Conservation of *Lactuca watsoniana* Trelease, an Azorean priority species: Phylogenetics, Population Genetics and Propagation

Tese de Doutoramento

Elisabete Furtado Dias

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Elisabete Furtado Dias

Orientadores

Professor Doutor Luís Silva
Professora Doutora Mónica Moura
Professor Doutor Hanno Schaefer

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RESUMO

Lactuca watsoniana Trel., é uma herbácea perene, endémica do Arquipélago dos Açores. Localmente denominada de "Alfacinha", pode ser encontrada em quatro das nove ilhas do Arquipélago dos Açores (Faial, Pico, Terceira e São Miguel), estando possivelmente extinta da ilha de São Jorge. O tamanho total estimado das populações é de 500 a 2000 indivíduos, mas observações recentes no campo indicam que poderão ser menos de 500 indivíduos. Considerada como *taxon* prioritário na Directiva Habitats (Annex B-II) bem como na Convenção de Berna em termos de conservação e está listada como ameaçada (EN B2ab (i, ii, iii); C2a(i)) na Lista Vermelha da IUCN 2017. Atualmente, a *Lactuca watsoniana* encontra-se restrita a zonas húmidas, ravinas profundas e estreitas, crateras zonas declivosas, prados naturais de *Festuca*, matos de *Erica* e florestas de *Juniperus* entre 600-800 m. Está ameaçada por perda e degradação de habitat, resultante de expansão dos pastos, invasão por espécies exóticas, introdução de herbívoros e distúrbio de áreas sensíveis por turistas e locais. Neste trabalho investigaram-se os seguintes tópicos: i) propagação vegetativa por sementes; ii) genética de populações; e iii) filogenética e filogeografia. Foi estabelecido um protocolo eficaz e exequível para propagação por via seminal, germinando as sementes com 0.1 mg l^{-1} GA_3, ethephon, e um filtro de luz vermelha e um regime de temperatura alterna de 15/10°C, com um fotoperíodo de 12 horas. Este protocolo permitiu a produção de centenas de plântulas viáveis que foram replantadas na população de origem. Dada a necessidade de proceder ao reforço das populações naturais em algumas ilhas, pretendeu-se definir se existiam riscos de contaminação genética resultante da troca de material entre as diferentes ilhas, para isso estudou-se a estrutura e variabilidade genética das

populações, usando 8 microssatélites nucleares, desenvolvidos especificamente para a *L. watsoniana*. Os resultados mostraram uma clara separação entre as várias ilhas dos Açores, estando a maior variação genética dentro das populações (55.0%), mas com níveis relativamente altos de variação entre os clusters (27.3%). Evidenciando um grau de diferenciação entre as populações elevado e fluxo genético com valores inferiores a 1, bem como a existência de isolamento e barreiras geográficas. De modo a clarificar a posição taxonómica da *L. watsoniana*, nomeadamente em relação a espécies próximas da Europa, Norte da América e de outros Arquipélagos da Macaronésia (Canárias), recorremos a marcadores moleculares nucleares (ITS) e quatro do cloroplasto. Na análise das sequências combinadas de ITS e do cloroplasto (Parsimónia, Máxima verosimilhança e Inferência Baeysiana) o taxon dos Açores, mostrou a existência de um padrão filogeográfico através do arquipélago, com a presença de 5 haplótipos distintos para a *L. watsoniana*, quatro endémicos em apenas uma ilha (Faial, Pico, Terceira e São Miguel) e um haplótipo com informação genética partilhada do Faial e Pico, bem como uma relação filogenética, bem suportada, com as *Lactuca* Norte Americanas.

ABSTRACT

Lactuca watsoniana Trel., is a perennial herb, endemic to the Azores archipelago. Locally called "Alfacinha", the species is restricted to only four of the nine islands of the archipelago (São Miguel, Terceira, Pico and Faial) and is possibly extinct in São Jorge Island. Estimates of its total population size are 500 to 2,000 individuals, but recent field observations indicate that it can be fewer than 500 individuals. It is therefore considered a priority species for conservation and was listed as endangered on the IUCN Red List 2017. Furthermore, it was included in Annex II of the EC Habitats Directive and is protected under the Berne Convention. *Lactuca watsoniana* is currently restricted to the steep slopes of craters and ravines in temperate juniper rain forest between 600-800 m of altitude. The species is threatened by habitat loss and degradation resulting from changes in land use, namely expansion of pasture, invasion by exotic species, consumption by introduced herbivores and disturbance of sensitive areas by tourists and locals.

In this thesis, we investigated the following topics: i) seed propagation; ii) population genetics; and iii) phylogenetics and phylogeography. We established and efficient and feasible seed propagation protocol by germinating the seeds germinate seeds with 0.1 mg l^{-1} GA_3, ethephon, a red-light filter and a temperature regime of 15/10°C, and 12 hours' photoperiod. This protocol allowed producing hundreds of viable seedlings that were reintroduced in the source population. Given the need to reinforce natural populations in some of the islands, we aimed to find out if any outbreeding risks existed, resulting from material exchanges between the different islands. For that, we studied the genetic structure and variability of the populations, using eight nuclear microsatellites, developed

specifically, for *Lactuca watsoniana.* Our findings showed a clear separation among the islands, with the largest proportion of genetic variation found within populations (55.0%) but with relatively high levels of genetic variation both among clusters (27.3%). Furthermore, a high variability between the populations, a gene flow value below one, and the presence of geographical isolation and barriers were estimated. In order to clarify the taxonomic position of *L. watsoniana*, namely the phylogeographic and phylogenetic relationship with the morphologically similar species from Europe, North America and the geographically close taxa from the Canary Islands, we used one nuclear region (ITS) and four chloroplast markers. Parsimony, Maximum likelyhood and Bayesian Inference analyses were conducted and revealed a strongly supported monophyletic *Lactuca clade*, and a clear separation of Macaronesian endemic *Lactuca* in sister clades. *Lactuca watsoniana*, endemic to Azores, showed a phylogeographic pattern across the archipelago, with the presence of five different haplotypes for *L. watsoniana*: four single island endemics (Faial, Pico, Terceira e São Miguel) and one occurring on both Faial and Pico and a strongly supported phylogenetic relationship with North America *Lactuca*

INTRODUCTION

The flora of oceanic islands has inspired research since the early scientific explorations. Islands can be considered ‘natural laboratories’ — simple systems with multiple replicates (Gillespie et. al. 2008).

Geological, geographical and ecological conditions, such as island age and area, geographical isolation by oceanic barriers, climatic stability, environmental heterogeneity, high habitat diversity and the absence of competitors, have been pointed out as key determinants of the speciation patterns observed within volcanic archipelagos (Gillespie and Baldwin 2009; García-Verdugo et al. 2014; Mairal et al. 2015). Therefore, in isolated habitats, ecological processes will become increasingly more likely to generate biodiversity than evolutionary processes which have been relatively more important in the past (Gillespie et. al. 2008). Biodiversity of remote islands has arisen with high levels of endemism displaying unique genetic patterns in comparison with continental relatives (Crawford et al. 1987; Stuessy and Ono 1998; Emerson 2002; Whittaker and Fernandez-Palacios 2007, Bramwell and Caujapé-Castells 2011; Silva et al. 2015; Takayama et al. 2015).

The Azores, Canary Islands, Cape Verde, Madeira and the Savage Islands, constitutes Macaronesia, a geographic region in the North Atlantic Ocean, recognized as an important component of the Mediterranean biodiversity hotspot (Myers et al. 2000), hosting ~3100 species of flowering plants, of which 1058 are endemic to the region (Fernández-Palacios and Dias, 2001, Jones et al. 2014). The Azorean vascular plant flora is currently thought to comprise c. 1110 species, of these, no more than 300 are considered as native, of which circa 80 are considered as endemic. However, these numbers likely underestimate the true diversity, since recent molecular studies have repeatedly revealed new

endemic taxa (Schaefer and Schönfelder 2009; Bateman et al. 2013; Moura et al. 2015a, b, c; Schaefer 2015), leading to the idea that there might be significantly more diversity in the archipelago's flora than has previously been documented, a case of the so-called "Linnean shortfall" (Schaefer et al. 2011). Therefore, preservation of these unique species is extremely important since each extinction represents a much larger percentage of biodiversity loss when compared to other island systems. In fact, according to IUCN's 2009 Red List of Threatened Species, 784 extinctions have been recorded since 1500, of which 79 are vascular plants, but many more likely have gone unnoticed (Martins 2012), and it is estimated that the current extinction rate is between 1000 and 10000 times higher than it would naturally be (IUCN 2017). Human colonization has had a strong impact on oceanic islands endemic plants since pristine ecosystems have evolved or persisted in the absence of novel or exotic disturbances (Connor et al. 2012).

Lactuca watsoniana Trel., the Azorean lettuce ("Alfacinha"), is an hemicryptophyte with anemochorous and hydrochorous dispersal, endemic to the Azores. It was probably first mentioned by Watson in his Catalogue of Azorean Plants published in the London Journal of Botany (1844) as "a large-leaved Composita from the Caldeira of Fayal" and was later described by Trelease (1897). It is an erect perennial with a corymbose panicle that is able to attain up to 2 m, presently restricted to four of the nine islands of the archipelago (Faial, Pico, São Miguel and Terceira). It is probably extinct in São Jorge Island, from where two specimens exist in the Lisbon University herbarium (LISU), collected between the valleys of "Ribeira do Salto" and "Ribeira de S. João". *Lactuca watsoniana* is mostly restricted to the steep slopes of craters, ravines, and

temperate juniper rain forest, between 600-800 m above sea level (Schaefer 2005; Silva et al. 2009; EFD, pers. obs.). Estimates for its total population size range from 500 to 2000 individuals (Schaefer 2005; Silva et al. 2009, 2011), but recent field observations indicate lower numbers (probably fewer than 500 mature individuals). Like many other endemic plants from oceanic islands (Francisco-Ortega et al. 2001; Caujapé-Castells et al. 2010) it is considered as a priority species for conservation and was listed as endangered (EN B2ab(i,ii,iii); C2a(i)) on the IUCN Red List 2017. Furthermore, it was included in Habitats Directive as a Priority species (Annex B-II) and also in Bern Convention (Appendix 1, Annex 1).

Lactuca watsoniana is threatened by habitat loss and degradation resulting from changes in land use, namely expansion of pastureland, invasion by exotic species, introduced herbivores and disturbance of sensitive areas by tourists and locals (Silva et al. 2009). Human colonization of previously pristine ecosystems, with large-scale changes in land use, originated habitat loss and fragmentation both of which contribute to a decline in biological diversity (Wilcox and Murphy 1985; Kuussaari et al. 2009; Krauss et al. 2010; Triantis et al. 2010). In the Azores, widespread and persistent vegetation and soil use changes in the last 500 years, together with the introduction of alien plants (Silva and Smith 2004; Connor et al. 2012; Costa et al. 2013), and herbivores (Moura et al. 2013; Dias et al. 2014; Silva et al. 2015), led to a decline in native vegetative cover and in the number of endemic plant populations and probably species throughout the archipelago. Therefore, extreme care should be taken regarding the implementation of augmentation conservation strategies. Although recovery plans for endangered plant taxa were developed in some Macaronesian

Archipelagos, namely in the Canary Islands, this is not a common practice in the Azores.

Besides its conservation interest, *Lactuca* belongs to an economically important family, Asteraceae or Compositae, providing products such as cooking oils, sunflower seeds, artichokes, sweetening agents, coffee substitutes, herbal teas and lettuce. Several genera are of horticultural importance, including *Calendula officinalis*, various daisies, fleabane, chrysanthemums, dahlias, zinnias, and heleniums. Asteraceae are important in herbal medicine, including *Grindelia*, yarrow, and many others, with more than 23600 currently accepted species, spread across 1620 genera. The genus *Lactuca* in its currently accepted circumscription comprises about 230 species, distributed in Europe, Africa, Asia, and North America (Kilian et al., 2009). *Lactuca watsoniana* is a distant wild relative of *L. sativa* (lettuce), an important crop species, with very lucrative commercial applications. While lettuce is well studied the same is not applied to the wild relatives, even though they are of potential value as sources for desirable traits in lettuce breeding (Sharrock et al., 2014), to prevent genetic diversity loss in the cultivated species. Moreover, previous studies with endemic Asteraceae in the Azores, namely the genera *Leontodon* and *Tolpis* showed high population genetic diversity and a complex genetic structure, with clear geographical-linked patterns (Dias et al. 2014; Borges Silva et al. 2016), with new species described (Moura et al. 2015).

Recently, Silva et al. (2015) proposed that a holistic approach should be used in the research devoted to rare island plants, population genetic, phylogenetic, vegetative propagation studies should be integrative in a more comprehensive and effective formulation of conservation strategies. Thus, the

main goal of this thesis is to present an integrated study devoted to the conservation of the highly endangered *L. watsoniana* providing a scientifically sound basis for a future recovery plan. Several questions were addressed along this research, including: i) vegetative propagation by seed; ii) population genetics; and iii) phylogeographic and phylogenetic between Macaronesian *Lactuca*, focusing on the taxonomic treatment of *L. watsoniana* within the Archipelago of Azores.

i) In Chapter 1, we address the issues regarding seed propagation. Classical propagation techniques for more common and abundant taxa, and in vitro propagation for highly threatened taxa is advisable (Cripps et al. 2006), and should be a priority to conserve evolutionary potential and enhance seed germination (Abdollahi et al. 2012). There is already a considerable body of knowledge for Azorean endemic plants (Moura et al. 2009, 2010, Martins et al. 2012). However, little was known about the vegetative propagation of *L. watsoniana* in natural populations or in the laboratory. Thus in the present work diaspore sampling of mature specimens established in natural communities was performed. Germination tests were conducted in acclimatized chambers (in Petri dishes). Dormancy break treatments were tested, where the effects of different concentrations of GA_3, different alternating temperature regimes, the addition of ethephon and various light wavelengths or interactions were tested on the germination of *L. watsoniana*. The main aim was to improve understanding of seed germination mechanisms in *L. watsoniana* and to establish an efficient propagation protocol, which could be used in ex situ conservation programmes.

ii) In Chapter 2, we address the issues regarding population genetics.

The occurrence of intra-archipelago genetic variability has implications in the definition of conservation strategies since, in order to preserve diversity among islands, no plant material should be exchanged. Furthermore, for those taxa which have very reduced populations, the evaluation of the genetic diversity and of the genetic divergence among populations will be important to define a recovery plan. It is the basis for establishing conservation effective strategies (Grassi et al. 2005). Also, for those taxa demanding the use of propagation methodologies, there is a need to use genetic markers to rule out the possibility of accidentally introducing hybrid individuals or those belonging to different genetic groups. Population genetics also produces important information to understand possible colonization patterns within the archipelago but also as a guide for conservation measures directed to endemic species (Silva et al., 2011, Martins et al., 2013, Moreira et al., 2013; Moura et al., 2013). Co-dominant markers were preferred due to the possibility of unbiased gene flow estimation among populations. The use of co-dominant markers such as microsatellites (SSR) is highly recommended for this purpose and newly developed SSR have been isolated for *L. watsoniana* (Chapter 2). Thus, the main goal of this study was to use reliable genetic markers in order to evaluate the intra and inter-population genetic variation patterns, to determine the population genetic structure, to identify populations with low genetic variability and inbreeding of *L. watsoniana* populations, and also to verify the occurrence of other putative operational taxonomic units. Beyond the conservation scope of *L. watsoniana*, the results of the population genetics study also allow to better understand the evolution of island plants in the Azores as well as in other relatively remote and geologically young island systems.

iii) In Chapter 3, we address issues regarding molecular systematics and phylogeography.

The molecular systematic characterization of Azorean endemic vascular plant taxa requires the use of DNA regions used in plant systematics and phylogenetic studies. Nuclear and chloroplast markers were used, namely:, 1) the commonly used ITS regions from the ribosomal DNA which have been used in other studies of Azorean taxa (Carine and Schaefer 2010; Schaefer et al. 2011), and in the first phylogeny of *Lactuca* (Koopman et al. 1998); and 2) plastid DNA regions, like the commonly used trnL-trnF spacer, which was also applied in a tribal phylogeny of Asteraceae (Bayer et al and Starr 1998), the BarCode of Life regions (matK and rbcL), and also the often highly variable "tortoise and hare" regions (Shaw et al. 2007). It should be noted that in a previous study of the tribe Lactuceae using AFLP fingerprints (Koopman et al. 2001), *L. watsoniana* was not included and some unclear groups still persist. In fact, *L. watsoniana* and *L. palmensis* (Macaronesian *Lactuca)* were not included in previous phylogenetic study of the genus *Lactuca* or the tribe. Therefore, phylogenetic relationships were tested and possible colonization routes for the Azorean Islands were proposed, taking into consideration divergence time estimation, geological island ages and haplotype network.

The thesis ends with a summary of the main conclusions resulting for this research dedicated to *Lactuca watsoniana.* I hope that the gathered knowledge during this work will contribute to the better protection of this beautiful and highly endangered plant and will promote guidelines for an integrated strategy for the conservation of *L. watsoniana*. And also contributed to increase the knowledge about genetic patterns and evolution of islands plants.