Estimation of genetic diversity in seedlings of *Plantago algarbiensis,* an endangered endemic species from the south of Portugal in risk of global extinction

NATACHA COELHO[1], CARMEN MARTÍN[2], MARÍA ELENA GONZÁLEZ-BENITO[2], ANABELA ROMANO[1,*]

[1]*Faculty of Sciences and Technology, MeditBio, University of Algarve, Campus de Gambelas, Ed. 8, 8005-139 Faro, Portugal*

[2]*Departamento de Biología Vegetal, Escuela Universitaria de Ingeniería Técnica Agrícola, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*

* Corresponding author: Anabela Romano, email: aromano@ualg.pt, Tel:+351289800910, Fax: +351.289.818419

Acknowledgements: N. Coelho acknowledges a grant from the Portuguese Science and Technology Foundation (FCT, grant SFRH/BD/63501/2009).

Abstract

Plantago algarbiensis is an endangered endemic species from the Algarve, the south of Portugal. In the present work the genetic diversity of three populations was assessed by RAPD markers. Samples were amplified using ten primers that generated 145 markers, 80% of which were polymorphic. Tunes population presented the highest polymorphism percentage (73.68%) and Algoz the lowest (67.67%). In the cluster analysis, two major groups were formed, one including individuals from Gambelas and the other clustered together individuals from the other populations. The highest level of genetic diversity, estimated by both Nei's gene diversity and Shannon's information measure, was found in Tunes and the lowest in Algoz. From the estimates of Shannon's index, the proportion of the diversity within populations was 86.12% and among populations was 13.88%. Similar results were obtained by AMOVA analysis. A correlation was found between geographic distance and genetic differentiation among populations. The N_m values obtained suggest a high level of gene flow among populations, which was inversely proportional to the distance between populations. RAPDs proved to be a useful tool to determine the genetic diversity of *P. algarbiensis* populations. The data obtained can be used to develop effective conservation strategies to prevent the declining of populations.

Keywords: conservation, gene flow, population, RAPD markers

Introduction

Plantago is the largest genus within the Plantaginaceae family comprising approximately 275 annual and perennial species distributed all over the world (Gonçalves and Romano 2016). This genus is characterized by small perennial or annual herbs, with alternate leaves forming a basal rosette and inflorescence as a bracteate spike (Castroviejo 2012). Most species are widely distributed and grow as weeds, but some others are restricted to a specific area, such as *Plantago algarbiensis* Samp. (Hoggard et al. 2003; ICN 2007). Although *Plantago* species are widely considered as weeds they have been used as medicinal plants for centuries. Some *Plantago* species are listed as safe herbs in the pharmacopoeias of numerous countries (Blumenthal 1998) while others are used as food and animal feed. *Plantago* species have been used as medicinal herbs to treat various diseases related to the skin, digestive tract, reproductive system, blood circulation disorders and cancer (Chiang et al. 2003). In addition, some species are tolerant to heavy metals in the soil (Remon et al. 2007; Malizia et al. 2012).

P. algarbiensis (Figure 1A) is an endangered endemic species to the Algarve, Portugal, limited to a few populations in a very small area. *P. algarbiensis* is a rosulate hemicryptophyte that grows up to 7 - 30 cm long and has woody stems, undivided or rarely bifid (Franco 1984). The species occurs on clay-rich soils that are temporarily flooded in winter and spring and it prefers areas that are located downstream from small springs or clearings containing acidophilic brushes (ICN 2007). The capacity of colonizing acid soils has been recently investigated by Martins et al. (2013a, b, c), indicating that *P. algarbiensis* accumulates considerable amounts of aluminium in its tissues. Of the, so far, three known populations, two are located in the interior of the region (Algoz and Tunes), widespread throughout an area of approximately 30 ha, and a third one (Gambelas) is located closer to the coast (Figure 1B). The major threats to this

species are mining of clay soils for the production of construction materials, urbanization, trampling and grazing by livestock (ICN 2007). Due to its restricted distribution worldwide, *P. algarbiensis* is in risk of global extinction and is considered endangered and legally protected by the European Habitats Directive 92/43/ CEE and Portuguese law (reference 140/99 of April 24)(ICN 2007). An *in vitro* propagation protocol has already been developed, as an *ex situ* conservation measure, to support legal protection in the preservation of *P. algarbiensis* (Gonçalves et al. 2009). However, for an effective implementation of any conservation programme, it is essential the understanding of genetic diversity and structure of natural populations. The information gathered can assist in the protection and management of populations and habitats, as well as in the strategy for germplasm collection, and genebank management.

Molecular markers present a wide range of applications and are a useful tool in the study of genetic diversity of plant populations. Random amplified polymorphic DNA (RAPD) is one of the most commonly used methods in the genetic characterization and analysis of plant species, including endangered species (Martín et al. 2008; Silva et al. 2011). This method consists in the amplification of genomic DNA with single, arbitrary primers resulting in different amplification products (Williams et al. 1990; Harris 1999). RAPD does not need large amounts of DNA for amplification reactions; neither requires previous knowledge of DNA sequences, which can be very useful when working with wild plant species with unknown genome; it is a simple, fast and more economical method comparing to other molecular markers (Harris 1999; Martín et al. 2008; Poczai et al. 2013). Besides, RAPD has advantages over specific markers [such as SSR (simple sequence repeat) or allozymes] for detecting genotyping variation due to the wider region of the genome they sample (Perez et al. 2015).

There are several studies reporting the use of RAPD to assess the genetic diversity of

Plantago species, such as *P. ovate* (Singh et al. 2009), *P. major* (Zubair et al. 2012) and others (Samantaray et al. 2010). Recently, Ferreira et al. (2013) reported the genetic diversity of *P. algarbiensis* and *P. almogravensis* (one population each) using RAPD and Inter-simple sequence repeat (ISSR) markers to provide information for their conservation but also to clarify their taxonomy, since in some literature both species are considered as one (Pedrol 2009). In this study we set out to evaluate the genetic diversity of the three known populations of *P. algarbiensis* and provide additional knowledge for the protection and management of this species.

Materials and methods

Plant material - Seeds were collected from three known wild populations in the Algarve region, Portugal (Algoz, Tunes and Gambelas) (Figure 1B). The number of individuals is estimated to be several thousand in Algoz population and less than 10,000 in Tunes population (ICN 2007). There are no official data concerning the number of individuals in Gambelas population, but the area of occupancy is lower than any of the other populations. Collection was made from 10 to 30 individuals randomly selected from each population and the distance between the individuals sampled was at least 1m. Seeds were germinated according to Martins et al. (2012). In short, for each population four replicates of 25 seeds were sown in glass Petri dishes on 0.6% (w/v) agar (Duchefa, The Netherlands) without nutrients and germinated at 15ºC with a 16 h light/ 8 h dark photoperiod (cool white fluorescent lamps, 69 μmol m^{-2} s^{-1}). Approximately eight weeks after sowing, the germination percentage was between 95 and 100% and 20 seedlings from each population were randomly selected and used for analysis.

DNA extraction and RAPD amplification - DNA was extracted from the seedlings

according to a slightly modified protocol described by Gawel and Jarret (1991). In short, seedlings were cut and placed on sterile eppendorf tubes and powdered with the help of a tissue lyser (Qiagen, Germany). Samples were incubated with CTAB (cetyl trimethylammonium bromide) buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl, 1% (p/v) PVP-40 (polyvinylpyrrolidone) and 0.2% β-mercaptoethanol] at 65ºC for 1 h. A chloroform/isoamyl alcohol (24:1) extraction was performed and DNA was precipitated by adding cold isopropanol (-20ºC). The precipitate was washed with 70% ethanol. The pellet was redissolved in 100 µl of sterile distilled water and RNA was removed by digestion with DNase-free RNase A. The DNA was quantified in a 0.8% agarose gel. Twelve arbitrary primers from Operon Technology (Alameda/CA, USA) were tested for RAPD amplification. Ten were selected to evaluate the genetic variability of the samples: OPO-5 (5' CCCAGTCACT 3'), OPO-7 (5' CAGCACTGAC 3'), OPO-10 (5' TCAGAGCGCC 3'), OPO-20 (5' ACACACGCTG 3'), OPF-1 (5' ACGGATCCTG 3'), OPF-3 (5' CCTGATCACC 3'), OPF-4 (5' GGTGATCAGG 3'), OPF-10 (5' GGAAGCTTGG 3'), OPE-19 (5' ACGGCGTATG 3') and OPA-11 (5' CAATCGCCGT 3'). DNA amplification reactions were performed in 25 µl reaction volume, containing approximately 10 ng template DNA, 0.4 or 0.8 µM primer, 5 µl 5x MyTaq reaction buffer and 1 U MyTaq DNA polymerase (Bioline, UK). Amplifications were performed in a thermal cycler (Eppendorf, Germany) with the following program: one cycle of 1 min at 95ºC, 35 cycles of 15 s at 95ºC, 15 s at 37ºC and 10s at 72ºC, and a final cycle of 10min at 72ºC. Amplification products were separated in a 1.5% (w/v) agarose gel in 1x TBE (Tris-borate-EDTA) buffer, followed by staining with ethidium bromide. The gels were visualised and photographed under UV light. Molecular weights were estimated by reference to a 100 Base-Pair Ladder (GE Healthcare, UK). All the amplifications were repeated at least twice and only bands reproducible in both PCR runs

were considered for analysis.

Data analysis - Amplified fragments from the RAPD analysis were scored as present (1) or absent (0). Genetic similarities were calculated using the Jaccard similarity coefficient (1908). The resultant matrix was subjected to cluster analysis by the unweighted pair group method analysis (UPGMA) and a dendrogram was constructed from the clustering results. These analyses were performed using the program NTSYS-PC version 1.80 (Rohlf 1992).

The data matrix resulting from the presence/absence of RAPD fragments was analysed using GenAlEx version 6.5 (Peakall and Smouse 2006, 2012) to estimate Nei's gene diversity (*h*) (Nei 1973) and to perform the analysis of molecular variance (AMOVA) (Excoffier et al. 1992).

From AMOVA analysis, Φ_{PT} estimator (which is an analogue of Fst) was calculated. Φ_{PT} estimates the proportion of the variance among populations. The use of Φ_{PT} values allowed for the estimation of the effective number of migrants (*Nm*) between populations [$Nm = ¼ (1 / \Phi_{PT} - 1)$] as an estimator of gene flow (Wright 1951; Freitas and Brehm 2001).

In addition, genetic diversity was estimated using Shannon's information measure, $H' = -\sum p_i \log_2 p_i$ (Lewontin 1972), where p_i is the frequency of a given RAPD fragment. Shannon index was calculated for two levels: the average diversity within populations (H'_{pop}), and the diversity within species (H'_{sp}). The proportion of diversity within populations was then estimated as H'_{pop}/H'_{sp}, and the proportion of diversity among populations as $(H'_{sp} - H'_{pop})/H'_{sp}$.

RESULTS

The RAPD profile - The ten primers used to amplify the 60 DNA samples from the three populations of *P. algarbiensis* (20 samples per population) produced 145 markers, ranging in size from approximately 290 to 1600 bp. From these markers, 116 were polymorphic (80%) and 29 (20%) were present in all individuals.

At the species level, 124 markers (85.52%) were shared by the three populations. Tunes population presented the highest polymorphism percentage (73.68%) and Algoz population the lowest (67.67%) (Table 1). Four markers (2.76%) were specific to Algoz population, six (4.14%) were only found in Gambelas population and two (1.38%) were exclusive to Tunes population (Table 1). None of these markers was monomorphic within the corresponding population.

Cluster analysis - In the dendrogram obtained from the RAPD data (Figure 2), all individuals presented a unique RAPD phenotype.

The cluster analysis revealed two major groups, which clustered according to their geographical locations. The samples from Algoz and Tunes populations, which are geographically closer, were grouped together (cluster I) and separated from Gambelas population (cluster II), which is located further to the east and closer to the coast in the Algarve region. According to the scale in the dendrogram, the two clusters were split at approximately 66% similarity.

Within cluster I, all individuals from Algoz population (except PA7 and PA19) clustered together in the same subgroup with a genetic similarity of 68.5%. The closest individuals in this group, PA14 and PA18, shared 85% similarity. Individuals from Tunes population, also included in cluster I, presented different levels of similarity between them. A subgroup of 12 individuals, Tunes I, was genetically closer to the Algoz subgroup, 68%, while the remaining 8 individuals, Tunes II, were 67% similar to Algoz and Tunes I. PT12

and PT13 were the closest individuals in Tunes population, sharing 79.5% similarity. Cluster II included individuals from Gambelas population (except PG18) with a similarity of approximately 67%. The highest homogeneity was between PG11 and PG13 with 86% similarity.

There were three individuals that were not grouped within their respective population: individual PG18, Gambelas population, was included in cluster I with Tunes population; and individuals PA7 and PA19, Algoz population, presented a similarity below 66% with all the other individuals and were not included in the two major clusters.

Genetic diversity **-** Genetic diversity in *P. algarbiensis* populations (Table 2) was estimated through Nei's gene diversity (*h*) (Nei 1973) and Shannon's information measure (*H'*) (Lewontin 1972). The Nei's gene diversity (*h*) ranged from 0.205 in Algoz population to 0.219 in Tunes. Similar results were obtained with Shannon's index, the lowest level of within-population variability was found in Algoz (H' = 3.222) and the highest diversity in Tunes (H' = 3.382). The mean diversity within species (H'_{sp}) and the mean diversity within populations (H'_{pop}) that resulted from the estimates of Shannon's index of phenotypic diversity was 3.845 and 3.311, respectively. The proportion of the diversity within populations was 86.12%, whereas the diversity among populations resulted in 13.88% of the total diversity. Estimation of genetic variation by AMOVA revealed similar values: 86.00% of total variation was found within populations and 14.00% among populations.

Relationships between populations **-** The distances among populations (Φ_{PT}) obtained from the AMOVA analysis are shown in Table 3. Φ_{PT} values ranged from 0.094 between Algoz and Tunes, to 0.175 between Algoz and Gambelas (Table 3). Each population was

clearly differentiated, since all distances between pairs of populations were significantly different from zero. Geographic distance can be correlated to the genetic differentiation among populations: the geographically closest populations (Algoz and Tunes) had the lowest genetic differentiation and the farthest populations (Algoz and Gambelas) the highest. Therefore, the genetic differences between populations of *P. algarbiensis* can be explained by geographic movement. N_m values were higher than 1.0 (Table 3), suggesting a high level of gene flow between populations. Gene flow was inversely proportional to the distance between populations and can also be correlated to the geographic distances between pairs of populations.

Discussion

The genetic diversity of three wild populations of *P. algarbiensis* was studied in the present work using RAPD markers. The high levels of polymorphism detected confirm that these markers are highly informative for the study of genetic parameters of *P. algarbiensis* populations. In addition, the analysis of these markers by different methods revealed very similar interpretations of the genetic structure of the populations studied. RAPD, due to their arbitrary nature, allows high variability detection compared to specific markers, since a higher genome proportion is analysed. For example, in the genetic study of the endangered endemism *Helianthemum juliae* RAPD markers showed differences among populations that allozymes did not distinguish (Pérez et al. 2015). On the other side, the dominant character of RAPD could lead to an underestimation of genetic diversity (Lee et al. 2002), although, in summary, RAPD markers can be considered appropriate to study genetic diversity in wild species, as in many instances no sequence information is available for the use of codominant markers. RAPD markers were also used to detect genetic differences between and within *P. major* populations (Zubair et al.

2012), the genetic variability of *P. ovata* accessions (Singh et al. 2009) and genetic relationships of several other *Plantago* species (Samantaray et al. 2010).

At the species level, the genetic diversity of *P. algarbiensis* populations, although geographically closed, can be considered high, as the results showed 80% of polymorphic bands. Ferreira et al. (2013) obtained percentages of polymorphic loci of approximately 74 and 61% for *P. algarbiensis* and *P. almogravensis*, respectively, in a study comparing the genetic diversity of the two species, using RAPD and ISSR markers. These results are contrary to the general view that rare species are considered to maintain low levels of genetic diversity (Hamrick and Godt 1989; Gitzendanner and Soltis 2000). *Tuberaria major*, another endemic species of the Algarve region, also presented high levels of genetic diversity, 98% of polymorphic bands using ISSR markers (Trindade et al. 2012). The *P. algarbiensis* population studied by Ferreira et al. (2013) was Algoz. They obtained, considering only RAPD data, higher percentage of polymorphic loci (83.7%) than in our study (67.7%), with the same number of primers and obtaining similar number of markers. These differences could be due to the number of individuals sampled.

Geographic distance can be correlated to the genetic differentiation among populations, the geographically closest populations (Algoz and Tunes) had the lowest genetic differentiation and the farthest populations (Algoz and Gambelas) the highest. The dendrogram obtained in the present study clearly distinguished *P. algarbiensis* populations. Individuals from Gambelas population, which is 32 and 29 km distant from Algoz and Tunes populations, respectively, formed a distinct group, sharing 66% similarity; while individuals from the other two populations, which are distant from each other 5 km, clustered together in the same group. Within this group, samples from Algoz population formed a well-defined sub-group and Tunes individuals were included in two different sub-groups. The high similarity found between individuals from Algoz and

Tunes can be explained by their geographic closeness. However, geographical range and population differentiation not always are correlated; environmental differences across the range of a species may be the origin of population differentiation (Loveless and Hamrick 1984). In this case the short distance between both populations is in accordance with a homogeneous environment.

The values that resulted from both the Nei's gene diversity (*h*) (Nei 1973) and Shannon's information measure (Lewontin 1972) analysis were in agreement, revealing a higher genetic variability in Tunes population than in the other populations. As for the study of the structure of the populations, obtained from both Shannon's index and AMOVA, a high proportion of genetic variation in *P. algarbiensis* occurs within populations (86%) than among populations (14%). Several factors can influence genetic diversity: migration, natural selection, genetic drift and reproductive system. Genetic differentiation among populations is promoted by natural selection and genetic drift, while allogamy and migration increase the levels of diversity within populations (Gómez-Gómez et al. 2012). The type of reproductive system in plants can therefore strongly affect the genetic diversity within and between populations. Mixed or outcrossing species present higher genetic variability within populations and less differentiation among populations than selfing species (Nybom and Bartish 2000; Nybom 2004). To the best of our knowledge, *P. algarbiensis* reproductive system is unknown, although according to our result would be presumable an outcrossing system of the species. Most *Plantago* species are described as allogamous (Pedrol 2009); however, the genus *Plantago* also comprises species with a wide range of mating systems, from inbreeders to obligate outcrossers (Zubair et al. 2012). *P. major*, for instance, is an inbreeding species and a recent study demonstrated that the genetic diversity within populations of this species was lower than between populations (Zubair et al. 2012).

Geographic distribution can also influence the genetic diversity of populations. The overgeneralization is that species with a wide geographic distribution have higher genetic diversity. Hamrick and Godt (1989) reported that endemic and narrowly distributed species exhibit significantly lower diversity than widespread species. However, Gitzendanner and Soltis (2000) verified that there is no association: some rare species have reduced genetic variation and others keep equal or higher levels of diversity compared to widespread species. Nybom and Bartish (2000) also found no correlation between geographic range and within population diversity. In the particular case of *P. algarbiensis*, as previously stated, the three populations are spread over a relatively small area and therefore geographically close. The genetic distances between populations, obtained in the AMOVA analysis, although differentiated each population, revealed a low genetic differentiation among populations. This fact suggests a high gene flow among populations, which was corroborated by N_m values. The level of differentiation among populations appears to be associated with the geographic distance between populations of *P. algarbiensis* and can therefore be explained by geographical trends.

Genetic diversity results from the genetic differences between individuals of a variety or population of a species (Brown 1983; Ramanatha Rao and Hodgkin 2002). The maintenance of genetic diversity in natural populations is one of the main objectives of nature conservation (Trindade et al. 2012). The determination of the extent and distribution of the different aspects of the genetic diversity in a species and structure of its natural populations is therefore an important and essential tool to create conservation strategies (Holsinger and Gottlieb 1991; Ramanatha and Hodgkin 2002). In addition, for an effective conservation, the different methods applied should preserve as much diversity as possible (Trindade et al. 2012; Ferreira et al. 2013).

Overall, the diversity measured in this work can be considered high. However, due to the

low populations' size and number, and their continuous declining, this level of diversity may be difficult to maintain. Genetic drift and inbreeding are some of the consequences associated with small and isolated populations that are responsible for a reduction of the genetic diversity (Ferreira et al. 2013). On the other hand, the survival of a species may not be directly related to its levels of diversity (Gitzendanner and Soltis2000) and in this particular case, the main threat comes from human activities. Though *P. algarbiensis* is a protected species (ICN 2007), other conservation strategies are urgently required to avoid the decrease of individuals and populations and ultimately to preserve this species.

From the results presented here, and taking into account the management of the endangered populations, some actions should be regarded. Due to the low genetic differentiation found among populations and the small area of occupancy of this species, the three populations could be managed as a same unit. Nevertheless, *ex situ* conservation strategies, such as seed collection and storage, *in vitro* propagation or cryopreservation, should consider the different populations. In addition, it is also important to define a strategy that includes the restoration of natural populations, using *in vitro* propagated plants. This approach could be easily accomplished since a protocol for *in vitro* propagation of *P. algarbiensis* using seeds as explants has been already developed and the plants produced were successfully transferred to field conditions exhibiting normal development (Gonçalves et al. 2009). Also the seed germination requirements were studied (Martins et al. 2012). The use of seeds as starting material allows the preservation of a wider range of genetic information of the species and a way to increase the genetic diversity and the adaptation capacity of *P. algarbiensis* would be by transfer of germplasm between populations. As a rule, the reintroduction of plants propagated *ex situ* should be primarily made in the populations with less variability, which in the present work were Algoz and Gambelas. However, due to the small number of individuals and

the endangered status of the species, the reintroduction of *ex situ* propagated plants in Tunes population should also be prioritized.

In conclusion, RAPD markers provided valid and useful genetic information about the structure of *P. algarbiensis* populations. The implementation of conservation approaches should comprise both *in situ* and *ex situ* methods and genetic markers are a valuable tool to develop appropriate strategies. The measures presented here, along with the legal protection hold by this species, can contribute to increase the number of individuals in all populations and to preserve the species genetic resources and finally to prevent its extinction.

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Figure legends:

Figure 1. *Plantago algarbiensis* wild plant from Algoz population (A) and map with the locations of the three populations studied in the present work (B).

Figure 2. Dendrogram based on UPGMA showing the genetic similarity (Jaccard coefficient) among *Plantago algarbiensis* samples obtained by RAPD analysis. PA: Algoz; PT: Tunes; PG: Gambelas.

Table 1. Total number of markers and number of polymorphic and population specific markers generated by RAPD primers for each *Plantago algarbiensis* population.

Population	**No. markers**	**Polymorphic markers**	**Polymorphism (%)**	**Population specific markers**
Algoz	133	90	67.67	4
Gambelas	137	93	67.88	6
Tunes	133	98	73.68	2

Table 2. Genetic variation of *Plantago algarbiensis* populations detected by RAPD markers.

Population	***H'***	***h***
Algoz	3.222	0.205
Gambelas	3.329	0.215
Tunes	3.382	0.219

H' - Shannon´s information measure (Lewontin 1972); *h* - Nei's gene diversity (Nei 1973).

Table 3. Genetic differentiation/gene flow (N_m) (below diagonal) and geographical distances (km) (above diagonal) between the three populations of *Plantago algarbiensis* studied.

Population	Algoz	Gambelas	Tunes
Algoz	-	32	5
Gambelas	0.175/2.353	-	29
Tunes	0.094/4.815	0.138/3.125	-

Figure

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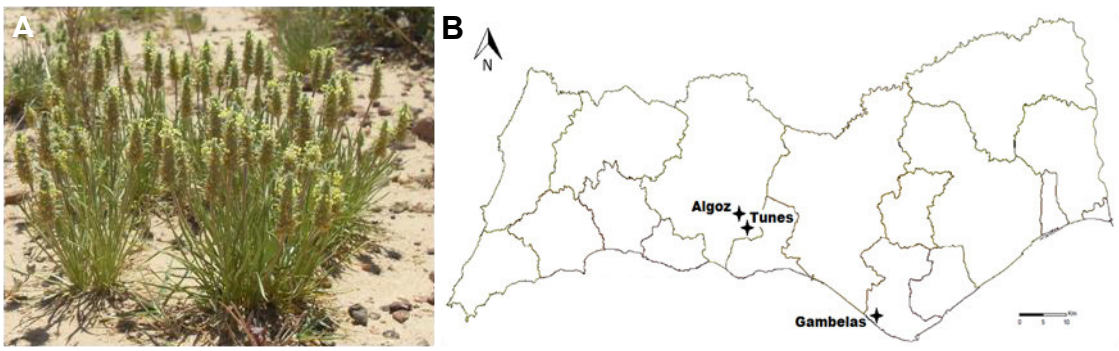

Figure

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