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Molecular characterization of the invasive aquatic macrophyte *Hydrilla verticillata* (Hydrocharitaceae) in Brazil

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Abstract: Invasive populations of macrophytes are widely distributed and have been successfully introduced and established in freshwater habitats. *Hydrilla verticillata* was first recorded in 2005 in the Upper Paraná River floodplain and in 2007 at the Itaipu Reservoir (Brazil-Paraguay border, ca. 300 km downstream from its first record). However, its genetic variability within different sites in South America is unknown. We used nucleotide sequences corresponding to the *trnL-trnF* fragment cpDNA to genetically characterize populations of *H. verticillata* in different ecosystems of the Upper Paraná River basin. The results indicated an absence of genetic differentiation within and between populations of the basin, and even individuals collected 600 km apart belonged to the same haplotype. Moreover, *H. verticillata* populations of the Upper Paraná River basin also matched the dioecious biotype haplotype of the Southern United States and Asia. The identification of this single haplotype suggests that one founder genotype was introduced and established successfully in the Upper Paraná River basin, then, as a consequence of vegetative reproduction and the dispersal of propagules, spread to different habitats. However, firm conclusions about this inference can only be obtained with markers of biparental inheritance.

Key words: cpDNA, genetic variability, *trnL-trnF*, Upper Paraná River basin.

INTRODUCTION

Hydrilla verticillata (L.f.) Royle (Hydrocharitaceae), here after hydrilla, is a rooted-submersed macrophyte that is established in all continents except Antarctica, most likely native to warmer regions of Asia, the Pacific Islands, eastern and northeastern Australia and Africa (Cook and Lüönd 1982). This species can be dioecious or monoecious and has a variety of reproductive strategies (e.g., fragmentation, production of turions, tubers and seeds) that enhance its

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establishment (Van 1989, Steward 1993, Langeland 1996). Attributes such as resistance organs, wide ecological amplitudes, high growth rates, high dispersion ability and the capacity to acquire and utilize resources at low levels provide hydrilla a high potential to successfully invade different habitats (Langeland 1996, Sousa 2011). This species spreads rapidly and infests large areas with dense biomass stands often resulting in important effects on abiotic and biotic environmental characteristics (Sousa 2011). Because hydrilla is highly invasive and can cause severe economic and ecological damages, it has raised great concerns from ecologists and environmental managers.

In the Americas, hydrilla was first recorded in North America in 1960 (Cook and Lüönd 1982, Cook 1985). However, the exact time when this species was first introduced in South America, more specifically into Brazilian inland waters, is unknown. It was reported for the first time in the Porto Primavera Reservoir (Upper Paraná Basin, Southeast Brazil) in March 2005 (Anderson et al. 2005) and in the Upper Paraná River floodplain (Sousa 2011), in a site at ca. 30 km downstream from this reservoir (S. M. Thomaz, personal observation) in June 2005. Hydrilla spread very quickly in the Paraná River basin and by January 2007 it was recorded in the Itaipu Reservoir, at a site ca. 300 km downstream from its first record (Thomaz et al. 2009). After the recent invasion of the Paraná River watershed, other ecologically and socially important aquatic systems in Brazil became more susceptible to invasion by hydrilla (Sousa 2011). This is a matter of concern due to the potential effect of hydrilla invasion on the highly diverse aquatic ecosystem of this region.

The origin of hydrilla has been investigated using molecular analyses in several regions, e.g., in the United States (Ryan et al. 1995, Madeira et al. 1997, 1999), New Zealand (Hofstra et al. 2000), and South Africa (Madeira et al. 2007). However, its origin as well as its genetic variability within different ecosystems in South America, are unknown. China is most likely the central area of genetic diversity for hydrilla, and this species probably originated in East Asia from where it dispersed throughout the world (Zhu et al. 2015). In contrast to native populations, low levels of genetic diversity have been reported for invasive aquatic species, most often attributed to events that cause population bottleneck (Amsellem et al. 2000, Li et al. 2006, Lambertini et al. 2010). However, lack of variability in hydrilla may also be due to the high degree of vegetative reproduction (Zhu et al. 2015) and/or the presence of only one dioecious sex and absence of sexual reproduction in invasive populations, as for example, in North America (Steward 1993).

In this study, we determined the genetic variability, at the haplotype level, of hydrilla recently introduced in the Upper Paraná River basin in Brazil. We analyzed accessions from several natural and artificial (reservoir) habitats, applying the region of the *trnL* intron and the *trnLF* intergenic spacer (*trnL-trnF*) of the chloroplast DNA. Because hydrilla was first recorded in the Upper Paraná River basin, we hypothesize that accessions found in habitats distributed in a large spatial scale (ca. 600 km along the river) are homogeneous, indicating a common source of this species in this basin.

MATERIALS AND METHODS

STUDY AREA AND SAMPLING

A total of 24 samples were taken in five distinct locations in the Upper Paraná River basin: Ilha Solteira Reservoir (Paraná River) (n=1), Três Irmãos Reservoir (Tietê River) (n=3), Porto Primavera Reservoir (Paraná River) (n=2), channels and floodplain lakes connected to the Paraná River (n=10) and Itaipu Reservoir (Paraná River) (n=8). The distance between the two furthermost sampling stations within these stations is of ca. 600

km. The plants were collected manually or with rakes. All samples were carried to the laboratory in plastic bags with water, inside an ice box and were maintained until the time of the extraction.

Genomic DNA from each sample was isolated according to an adaptation of the protocol from Lodhi et al. (1994). The purified DNA was quantified using agarose gel (0.8%) electrophoresis and stored in an ultracold (-80ºC) freezer.

AMPLIFICATION OF cpDNA SEQUENCES, SEQUENCING AND GENBANK SEQUENCES

The *trnL-trnF* fragment was amplified by PCR (*Polymerase Chain Reaction*) with a pair of specific primers: *trn-c-F* and *trn-f-R* (Holt et al. 2004). Amplification reactions were in a volume of 25 µL, with Tris-KCl buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), $MgCl_2$ (2.5 mM), 1.5 µM of each primer (trn-cF, 5'-GGAAATCGGTAGACGCTACG-3 ', trn-fR, 5'-ATTTGAACTGGTGACACGAG-3'), dNTP (0.1 mM of each), 1 U of Taq DNA polymerase and 15 ng template DNA. PCR amplifications were carried on MJ Research PTC-100 thermal cycler and comprised a cycle of four minutes at 92º C, 40 cycles at 94º C for 15 seconds, 59º C for 30 seconds and 72º C for two minutes, followed by a final extension of 72º C for ten minutes. Negative controls were also included in each set of amplifications.

Aliquots of the reaction product from each sample were visualized, measured and photographed by routine methodology (Madeira et al. 2004). The products of PCR were purified according to the protocol of Rosenthal et al. (1993) and sequenced separately with the primers *trn-c-F* and *trn-f-R,* using the MegaBace platform (Amersham), following the instruction of the manufacturers. In addition to the sequences generated in this work, other sequences of hydrilla for the cpDNA region *trnL-trnF* were obtained from GenBank.

DATA ANALYSIS

The sequences from Upper Paraná River and those taken from GenBank (*Hydrilla verticillata* haplotypes native and introduced wordwide, n=41) were edited manually with the program BioEdit (Hall 1999) and aligned with MAFFT using the site https://www.ebi.ac.uk/Tools/msa/mafft/. Gaps with two or more base pairs were coded as single mutation events and when overlapping indels occurred, the overlap portion was considered a single event, according Zhu et al. (2015). The number of polymorphic nucleotide and indels were calculated with DnaSP 5.1 (Librado and Rozas 2009). The distances-*p* (percentage of polymorphic sites) among all haplotypes of hydrilla*,* taken two by two, were calculated with the program MEGA 6 (Tamura et al. 2013).

RESULTS

Fragments of 1132 bp corresponding primarily to the *trnL-trnF* region of 24 samples of hydrilla collected in the Upper Paraná River basin were compared with 41 samples taken from GenBank. Among the individuals collected in the Upper Paraná River basin, neither base substitutions nor deletions were found and all present the same haplotype. Among the samples from the Paraná basin that were compared to those from GenBank, 13 base changes and 81 deletions were observed. The sequences were collapsed into 14 haplotypes (Supplementary Material - Table SI).

The haplotype that characterized hydrilla from the Upper Paraná basin corresponds to a haplotype identified by Madeira et al. (2007), which includes the dioecious accessions of hydrilla from the United States (Florida, California, Louisiana and Texas) and some locations of Asia: India (Bangalore, Kashmir, New Delhi, Rajasthan), China, Nepal North Vietnam and Pakistan. The p-distance (the proportion (p) of nucleotide sites at which two

sequences being compared are different) between the haplotype from these regions and our haplotype was equal to zero. In addition, this haplotype includes sequences identified by Zhu et al. (2015) in southern part of East Asia (Haplotypes B1/H1 – Table SI).

Among haplotypes collected in the Paraná basin and those from the GenBank, the p-distance values ranged from zero to 0.018. Excluding those accessions identical to ours, the accessions that showed the lowest p-distance values compared to the ones from the Upper Paraná basin were KM982399 (Haplotype H2/B2) (Lake Tanganyika Burundi/south of Yangtze River, China) and EF458072 (Haplotype H4) (L.Cairns, Queensland, Australia) (p-distance = 0.001) (Figure 1). The highest p-distance values compared to the Upper Paraná basin were from the accessions EF458053 (Haplotype H8) (Kobe, Japan) and EF458054 (Haplotype H9) (Lake Krulak, Poland), equal to 0.018 and 0.017, respectively (Figure 1).

DISCUSSION

The results indicated an absence of genetic differentiation at the haplotype (phylogenetic) level within and between populations in the Upper Paraná River basin and even individuals collected 600 km apart. Because we found a single haplotype in the Upper Paraná basin, we suggest that this region was invaded by hydrilla originating from a common source. This introduction history differs from the one of hydrilla in the North America, where the first introduction was the dioecious female biotype, in 1950, from Sri Lanka (Southeast Asia) into Florida (Schmitz 1990). Later, in 1976, the monoecious biotype was introduced into the State of Delaware and, subsequently, into the Potomac River, Washington, D.C. (Haller 1982, Steward et al. 1984, Anderson 1996, Madeira et al. 2004). Currently, populations of dioecious and monoecious biotype are also present in other US states (Madeira et al. 2000, 2007, True-Meadows et al. 2016). The monoecious biotype generally shows a more northern distribution in the USA while the dioecious hydrilla occurs mostly in the Southern Atlantic and Gulf basins (Figure 1).

Hydrilla samples from the Upper Paraná basin presents the same haplotype as dioecious plants from the United States and Asia and, for this reason, it is not possible to determine objectively the origin of hydrilla in Brazil. This information could possibly be obtained with more sensitive markers such as microsatellites. However, the proximity between the USA and Brazil and the greater exchange of people and commerce between them, as compared with Asian countries (Embratur 2005), would favor the argument that propagules from the USA were introduced in Brazilian ecosystems. Besides that, Florida is a traditional center of ornamental aquaculture farming and trade (Chapman et al. 1997).

The genetic diversity in aquatic plants is generally lower than in terrestrial plants, and it is often identified among populations, and not inside them, due to the dominance of vegetative reproduction (Nakamura and Kadono 2000). Moreover, a population´s colonization by a single founder genotype which reproduces via cloning results in populations that are genetically uniform (Burden and Marshall 1981, Hofstra et al. 2000). For example, more than three-fourths of the hydrilla populations sampled throughout China belong to a single haplotype and lack of intra-population variation of these populations seems frequent (Zhu et al. 2015). The absence of variability in the nucleotide sequences of the populations from the Upper Paraná basin, besides indicating that there was a single genotype founder, probably can be a consequence of the maintenance of populations via vegetative reproduction and the dispersal of propagules to the different habitats. Only pistillate flowers of *H. verticillata* have been

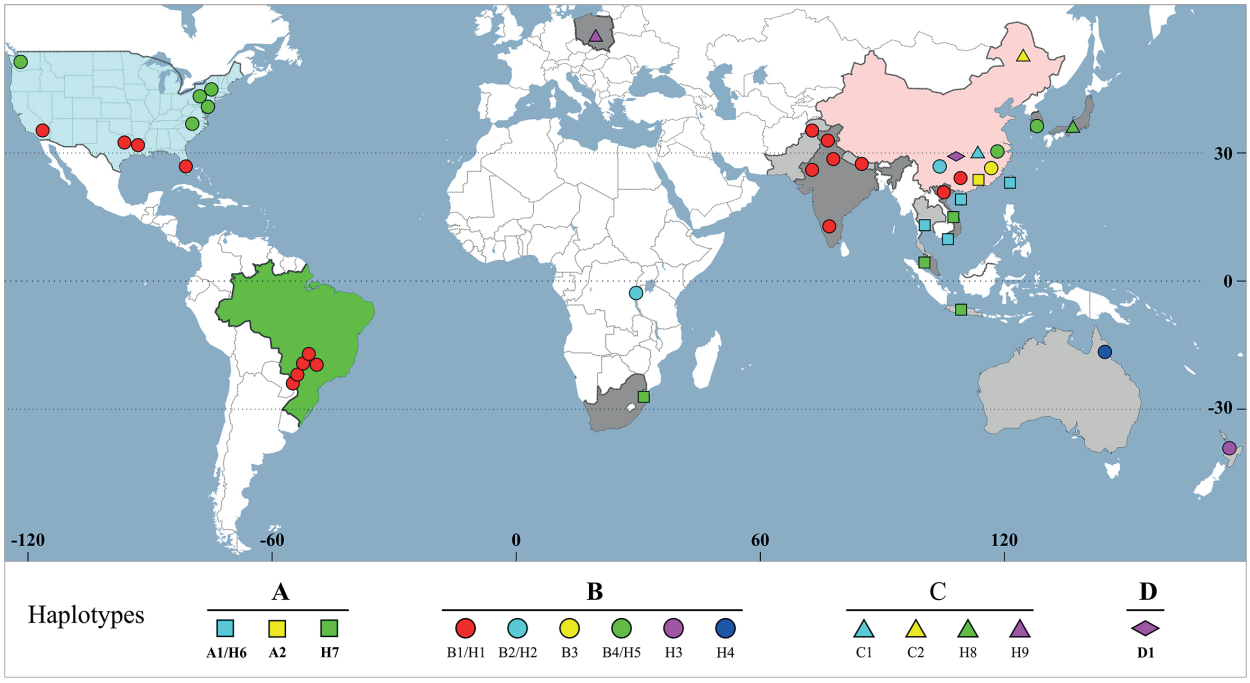

Figure 1 - Distribution of the *Hydrilla verticillata* haplotypes native and introduced worldwide. Our data (Brazil) compared with world data retrieved from Madeira et al. (2004, 2007) and Zhu et al. (2015).

collected in the Upper Paraná River, providing evidence that all individuals belong to a dioecious female (Sousa 2011) and thus, they are not able to reproduce sexually. In addition, the temporal sequence of invasions going downstream (Porto Primavera Reservoir – Paraná River floodplain - Itaipu Reservoir) (Thomaz et al. 2009) is also an indication that dispersal of fragments is the probable means of spread of this plant in the basin. Despite these indications that hydrilla is maintained via vegetative reproduction in the Upper Paraná basin and because cpDNA is of maternal inheritance, firm conclusions about this inference can only be reached with studies involving nuclear genome analysis.

In conclusion, our data supports the hypothesis of a single founder genotype in the introduction of hydrilla to the Upper Paraná, which then spread rapidly becoming a successful invader of a variety of ecosystems distributed over a large spatial scale. The lack of genetic variability in the hydrilla populations found in Brazil has to be taken into account if management is necessary.

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AUTHOR CONTRIBUTIONS

LCL, SMT and AJP conceived the work. LCL and SMT collected the samples. LCL conducted the molecular analysis, with the help of TAB, SMAPP and AVO. LCL wrote the first draft of the manuscript, which was critically revised and

improved by SMT, TAB, AVO, SMAPP and AJP. All authors read the last version of the manuscript.

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SUPPLEMENTARY MATERIAL

Table SI - A fragment nucleotide polymorphism corresponding to the *trnL-trnF* region of cpDNA from the *H. verticillata* accessions from Genbank and the Upper Paraná basin (Brazil). Data retrieved from Madeira et al. (2004, 2007) and Zhu et al. (2015).