

Morphological and genetic characterization of four populations of *Corema album* (L.) D. Don

João Miguel Antunes Jacinto

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Engenharia Agronómica

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Resumo

Corema album (L.) D. Don 1830, é um arbusto dioico da família Ericaceae, que se desenvolve costa Atlântica de Portugal e Espanha, em ecossistemas dunares e de cobertos costeiros de pinheiro. Existe um potencial por parte desta espécie para integrar o mercado dos pequenos frutos.

Na primeira parte deste trabalho, oitenta plantas provenientes de Monte Clérigo, Comporta, Aldeia do Meco e Dunas de Quiaios foram amostradas. A fenologia reprodutiva de seis plantas da Aldeia do Meco foi seguida desde do mês de Março até Agosto. Finalmente, vinte e nove características morfológicas foram avaliadas, entre as quatro populações.

Na segunda parte do trabalho, seis ISSR foram utilizados para caracterizar a diversidade genética entre populações. Quinze caracteres morfológicos foram escolhidos e a sua diversidade foi avaliada, através de análises aglomerativas. Cinco variáveis bioclimáticas foram utilizadas para procurar correlações com os dados morfológicos e moleculares.

No primeiro trabalho foram encontradas diferenças a nível da fenologia das plantas estudadas na Aldeia do Meco. Foi também encontrada diversidade morfológica dentro e entre populações.

O segundo trabalho demonstrou uma falta de agregação populacional, por parte das características morfológicas, mas o mesmo não ocorreu com os dados moleculares. No entanto, uma baixa correlação foi encontrada entre eles.

A ACC realizada entre dados morfológicos e bioclimáticos demonstrou que aproximadamente 50% da variação morfológica é explicada pelas variáveis bioclimáticas, no entanto a sua correlação não foi significativa. A mesma análise entre dados moleculares e bioclimáticos, obteve uma correlação positiva, de baixo valor, revelando que o ambiente também tem influência na genética das espécies.

Ambos os trabalhos foram importantes para aumentar o conhecimento sobre *C. album*, tendo em vista o possível programa de pré-melhoramento.

Palavras-chave: *Corema album*, camarinhas, caracterização morfológica, caracterização molecular, índices bioclimáticos

Abstract

Corema album (L.) D. Don 1830, is a dioecious shrub from the Ericaceae family, that can be found in the coastal Atlantic dune systems and coastal pine tree forests of Portugal and Spain with a potential to integrate the berry market.

For the first work eighty plants were sampled from Monte Clérigo, Comporta, Aldeia do Meco and Dunas de Quiaios. Six plants from Meco were chosen to follow their reproductive phenology from March to August. Finally twenty-nine morphological traits were evaluated, both from vegetative and reproductive organs, in order to compare them between populations.

On the second part, six Inter Simple Sequence Repeats (ISSRs) were used to characterize the genetic diversity among populations. Fifteen morphological traits were chosen and their diversity was evaluated, through cluster analyses. Five bioclimatic indexes were used to seek correlations between morphological and molecular data.

On the first work, differences between plant phenology from Aldeia do Meco were found. Morphological diversity was found between and within populations.

The second work showed no geographic aggregation, regarding morphological data. The same did not occur with molecular data. However, when comparing the last two types of data, a low correlation between them was found.

The CCA between morphological traits and bioclimatic indexes showed that almost 50% of the morphological variation was explained by the bioclimatic data; however, with no the significant correlation. The same analyses between molecular and bioclimatic data, showed a significant correlation, but with a low value, revealing that the environment also as an influence in species genetics.

Both these works were important to establish new insights about *Corema album*, and to understand more about this species, always aiming to a possible pre-breeding program.

Key words: *Corema album*, White crowberry, morphological data, molecular data, bioclimatic variables

Resumo alargado

A espécie *Corema album* (L.) D. Don 1830, conhecida como camarinheira ou camarinha, pertence à família das Ericaceae e é um arbusto dióico endémico de ecossistemas dunares da costa atlântica de Portugal e Espanha, podendo mesmo ocorrer em cobertos arbóreos onde domina o pinheiro bravo. *C. album* possui duas subespécies, a que se encontra na Península Ibérica (*Corema album* subsp. *album*) e uma nativa do arquipélago dos Açores (*Corema album* subsp. *azoricum*). O género a que pertence a camarinha apenas alberga mais uma espécie, *Corema conradii*.

Esta espécie desenvolve umas bagas de distinta cor branca em forma de drupa, possuindo um sabor relativamente doce e agradável. Estudos bioquímicos revelaram que estes frutos são ricos em antioxidantes, bem como em outros compostos com interesse para a saúde.

Devido ao conhecimento das características do fruto, tem havido um interesse crescente na intensificação e diversificação da cultura desta espécie, a fim de aumentar a produção e promover a comercialização no mercado dos pequenos frutos.

O estabelecimento desta espécie como uma nova cultura que reúna as características desejáveis à comercialização constitui um grande desafio a ser enfrentado e implica a aplicação do conhecimento obtido em vários domínios de investigação que ofereçam suporte a um programa de melhoramento.

Neste sentido, vários estudos têm sido desenvolvidos, desde a fenologia vegetativa e reprodutiva, a reprodução sexuada, anatomia e dimorfismo sexual, a propagação vegetativa e seminal, a ecologia e ecofisiologia, o estabelecimento das plantas em cultura, no sentido de aprofundar o conhecimento sobre a morfologia da espécie.

Outro domínio prioritário a desenvolver relaciona-se com o dimorfismo sexual da espécie e com a necessidade de identificar, precocemente, as plantas femininas e masculinas. Para a *Corema album*, não existem caracteres morfológicos e fenotípicos que possam diferenciar os indivíduos femininos e masculinos na fase jovem, o que limita a adoção de estratégias de melhoramento que envolvam a seleção ou amostragem de plantas na fase ainda não reprodutiva. Nesta espécie dióica, a identificação do sexo das plantas no campo só pode ser visualizada na época da primeira floração que ocorre após 5 a 6 anos, quando de propagação seminal, o que implica a necessidade de aumentar o número de plantas no campo, o que termina por contribuir para o encarecimento dos custos de produção.

Neste sentido, foi já desenvolvida uma abordagem molecular com o objetivo de identificar um marcador genético que permita a identificação do sexo das plantas num estágio inicial de desenvolvimento, ou seja, antes da floração.

Para o desenvolvimento do programa de melhoramento, é necessário ainda avaliar a potencialidade desta espécie através da caracterização da diversidade genética dirigida ao melhoramento, identificar correlações quer entre a variação genética e a variação fenotípica quer entre a variação geográfica e a climática.

Neste trabalho analisaram-se duas áreas, a caracterização morfológica e a diversidade genética, porque se tratam de áreas complementares e que contribuem com informação

fundamental para a tomada de decisões relativamente ao programa de melhoramento e também conservação desta espécie.

Assim, em quatro populações distintas localizadas ao longo da costa oeste de Portugal: Aldeia do Meco, Dunas de Quiaios, Comporta e Monte Clérigo, na época de floração foram selecionadas e georreferenciadas 80 plantas femininas (20/população). Nesta época, foram colhidas folhas para a análise molecular utilizando marcadores ISSR, e foram também analisadas características morfológicas a nível vegetativo (altura, dois diâmetros e volume da planta; número de crescimentos vegetativos; número médio de ramos; tamanho médio dos crescimentos anuais; comprimento, largura e área da folha; número de folhas por verticilo; tamanho do pecíolo; cor do pecíolo; forma do pecíolo e da folha; tamanho da abertura da folha provocada pelo enrolamento) e reprodutivo (número de inflorescências; número médio de inflorescências; número de flores; rácio entre número de flores e inflorescências). Na época de frutificação foram colhidos os frutos, tendo sido separados por calibres; registado o peso dos frutos pelos respetivos calibres; calculado a percentagem de frutos brancos e translúcidos e calculado o rácio peso seco/fresco dos frutos. No total foram analisadas 29 caracteres morfológicos. Com base nos resultados obtidos foram analisadas as diferentes correlações.

Para aprofundar o conhecimento sobre a diversidade morfológica dentro e principalmente entre as populações, a análise de algumas características morfológicas e fenológicas, na plantas, foi realizada de acordo com a distribuição espacial (Norte, Este, Sul e Oeste) em cada indivíduo.

Foram ainda efetuados cortes histológicos a secções da folha e do caule, para verificar a adaptabilidade desta planta ao ambiente em que esta se encontra.

A realização deste estudo permitiu concluir que:

Relativamente à fenologia reprodutiva, foram registadas diferenças entre as plantas que se encontravam na duna sul e norte da praia da Aldeia do Meco. No entanto, no decorrer da maturação dos frutos, existe um estágio de desenvolvimento em ocorre uma homogeneização, não se detetando diferenças entre os quatro quadrantes cardinais nas plantas. A comparação entre as quatro populações também demonstrou haver uma antecipação do desenvolvimento nas plantas localizadas a sul do que nas plantas localizadas a norte.

Através da observação dos cortes histológicos, diversas estruturas como o enrolamento da folha, a cutícula e os tricomas demonstraram a adaptação desta espécie ao ambiente em que se encontra.

Relativamente à morfologia, não foram encontradas diferenças significativas entre os caracteres analisados entre os quatro pontos cardiais. No entanto, a diversidade morfológica dentro das populações foi maior na Aldeia do Meco e na Comporta, e foram encontradas diversas diferenças entre populações em ambos os níveis vegetativo e reprodutivo. A população das Dunas de Quiaios destacou-se das restantes no desenvolvimento vegetativo, reduzindo o investimento na reprodução que se reflete na produção de fruto. Esta diferença parece estar relacionada com o coberto arbóreo, uma vez que esta população se encontra num pinhal e o coberto arbustivo teve de investir mais no seu desenvolvimento.

A análise de componentes principais (ACP) também demonstrou que algumas características estudadas estavam mais associadas a certas populações que a outras, nomeadamente a percentagem de frutos translúcidos e o número de ramos à população de Monte Clérigo, bem como a média dos crescimentos vegetativos anuais e o rácio peso seco/fresco dos frutos à população das Dunas de Quiaios.

Relativamente à avaliação da diversidade genética foram utilizados seis marcadores ISSR, a análise aglomerativa mostrou que as quatro populações se agruparam em cinco grupos, demonstrando uma aglomeração por localização geográfica. Verificou-se também que as populações com maior diversidade genética foram a Comporta e a Aldeia do Meco, enquanto que as que tiveram menos diversidade foram, Monte Clérigo e Quiaios. As populações com maior diversidade genética, foram aquelas que também apresentaram maior diversidade morfológica.

A análise de variância molecular (AMOVA) demonstrou que a variância é maior entre populações (62.4%), do que dentro de populações, onde foi de 37.6%.

Com o intuito de esclarecer a origem da elevada variabilidade morfológica foram avaliadas as correlações entre cinco variáveis bioclimáticas com as variáveis morfológicas e os dados moleculares, a fim de avaliar a variação atribuível ao genoma e/ou influências do meio ambiente, recorrendo-se a análises canónicas de correspondência (ACC).

Os resultados mostraram que as variáveis bioclimáticas explicam cerca de 50 % da variação morfológica, mas a correlação entre estas duas bases de dados não foi significativa. Provavelmente outros fatores para além da temperatura e precipitação; parâmetros com que são calculadas estas variáveis bioclimáticas; poderão ter uma correlação mais significativa como é o caso do vento ou a presença de ensombramento nas plantas estudadas.

Relativamente aos dados moleculares, os resultados mostraram que a correlação com as variáveis bioclimáticas foi significativa, embora com valores baixos, demonstrando que o ambiente também tem influência na genética da espécie.

Palavras-chave: *Corema album*, camarinhas, caracterização morfológica, caracterização molecular, índices bioclimáticos

Index

Acknowledgments	v
Resumo	vii
Abstract	ix
Resumo alargado	xi
Figure Index	xvii
Tables	xxi
Abbreviations and Symbols	xxiii
Scientific Binomials list	xxv
Introduction	1
Aim of this study	2
Dissertation Structure	2
Chapter 1: <i>Corema album</i> - a multidisciplinary insight	5
The Species and the agronomical approach	5
Taxonomy and Morphology	5
Ecophysiology	7
Geographical distribution	7
Ecosystem structure	8
DNA-based markers - The case of inter simple sequence repeat (ISSR)	9
Bioclimatology	10
References	12
Chapter 2: Variability of agro-morphological traits in <i>Corema album</i> (L.) D. Don in four coastal Atlantic populations	17
Abstract	17
Introduction	18
Materials and Methods	18
Biological Material	18
Phenological Data	19
Histological Data	19
Morphological Data	20
Statistical Analyses	22
Results and discussion	22
Plant Phenology	22
Histological data	24
Morphological data	25

Conclusions	32
Sources of Funding.....	33
Acknowledgments.....	33
References	33
Chapter 3: Genetic Divergence of <i>Corema album</i> genotypes revealed by DNA-based markers and agro-morphological traits	39
Abstract	39
Introduction.....	40
Material and Methods	40
Plant material and sampling sites	40
Molecular data: DNA isolation and amplification.....	41
Morphological data	42
Bioclimatic Indexes.....	42
Statistical Analyses	43
Results	44
Discussion	49
Conclusion.....	51
Sources of Funding.....	51
Acknowledgments.....	51
References	52
Chapter 4: Conclusion and Perspectives.....	57

Figure Index

Chapter 1

Fig. 1 - Left: male *Corema album* inflorescences in a late state of development. Right: female inflorescences, in a late state of flowering and beginning of sprouting..... 6

Fig. 2 - Current and potential distributions of *Corema album* according to several authors. It is also shown archaeological evidences of this species (Lopez-Doriga *et al.*, 2018)..... 8

Chapter 2

Fig. 1 - Location of studied populations sampled, with their annual temperature (°C) and precipitation (mm) variation, as well as their average temperature and precipitation [22].....19

Fig. 2 - Phenological stages regarding six Meco plants in the four cardinal quadrants from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.....22

Fig. 3 - Phenological stages regarding the mean between six Meco plants in the four cardinal quadrants from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.23

Fig. 4 - Phenological stages regarding the mean between the four cardinal quadrants of the six Meco plants, from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.23

Fig. 5 - Phenological stages regarding the mean between the six plants of the four populations, from the 16th of March 2018 (Week 11) and to the 20th of September 2018 (Week 40). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 71 was fruits with 10% of their total size; number 73 was fruits with 30% of their total size; number 75 was the moment when fruits had half their size, number 76 was fruits with 60% of their total size; number 79 was the moment when they achieved their full size and 87 was the beginning of senescence.....24

Fig. 6 - A: Transversal histological section of a stem of a plant which had four leaves per whorl, appearing the vascular system in a square shape. **B:** Transversal histological section of a stem of a plant which had three leaves per whorl, appearing the vascular system in a triangular shape. Both preparations were coloured with Astra Blue.24

Fig. 7 - A: Longitudinal leaf section, showing the leaf compartmentalization in chambers. B: Transversal section of a leaf, showing the groove that separates both extremities. st: stomata. Either both preparations were coloured with Astra Blue.	25
Fig. 8 - A: Transversal leaf section showing detail leaf structure with Sudan Black, showing the lipid content of the cuticle. B: Transversal leaf section showing detailed leaf structure coloured with Astra Blue. st: stomata; cu: cuticle; tm: capitate glandular trichomes; tu: erect uniseriate non-glandular trichomes; ox: druses calcium oxalate crystals.	25
Fig. 9 - A: Plant volume in the four populations. B: Average length of the annual growths in the four populations. C: Average branching number in the four populations (n=20).	27
Fig. 10 - Number of leaf per whorl in <i>Corema album</i> . A: Three leaves per whorl; B: Three and four leaves per whorl; C: Four leaves per whorl; D: Four and five leaves per whorl; E: Five leaves per whorl.	27
Fig. 11 - A: Average leaf width between the four populations. B: Average leaf length in the four populations. C: Average leaf area in the four populations (n=20).	28
Fig. 12 - A: Petiole length between the four populations. B: Groove distance in abaxial leaf surface between the four populations (n=20).	28
Fig. 13 - A: Number of inflorescences between the four populations. B: Number of flowers between the four populations (n=20).	29
Fig. 14 - A: Number of fruits on each calibre between the four populations. The variables "x.10.25"; "x.7.5" and "xR", represent the fruit calibres "NFCM"; "NFCS" and "NFCR", being the "x" correspondent to each population (M: Meco; C: Comporta; MtC: Monte Clérigo and Q: Quiaios). B: Weight of the fruits per calibre between the four populations. The variables "x.10.25"; "x.7.5" and "xR", represent the fruit calibres "WFM"; "WFS" and "WFR", being the "x" correspondent to each population (M: Meco; C: Comporta; MtC: Monte Clérigo and Q: Quiaios) (n=20).	29
Fig. 15 - Dry and fresh weight ratio between the four populations (n=20).	30
Fig. 16 - Principal component analysis (PCA) showing the dispersion of the individual of the four populations sampled, as well as what were the traits that had the biggest influence on itch population. The variables "NFCM"; "NFCS"; "WFM" and "WFS" were represented as "NFC.10.25"; "NFC.7.5"; "WF.10.25" and "WF.7.5", respectively.	32

Chapter 3

Fig. 1 - Dendrogram obtained with UPGMA method using the Nei & Li coefficient for 71 plants of the four populations (r=0.729).	45
Fig. 2 - Principal Coordinates Analyses (PCoA) of the molecular data, which explained 22% of the variation amongst populations.	46
Fig. 3 - Dendrogram obtained with the UPGMA method using the Euclidian distance of the morphological data of the 71 plants. Each colour represents a cluster. (r=0.879).	47

Fig. 4 - Canonical correspondence analyses (CCA) between the morphological data and the bioclimatic data, showing that 46% of the variation of the morphological data was related to bioclimatic variables.48

Fig. 5 - Canonical correspondence analyses (CCA) between the molecular data and the bioclimatic data, showing that 23% of the variation of the molecular data was related to bioclimatic variables.49

Tables

Chapter 1

Table 1 - Indexes used in worldwide bioclimatic classification systems (Rivas- Martínez <i>et al.</i> , 1999).....	11
---	----

Chapter 2

Table 1 - Morphological traits observed for the characterisation of <i>Corema album</i>	20
--	----

Table 2 - Summary table with means and standard deviations (SD) of the only significant trait, number of vegetative growths, in the four populations and in all populations, in each cardinal quadrant.....	26
--	----

Table 3 - Summary table with means and standard deviations (SD) of the twenty three traits in the four cardinal populations.....	26
---	----

Table 4 - Spearman correlation matrix ($\alpha=0.05$) between the twenty three traits in this study in all the populations.....	31
--	----

Chapter 3

Table 1 - Origin (collection site), number of sampled individuals (n) and geographical coordinates of <i>C. album</i> populations.....	41
---	----

Table 2 - List of primers used for ISSR amplification, sequences and annealing temperatures.....	41
---	----

Table 3 - Morphological traits for the characterization of wild plants.....	42
--	----

Table 4 - Bioclimatic parameters and indexes applied to the study area, according to Rivas- Martínez <i>et al.</i> (1999).	43
---	----

Table 5 - Genetic diversity estimates of <i>C. album</i> populations including NB : number of bands; NPB : Number of polymorphic bands; PPB : Percentage of polymorphic bands; PIC : Polymorphism information content; MI : Marker index; RP : Resolving power and ISSR populations : Number of different ISSR profiles among populations and primers.....	44
--	----

Abbreviations and Symbols

BBCH-scale: Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie - scale

GA₃: Gibberellic acid

SSS: Spatial Segregation of Sexes

Δ₁₃C: Carbon-13 isotope

CO₂: Carbon Dioxide

ISSR: inter simple sequence repeat

PCR: Polymerase Chain Reaction

I_{tc}: Compensated Thermicity Index

I_o: Annual Ombrothermic Index

I_{os2}: Ombrothermic Index of the Summer Bimonth

I_{os3}: Ombrothermic Index of the Summer Trimester

I_{osc4}: Summer Compensated Ombrothermic Index

INIAV: Instituto Nacional de Investigação Agrária e Veterinária, I.P.

D_{mNS}: plant North-South orientation diameter

D_{mEW}: Plant West-East orientation diameter

H_t: Plant Height

V_l: Plant Volume

NI: Number of inflorescences

NV: Number of vegetative growths

TNF: Number of flowers in twenty cuttings

AI: Average number of inflorescences in twenty cuttings

RFI: Ration between number of inflorescences and number of flowers

ALL: Average leaf length

ALW: Average leaf width

ALA: Average leaf Area

NLW: Number of Leaves per whorl

LS: Leaf Shape

PL: Petiole Length

PS: Petiole Shape

PC: Petiole Colour

DG: Groove distance on the apparent abaxial leaf surface

ALG: Average length of annual vegetative growths

ANG: Average branching number

NFCM: Number of fruits with more than 10.25 mm in diameter

NFCS: Number of fruits between 10.25 and 7.5 mm

NFCR: Number of Rejected fruits

PTotBr: Percentage of white fruits

PTotTr: Percentage of translucent fruits

WFM: Weight of the fruits with more than 10.25 mm in diameter

WFS: Weight of the fruits between 10.25 and 7.5 mm in diameter

WFR: Weight of the rejected fruits

RP: Dry/fresh weight ratio

PCA: principal component analyses

st: stomata;
cu: cuticle;
tm: capitate glandular trichomes;
tu: erect uniseriate non-glandular trichomes;
ox: druses calcium oxalate crystals.
SD: Standard deviation
bp: Base pairs
DNA: Desoxyribonucleic Acid
dNTP: Deoxynucleotide triphosphate
GPS: Global Position System
TBE: Tris/Borate/EDTA Buffer
kb: kilobase pair
PIC: Polymorphism Information Content
RP: Resolving Power

MI: Marker Index
UPGMA: Unweighted Pair-group Method Analysis
PCoA: Principal Coordinates Analyses
AMOVA: Analysis of Molecular Variance
NF: Total Number of Fruits
WF: Total Fruit weight
CCA: Canonical Correspondence analyses
NB: Number of bands
NPB: Number of polymorphic bands
PPB: Percentage of polymorphic bands
SNP: Single Nucleotide Polymorphism
GBS: Genotyping by Sequencing
GWAS: Genome Wide Association Study

Scientific Binomials list

Corema album subsp. *album* (L.) D. Don

Corema album subsp. *azoricum* P. Silva

Corema conradii (Torr.) Torr. ex. Loudon

Arbutus unedo L.

Acacia melanoxylon R. Br. in W.T. Aiton

Acacia longifolia (Andrews) Willd

Acacia dealbata Link

Carpobrotus edulis (L.) N.E. Br

Vaccinium vitis-idaea L. 1753

Vaccinium stamineum L.

Vaccinium macrocarpon Ait.

Vaccinium corymbosum L.

Pinus pinaster Ait.

Rubia peregrina L.

Vaccinium myrtillus L. 1753

Vaccinium angustifolium Ait. 1789

Solanum pimpinellifolium L. 1755

Introduction

Corema album (L.) D. Don, known as 'white crowberry', is one of two species in the genus *Corema*, in the tribe Empetraceae, of the subfamily Ericoideae, of the Ericaceae family.

Corema album subsp. *album* occurs on the Atlantic coast of the Iberian Peninsula from Gibraltar to Finisterre, and subsp. *azoricum* in the Azores. There are large populations in the three large dune systems, Aspeillo in Donana National Park in the south of Spain, from Sines to Troia in south-west of Portugal, and from Nazare to Ovar in central-north Portugal. Disjunctive populations exist on cliffs and isolated sand dunes throughout the rest of the range (Castroviejo *et al.*, 1993; Lopez-Doriga, 2018).

Other berry crops can be found in the Ericaceae family, like the blueberries, which are already a well established culture in some parts of the world (Eck, 1988). *Arbutus unedo* L., the strawberry tree, also belongs to this family and some research has been done to turn this into a new crop (Mulas & Deidda, 1997; Celikel *et al.*, 2008; Gomes *et al.*, 2010; Ruiz-Rodríguez *et al.*, 2011).

The plant is a dioecious, perennial shrub, and the fruit, produced in summer, is a drupe shape berry (diameter 5–8 mm), white or pinkish white when ripe, with a mild acidic lemony flavor. These berries contain many anti-oxidants, flavinol, chlorogenic acid derivates and phenolic acid and low amounts of anthocyanins (León-González *et al.*, 2013; Andrade *et al.*, 2017b). These drupes could also be a very important source of fibbers and sugars (Andrade *et al.*, 2017a). These important characteristics can lead *C. album* to be a very interesting species to adapt as a new crop.

Historically these fruits have been eaten fresh for many centuries, and nowadays can be found in some markets, namely in Galicia. It was also used in traditional medicine for many purposes (Gonzalez, 2001).

Several research studies in vegetative and reproductive phenology, anatomy and sexual dimorphism, vegetative and seminal propagation, ecology and ecophysiology, have been performed on this plant, in order to deepen knowledge about the behavior and characterization of this species under agricultural production and for future inclusion in the small fruits market (Oliveira & Dale, 2012).

Some fundamental results have already been achieved. For example, a BBCH-scale to identify the phenological development/growth stages was established (Magalhães, 2015), adapted seminal (Lisboa, 2017; Santos, 2013) and vegetative propagation methods (Magalhães, 2015), were performed.

The origin of plant material seemed to affect the rates of success, either by seed germination or the rooting of the vegetative propagation.

Regarding to the molecular approaches for this species, a study using Inter Simple Sequence Repeat (ISSR) markers was carried out, in order to design a molecular marker related to plant sex identification in a young development (pre-flowering) stage (Nóbrega, 2016). This aspect is very important because it will allow cost reductions, associated with the

maintenance (nursery space, labour and production inputs) of the male plants, which are less interesting and needed in more reduced density.

Besides that, interesting plants from most promising genotypes can then become the starting material for a specific breeding programme.

However, in order to implement a breeding program for this crop, it is fundamental to evaluate the plant variability, either by morphological either molecular characterization (Aremu, 2017; Burgos *et al.*, 2018).

Aim of this study

The present investigation has been carried out to assess the level of phenotypic and genotypic variability associated to *Corema album* populations in Portugal for creating the first data base consisting of representative genetic material available to initiate a breeding program.

Thus, the distinct objectives were:

- I. Phenotyping all berry genotypes, located in the four *Corema album* populations for a number of traits including the ability to set fruits, growth habit, yield and fruit quality parameters;
- II. Determine the genetic diversity and relationships among the same populations along the west Portuguese coast;
- III. Establish correlations between all data.

Dissertation Structure

The current study comprises four chapters, with the results presented in the form of two scientific papers. Each paper follows the journal's specific guidelines consisting of an introduction, material and methods, results, discussion of results and literature cited.

Chapter 1 reviews the current knowledge of the species, with particular focus on the agronomic approach and the respective results, the taxonomical and morphological characterization, as well as its ecophysiology, the geographical distribution and its ecosystem structure, a review about ISSR-markers and bioclimatic indexes.

Chapter 2 analyzes the evaluation of the morphological variation between populations, using several plant traits, some of which with an agronomic potential and also the phenology of six female plants from one population using a BBCH-scale.

Chapter 3 focuses on the evaluation of the genetic diversity of *C. album* using ISSR markers and in the determination of the genetic and agro-morphological relationships among and within four populations.

The core body of the thesis will be divided into two scientific papers. The main goals of the first one will be to:

- Follow the phenology of 6 plants from Aldeia do Meco;
- Seek morphological diversity regarding vegetative and reproductive traits within and between populations.

The aims of the second scientific paper will be to:

- Seek molecular differences between and within populations, with the use of Inter Simple Sequence Repeat markers (ISSR);
- Seek a correlation between morphological and molecular data;
- Seek if the morphological data was influenced by bioclimatic.

Chapter 4 focus on the main conclusions and present suggesting future lines of research.

The insights provided by this work will hopefully help to choose the best provenance locations, as well as the best plants featuring interesting agronomic traits and if those traits will be maintained under new environmental pressures.

Chapter 1: *Corema album* - a multidisciplinary insight

The Species and the agronomical approach

The 'white crowberry' (*Corema album* (L.) D. Don), known in Portuguese as 'camarinhas', is a native plant that is starting to get some attention from the agronomic point of view, due to its edible fruits and their unique form and colour, resembling "white pearls". The crowberry flavour is considered to be mildly acid, with a lemony flavour, and the berries content in antioxidants is often mentioned as well (Oliveira & Dale, 2012; Andrade *et al.*, 2017).

Considering the agronomic possibilities related to reproduction and propagation, the development of different germination experiments was performed (Costa, 2011; Santos *et al.*, 2014; Magalhães, 2015 and Lisboa, 2017).

Some studies, mostly master dissertations, have been done to understand the reproduction and propagation of this species, either in a vegetative or seminal way.

Costa (2011) observed that the germination of *C. album* seeds was influenced by the stage of maturation of the fruit and that the chemical scarification with sulphuric acid is a promising method for improving seeds germination.

Regarding seed germination and different treatments, Santos (2013) showed there was a variation in site-to-site during the year and in different years. It was showed that the rupture of the seed coat and endosperm was two sequential events during the germination process. It was also suggested that seeds have morphophysiological dormancy. All results demonstrated that germination process and seedling survival were very low.

Regarding the vegetative propagation, important results indicated that vegetative growth in both male and female individuals was similar, and they grow from February to July, with different timings. Flowering was synchronous, from February to April. A BBCH-scale for this species was also proposed (Magalhães, 2015).

Seed morphology accessed by Lisboa (2017), lead to the conclusion that embryos do not have a morphological dormancy, due to the presence of formed cotyledons and that seeds had a structure in their epidermis (micropyle), in order to make gas and water exchanges with the environment. It was also showed that seeds submitted to a hot and cold stratification and with GA₃ application, had higher germination percentages. And finally that seeds can't tolerate high temperature before germination.

No studies or records of diseases and pests in *Corema album* (Oliveira & Dale, 2012).

Taxonomy and Morphology

The genus *Corema* only has two species, *Corema album* (L.) D. Don and *Corema conradii* (Torr.) Torr. ex. Loudon. *C. conradii* is found in eastern North America (Oliveira & Dale, 2012). Morphologically speaking, *C. conradii* compared with *C. album*, has smaller fruits, with no fleshy part and covered with elaiosomes (oily appendages associated with ant dispersal) (Martine *et al.*, 2005). Leaf size is different, *Corema album* has bigger leaves and some

differences regarding their morphology (McEwen, 1894). The species *Corema album* has two subspecies, subsp. *album* and subsp. *azoricum* P. Silva (Valdés *et al.*, 1987).

The genus *Corema* belongs to the tribe Empetraceae, which contains two other genera, *Ceratiola* and *Empetrum*, both morphologically very similar. Phylogenetic studies suggest that *C. conradii* might be a hybrid between ancestral populations of *C. album* and *Ceratiola* (Li *et al.*, 2002).

Several morphological aspects of *C. album* were described in Franco (1984) and Tutin (1972).

Although this is a dioecious plant, it was suggested that 1 to 4% of the male plants from some populations in the southwest of the Iberian Peninsula (Vila Real de Santo António and Doñana), had hermaphrodite inflorescences (Zunzunegui *et al.*, 2006). Its flowers are actinomorphic, in other words they can be divided in 3 or more identical radially symmetrical sectors, and have a terminal inflorescence, usually containing 4 to 14 flowers, but never with more than 20, the number depend on the sex of the individuals (Álvarez-Cansino *et al.*, 2010a; Guitián *et al.*, 1997). Male flowers have 3 suborbicular and pubescent sepals with 2 to 3 mm, 3 petals in tones of pink with 3 to 5 mm, and 3 stamens growing between 5 and 6 mm with red anthers that can be seen in naked eye. Female flowers are smaller, their sepals have 1 to 2 mm, and their petals have 1 mm. They have a superior ovary with a style and the stigma has 3 red linear-oblong lobes (Fig. 1) (Simmonds 1979; Tutin *et al.*, 1972). These plants are wind-pollinated (Álvarez-Cansino *et al.*, 2010a; Guitián *et al.* 1997).



Fig. 1 - Left: male *Corema album* inflorescences in a late state of development. Right: female inflorescences, in a late state of flowering and beginning of sprouting.

The fruits of *C. album* are small and in a form of a drupe. They appear in the middle of the branch as the terminal bud continues to grow. The berries have a diameter between 5 and 8 mm, and are white or pinkish-white. They have 3 usually seeds, called pyrenes, but it can range from 2 to 9 (Simmonds, 1979; Tutin *et al.*, 1972). The berries are ripe in the summer, between August and September, (Díaz-Barradas *et al.*, 2016) and its colour can vary

between the usual white, a translucence form when you can see the seeds, or even a red or greenish colours (Oliveira & Dale, 2012).

The investment of this species in reproduction is really high, either in the number of fruits produced, or the time dedicated to reproductive growth, being it related to the aggressive conditions of the environment. These conditions promote a strong vegetative growth in simultaneous with the flowering, increasing the duration and the number of flowers, in order to enhance the probability of reproduction. (Magalhães, 2015).

Ecophysiology

Due to the extreme environment on which this species inhabit, it has developed adaptations to drought, as well to low levels of moisture in the soil. It is the only species that is known to disconnect their ecophysiological functions from the access to ground water, when under low water availability. It was also proved that gender plays a major role in the variation of fruit production and dry matter. Female plants are able to reach a deeper soil layer, which indicates a possible mechanism to compensate for their higher reproductive efforts (Álvarez-Casino *et al.*, 2010b; Máguas *et al.*, 2011). It was also shown that could exist a trade-off between reproductive and vegetative biomass, demonstrating that reproduction and growth depend on the same resources (Álvarez-Casino *et al.*, 2010a). Besides the higher reproductive effort and costs that female plants endure, they display lower water and nutrient use efficiency and growth. Climate influences gender responses, because they are site-specific, occurring variations in the physiological performance and growth of this species. Population structure could change because of the responses that both may have to climate changes (Álvarez-Casino *et al.*, 2012). This difference between sexes is called spatial segregation of the sexes (SSS). The work done by Martins (2017), showed that the sexual dimorphism in *C. album* allowed to adapt do the environment avoiding SSS.

Despite the fact that in this dioecious plant gender recognition can only be done during the flowering season Díaz-Barradas *et al.* (2018), proved that through $\Delta_{13}\text{C}$ and in leaf water potential in the dry season are ways to distinguish between both genders. Female plants have lower integrated water use efficiencies to maintain fruit production. Like it was said previously, female plants might have deeper roots than males, as a compensatory mechanism, presenting underground SSS.

Research showed that due to *C. album* leaf morphology, a big stomatal control can be found, since their leaves roll dorsoventrally forming a closed chamber, contributing to the reutilization of CO_2 and a higher photosynthetic capacity. Inside the stomatal grooves glandular and non-glandular trichomes cover that area, in order to control plant water content and regulate leaf temperature (Antunes *et al.*, 2018). *C. album* also has a thick cutinized upper epidermal cell wall to protect against water deficit and higher solar radiation (Antunes *et al.*, 2018).

Geographical distribution

Corema album it is known to inhabit the dunes of the Atlantic cost of the Iberian Peninsula, being one the most characteristic endemic species. It can be found in rocky cliffs and even in pine forest understorey. The subspecies *azoricum* appears in volcanic fields (Fig. 2)

(Calviño-Cancela, 2004). In the Iberian Peninsula, along the Atlantic coast, this species of berries can be found in semi-fixed dunes, behind the mobile dune, and in hind dunes. It could also be found in coastal barrens and cliffs (Gutián *et al.*, 1997; Zunzunegui *et al.*, 2006).

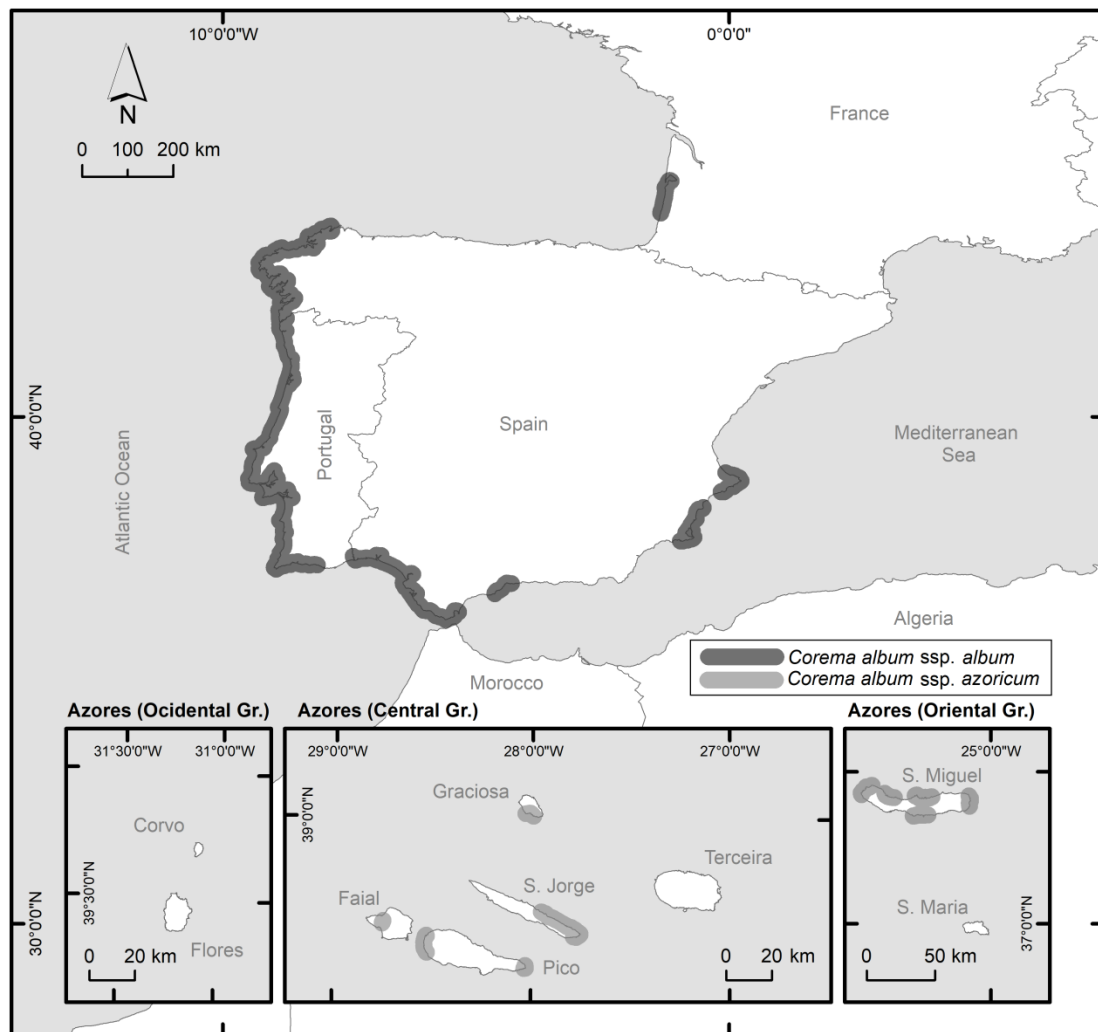


Fig. 2 - Current distributions of *Corema album* sp. *album* and *Corema album* sp. *azoricum*. Adapted from Lopez-Doriga *et al.*, 2018.

Corema album was initially found in Portugal and Spain. Lopez-Doriga (2018) cited that new populations have been described by Marques (2007) in the Spanish Province of Alicante and by Bock (2016) in France, this latter driven by an introduction and naturalization. It was also mentioned in her article, a proposal for a possible range of this species, based on the current distribution, a small population located in the province of Valencia in Spain, according to Aguiella *et al.* (2009) and the case cited from Bock's work.

Regarding the subspecies *azoricum* like it is suggested by the name, it can be found in the archipelago of Azores, in six of the nine islands, below the 200 m (Franco, 1984).

Ecosystem structure

In the sand dunes, it occurs in pioneer and mature stages of shrub succession, in fixed dunes, (Gutián *et al.*, 1997) being the dominant shrub in some of those areas (Álvarez-

Cansino *et al.*, 2010a). Although they grow in protected dune systems, it is not an endangered species, however the subspecies that grows in Azores, is one of the plants that is a priority, in terms of conservation, according to the European project BIONATURA (Decreto Legislativo Regional n. 15/2012/A, 2012; Ministerio de Medio Ambiente, 1990–2006).

According to the syntaxonomical classification of vegetation, *Corema album* appears in two vegetation categories: the heathland, dwarf scrub and scrub vegetation, and the Eurosiberian and Mediterranean climatic zonal and potential natural vegetation. It is considered a characteristic species in the scrubland of the alliance *Coremation albi* Rothmaler 1943, and a dominant species in the two associations: the *Festuco-Corematetum albi* M.A. Giménez & J.M. Losa in J.M. Losa 1975, and the *Stauracantho genistoidis-Corematetum albi* Br.-Bl., P. Silva & Rozeira 1965. In the Mediterranean climatic zonal vegetation it is a dominant species in the association *Coremato albi-Juniperetum macrocarpae* ass. Nova (alliance *Juniperion turbinatae* Rivas-Martínez 1975 corr. 1987) (Rivas-Martínez *et al.*, 2001).

Regarding the seeds studies show that germination increases when fruits are ingested by animals, and are thus dispersed, often being deposited in other suitable sites. Many animals ingest this seeds especially foxes, gulls, blackbirds and rabbits (Calviño-Cancela, 2002, 2004; Fedriani & Delibes, 2009; Larrinaga, 2010).

The dune ecosystems are deeply at risk due to human activities, like urbanization, golf fields, road construction and tourism. Other major concern is the impact of invasive species, which have higher growth rates compared to the native ones and are used to stabilize the modile sandy substrate and to enhance soil fertility where it is poor (Clavijo *et al.*, 2002).

Such is the case of some *Acacia* species. *Acacia melanoxydon* R. Br. in W.T. Aiton, *Acacia longifolia* (Andrews) Willd and *Acacia dealbata* Link, are the most problematic invaders in Portugal (Marchante *et al.*, 2003). Studies on the direct impact of *A. longifolia* in the native *C. album*, in an early state of invasion showed that the invasive *A. longifolia* had a positive influence in *C. album* providing nitrogen for the development of the native species. (Hellmann *et al.*, 2007; Ulm *et al.*, 2017).

Other species with a major concern of its impact in *C. album*, is *Carpobrotus edulis* (L.) N.E. Br. in E. Phillips, 1926, also an invader used to fixate sand in dune ecosystems (Clavijo *et al.*, 2002; Lopes, 2015).

DNA-based markers - The case of inter simple sequence repeat (ISSR)

For the genotype identification and assessment of genetic relationships within and among natural populations from different geographical regions, DNA based molecular markers offer a powerful means of analysing genetic diversity (Agarwal *et al.*, 2008).

Molecular markers have some advantages, since they allow the recognition of polymorphic regions within the genome excluding the influence of environmental effects or the developmental state of the individual (Weising *et al.*, 1995; Reddy *et al.*, 2002).

Inter Simple Sequence Repeat - ISSR - (Zietkiewicz *et al.*, 1994) employs the polymerase chain reaction (PCR) and microsatellite sequences to generate multilocus markers with a high degree of reproducibility. Some of the advantages related to this technique include low costs and the possibility of implementation without prior knowledge of the plant genome (Reddy *et al.*, 2002).

For berries that belong to the Ericaceae family, the same family as *Corema album*, several studies has been widely employed in the analysis of genetic variability using the technique ISSRs (Reddy *et al.*, 2002), mainly in species of the genus *Vaccinium*: lingonberry (*Vaccinium vitis-idaea* L.) (Debnath, 2007, 2009); deerberry (*Vaccinium stamineum* L.) (Yakimowski & Eckert, 2008); cranberry (*Vaccinium macrocarpon* Ait.) (An *et al.*, 2015) and northern highbush blueberry (*Vaccinium corymbosum* L.) (Gawroński *et al.*, 2017).

The first study conducted in white crowberry using ISSRs was made by Nóbrega *et al.* (2016), distinguishing between male and female plants before flowering during an early development period, screening twelve primers. This is important since this plants take from five to six years to have their first flowering season. Two of the twelve showed a high amount of polymorphisms between male and female plants being able to tell apart both genders. It was also developed approaches in order to study the protein storage and the detection of polymorphisms associated genes related to embryo growth and degradation of endosperm, both important to understand seed changes during germination process.

Bioclimatology

Bioclimatology is a science which studies the relation between climate and the distribution of living organisms over an extended period of time. In terms of Vegetation Science, this discipline deals with the interactions between climate and plant distribution on Earth (Rivas-Martínez, 1999).

Bioclimatic variables hold the characteristics of the climate that are relevant to some traits, like plant growth and distribution. In fact this variables are used in several studies, like spatial distribution patterns and vegetation mapping (e.g. Franklin, 1995, cited in Mesquita & Sousa, 2009); prediction of the consequences related to climate change in species distribution (Sykes *et al.*, 1996; Peng, 2000; Walther *et al.*, 2005; cited in Mesquita & Sousa, 2009) and other cases.

Since 1981, Rivas-Martínez started a work regarding a bioclimatic classification system focusing in vegetation science, to achieve a worldwide bioclimatic typology. The main goal was a quantifiable typology, expressed by some climatic parameters or indexes that are easily calculated. (Rivas-Martínez, 1999) The latest version of this classification system includes five macrobioclimates (tropical, mediterranean, temperate, boreal and polar), subdivided into twenty-seven bioclimates and five bioclimatic variants. Beyond that, in each bioclimate, bioclimatic belts are defined, which reflects the variations in temperature and rainfall regimes – thermotypes and ombrotypes. (Mesquita & Sousa, 2009)

Temperate and Mediterranean territories can be found in mainland Portugal. To distinguish between these territories, it is necessary to define ombrothermic belts. (Mesquita & Sousa, 2009) The indexes used for these calculations are in Table 1.

Table 1- Indexes used in worldwide bioclimatic classification systems (Rivas- Martínez, 1999).

Indexes	Goals	Formula	Variables
Compensated Thermicity Index (Itc)	Summarize the thermic constraints of climate to plant growth	$I_{tc} = (T + m + M) 10 + C$	T is the monthly mean annual average temperature (°C)
			m is the mean minimum temperature of the coldest month of the year (°C)
			M is the average maximum temperature of the coldest month of the year (°C)
			C is a compensation parameter, used if the climate is too continental or too oceanic
Annual Ombrothermic Index (I _o)	Expresses the average annual water availability to plants	$I_o = (P_p/T_p) \times 10$	P _p is the total rainfall of all months with positive average temperature (mm)
			T _p is the sum of the monthly average temperature of all months with positive average (°C)
Ombrothermic Index of the Summer Bimonth (I _{os2})	Similar to (I _o); Calculated for the two consecutive warmer months of the year	$I_{os2} = (P_{p2}/T_{p2}) \times 10$	T _{p2} is the sum of the monthly average temperature of July and August (in tenths of degrees Celsius)
	Based on the definition of Mediterranean Bioclimate, and is used for defining its boundaries		P _{p2} is the total rainfall of July and August (mm)
Ombrothermic Index of the Summer Trimester (I _{os3})	Similar to (I _{os2}); Calculated for the three consecutive warmer months of the year	$I_{os3} = (P_{p3}/T_{p3}) \times 10$	T _{p3} is the sum of the monthly average temperature of June, July and August (in tenths of degrees Celsius)
	Analyse territories with a summer drought of two consecutive months and whether this dryness is compensated by the previous month's rainfall		P _{p3} is the total rainfall of June, July and August (mm)
Summer Compensated Ombrothermic Index (I _{osc4})	Similar to (I _{os3}); During a period of four consecutive months	$I_{osc4} = (P_{p4}/T_{p4}) \times 10$	T _{p4} is the sum of the monthly average temperature of May, June, July and August (in tenths of degrees Celsius)
			P _{p4} is the total rainfall of May, June, July and August (mm)

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Chapter 2: Variability of agro-morphological traits in *Corema album* (L.) D. Don in four coastal Atlantic populations*

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Abstract

BACKGROUND: *Corema album* is a dioecious shrub that inhabits the coastal Atlantic systems, dune and pine tree woodlands of Portugal and Spain. Its distribution ranges more than 600 km along the north-south direction, which could lead to different developmental conditions.

OBJECTIVE: The main goal of this work was to find morphological variability between populations of this species.

METHODS: Twenty plants from four populations scattered on the Atlantic coast of Portugal, in different habitat conditions, were sampled. Furthermore, the phenology of 6 female plants was followed in one location from March to August. Histological sections of leaves and stems were performed. Twenty-nine morphological traits in vegetative and reproductive organs were quantified in order to compare them within and between populations.

RESULTS: Regarding the reproductive phenology, differences in north and south of Meco sampling site were obtained. Morphological traits were different between some populations. The population located under a pine tree forest developed poorly reproductive organs and subsequently fruit production was lower than in other populations. Conversely, an investment in vegetative development was observed, which could be influenced by shading.

CONCLUSIONS: Different phenotypic responses were found depending on the habitat. Further research should be made, in order to see if these differences were a response to the environment or due to genetic variability.

Key-Words: White crowberry, plant plasticity, phenotypic responses, morphological traits.

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Introduction

Morphological plasticity is very common between plant species. The reasons could vary due to altitude changes; latitude or longitude variations; environmental conditions or could even be stress related [1, 2, 3, 4, 5].

Corema album (L.) D. Don, the white crowberry, is a dioecious plant that inhabits the Atlantic coastal dunes of Portugal and Spain. These plants have edible fruits, which are unique due to their white colour and their flavour is considered to be mildly acid with a lemony flavour [6]. They also have high amounts of anti-oxidants, flavinol, chlorogenic acid derivatives and phenolic acid and low amounts of anthocyanins [7, 8]. The white crowberry could also be a very important source of fibers and sugars [9].

This shrub was initially described in Portugal named by Don [10] and later referred as native from the coastal Atlantic dunes of Iberian Peninsula [11, 12]. In the work published by Lopez-Doriga [13], new populations have been described in the Spanish Province of Alicante [14] and in France, this latter driven by an introduction and naturalization [15]. It was also showed a proposal for a possible range of this species, based on the current distribution, a small population located in the province of Valencia in Spain [16], and the French population [15].

Besides the range where this species could grow, the environment on which they live present some differences since they can be found in semi-fixed dune and in hind dunes, as well as on coastal cliffs and barrens [17, 18].

The white crowberry can reach a height of 1 m, but the average height stays between 30 and 75 cm. This shrub has many branches, where the leaves present themselves in whorls of 3 or 4, with short petioles [19, 20]. Flowers appear in terminal inflorescences, in groups of 4 to 14 flowers, but never more than 20 and their number depend on the sex [17, 21]. The fruits are small drupes that develop in the middle of the branch as the terminal bud continues to grow. The berries have a diameter that range from 5 to 8 mm and have usually three seeds, called pyrenes, with variable sizes and not proportional to fruit size [12, 20].

Driven by the novelty of this berries, germplasm selection from wild populations is important to start a pre-breeding program. The aims of this work were to sample four Portuguese populations of *Corema album* and sought morphological variability in traits of agronomical interest, in order to choose a possible location to collect germplasm for posterior replication.

Materials and Methods

Biological Material

The biological material was collected from four populations along the Atlantic coast of Portugal. These populations were Meco (38°29'15"N; 9°11'2"W), Comporta (38°22'55"N; 8°48'06"W), Quiaios (40°13'52"N; 8°50'59"W) and Monte Clérigo (37°20'29"N; 8°51'11"W) (Fig. 1), all of them having *C. album* as the most abundant shrub. All of populations were located in places under Mediterranean climate conditions. Their annual precipitation and temperature variation, as well as their average precipitation and temperature, were represented in Fig. 1 [22].

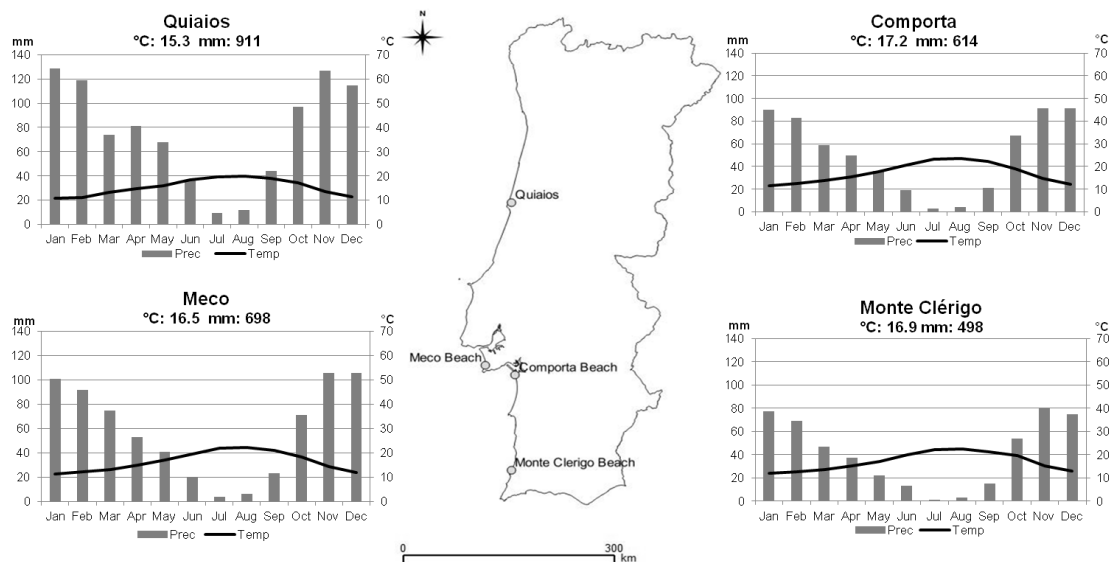


Fig. 1 - Location of studied populations sampled, with their annual temperature (°C) and precipitation (mm) variation, as well as their average temperature and precipitation [22].

Twenty *C. album* female plants were marked in each site. Meco and Comporta were both dunes with woodlands further inland. Quiaios' population was the only population in pine tree woodland. In the previous three sites the woodlands are composed by *Pinus pinaster* Ait.. Monte Clérigo is the only population that doesn't have woodlands, but only shrub size vegetation.

Phenological Data

The phenology of six female plants was followed in Meco, in order to evaluate their development, based on phenological stages described by Magalhães [23]. The first phenological stage considered was elongation of the stigmata (627); the second fruit setting (70); fruits with the half of their total size (75) and finally fruits completely developed (79). Weekly follow-ups were made to observe the development of both flowering and fruiting periods. First data was registered in March 16th (Week 11) and the last week was August 24th, 2018 (Week 34). In the two sampling moments of the other three populations, the phenological stage of six plants per population was also accessed. Meco stages were compared to the other three populations.

In Meco differences between four cardinal quadrants in the six plants studied were considered, as well as, the mean between quadrants and the mean between plants.

Histological Data

Samples of leaves and stems collected at Comporta were fixed in formaldehyde - acetic acid - ethanol (5: 5: 90 vol) and followed by a dehydration of a graded ethanol series (with 70%, 80%, 96% and absolute ethanol). Biological material was clarified using Histo-Clear and then embedded in paraffin. Sample sections with 10 μ thickness were obtained by using Leica RM2255 rotary microtome (Leica, Germany). Transversal sections of the stems were stained using Astra Blue and both leaves longitudinal and transversal sections were stained with Sudan Black and Astra Blue. Sections were observed under Leica DM1000 stereo microscope (Leica, Singapore) and Olympus BX51 microscope (Olympus, Germany).

Morphological Data

Morphological traits were gathered during two field visits in 2018. The first was between March and April for southern populations (Meco, Comporta and Monte Clérigo) and May for Quiaios (the further north population), during flowering season and a second visit between August and September during fruiting season (Table 1).

Table 1 - Morphological traits observed for the characterisation of *Corema album*.

Trait description	Trait symbol
1 - Height	Ht
2 - Diameter N-S	DmNS
3 - Diameter W-E	DmEW
4 - Volume	VI
5 - Number of inflorescences	NI
6 - Number of vegetative growths	NV
7 - Total number of flowers in twenty cuttings	TNF
8 - Average of inflorescences in twenty cuttings	AI
9 - Flower/inflorescences ratio	RFI
10 - Average of leaf width	ALW
11 - Average of leaf length	ALL
12 - Average of leaf area	ALA
13 - Distance between groove on the apparent abaxial surface	DG
14 - Leaf shape	LS
15 - Number of leaves per two whorls	NLW
16 - Petiole colour	PC
17 - Petiole length	PL
18 - Petiole shape	PS
19 - Average branching number	ANG
20 - Average length of annual growths	ALG
21 - Number of fruits with more than 10.25 mm in diameter	NFCM
22 - Number of fruits between 10.25 mm and 7.5 mm	NFCS
23 - Number of Rejected fruits	NFCR
24 - Percentage of white fruits	PTotBr
25 - Percentage of translucent fruits	PTotTr
26 - Weight of the fruits with more than 10.25 mm in diameter	WFM
27 - Weight of the fruits between 10.25 mm and 7.5 mm in diameter	WFS
28 - Weight of the rejected fruits	WFR
29 - Dry/fresh weight ratio	RP

On the first field work traits from 1 to 18 were collected (Table 1). Field measurements were taken in order to calculate the volume of the shrub, using two diameters, one regarding the north-south orientation ($DmNS$) and other the west-east orientation ($DmEW$), and the height (Ht). Volume was calculated according to this formula:

$$Vl = \frac{2}{3} * \frac{DmNS}{2} * \frac{DmEW}{2} * Ht * \Pi$$

On site, in a delimited plant area of 10x10 cm, the number of terminal growths, that had inflorescences (NI) or only terminal vegetative growths (NV), in all four cardinal quadrants were registered.

Twenty cuttings that had inflorescences were collected, and the total flower number (TNF) in the cuttings was assessed with the aid of a stereo microscope Olympus SZ40 (Olympus, Japan). The average of inflorescences in the twenty cuttings (AI) was also taken into account. Ratio between flower number and inflorescence number (RFI) was calculated.

Fifty leaves per plant were measured concerning their length (ALL), width (ALW) and area (ALA). To get these measurements all leaves were digitalized and the freeware ImageJ [24] was used to obtain all measurements. All leaves measured were from the previous year growths.

The stereomicroscope Leica DM1000 (Leica, Singapore) and the 3D acquisition software "Leica Application Suite V4.04", were used to collect data regarding the number of leaves per two whorls (NLW), leaf shape (LS), petiole length (PL), petiole shape (PS), petiole colour (PC) and groove distance on the apparent abaxial surface (DG). LS, PC and PS were qualitative traits and in order to compare them, numbers were attributed to their characteristics. Leaf shape was divided into three different characterizations (1-Concave leaves; 2-Straight leaves and 3-Convex leaves); the same was done to Petiole colour (1-Yellow; 2-Red and 3-Green) and finally to Petiole shape (1-Straight and 2-Curved).

On the second field work, traits from 19 to 29 were collected (Table 1). To have similar data in terms of yield per quadrant and per plant, branches with a diameter between 5.50 mm and 6.99 mm were chosen. In each branch, the principal growth axis was identified and the average number of nodes per branch was counted (ANG). Ten vegetative year growths were also measured (ALG).

Fruits from each branch were collected and separated in to three categories, according to their calibre using three different sieves; bigger than 10.25 mm (NFCM), with less than 10.25 mm and more than 7.50 mm (NFCS) and smaller than 7.50 mm (NFCR). In the last category it could be found fruits that stopped developing, fruits that were still developing and fruits from a previous year that were still in the shrub. The percentage of white (PTotBr) and translucent (PTotTr) fruits was calculated in the other two calibres. Fruits in the three calibres were weighted (WFM; WFS and WFR).

Fresh and dry weight of fifty fruits per plant was registered. Dry weight was obtained after a period of three weeks at 70°C in a eat chamber Memmert UL60 (Mermmet, Germany). The ratio between dry and fresh weight was calculated (RP).

Statistical Analyses

Statistical analyses were performed with RStudio USA [25]. Non-parametric analyses were performed, a Kruskal-Wallis test, to test if there were any differences between the 4 cardinal quadrants in each population (n=20), between the 4 cardinal quadrants in all plants sampled (n=80) and between the 4 populations (n=20), at a significance level of $\alpha=0.05$. A Fisher's post hoc test ($\alpha=0.05$) was conducted for mean separation. The *agricolae* R package [26] was used. Boxplots of the interest traits were made for a more visual effect using stats R package [27].

In order to seek relations between variables in all plants of the 4 populations (n=80) and then in each population (n=20), Spearman's correlation ($\alpha=0.05$) was used from *Hmisc* R package in twenty-three of the variables, only being present the variables that had a correlation with other variables [28].

From the traits correlated, fifteen were chosen not only due to their high statistical significance, but also due to their agronomic interest, being used to perform principal component analyses (PCA), with *Factorextra* R package, in order to find which traits differentiate better each population [29].

Results and discussion

Plant Phenology

Phenological development stages [23] observed in Mecos showed heterogeneity between plants, within the same plant and between cardinal quadrants (Fig. 2).

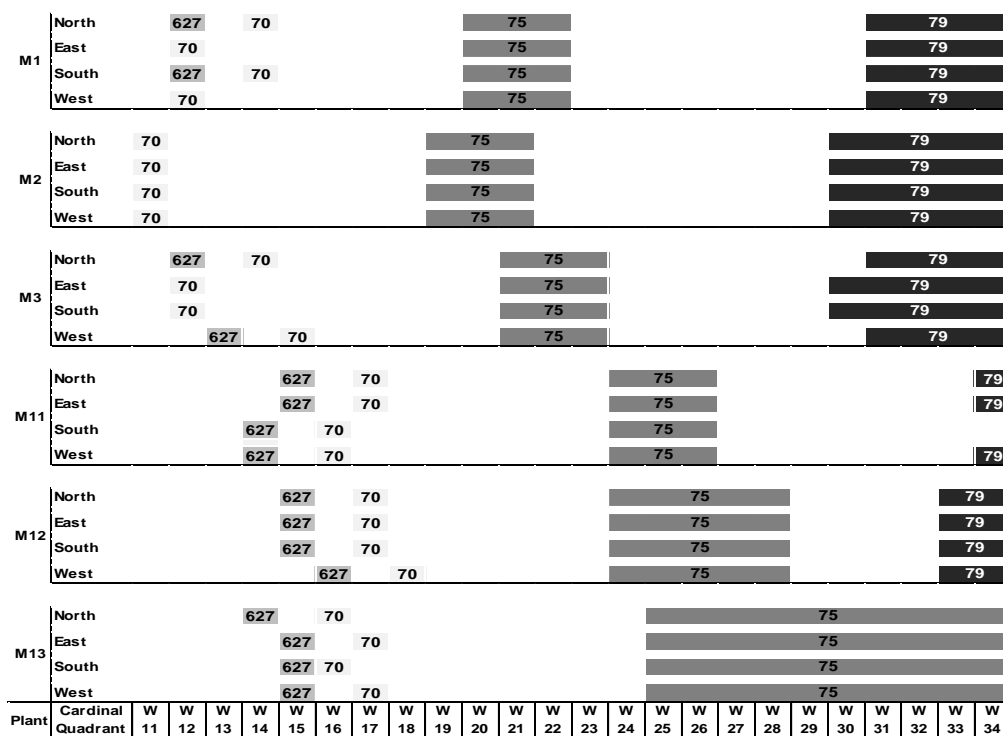


Fig. 2 - Phenological stages regarding six Mecos plants in the four cardinal quadrants from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by

Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.

When the fruits achieved half their size, it seemed that this was the stage where plants started to homogenize in all quadrants (Fig. 3). A major difference was found between north and south sampling sites. Plants from the southern dune were delayed in comparison to the plants of the northern one (Fig. 4).

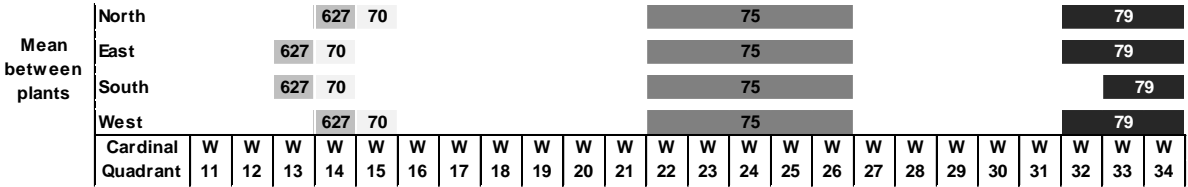


Fig. 3 - Phenological stages regarding the mean between six Mecos plants in the four cardinal quadrants from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.

The plant M13 stayed in the stage 75 till the end of the monitoring. An explanation for this might be because it was the only sampled plant in Mecos that was under a pine tree canopy. The lack of mature fruits in this plant could be related to the shading provide by the trees, leading to an under development of the berries. Another possibility was the susceptibility of that dune to winds, or even higher humidity coming by the sea [30].

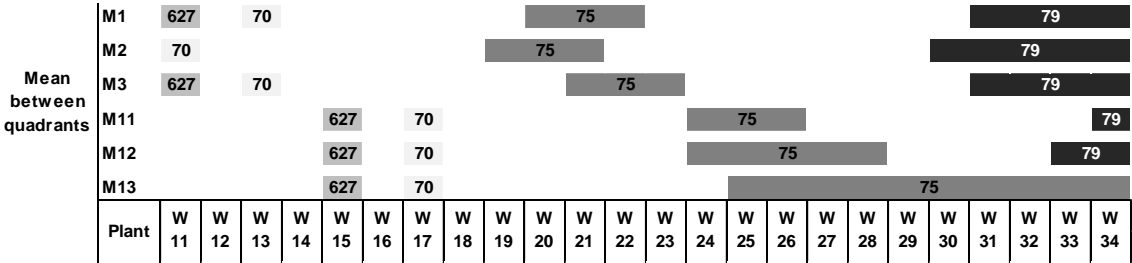


Fig. 4 - Phenological stages regarding the mean between the four cardinal quadrants of the six Mecos plants, from the 16th of March 2018 (Week 11) and to the 24th of August 2018 (Week 34). The numbers attributed to these stages were given by Magalhães, 2015 [23]. Number 627 was the moment of stigmata elongation; number 70 was the fruit setting; number 75 was the moment when fruits had half their size and number 79 was the moment when they achieved their full size.

When populations were compared Comporta and Mecos had similar phenological stages timings, (Fig. 5). Monte Clérigo population was more phenologically advanced compared to the other three populations, which was expected once this was the population further south. The stage of fruit development at Quiaios was much delayed. A reason for this could be due to the shading provide by the pine trees, leading to a lack of development of these fruits, as happened with the plant M13, in Mecos [31].

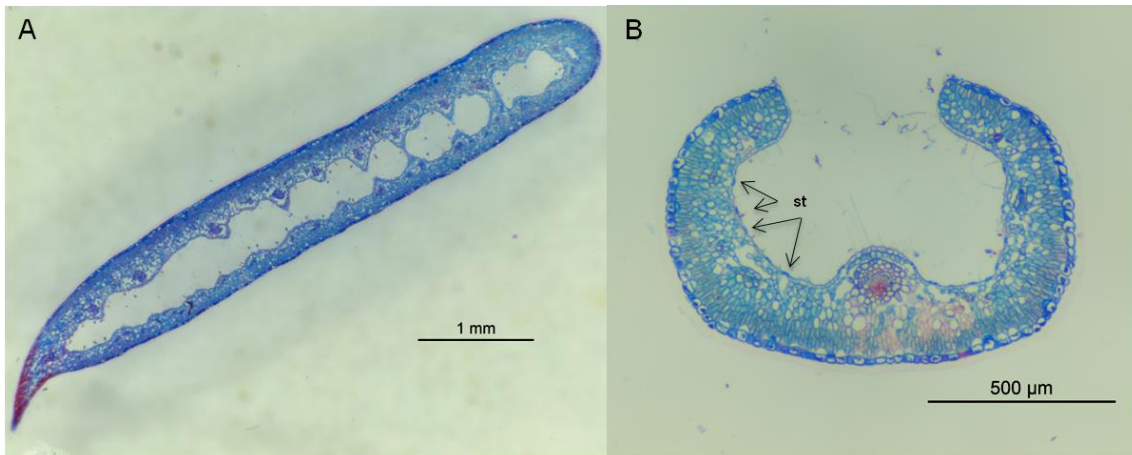


Fig. 7 - A: Longitudinal leaf section, showing the leaf compartmentalization in chambers. **B:** Transversal section of a leaf, showing the groove that separates both extremities. **st:** stomata. Either both preparations were coloured with Astra Blue.

In Fig. 8A, the cuticle was stained with Sudan Black, showing the lipid content of this structure. Other structures inside the leaf chamber that help to maintain the humid environment were the erect uniseriate non-glandular trichomes and the capitate glandular trichomes, with three-five cells. These adaptations, both the cutinized cell walls and the trichomes, were a strategy to protect against water deficit and solar radiation. All these observations are in line with other authors [10, 32]. It was also possible to see the formation of deposits of calcium oxalate druses, which could be linked either to genetic or environmental factors [33] (Fig. 8B).

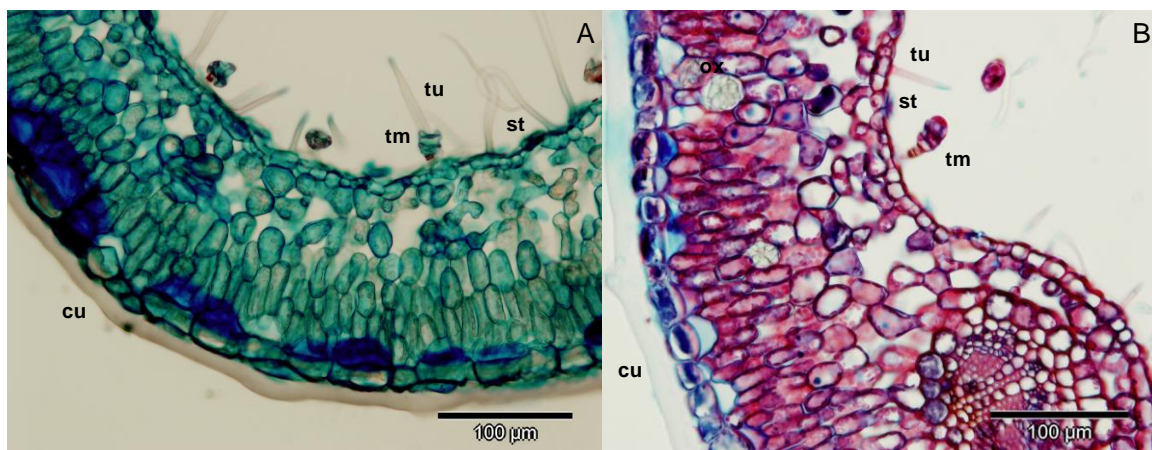


Fig. 8 - A: Transversal leaf section showing detail leaf structure with Sudan Black, showing the lipid content of the cuticle. **B:** Transversal leaf section showing detailed leaf structure coloured with Astra Blue. **st:** stomata; **cu:** cuticle; **tm:** capitate glandular trichomes; **tu:** erect uniseriate non-glandular trichomes; **ox:** druses calcium oxalate crystals.

Morphological data

The comparison between cardinal quadrants in Mecó showed that there were no significant differences in the studied variables. The number of vegetative growths (NV) was the only variable that showed differences between the cardinal quadrants of Comporta, Monte Clérigo and Quaios; however, the differences did not appear in the same quadrants along these three populations (Table 2). When the seven variables were compared using the total

number of plants sampled and the cardinal quadrants, no significant differences were found, concluding that there were no significant differences, in the four cardinal quadrants, within a plant (data not showed).

Table 2 - Summary table with means and standard deviations (SD) of the only significant trait, number of vegetative growths, in the four populations and in all populations, in each cardinal quadrant.

Populations	Traits	Cardinal Quadrants			
		North	East	South	West
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Meco		1.90 ± 3.55 a	3.55 ± 6.00 a	2.35 ± 4.39 a	1.30 ± 2.43 a
Comporta		1.80 ± 3.61 ab	3.75 ± 5.41 ab	4.35 ± 5.64 a	1.50 ± 2.96 b
Monte Clérigo	NV	6.75 ± 3.61 a	3.75 ± 2.63 b	4.50 ± 4.86 b	5.40 ± 4.75 ab
Quiaios		5.05 ± 4.31 ab	4.60 ± 5.78 b	5.60 ± 4.16 ab	7.70 ± 7.43 a
All Populations		3.88 ± 4.28 a	3.91 ± 5.06 a	4.20 ± 4.85 a	3.98 ± 5.44 a

Fisher's post hoc test ($\alpha=0.05$) was performed and if there were significant differences between rows a different letter was shown. Meco, Comporta, Monte Clérigo and Quiaios with $n=20$ and all populations with $n=80$.

Plants from each site were considered and differences between populations were found in several traits (Table 3).

Table 3 - Summary table with means and standard deviations (SD) of the twenty three traits in the four cardinal populations.

Traits	Populations			
	Meco	Comporta	Monte Clérigo	Quiaios
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
VI	3.158 ± 1.833 b	6.851 ± 4.361 a	1.971 ± 1.272 c	1.784 ± 1.162 c
NI	176.050 ± 96.922 a	112.900 ± 39.671 b	109.850 ± 32.605 b	50.950 ± 20.972 c
NV	9.100 ± 10.310 b	11.400 ± 11.953 b	20.400 ± 11.794 a	22.950 ± 13.296 a
TNF	147.650 ± 42.972 ab	160.750 ± 47.933 a	127.450 ± 36.611 b	151.400 ± 33.203 a
AI	1.088 ± 0.202 a	1.100 ± 0.280 ab	1.020 ± 0.064 ab	1.000 ± 0.000 b
RFI	6.731 ± 1.089 ab	7.352 ± 1.616 a	6.230 ± 1.695 b	7.570 ± 1.660 a
ALW	0.652 ± 0.100 b	0.763 ± 0.189 a	0.613 ± 0.053 c	0.691 ± 0.067 ab
ALL	1.557 ± 0.163 a	1.486 ± 0.338 bc	1.370 ± 0.173 c	1.475 ± 0.126 ab
ALA	0.490 ± 0.110 a	0.581 ± 0.335 a	0.395 ± 0.075 b	0.478 ± 0.065 a
DG	246.887 ± 153.913 c	357.034 ± 194.157 b	345.287 ± 154.745 b	479.314 ± 212.308 a
PL	1.089 ± 0.266 a	0.891 ± 0.153 c	0.934 ± 0.217 bc	0.991 ± 0.109 ab
NLW	6.550 ± 0.887 bc	7.050 ± 1.356 ab	6.150 ± 0.489 c	7.400 ± 0.995 a
ALG	2.069 ± 0.519 c	2.649 ± 0.818 b	1.268 ± 0.429 d	3.549 ± 0.700 a
ANG	12.525 ± 2.561 b	12.088 ± 4.165 b	15.613 ± 2.883 a	8.788 ± 2.038 c
NFCM	6.400 ± 12.496 b	25.200 ± 26.483 a	4.550 ± 5.790 b	0.050 ± 0.224 c
NFCS	135.550 ± 147.892 a	108.550 ± 72.719 a	89.000 ± 51.441 a	16.200 ± 27.714 b
NFCR	133.750 ± 94.377 ab	181.300 ± 119.710 a	86.300 ± 77.988 bc	63.550 ± 38.582 c
PTotBr	62.571 ± 21.789 a	48.562 ± 25.284 a	23.100 ± 20.488 b	58.926 ± 28.622 a
PTotTr	37.429 ± 21.789 b	51.438 ± 25.284 b	76.901 ± 20.488 a	41.074 ± 28.622 b
WFM	3.345 ± 6.648 b	14.327 ± 15.036 a	2.598 ± 3.588 b	0.023 ± 0.101 c
WFS	42.594 ± 52.094 a	37.219 ± 25.588 a	28.784 ± 17.262 a	4.087 ± 7.477 b
WFR	12.775 ± 8.046 a	16.990 ± 10.978 a	7.208 ± 4.988 b	4.584 ± 3.247 b
RP	0.217 ± 0.037 ab	0.183 ± 0.028 c	0.198 ± 0.026 bc	0.242 ± 0.052 a

Fisher's post hoc test ($\alpha=0.05$) was performed and if there were significant differences between rows a different letter was shown ($n=20$).

Comporta showed plants with the higher volume (VI). Quiaios plants were smaller as a result of pine trees shading [30] (Fig. 9A). Despite the fact that they had a smaller VI, plants from Quiaios seemed to develop a strategy, in order to overcome the shading problem.

Comparing to other populations, plants from Quiaios had higher average length of annual growths (ALG) (Fig. 9B), smaller average branching number (ANG) (Fig. 9C) and a higher number of leaves per whorl (NLW).

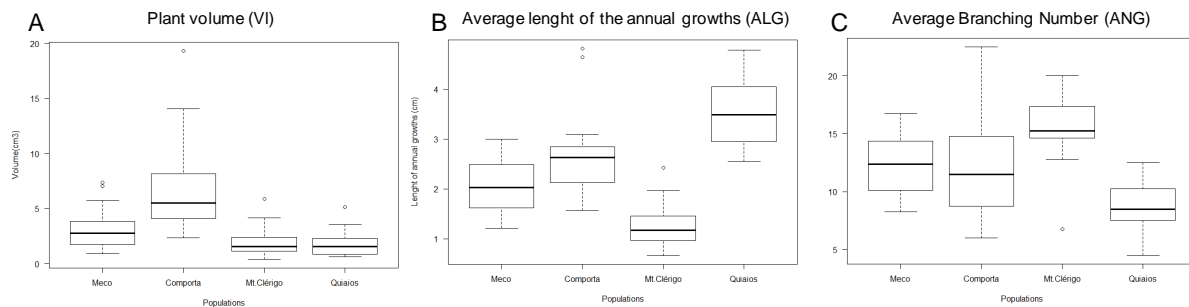


Fig. 9 - A: Plant volume in the four populations. **B:** Average length of the annual growths in the four populations. **C:** Average branching number in the four populations (n=20).

Regarding this last trait, NLW, in the species description, plants could have three (Fig. 10A) or four (Fig. 10C) leaves per whorl, being the most common [19, 20]. However, in this work it was found that besides these leaf rearrangements, they could also have, in the same branch whorls with three and four leaves (Fig. 10B), whorls with four and five leaves in the same branch (Fig. 10D) and whorls with five leaves (Fig. 10E).

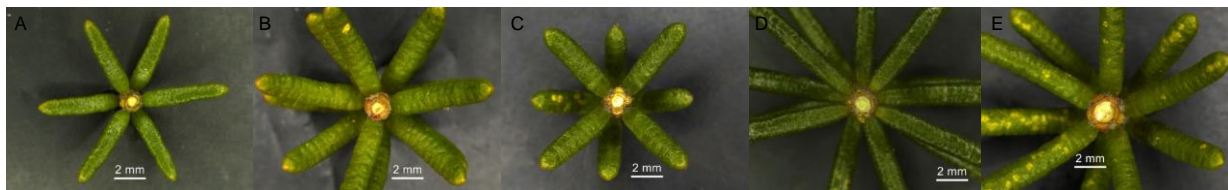


Fig. 10 - Number of leaf per whorl in *Corema album*. **A:** Three leaves per whorl; **B:** Three and four leaves per whorl; **C:** Four leaves per whorl; **D:** Four and five leaves per whorl; **E:** Five leaves per whorl.

Several are the taxa that showed differences in the number of leaves per whorl and therefore the explanation for these adaptations were different as well [34]. Unlike Quiaios, that had more plants with four leaves per whorl, in Monte Clérigo three leaves per whorl was more common, perhaps due to solar radiation exposition. In Monte Clérigo, besides the less number of leaves per whorl, they had also smaller ALG (Fig. 9B) and higher ANG (Fig. 9C). Even though Meco and Comporta had similar behaviours, in Meco some of their values were closer to Monte Clérigo beach and Comporta's values were closer to Quiaios.

Regarding leaf traits, average leaf width (ALW) was higher in Comporta and Quiaios and smaller in Monte Clérigo (Fig. 11A), average leaf length (ALL) was higher in Meco and Quiaios and smaller in Monte Clérigo (Fig. 11B) and finally average leaf area (ALA) was smaller in Monte Clérigo and the other three populations did not show any significant differences between them (Fig. 11C).

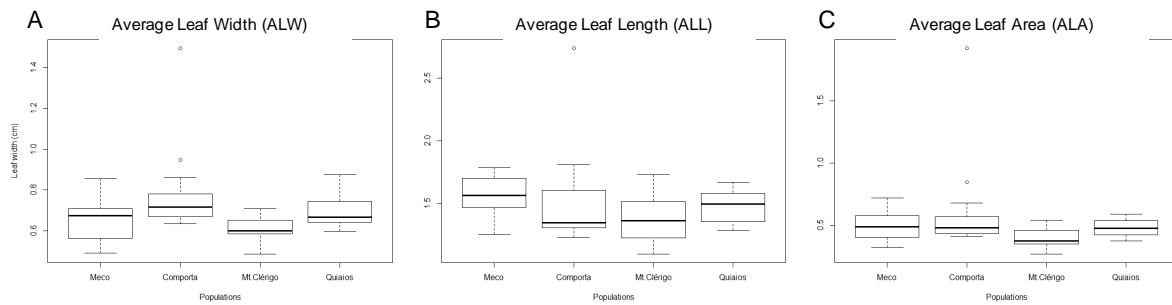


Fig. 11 - A: Average leaf width between the four populations. **B:** Average leaf length in the four populations. **C:** Average leaf area in the four populations (n=20).

The values of ALA followed what was already found in other species, i.e., in *Rubia peregrina* the values were higher in plants that were affected by shading [35]. Smaller leaves are better to regulate temperatures and water-use efficiency in a high solar radiation environment [36, 37]. Another trait, petiole length (PL) showed that Meco and Quiaios had the highest values and Monte Clérigo and Comporta had the lowest. (Fig. 12A) The petiole length influences the leaf angle insertion and can help in light capturing, like it was confirmed in other works [38, 39], which was the case of Quiaios. Groove distance in the apparent abaxial leaf surface (DG) was higher in Quiaios and smaller in Meco (Fig. 12B).

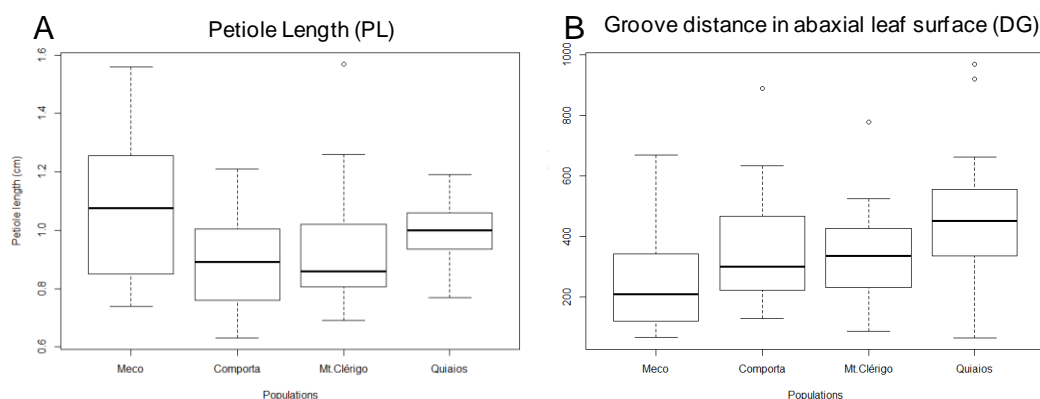


Fig. 12 - A: Petiole length between the four populations. **B:** Groove distance in abaxial leaf surface between the four populations (n=20).

The Quiaios values could be related to lack of direct solar irradiation. Plants from other populations than Quiaios had more direct sun exposure, leading to a more sealed chamber in order to control water content and leaf temperature [10, 32, 40, 41].

In terms of reproductive organs, the number of inflorescences (NI) was higher in Meco and lower in Quiaios, (Fig. 13A) which confirm the investment of the Quiaios plants in the vegetative growth instead of the reproductive organs [42]. Experiments involving the removal of inflorescence buds in *C. album* resulted in elongated shoots [21]. However in Quiaios, the number of flowers (TNF) was not significantly different from Meco and Monte Clérigo which had the lowest number of flowers (Fig. 13B). The ratio between flower and inflorescence number (RFI) was higher at Quiaios but was not significantly different from Meco. Monte Clérigo had fewer flowers per inflorescence but it was also not significantly different from Meco.

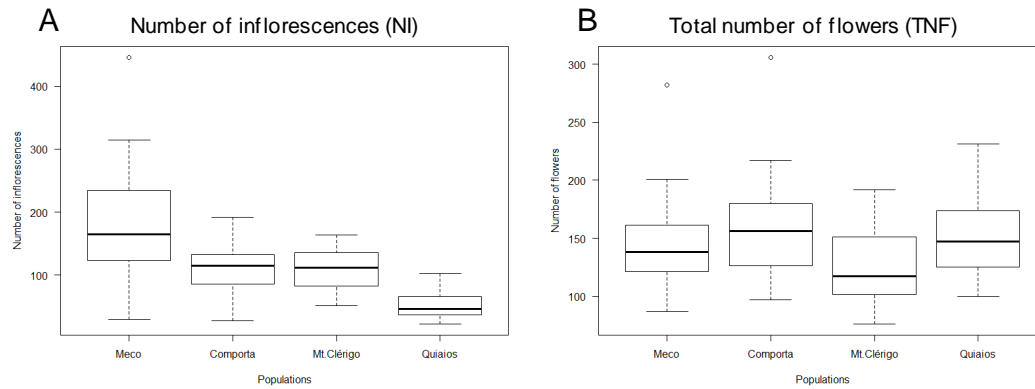


Fig. 13 - A: Number of inflorescences between the four populations. **B:** Number of flowers between the four populations (n=20).

Fruit production was higher in Comporta and lower in Quiaios (Fig. 14). In terms of fruit classes, fruit number (NFCM) and fruit weight (WFM) with more than 10.25 mm in diameter was higher in Comporta and lower in Quiaios. Differences between populations in fruit size were also found in other studies [43]. Several factors could be influencing fruit size, like anthropogenic pressures [44]; environmental conditions [11, 21, 45]; soil type [14, 46] or even the type of animal dispersers [11, 47, 48, 49]. The lower number of fruits in Quiaios could also be related to pollen production since a previous study showed that plants growing under low light conditions produce less pollen than plants that grew under high solar radiation [50].

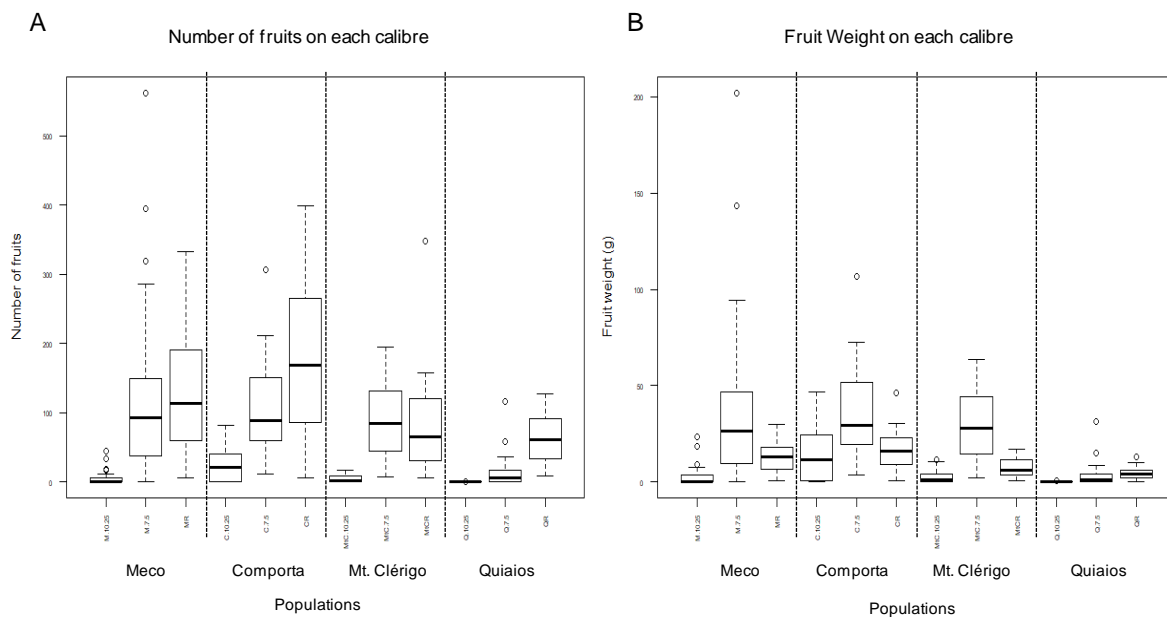


Fig. 14 - A: Number of fruits on each calibre between the four populations. The variables "x.10.25"; "x.7.5" and "xR", represent the fruit calibres "NFCM"; "NFC5" and "NFCR", being the "x" correspondent to each population (M: Meco; C: Comporta; MtC: Monte Clérigo and Q: Quiaios). **B:** Weight of the fruits per calibre between the four populations. The variables "x.10.25"; "x.7.5" and "xR", represent the fruit calibres "WFM"; "WFS" and "WFR", being the "x" correspondent to each population (M: Meco; C: Comporta; MtC: Monte Clérigo and Q: Quiaios) (n=20).

The percentage of white fruits was not significantly different between Meco, Comporta beach and Quiaios. Monte Clérigo had a lower percentage of white fruits, but this might have happened because the fruits start to turn translucent when they get over ripe [6].

The dry/fresh weight ratio of the fruits (Fig. 15) gave the pulp quantity. Quiaios and Meco had higher ratios which could mean smaller seeds compared to the pulp. In Comporta this ratio was leading to the assumption that seeds were bigger compared to the fleshy part of the fruit.

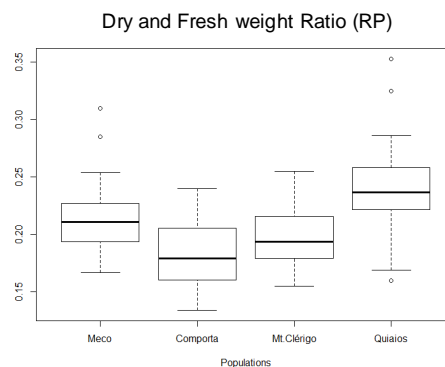


Fig. 15 - Dry and fresh weight ratio between the four populations (n=20).

Spearman's correlation from all populations (Table 4) showed a positive correlation between VI and NI and as well as VI and all the fruit categories, which was expected. Regarding VI, it was negative correlated to DG, which corroborates the fact that plants from Quiaios, which had higher values of DG had also smaller volumes.

Number of inflorescences (NI) was negative correlated to the number of vegetative growths (NV), to the number of leaves per whorl (NLW) and the average length of annual growths (ALG), which could be related, to the effort that the plant need to develop reproductive organs instead of vegetative ones [17, 42, 51, 52, 53]. NI was positive correlated to all the classes of fruits, both their number and their weights. The number of inflorescences was also positive correlated with the average branching number (ANG).

The positive correlation between ALG and NLW, as well as the negative correlation between the NLW and ANG, gave once more the evidence of strategies from plants that were in shaded populations and plants that were in open sun light exposure. This strategy it was also applied to ALA, for example, plants from Monte Clérigo, which had smaller ALA, had bigger ANG and smaller ALG. These correlations were expected, like it was described in other works. Plant morphological characteristics were different between locations, in order to achieve better optimization of solar radiation capture. The reduced branching and the elongation of vegetative growths were an example of these characteristics [54, 55].

All fruit calibres had a negative correlation to ALG, although only one of them had a significant value (NFCS). Some studies had already suggested that female plants had smaller growths than the male plants and higher water stress in fruiting season [18, 21]. This trade-off mechanism between reproductive investment and vegetative biomass was already documented in *C. album*. Female plants had to compensate by having a larger root system [17, 42, 51, 52, 53]. Correlations within each population were also sought; however, the results were similar to the all populations' analyses.

Table 4 - Spearman correlation matrix ($\alpha=0.05$) between the twenty three traits in this study in all the populations.

r \ p	VI	NI	NV	TNF	AI	ALA	DG	LS	NLW	PC	PL	PS	ANG	ALG	NFCM	NFCS	NFCR	PTotBr	PTotTr	WFM	WFS	WFR	RP	
VI		0.002	0.004		0.009		0.049								2.80E-05	0.007	0.018			2.70E-05	0.006	0.001	0.003	
NI	0.337		0.001		0.006			0.018	0.001			0.019	4.55E-04	9.85E-06	0.001	4.09E-06	0.006			0.001	5.40E-06	9.06E-05	0.002	
NV	-0.316	-0.351		0.001	0.022	0.034		0.039								0.022		0.021	0.021		0.022		0.008	
TNF	0.162	0.149	-0.370		9.87E-05	3.08E-05							0.011	0.015				0.005	0.005					
AI	0.289	0.307	-0.255	0.422		0.024									0.007					0.008			0.040	
ALA	0.104	-0.085	-0.237	0.448	0.252		0.005						2.08E-05	1.19E-05				0.026	0.026					
DG	-0.221	-0.197	0.140	0.091	-0.066	0.308						0.038	0.007	0.014									0.047	
LS	0.098	0.263	-0.231	0.161	0.192	0.031	-0.063				0.001					0.002					0.002	0.035		
NLW	-0.056	-0.370	0.003	0.190	-0.030	0.163	-0.001	-0.027					5.04E-05	6.20E-05										
PC	-0.074	-0.103	-0.095	0.044	-0.043	0.108	-0.003	0.046	-0.005															
PL	-0.050	0.013	-0.183	0.182	-0.074	0.157	0.121	0.371	-0.011	0.027		0.002						0.024	0.019	0.019			0.030	0.040
PS	-0.086	-0.261	0.092	0.037	-0.154	-0.050	0.233	-0.114	-0.037	0.048	0.347													
ANG	0.116	0.383	-0.080	-0.282	0.008	-0.457	-0.301	0.151	-0.437	-0.216	-0.169	-0.096		0.000		7.04E-05		0.008	0.008	0.043	8.25E-05		0.030	
ALG	0.046	-0.472	-0.023	0.271	0.035	0.468	0.273	-0.147	0.432	0.141	0.088	0.036	-0.716			8.63E-05		0.003	0.003		5.69E-05			
NFCM	0.450	0.354	-0.213	0.123	0.297	0.159	0.062	0.126	-0.062	0.054	-0.019	-0.203	0.220	-0.189		8.53E-09				0.000	2.18E-11		3.52E-06	
NFCS	0.298	0.489	-0.256	0.128	0.220	-0.015	-0.198	0.349	-0.201	-0.001	-0.080	-0.177	0.429	-0.425	0.590		2.68E-04			1.49E-08	0.000	4.68E-07	1.29E-07	
NFCR	0.264	0.303	-0.133	-0.025	0.072	-0.091	-0.204	0.191	0.075	0.060	-0.253	-0.102	0.215	-0.126	0.064	0.397					0.001	0.000		
PTotBr	0.062	0.038	-0.269	0.323	0.021	0.260	0.011	0.047	0.180	0.109	0.274	0.036	-0.307	0.345	0.009	0.016	-0.108		0.000					
PTotTr	-0.062	-0.038	0.269	-0.323	-0.021	-0.260	-0.011	-0.047	-0.180	-0.109	-0.274	-0.036	0.307	-0.345	-0.009	-0.016	0.108	-1.000						
WFM	0.451	0.357	-0.212	0.121	0.297	0.143	0.059	0.120	-0.061	0.055	-0.031	-0.206	0.227	-0.200	0.997	0.582	0.084	-0.007	0.007		3.60E-11		3.85E-06	
WFS	0.303	0.484	-0.256	0.121	0.212	0.000	-0.166	0.337	-0.206	0.022	-0.092	-0.213	0.426	-0.434	0.663	0.988	0.371	-0.002	0.002	0.657		1.84E-06	3.67E-08	
WFR	0.354	0.424	-0.136	-0.020	0.109	-0.042	-0.223	0.235	0.010	0.064	-0.243	-0.145	0.214	-0.186	0.176	0.529	0.943	-0.042	0.042	0.193	0.504			
RP	-0.340	-0.360	0.308	-0.146	-0.241	0.098	-0.021	-0.107	0.075	0.019	0.241	0.176	-0.254	0.201	-0.513	-0.571	-0.163	0.053	-0.053	-0.511	-0.591	-0.176		

In the lower part of the matrix there was the correlation value and in the upper part only the significant p-values (n = 80).

From the twenty-three traits correlated, fifteen were chosen to perform a principal component analysis (PCA) (Fig. 16).

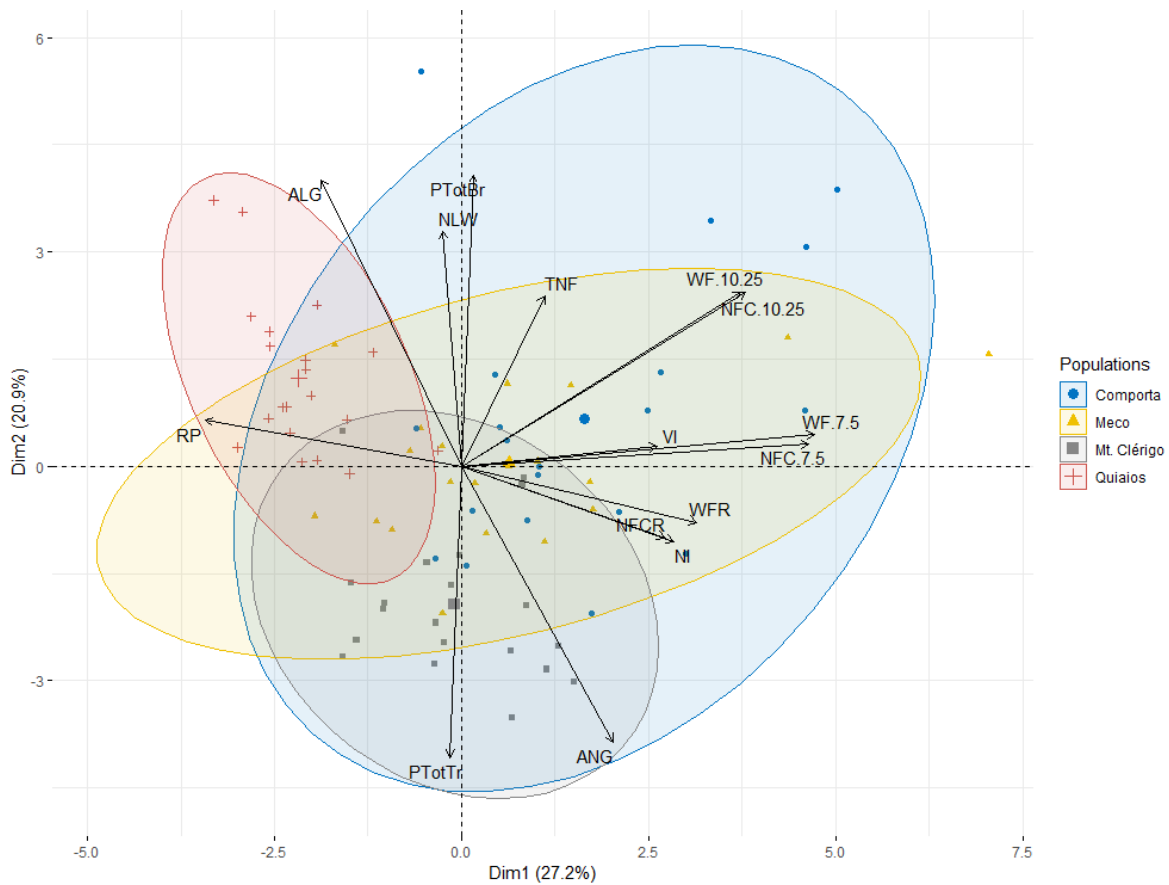


Fig. 16 - Principal component analysis (PCA) showing the dispersion of the individual of the four populations sampled, as well as what were the traits that had the biggest influence on it. The variables "NFCM"; "NFC"; "WFM" and "WFS" were represented as "NFC.10.25"; "NFC.7.5"; "WF.10.25" and "WF.7.5", respectively.

The first 2 axes of the PCA explained 48.1% of the variation, regarding morphological variability. From this analysis it was possible to visualize that Comporta was the population with more diversity, followed by Meco beach. All the traits related to fruit number, as well as fruit weight and plant volume were the ones that have more weight to Comporta and Meco populations.

Both Monte Clérigo and Quiaios, although they overlap, they tend to occupy different quadrants of the graph. The traits that best explain the position of Monte Clérigo were the percentage of translucent fruits and the average branching number (ANG), which was the almost the symmetric variable of the average length of annual growths (ALG), one of the traits that best explain Quiaios' population. Another explanatory trait of the latter population was also the ratio between dry/fresh weights (RP).

Conclusions

Corema album showed a diverse phenotypic plasticity depending on the environment in which they inhabit. Finding variability was key, in order to seek relevant agro-morphological traits for a future use in a culture like environment.

The main issues of this potential crop are fruit size, the existence of large seeds and translucent fruits. The results from the PCA showed that Aldeia do Meco and Comporta could be a source for plants with higher calibres; Quiaios had plants with higher ratios of dry/fresh fruit weight, leading to fruits with smaller seeds and Comporta had a higher percentage of white fruits, which is a desirable trait.

However it would be important to find if this diversity is linked to a genetic variability or if it is only a camouflage from the environmental pressures in which they live. Either way, this was an important step to find phenotypic diversity, for future germplasm selection.

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Chapter 3: Genetic Divergence of *Corema album* genotypes revealed by DNA-based markers and agro-morphological traits

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Abstract

Corema album (L.) D. Don is the only species of the genus throughout the Atlantic coast of the Iberian Peninsula. *C. album* is an important species in sand dune ecosystems, both ecologically for the landscape conservation and economically for commercialization of its edible blueberry-like fruits. Recently there have been efforts to introduce this blueberry-like fruit as a commercial crop and fill a new market niche for berries of white color. For the establishment of this species as a new culture and for the market development to be a success, a first step is to assess the genetic diversity of wild crop germplasm for *in situ* conservation, on-farm management and development of plant breeding programs.

Considering the lack of information of on genetic studies of this species, the aim of this research was to evaluate the genetic diversity among Portuguese populations of *C. album*. Six selected Inter-Simple Sequence Repeat (ISSR) primers were used to assess the genetic diversity within 71 individual female plants collected from four different sampled sites: Aldeia do Meco, Dunas de Quiaios, Comporta and Monte Clérigo. These primers allowed the amplification of 50 polymorphic loci which, based on analysis of clustering and principal coordinate analysis, indicated the existence of five distinct groups according geographic regions. The genetic variation among populations was high (62.40 %) whereas only 37.6% occurred within populations. Correlations between 6 morphological characteristics and 5 bioclimatic variables with molecular data were also obtained. There were no significant correlations. Both molecular and morphological data suggested the *C. album* is rich in genetic diversity.

To our knowledge this is the first study on the assessment of the genetic diversity in this species.

Key words: White crowberry, genetic diversity, ISSR, clustering analyses.

Introduction

Corema album (L.) D. Don belongs to the Ericaceae family and is one of two species of the genus *Corema*. Its populations are a vegetation component of fragile ecosystem, such as dry sand dunes in coastal regions and thus are ecologically of great importance (Gutián *et al.* 1997; Zunzunegui *et al.* 2006).

Corema album is considered endemic to the Atlantic shorelines of the Iberian Peninsula and Azores Islands. The edible white berries have been highly appreciated and exploited (Oliveira and Dale 2012) due to the high nutritional value and important antioxidant properties. These fruits are also rich in compounds like anti-oxidants, flavinol, chlorogenic acid derivatives and phenolic acid and have low amounts of anthocyanins (León-González *et al.* 2013; Andrade *et al.* 2017b) and are an important source of fibers and sugars (Andrade *et al.* 2017a).

Despite the health-related properties, this species is still poorly exploited from a commercial point of view, being still harvested in forest fields without any cultivation. For the establishment of this species as a new domesticated culture with fruit of high quality, a germplasm collection with a considerable amount of genetic diversity is a prerequisite. The amount and genetic variability has been evaluated by the use of morphological characters but molecular markers have become an important tool for characterization of wild and cultivated germplasm, within and among natural populations (Agarwal *et al.* 2008). Inter-simple sequence repeat (ISSR) markers have shown great potential for assessing the genetic diversity of wild species and structuring of natural populations (Zietkiewicz *et al.* 1994; Ueno *et al.* 2015; Zoratti *et al.* 2015). The ISSR technique is simple, fast and has high reproducibility, low costs and no genome knowledge is needed for its implementation (Reddy *et al.* 2002).

These markers have also been used in genetic studies of populations of species of the genus *Vaccinium*, which also belong to the Ericaceae family (An *et al.* 2015; Debnath 2007, 2009; Gawroński *et al.* 2017 and Yakimowski & Eckert, 2008). For *C. album*, only a molecular approach, using ISSR, was carried out among male and female plants to identify a putative sex-specific marker (Nóbrega *et al.* 2016).

Considering the lack of information on the genetic diversity and the importance for contributing with knowledge of the plants that produce high yields and high-quality fruits, ISSR markers were chosen for our study where the aims were to (i) test the applicability of ISSR markers on *C. album* and (ii) determine genetic relationships and diversity among populations along the distribution area of the species.

Material and Methods

Plant material and sampling sites

The study was developed in 80 female *C. album* plants collected from four populations (Table 1) located in different biogeographical areas along the Atlantic coast of Portugal were evaluated in this study. During the collections geographical coordinates for all plants were recorded using the global position system (GPS).

Table 1 - Origin (collection site) and geographical coordinates of *C. album* populations.

Code	Origin	Coordinates	
		Latitude (N)	Longitude (W)
M	Aldeia do Meco	38°29'15"	9°11'02"
C	Comporta	38°22'55"	8°48'06"
MC	Monte Clérigo	37°20'29"	8°51'11"
Q	Duna de Quiaios	40°13'52"	8°50'59"

In all populations *C. album* is the dominant shrub. *Pinus pinaster* Ait. was present further inland of the dune systems, except in Monte Clérigo. In Comporta and Aldeia do Meco, the populations were on the interface between the dunes and the pine woodland, whereas the population of the Duna de Quiaios was under the canopy of the pine woodland.

Molecular data: DNA isolation and amplification

Fresh and healthy leaves were ground to a fine powder in liquid nitrogen using pre-cooled mortar and-pestle and then stored at -80°C until use for DNA extraction.

Total genomic DNA was isolated from approximately 100 mg of leaf powder using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration of DNA was estimated with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

Twenty ISSR primers were screened and six primers that gave clear and polymorphic banding patterns were selected for the final study. Table 2 shows the list of primers used with their respective sequences and annealing temperature.

Table 2 - List of primers used for ISSR amplification, sequences and annealing temperatures.

Primer name	Sequence (5' - 3')	Annealing temperature °C
UBC840	(GA) ₈ YT	49
UBC845	(CT) ₈ RC	50
UBC848	(CA) ₈ RG	50
UBC855	(AC) ₈ YT	50
UBC891	HVH(TG) ₇	50
MR	GAGGGTGGCGTTCT	55

(Y=C,T; R=A,G; H=A,C,T; B=C,G,T; V=A,C,G; D=A,G,T)

PCR reactions were performed in a 25 µl final volume of using the Promega GoTaq Flexi DNA Polymerase Kit (Promega, Madison, USA), containing 1 µl template DNA, 5 µl GoTaq Flexi PCR buffer, 1.5 mM MgCl₂, 0.20 mM each dNTPs, 1.25 U GoTaq Flexi DNA Polymerase (Promega, Madison) and 0.4 µM of each primer in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany), in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) (Nóbrega *et al.* 2016).

Amplification profile consisted of an initial denaturation of 3 min at 94°C, followed by 40 cycles for 1 min at 94°C for denaturation, 1 min at the annealing temperature, 2 min at 72°C

for extension step. The final extension step was done for 10 min at 72°C and the reactions were kept at 4°C. The annealing temperature depended on the primers used (Table 2).

Amplicons were separated by electrophoresis at 5 Vcm⁻¹ in agarose gel (1.5%) containing 0.5 µg/mL ethidium bromide and 1x TBE running buffer.

From the 80 plants sampled three from Meco, two from Monte Clérigo and four from Quiaios, did not show any amplification, being used a total of 71 accessions.

Morphological data

Fifteen morphological characteristics were evaluated during flowering (March to May) and fruiting season (August and September) in all plants (Table 3).

The traits include: plant volume (VI); number of inflorescences (NI); number of flowers in twenty cuttings (TNF); number of leaves per whorl (NLW); average length of annual growths (ALG); average branching number (ANG); number of fruits with more than 10.25 mm in diameter (NFCM); number of fruits between 10.25 mm and 7.5 mm (NFCS); number of rejected fruits (NFCR); percentage of white fruits (PTotBr); percentage of translucent fruits (PTotTr); weight of the fruits with more than 10.25 mm in diameter (WFM); weight of the fruits between 10.25 mm and 7.5 mm in diameter (WFS); weight of the rejected fruits (WFR) and fruit dry/fresh weight ratio (RP), and were used for further analysis (Table 3).

Table 3 - Morphological traits for the characterization of wild plants.

Character	Acronym
Volume	VI
Number of inflorescences	NI
Total number of flowers in twenty cuttings	TNF
Number of leaves per to whorls	NLW
Average length of annual growths	ALG
Average branching number	ANB
Number of fruits with more than 10.25 mm in diameter	NFCM
Number of fruits between 10.25 mm and 7.5 mm	NFCS
Number of Rejected fruits	NFCR
Percentage of white fruits	PTotBr
Percentage of translucent fruits	PTotTr
Weight of the fruits with more than 10.25 mm in diameter	WFM
Weight of the fruits between 10.25 and 7.5 mm in diameter	WFS
Weight of the rejected fruits	WFR
Fruit dry/fresh weight ratio	RP

Bioclimatic Indexes

Five bioclimatic indexes based on the Rivas-Martínez classification system (Rivas-Martínez, *et al.* 1999) were used in order to evaluate their influence in the morphological and molecular data.

The indexes used were summarized in the Table 4: Compensated Thermicity Index (l_{tc}); Annual Ombrothermic Index (l_o); Ombrothermic Index of the Summer Bimonth (l_{os₂}); Ombrothermic Index of the Summer Bimonth (l_{os₃}) and Summer Compensated Ombrothermic Index (l_{oc₄}). These variables were collected where the populations are located. A summary of the indexes and their meaning are presented in Table 4.

Table 4 - Bioclimatic parameters and indexes applied to the study area, according to Rivas-Martínez et al. (1999).

Index	Acronym	Goals
Compensated Thermicity Index	l _{tc}	Summarize the thermic constraints of climate to plant growth
Annual Ombrothermic Index	l _o	Expresses the average annual water availability to plants
Ombrothermic Index of the Summer Bimonth	l _{os₂}	Similar to (l _o); Calculated for the two consecutive warmer months of the year
		Based on the definition of Mediterranean Bioclimate, and is used for defining its boundaries
Ombrothermic Index of the Summer Trimester	l _{os₃}	Similar to (l _{os₂}); Calculated for the three consecutive warmer months of the year
		Analyse territories with a summer drought of two consecutive months and whether this dryness is compensated by the previous month's rainfall
Summer Compensated Ombrothermic Index	l _{oc₄}	Similar to (l _{os₃}); During a period of four consecutive months

Statistical Analyses

Data analysis was performed using the software RStudio (RStudio Team, 2015).

Molecular data analysis was performed based on a matrix with presence (1) or absence (0) of bands, considering only the robust and unambiguous bands. Genetic diversity was estimated by three indexes calculated for each ISSR marker: the polymorphism information content (PIC) (Roldán-Ruiz *et al.* 2000), the resolving power (RP) (Prevost and Wilkinson 1999) and the marker index (MI) (Powell *et al.* 1996) and by the parameters: number of bands, number of polymorphic bands and percentage of polymorphic loci.

Cluster analysis was performed using the matrix of dissimilarities achieved from the *stats* R package (R Core Team, 2013), considering the percentage of common fragments (Nei & Li, 1979). The dendrogram was performed based on the average method, similar to the UPGMA method (Unweighted Pair-group Method Analysis) using the *factoextra* R package (Kassambara & Mundt, 2017).

The genetic structure and the differences between populations were also evaluated using a Principal Coordinates Analyses (PCoA) using the *vegan* R package (Oksanen *et al.* 2013). A hierarchical analysis of molecular variance (AMOVA), using *pegas* R package (Paradis *et al.* 2018) was also performed.

Regarding to the morphological data, cluster analysis was performed based on the dissimilarity matrix obtained using the Euclidian distance. Dendrogram was built also using the average method from the *factoextra* R package (Kassambara & Mundt, 2017).

From the fifteen morphological traits, regarding the six fruit traits (three for fruit number and three for weight class), classes were dismissed and only two fruit traits were taken into account (fruits number (NF) and fruit weight (WF)). A further selection was made between morphological traits and only the ones that were higher statistical significance were chosen (number of inflorescences (NI), average length of annual growths (ALG); average branching number (ANB), number of leaves per whorl (NLW), fruit number (NF) and fruit weight (WF)) to perform a canonical correspondence analyses (CCA) with the molecular data, as well as the bioclimatic data. For these analyses the *vegan* R package was used (Oksanen *et al.* 2013).

Correlations between matrixes were achieved through a Mantel's test, one from genetic dissimilarities and the other two resulting from morphological traits and the bioclimatic data, which were estimated using the *dendextend* R package (Galili, 2015).

Results

The six ISSR primers produced distinct reproducible polymorphic banding patterns with a total of 51 bands, ranging from 6 to 10 bands per primer and the amplification products are in the range of 500 bp to 2.0 kb (Table 5).

The electrophoretic bands ranged from 6 to 10 bands for each of the primers, with an average of 8 bands per primer. The Polymorphism Information Content (PIC) values of the primers varied between 0.16 (primer M13) and 0.37 (primer UBC855) with an average of 0.31. The highest Marker index (MI) was showed in primer M13 (17.41), with an average of 10.1 per primer. The highest resolving power was from UBC840 (17.24), with an average per primer of 11.26. Regarding ISSR banding profiles the genotypes from Aldeia do Meco and Comporta presented higher diversity than the genotypes from Monte Clérigo and Duna de Quiaios.

Table 5 - Genetic diversity estimates of *C. album* populations including **NB**: number of bands; **NPB**: Number of polymorphic bands; **PPB**: Percentage of polymorphic bands; **PIC**: Polymorphism information content; **MI**: Marker index; **RP**: Resolving power and **ISSR populations**: Number of different ISSR profiles among populations and primers.

Primers	ISSR populations									
	NB	NPB	PPB	PIC	MI	RP	Aldeia do Meco (n=17)	Comporta (n=20)	Mt. Clérigo (n=18)	Duna de Quiaios (n=16)
UBC 840	6	6	100	0.34	9.04	17.24	10	13	7	7
UBC 845	10	10	100	0.35	7.69	10.06	13	9	12	9
UBC 848	8	7	87.5	0.30	6.93	6.89	14	13	9	6
UBC 855	8	8	100	0.37	8.00	12.37	14	12	13	3
UBC 891	9	9	100	0.32	11.27	12.49	12	12	6	11
M 13	10	10	100	0.16	17.41	8.51	10	7	5	7

The dendrogram generated from cluster analysis based on 51 ISSR markers is shown in Fig. 1. Five distinct clusters were identified. Cluster 4 consisted of accessions from Duna de Quiaios and cluster 5 with the accessions from Monte Clérigo. Accessions from Aldeia do Meco and Comporta were dispersed in clusters 1, 2, and 3.

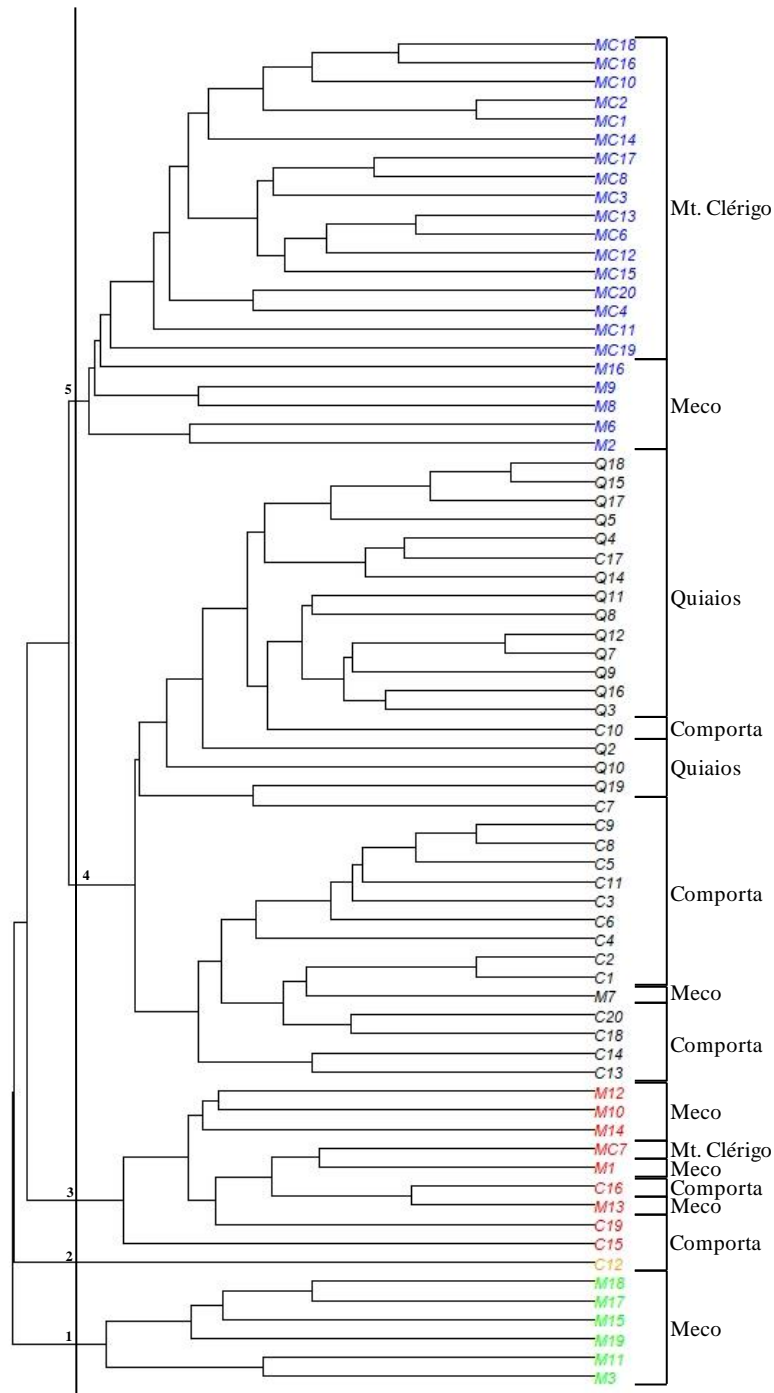


Fig. 1 - Dendrogram obtained with UPGMA method using the Nei & Li coefficient for 71 plants of the four populations ($r=0.729$).

PCoA analysis performed on the ISSR dissimilarity matrix among all genotypes was presented in Fig. 2.

Axis 1 and 2, counting 22% of the variation observed. Accessions from Duna de Quiaios, Comporta and Monte Clérigo were clearly discriminated. Accessions from Aldeia do Meco overlapped Monte Clérigo and the Comporta populations. Comporta was the most scattered population and Duna de Quiaios had a smaller group.

AMOVA analysis showed that the proportion of variation attributable to among populations was high (62.40 %) whereas only 37.6% occurred within populations.

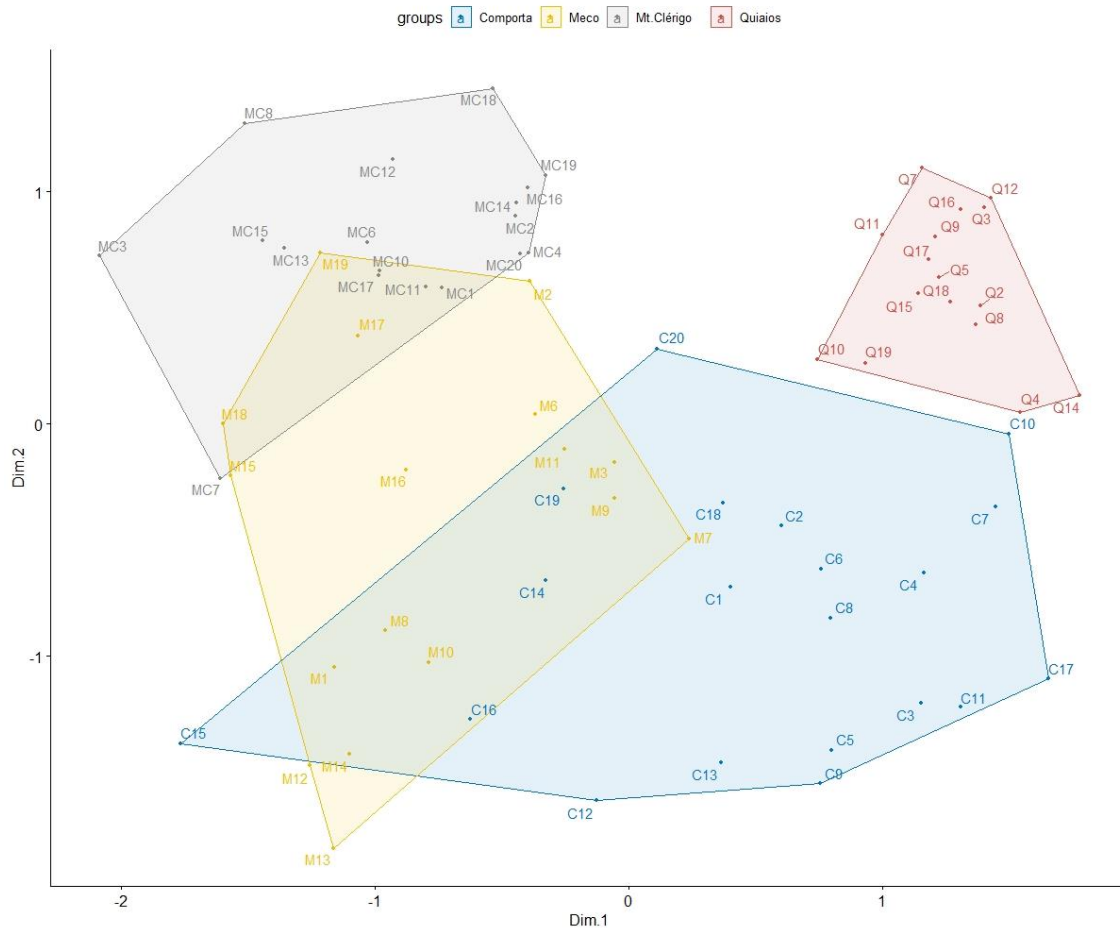


Fig. 2 - Principal Coordinates Analyses (PCoA) of the molecular data, which explained 22% of the variation amongst populations.

The tanglegram performed data (data not showed) lead to a low cophenetic correlation coefficient between both (cophenetic correlation = 0.031). Mantel test between the morphological and molecular data showed a low correlation between dissimilarity matrixes ($r=0.115$; $P=0.027$).

Cluster analysis based on morphological traits for the 71 genotypes produced the dendrogram presented in Fig. 3. The morphological traits varied widely and the highest polymorphism was observed for Comporta and Aldeia do Meco. However, this general distribution of the genotypes did not reflect the geographical origin. Monte Clérigo and Duna de Quiaios was the population had its accessions belonging to the same cluster, showing a similarity between these population's accessions, with the same results to Dunas de Quiaios.

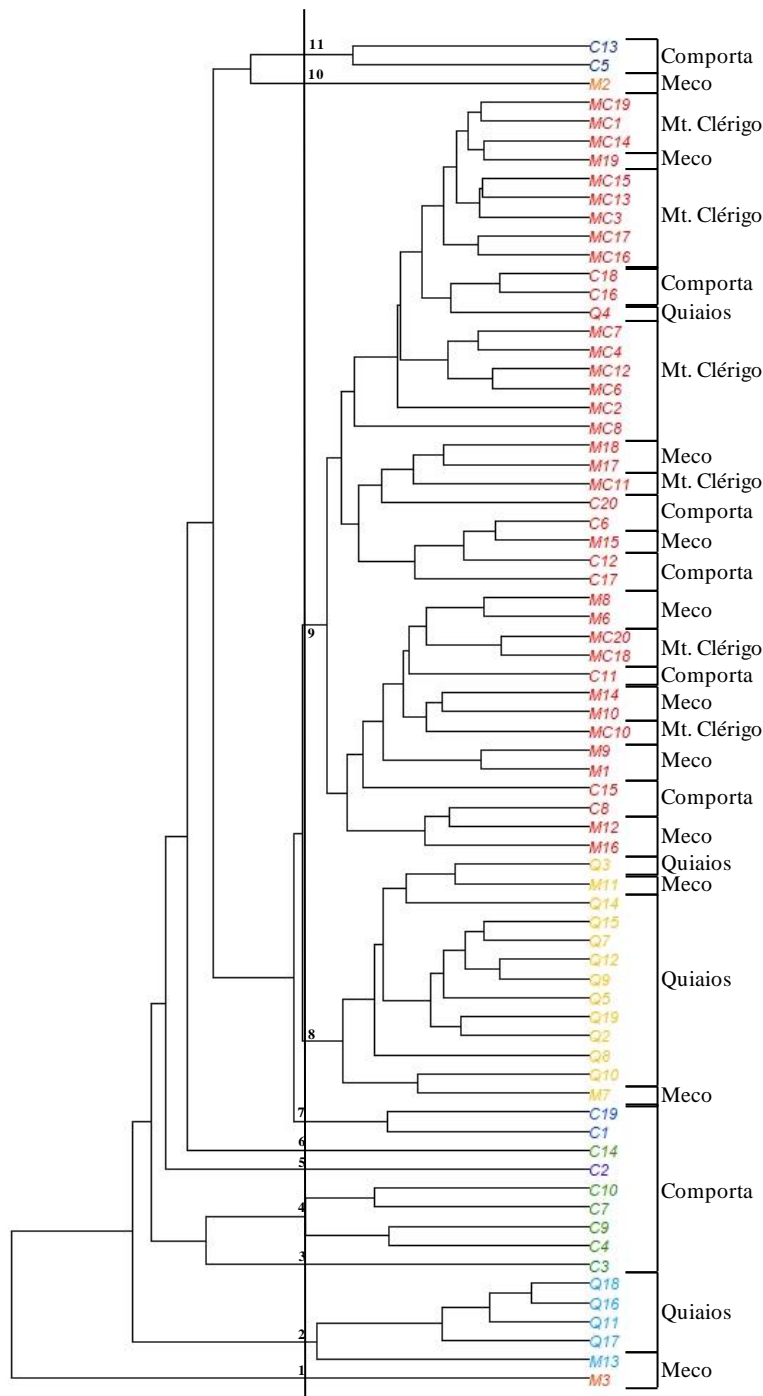


Fig. 3 - Dendrogram obtained with the UPGMA method using the Euclidian distance of the morphological data of the 71 plants. Each colour represents a cluster. ($r=0.879$).

The degree of association between the local environmental variables and the morphology for each genotype from each geographic area was evaluated among the five bioclimatic indexes and six morphological traits.

Canonical Correspondence Analysis (CCA) showed that 46 % of the variance related to these morphological traits was affected by the environmental factors (Fig. 4).

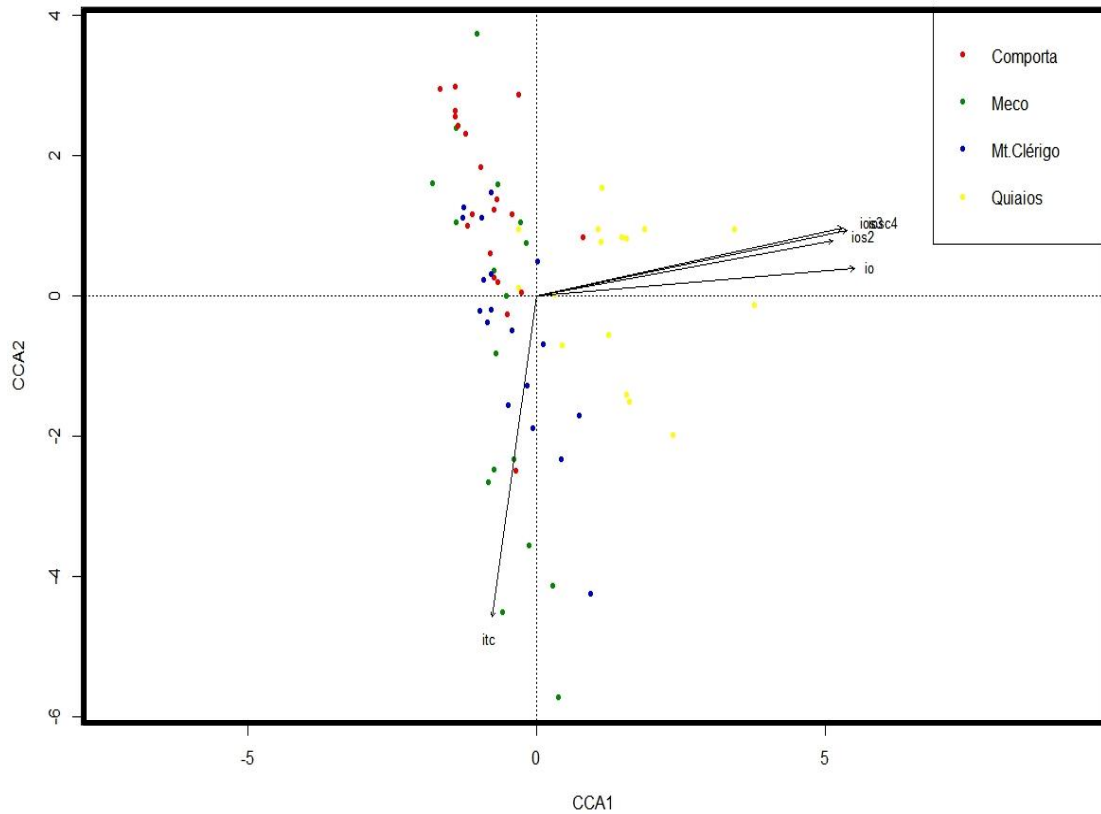


Fig. 4 - Canonical correspondence analyses (CCA) between the morphological data and the bioclimatic data, showing that 46% of the variation of the morphological data was related to bioclimatic variables.

Similar analysis between molecular and bioclimatic data was performed. A total of 23 % of the variance was explained by the first two CCA axes (Fig. 5).

Both analyses showed that the variables Annual Ombrothermic Index (io), Ombrothermic Index of the Summer Bimonth (ios₂), Ombrothermic Index of the Summer Trimester (ios₃) and Summer Compensated Ombrothermic Index (iosc₄) played a significant influence in genotypes from Duna de Quiaios.

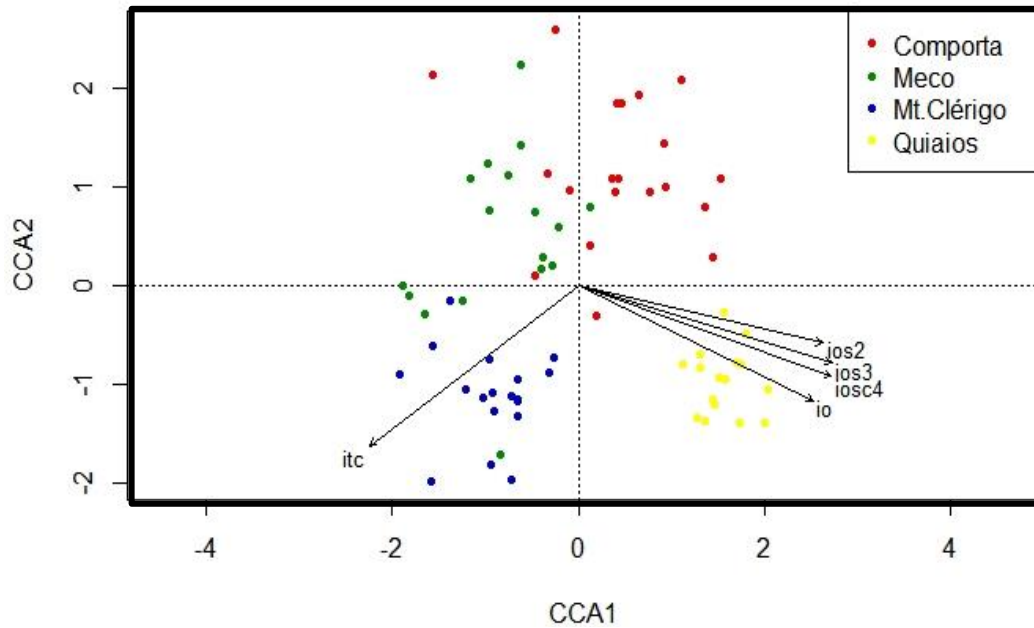


Fig. 5 - Canonical correspondence analyses (CCA) between the molecular data and the bioclimatic data, showing that 23% of the variation of the molecular data was related to bioclimatic variables.

The Mantel test performed between the morphological traits and the bioclimatic data revealed no significant correlation ($r = 0.084$; p -value = 0.096) and between molecular data and the same bioclimatic data showed a significant but low correlation ($r = 0.189$; p -value = 0.0002).

Discussion

The genetic improvement of any crop is dependent on the utilization of well characterized wild relatives and breeding techniques. The assessment of genetic diversity is a requirement to select high yielding genotypes.

In this study, the cluster analysis of morphological traits revealed that there is no distinct separation among the *C. album* genotypes grown in different geographic areas. This variability within and among populations was also found in other studies (Burgos *et al.* 2018). The morphological traits of the species did not show any accordance to its geographical distribution, similar to Solouki *et al.* (2008). However, this could be linked to evolutionary elements which could be related to environmental factors and human activities (Martins *et al.* 2006).

Molecular analysis showed more clear information. Fifty alleles were identified by six ISSR markers proving their ability to be used as polymorphic markers in *C. album* accessions.

Although ISSR markers have been extensively used to assess genetic diversity, there are no reports in *Corema* genus, which include only two species. Thus, to our knowledge this is the first study on the assessment of the molecular genetic diversity in this species.

Regarding molecular data the clustering analyses showed that Aldeia do Meco and Comporta were the populations with more dispersed accessions in the dendrogram, which could lead to a higher genetic diversity compared to Monte Clérigo and Duna de Quiaios.

The PCoA analysis and phylogenetic data obtained based on ISSR markers generated a specific clustering patterns which revealed geographical variation due to environmental conditions and the three groups obtained in a dendrogram demonstrated the genetic relationship among the germplasm. Such characterization of genetic resources forms an important factor for crop improvement program.

In fact, the PCoA analysis obtained based on ISSR markers showed that the Comporta and Aldeia do Meco populations present a higher scattering compared to the other populations. This analyses showed three distinct groups (Monte Clérigo, Comporta and Duna de Quiaios), and an overlap of Aldeia do Meco with Monte Clérigo and Comporta. The northern population (Duna de Quiaios) formed a different group from the three southern populations, perhaps due to lack of genetic information exchange with the southern populations or due to the different environment in which these plants were found. However, the overlapping between Aldeia do Meco and Monte Clérigo was unexpected, since the population which was geographically closer to Monte Clérigo was Comporta. This might be linked to seed dispersers. Some studies indicate that one of the main disperser of *C. album* were seagulls (Calviño-Cancela 2002; 2004), and their capability to travel long distances could favour gene flow among populations (Calviño-Cancela 2011).

The values of the AMOVA were different from what was observed in other species of the Ericaceae family. Values were higher for within population variance, also using ISSR markers, in *Vaccinium myrtillus* (Zoratti *et al.* 2015), *Vaccinium angustifolium* (Debnath 2009) and *Vaccinium vitis-idaea* (Debnath 2006), perhaps due to their self-pollination capabilities. In the other hand, *C. album* is cross-pollinated through wind (Álvarez-Cansino *et al.* 2010; Guitián *et al.* 1997) and with a low germination percentage (Calviño-Cancela 2004), leading to a higher variation between populations.

The result regarding the Mantel test between morphological and molecular data was quite low, being the values of this correlation also similar in other studies (Fanizza *et al.* 1999; Allel *et al.* 2017; Giordani *et al.* 2017; Burgos *et al.* 2018). Several factors could indicate the low correlation. Molecular markers could be covering parts of the genome with coding and non-coding regions and could be less subjected to artificial selection compared to morphological markers (Burgos *et al.* 2018). Morphological traits could also be influenced due to natural and artificial pressures, whereas microsatellites cannot (Semagn 2002). Both types of data could also be following different evolutionary paths (Wendel & Doyle 1998). Other explanation could be related to a correlation of the morphological traits with the environmental conditions (Johns *et al.* 1997).

Interaction between environmental factors could be very important to the success of an organism, which could vary between ecosystems (Agrawal 2007).

The CCA performed between the six chosen morphological and bioclimatic variables showed that 46% of the morphological variation was explained by the bioclimatic variables. The Duna de Quiaios population was the one which was more influenced by the bioclimatic variables. Taken into account that Duna de Quiaios population was the furthest population north, precipitation and temperature were relatively different (higher precipitation and lower

temperatures), compared to the other 3 populations. However the Mantel test between those matrixes had a lower correlation rate and a p-value that was not significant ($r=0.084$; $p\text{-value}=0.096$). Perhaps other environmental variables had higher influence in the morphological traits like wind and light intensity (Giordani *et al.* 2017).

Canonical correlation analyses between molecular and bioclimatic variables showed that the 23% of the molecular variation was explained by environmental factors. Such result was also higher in works with barley landraces from Ethiopia (Abebe *et al.* 2015) and in wild soybean (Leamy *et al.* 2016). Regarding to the Mantel test between the two matrixes ($r=0.189$; $p\text{-value}=0.0002$) was significant and it was also proven in wild barley (Pournosrat *et al.* 2018), in wild soybean (Leamy *et al.* 2016) and also in *Solanum pimpinellifolium* (Zuriaga *et al.* 2009); showing the impact that precipitation and temperature might have in the population genetics.

Conclusion

Molecular marker-based cluster analysis reveled there was separation among the *C. album* genotypes in different geographic areas in Portugal.

There are no reports on the genetic diversity of *C. album* accessions using morphological traits and molecular markers so far. This remains the first study using morphological and genetic diversity characterization of *C. album* in Portugal and the study reveals a high degree of diversity among the accessions which can be further used for crop improvement. This may provide an opportunity to enhance and boost the breeding strategy.

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Chapter 4: Conclusion and Perspectives

To our knowledge, this is the first study assessing the morphological and genetic characterization of *Corema album* in Portugal.

In this first approach, we analyzed both phenotypic variability and genetic diversity using ISSR markers in four populations along the west coast of Portugal, in order to examine the effects of genetic variation and environmental conditions on the phenotypic variation within and among populations, and also the effects of environment on the genetic variation of the species.

The phenological stages in Aldeia do Meco within the six plants studied, showed a difference between the northern and southern plants. However, no differences were present, within plant, regarding the cardinal quadrants. The comparison between populations phenological stages, demonstrated that the southern population (Monte Clérigo), was further advanced regarding their phenology, compared to the northern population (Dunas de Quiaios).

Comparison between populations regarding the morphological traits showed that Comporta and Aldeia do Meco were the populations with higher morphological diversity. The population of Quiaios had a different behaviour compared to the other three populations, once these plants inhabit under pine tree woodland, making a priority in developing vegetative organs instead of reproductive ones.

Morphological cluster analysis revealed there was no separation among the *C. album* genotypes in different geographic areas in Portugal.

Meanwhile cluster analysis to molecular data showed more clear information, clustering the populations according to their geographical distribution. Comporta and Aldeia do Meco were also the populations with more genetic diversity, compared to Monte Clérigo and Duna de Quiaios.

It was also possible to identify that molecular variation was higher between populations, instead of within populations.

Correlation analysis among morphological traits and molecular data, revealed a low correlation between them, which could be explained by several factors.

CCA analyses between morphological traits and bioclimatic variables showed that the environment had an impact on morphological data; however the correlation was not significant.

The same canonical correspondence analyses between genetic and bioclimatic indexes showed a low influence between them, but with a significant correlation.

It will be needed a deep knowledge about the genetic diversity and the population structure in order to characterize the genotypes for practical breeding purposes. For a successful expansion of this species, the selection of genotypes well characterized it will be also need, in order to investigate the adaptation to the different environmental conditions.

Based on that, a future aim will be a genomic approach for the evaluation of genetic diversity using the single nucleotide polymorphism (SNP) variation supplied by GBS. This knowledge is very important for future genetic analyses such as genomic selection studies, GWAS and marker-assisted selection for a breeding program.

On the other hand, further investigation about other environmental data should be done, such as the wind and solar irradiation, since this species is wind-pollinated, and, can grow in dunes near the beach or even in a pine forest.

Studies about agronomical approaches to this species should also be taken into account, mainly regarding planting systems, pruning techniques, fertilization and irrigation.

This kind of study should be made in other population, especially further north and south from the studied populations, in plants in other environmental conditions and perhaps even from Spanish populations, once they also have large populations of this species in Galicia and in the south of Spain.

With the knowledge provided by this study and future insights, more chances will be given to this berry to enter in the small fruit market.