC012915
Myriophyllum aquaticum (Vell.) Verdc. (MAS3)*	KC012916
Myriophyllum aquaticum (Vell.) Verdc. (MAS4)*	KC012917
Myriophyllum aquaticum (Vell.) Verdc. (MAS8)*	KC012918
Myriophyllum crispatum Orchard	EF178721
Myriophyllum drummondii Benth.	EF178725
Myriophyllum farwellii	EF178731
Myriophyllum heterophyllum Michx.	AF513824
Myriophyllum hippuroides Nutt.	FJ870946
Myriophyllum humile (Raf.) Morong	EF526393
Myriophyllum latifolium F. Muell.	EF178729
Myriophyllum laxum Shuttlew. ex Chapm.	FJ870948
Myriophyllum limnophilum Orchard	FJ870949
Myriophyllum lophatum Orchard	EF178718
Myriophyllum papillosum Orchard	EF178724
Myriophyllum pinnatum (Walter) Britton, Sterns & Poggenb.	FJ870966
Myriophyllum quitense Kunth	FJ870954
Myriophyllum robustum Hook. f.	FJ870955
Myriophyllum sibiricum Kom.	FJ426352
Myriophyllum simulans Orchard	EF178719
Myriophyllum spicatum L.	EF526362
Myriophyllum tenellum Bigelow	EF526410
Myriophyllum ussuriense Maxim.	EF178726
Myriophyllum variifolium Hook.f.	FJ870959
Myriophyllum sp (Aq-red1)†	JF717420
Myriophyllum sp (red1)°	FJ870962
Gonocarpus montanus (Hook. f.) Orchard	EF178770
Haloragis odontocarpa F. Muell.	EF178747
Laurembergia repens (L.) P.J. Bergius	EF178735

*Sequence generated in the present study, and submitted to GenBank; †Thum et al. (2012); °Moody and Les (2010). Abbreviations MAS2, MAS3, MAS4, and MAS8 next to the name of the taxon represents the accessions of *Myriophyllum aquaticum* collected from aquatic habitats of Kashmir Himalaya.

RESULTS AND DISCUSSION

Field surveys of all investigated lakes and wetlands in the Kashmir Himalaya revealed a high incidence of *M. aquaticum* with a distinct colonization preference along the littoral regions and more shallow waters of invaded habitats. In nearly all surveyed water bodies, *M. aquaticum* exhibited formation of green mat-like homogenous patches with a fast-spreading tendency and development of inroads from littoral marshy regions to more open water areas (Figure 1).

Figure 1. A view of invasion by *Myriophyllum aquaticum* from Shallabugh wetland of Kashmir Himalaya.

Morphological investigation showed bright green and slender stems, stem leafy throughout and bearing simple, oblong, feathery, and whorled leaves. Both submersed and emergent leaves were present, inflorescence was generally emergent, the plants were dioecious and terminal with inconspicuous flowers, and without petals borne in emergent leaf axils.

Parsimony analysis of the entire ITS region resulted in 26 maximally parsimonious trees with a total length of 331 steps, consistency index of 0.639, composite index of 0.598, and retention index of 0.849 (Figure 2). The *Myriophyllum* accessions, which included MAS2, 3, 4, and 8, showed proximity (bootstrap support 99%) to the accessions of *M. aquaticum* from GenBank (FJ870962, EF526367, and F717420). The ITS genotypes of sequenced accessions of *Myriophyllum* were identical to GenBank accession EF526367; however, the sequences differed from FJ870962 by 1-2 nucleotide substitutions according to sequence alignment at various positions, including 39, 104, 112, 144, 147-148, 157, 172, 180, 215, 218, 219, 420, and 553, and 3 small indels of 1-4 base pairs each, including at sequence alignment position 86-90, 141, and 153-155. Of these, nucleotide substitutions at sequence alignment position 39 and 111 were not consistent with those of F717420. These differences in ITS genotypes may be due to polymorphisms, which are frequently observed in this gene (Bailey et al., 2003; Pandey and Ali, 2006; Xiao et al., 2010); however, the short sequence length of JF717420 at

the beginning and towards the end as compared with the other GenBank accessions of *Myriophyllum* is interesting and should be further evaluated.

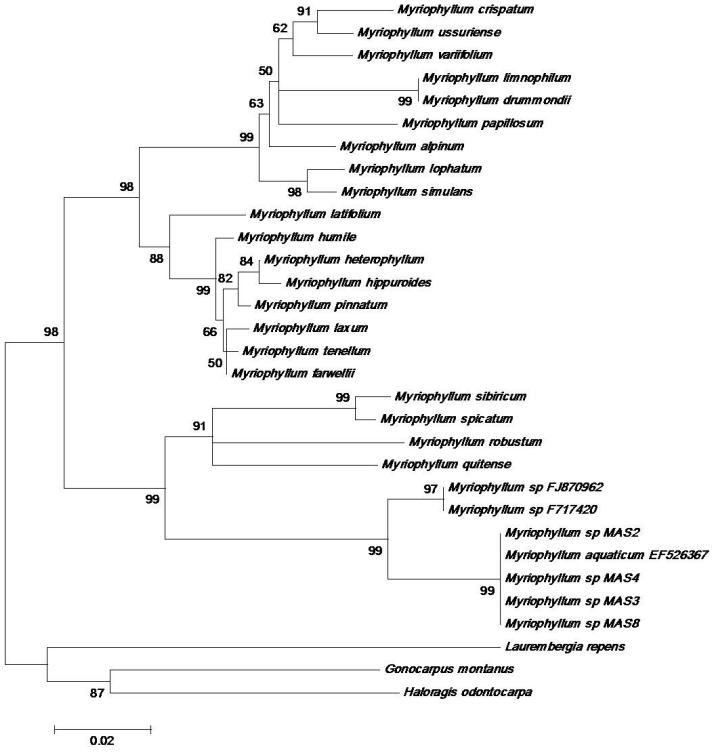

Figure 2. Maximum parsimony tree inferred from analyses of internal transcribed spacer sequence of nuclear ribosomal DNA. Taxon nomenclature and accession name follow Moody and Les (2010) and Thum et al. (2012) respectively. Numbers on the nodes are bootstrap support under 1000 bootstrap replicates for maximum parsimony analysis.

Precisely identifying *M. aquaticum* in Kashmir Himalaya using both morphological and molecular phylogenetic approaches is important because invasion managers are unambiguously using targeted biological and chemical control strategies against *M. aquaticum*. Specific biological control agents have been already developed to combat *M. aquaticum* (Cilliers, 1999; Gassmann et al., 2006). After clear delimitation of *M. aquaticum*, taxonomists and wetland managers in Kashmir, who quite often overlap the taxonomic identity of different species of *Myriophyllum*, can use specific biocontrol agents to manage this species.

Because of its widespread dominance in all the aquatic habitats, particularly in the Kashmir Himalaya, *M. aquaticum* has recently been classified (Shah and Reshi, 2012) as Stage V species (Colautti and MacIsaac, 2004). Climate changes have caused some tropical species such as *Azolla cristata* Kaulf. to expand their ranges to typically temperate regions, including Kashmir Himalaya. Although *M. aquaticum* is native to tropical South America, it behaves as a global pest species, invading many temperate regions as well. This further complicates the delimitation of different species of *Myriophyllum* as they appear similar to other tropical species of the family Haloragaceae, such as *L. repens*, which may be present in the Kashmir valley.

In conclusion, in addition to morphological attributes, molecular phylogenetic analysis based on nrDNA authenticated the identification of *M. aquaticum* in Kashmir Himalaya. The results have important taxonomic ramifications and can be used to guide biodiversity managers in effective and targeted management of this aggressive invasive species in the Himalayan region.

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