Aquatic Botany 111 (2013) 35–42

Contents lists available at ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

A multi-level analysis to evaluate the extinction risk of and conservation strategy for the aquatic fern *Marsilea quadrifolia* L. in Europe

I. Bruni[a,1], R. Gentili[b,1], F. De Mattia[a], P. Cortis[c], G. Rossi[d], M. Labra[a,*]

[a] Università degli Studi di Milano Bicocca, ZooPlantLab, Dipartimento di Biotecnologie e Bioscienze, Piazza della Scienza 2, 20126 Milan, Italy
[b] Università degli Studi di Milano Bicocca, Dipartimento di Scienze dell'Ambiente e del Territorio, Piazza della Scienza 1, 20126 Milan, Italy
[c] Università degli Studi di Cagliari, Dipartimento di Scienze della Vita e dell'Ambiente Macrosezione Botanica ed Orto Botanico, Viale S. Ignazio 13, 09123 Cagliari, Italy
[d] Università degli Studi di Pavia, Dipartimento di Scienze della Terra e dell'Ambiente, Via S. Epifanio 14, 27100 Pavia, Italy

ARTICLE INFO

Article history:
Received 12 February 2013
Received in revised form 22 August 2013
Accepted 26 August 2013
Available online 10 September 2013

Keywords:
Marsilea quadrifolia L.
Herbicides
AFLP
Agro-ecosystem
Restoration genetics
Plant conservation

ABSTRACT

This study investigated the conservation status of *Marsilea quadrifolia*, an endangered fern found in paddy fields, irrigation ditches and ponds. An evaluation at the European level based on IUCN criteria showed that the extent of occurrence (EOO) of *M. quadrifolia* has decreased from 5,930,000 km^2 to 5,774,000 km^2 during the past decade, whereas its area of occupancy (AOO) has decreased from 620 to 400 km^2 (approximately 35.5%). These findings allowed the species to be upgraded from the IUCN classification of Near Threatened to Vulnerable. The agricultural chemical treatments seem to be the main extinction cause of *M. quadrifolia*, therefore we performed toxicological with 7 most common rice herbicides. Young plantlets were incubated for 96 h with each herbicide at three different concentrations: TQ (Tale Quale, chosen in accordance with the suggested dose for rice fields described on the product label), 1:100 and 1:1.000. Results suggested that survival of the plantlets depended on the herbicide and concentration used, and ranged between 0 and 80%, and no survival at ambient concentrations for 4 (Aura, Aura + Dash, Clincher and Viper) out of the 8 chosen herbicides. We conclude that herbicides represent one of the principal threats to the survival of this species. Finally, a DNA analysis using the AFLP approach was employed to identify the most suitable genetic pool for plant reintroduction efforts. The data show that the analysed populations of *M. quadrifolia* suffered from low genetic variability (Nei's gene diversity varied from 0.025 to 0.036). However, the analysis of the distribution of genetic variability suggested that 4 populations were characterised by different genetic traits that are useful in defining a genetic pool for plant conservation. This study highlights a strategy for implementing a plan of action for species growing in agro-ecosystems based on an integrated approach that is able to clarify the species conservation status, the principal threat factor and the genetic pool to be used for species conservation and reintroduction.

1. Introduction

In many countries, aquatic plants are particularly affected by such threats as the geological modification of freshwater basins, eutrophication (Gücker et al., 2009; Bornette and Puijalon, 2011; Barni et al., 2013) and contamination by xenobiotic factors (Labra et al., 2007; Eullaffroy et al., 2009; Vannini et al., 2011). Consequently, these plants are among the most jeopardised groups of organisms (Gücker et al., 2009; Kozlowski and Vallelian, 2009). Among the European endangered species, *Marsilea quadrifolia* L. is an ancient amphibious leptosporangiate fern that is characterised by unusual reproductive structures and heterospory (Iamonico, 2012). Its principal habitats are paddy fields, irrigation ditches and ponds. *M. quadrifolia* has a widespread distribution extending from its natural range in the Eurasian region to tropical and warm temperate regions in eastern Asia (India) and to North America, where it is considered an alien species (Takematsu and Ichizen, 1997; Benson et al., 2004). In recent decades, this fern has been included in several national Red Lists in western Europe in high-risk categories. In Italy, *M. quadrifolia* has recently been assessed as Endangered (EN) (Gentili et al., 2010a), while it is

Abbreviations: AFLP, amplified fragment length polymorphism; AOO, area of occupancy; CAP, Common Agricultural Policy; EOO, extent of occurrence; IUCN, International Union for Conservation of Nature; NAP, National Action Plans; TQ, Tale Quale.

* Corresponding author. Tel.: +39 02 64483472; fax: +39 02 64483450.
E-mail address: massimo.labra@unimib.it (M. Labra).

[1] These authors contributed equally to this research.

0304-3770/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquabot.2013.08.005

considered Critically Endangered (CR) in Portugal and Extinct in the Wild (EW) in Spain. Although *M. quadrifolia* is rapidly disappearing from areas where it was once common, it has only been evaluated as Near Threatened (NT) in the recent European IUCN Red List; this evaluation is most likely due to a lack of exhaustive evaluation regarding the current species distribution and real extinction risks (Bilz et al., 2011). In certain European countries, such as France, several historical populations have disappeared in the past few years (Dehondt et al., 2005); however, these data were not included in the EU IUCN evaluation. Moreover, data regarding the species distribution in most eastern European countries (i.e., Bulgaria, Croatia and Romania) are partial, approximate and not up to date (see www.iucnredlist.org/details/161864/1). Due to the ecological importance of this species for aquatic habitats, its significance in the evolutionary history of plants (Nagalingum, 2007) and its unsatisfactory conservation status, it was listed as a conservation priority species by the Bern Convention and European Union's "Habitat" Directive 92/43 CEE. If the conservation status of this species appears to be relatively positive inside the protected areas of European interest (Natura 2000 and Emerald networks), this is not the case in cultivated areas or those areas otherwise inhabited or affected by humans. As a consequence, its distribution is very scattered and not widely known. Moreover, active conservation strategies for *M. quadrifolia*, such as plant relocation and reintroduction, need to be implemented (at least in Western Europe) to improve its survival rate.

However, to ensure success, reintroduction and reinforcement strategies must be based on scientific practices (Godefroid et al., 2011; Chau and Reyes, 2013). Thus, we conducted a multidisciplinary study to define a suitable conservation strategy for this species. First, we analysed the distribution and conservation status of *M. quadrifolia* in European countries based on IUCN categories and criteria (IUCN, 2001) to define an updated distribution map. Second, we evaluated the anthropogenic and natural pressures that threaten the survival of this species. Recent investigations suggest that herbicides discharged into natural aquatic systems, primarily through water runoff, could be one of the top risk factors for species survival (Eullaffroy et al., 2009; Casanova, 2012). Accordingly, we tested the effect of the most common and modern-day European herbicides on *M. quadrifolia*. Third, a DNA molecular analysis was used to study the genetic pattern of several surviving *M. quadrifolia* populations with the principal goal of selecting a gene pool that would be useful for reintroduction programmes (McKay et al., 2005; Gentili et al., 2010a; Godefroid et al., 2011). This analysis was based on the AFLP approach, which screens a large number of genetic loci and does not require knowledge of the genome sequence of the analysed species. This method is considered the most effective tool for revealing variability and population structure within a single species (Grassi et al., 2004). The multi-level analysis performed on *M. quadrifolia* helps to define the most useful guidelines for the preservation of this ancient fern in its natural, semi-natural and human-made habitats.

2. Materials and methods

2.1. Analysis of conservation status according to IUCN criteria and categories

To update the conservation status of *M. quadrifolia* at the European level, IUCN criteria and categories were used (IUCN, 2001, 2013) based on the guidelines for a regional-level evaluation (IUCN, 2003). We considered criteria A and B for evaluating the species trend at the temporal and spatial scales. In detail, criterion A assesses the species decline in the past ten years through the analysis of population size reductions (%) over that period (IUCN, 2001). This criterion was estimated based on the evaluation of the past and present area of occupancy (AOO) of the species at the European level (criterion A3). Criterion B refers to the two spatial measures related to the geographic range of the species, the EOO and AOO, based on different thresholds set for the different threat categories and on the number of locations and/or quality of habitats (IUCN, 2001, 2011). To calculate EOO and AOO, we collected data on the distribution of *M. quadrifolia* from the following different sources: (i) national Red Lists and Red Books of European countries, (ii) web data sources of protected areas and environmental agencies and institutions at the national level, (iii) specialised literature and (iv) direct field analysis and information from local specialist botanists. Data from the past 10 years were considered in defining the current distribution of the species in EU countries (see Supplementary materials S1). The geographical information on each population was introduced into our GIS database to define a georeferenced distribution map. However, data related to recent species reintroduction actions (approximately the past 5 years) were not considered, as these populations can be treated as wild populations only if they are self-sustaining (IUCN, 2013). The map obtained was compared to the past species distribution map described by Jalas and Suominen (1972) to calculate the past and present EOO and AOO. The EOO value was obtained based on the minimum convex polygon (IUCN, 2013) generated using the GIS database. The AOO value was obtained by bounding a 2 km × 2 km grid on the distribution range of the species and then summing the surface of the 2 km × 2 km cells in which the species was present (IUCN, 2001, 2013).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2013.08.005.

For each EU country, we also evaluated the IUCN category and the principal threats to the species according to the IUCN Threats Authority File (Version 3.0; www.iucnredlist.org/static/major_threats).

2.2. Analysis of herbicide effects on M. quadrifolia

To perform the toxicological test, young *M. quadrifolia* plantlets were collected from the Botanical Garden in Pavia, Italy. The original population was collected from Mede – Pavia, Italy (4995596 N–478417 W). The plantlets were first acclimatised for 4 days in ½-strength MS medium (Murashige and Skoog, 1962) in a temperature-controlled room (20 ± 3 °C) using a 14-h light/10-h dark cycle and a fluorescent illumination system producing 220 ± 20 μmol m^{-2} s^{-1} (36 GRO-LUX F30/GRO lamps, Sylvania). Toxicological tests were performed with the following 7 most common rice herbicides, starting at the TQ concentration, which was chosen in accordance with the suggested dose for rice fields described on the product label: glyphosate (Glycine class, 10 mg/L), Clincher (aryloxyphenoxypropionate class, 0.5 mg/L), Aura (ciclohexenone class, 10 mg/L), Viper (sulfonamide and triazolopyrimidine class, 20 ml/L), Command (iso-oxazolidinone class, 0.5 mg/L), Aura + Dash (20 mg/L), Gulliver (sulfonylurea class, 15 mg/L) and Most MC (dinitroaniline class, 20 mg/L). The herbicides were tested at the TQ concentration and at two different dilutions: 1:100 (T1) and 1:1000 (T2). For each herbicide concentration, a test was performed on 10 individuals in a single tube with the rhizomes and roots completely submerged in the medium. All tests were performed in triplicate. Plants growing in ½ MS medium alone were used as control (C). The per cent plant survival of *M. quadrifolia* was analysed after 96 h of incubation, corresponding to the herbicides' peak of action and maximum persistence in the field (Cattaneo et al., 2012). Plants showing necrosis in more than 70% of leaves were considered as dead. Statistical analyses were performed on the mean values of the per cent plant survival in response to the different herbicides at different dilutions. These