INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Caracterização Morfológica, Molecular e Química de *Arbutus unedo* L. com vista à selecção de genótipos de superior qualidade

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Dissertação apresentada à Escola Superior Agrária de Bragança
para obtenção do Grau de Mestre em Qualidade e Segurança Alimentar

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Bragança
2010

Aos meus Pais

Agradecimentos

É com enorme prazer, que ao entregar este trabalho, agradeço a todos aqueles que de alguma forma contribuíram e ajudaram à sua realização e conclusão.

Em primeiro lugar, agradeço às minhas orientadoras. À Professora Doutora Paula Cristina Santos Baptista, da Escola Superior Agrária, pelos conhecimentos transmitidos, por toda a ajuda e incentivo na realização do trabalho prático, de campo e escrito, pela permanente disponibilidade e acima de tudo pela amizade demonstrada.

À Professora Doutora Ana Maria Quintana Vivar, da Escola Superior Politécnica de Zamora, pela disponibilidade, pelos conhecimentos transmitidos e pelo auxílio prestado.

Ao Professor Doutor José Alberto Pereira, da Escola Superior Agrária, pelos conhecimentos transmitidos, pelo apoio, disponibilidade, incentivo e pela amizade demonstrada.

À Professora Doutora Sónia Santos, pela ajuda no tratamento estatístico dos dados, pelo apoio e pelas sugestões que permitiram melhorar este trabalho.

Aos meus colegas de laboratório, Ricardo Malheiro, Ivo Oliveira, Valentim Coelho e Lucas Lopes pela ajuda, apoio e amizade incondicionais.

Por último, mas não menos importante, agradeço a todas as pessoas da minha família e amigos que me apoiaram incondicionalmente. Ao meu irmão pela paciência, coragem e apoio. À minha mãe por todo o apoio, coragem, força e amor incondicional, por nunca me deixar desistir e por ter sempre uma palavra positiva. Ao meu pai, que à sua maneira soube demonstrar o seu carinho, apoio e amor sem limites. Aos dois agradeço por chegar até aqui.

A todos vocês, um Muito Obrigada!

Trabalho financiado em parte pelo Projecto **"Prospecção e caracterização do medronheiro, *Arbutus unedo* L., em Trás-os-Montes com viata à sua valorização"** financiado pela Caixa de Crédito Agrícola Mútuo da região de Trás-os-Montes e Alto Douro.

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Resumo

O medronheiro (*Arbutus unedo* L.) é uma árvore fruteira de elevado valor ornamental, ambiental, económico e medicinal devido às propriedades atribuídas às flores, frutos e folhas. No entanto, nos últimos anos não tem sido atribuída a devida importância a esta espécie na região de Trás-os-Montes, ao contrário do que se verifica no resto do país, registando-se um declínio da área ocupada por *A. unedo* nesta região. Com o objectivo de preservar e potenciar a produção desta espécie procedeu-se, numa primeira fase, à avaliação da diversidade de 4 populações naturais de *A. unedo* do interior Norte e Centro de Portugal, através da caracterização morfológica e molecular. Numa segunda fase, avaliaram-se as propriedades químicas de 19 genótipos provenientes da região de Bragança, com vista a seleccionar os de superior qualidade. Desta forma espera-se contribuir para a multiplicação e valorização de populações de maior interesse.

A diversidade de populações naturais de *A. unedo* foi avaliada através da análise morfológica e genética pela utilização de marcadores moleculares, num total de 46 genótipos. Para tal, foram colhidas 40 folhas aleatoriamente em cada árvore, nas quais se mediu o comprimento, largura, peso seco, comprimento do pedúnculo e se estimou a relação comprimento/largura. Na análise genética utilizaram-se dois marcadores moleculares, amplificação aleatória de DNA polimórfico (RAPD) e sequencias simples repetidas (ISSR). As características morfológicas analisadas, e em especial o peso seco e comprimento do pedúnculo das folhas, permitiram diferenciar os vários genótipos. Os resultados decorrentes da análise genética mostraram que a população de Bragança foi a que apresentou maior diversidade genética (Na=1,93; Ne=1,71; Ho=0,39; Io=0,5; P=93,02), e Castelo Branco foi a que apresentou menor diversidade (Na=1,43; Ne=1,29; Ho=0,16; Io=0,24; P=43,02). Cada população foi caracterizada como um "pool" génico distinto, com baixa variabilidade intrapopulacional e com um grau de diferenciação bastante elevado. Este resultado sugere a necessidade de se proceder à conservação de cada população individualmente. O teste de Mantel evidenciou a inexistência de correlação entre as matrizes de distância calculadas com base nos marcadores RAPD e ISSR bem como entre estes e os dados morfológicos.

A avaliação da actividade antioxidante, foi testada em extractos aquosos das folhas de 19 genótipos de *A. unedo,* pelos métodos do Poder Redutor e efeito bloqueador de radicais livres DPPH. O teor em fenóis totais foi avaliado pelo método de Folin-Ciocalteau. Os extractos provenientes das amostras de Vila Verde e Donai foram os que apresentaram maior actividade antioxidante (EC_{50} de 0,233 e 0,245 mg/ml para o poder redutor e 0,088 e 0,090 mg/ml para o DPPH), enquanto que a amostra proveniente de Vila Boa 2 foi aquela que apresentou maiores valores de EC_{50} (0,378mg/mL para o poder redutor e 0,142 mg/mL para o DPPH) revelando assim menor potencial antioxidante. Relativamente ao teor em fenóis totais a amostra de Bragança 1 foi a que reportou maior valor (215,0 mg. Equivalentes de acido gálico/g extracto) e a amostra de Vila Boa 4 a que apresentou o menor valor (148,0 mg. Equivalentes de acido gálico/g extracto).

Palavras-chave: *Arbutus unedo* L., actividade antioxidante, fenóis totais, diversidade genética, RAPD, ISSR, conservação.

Abstract

The strawberry tree (*Arbutus unedo* L.) is a fruit tree with high ornamental, environmental, economical and medicinal value due to the attributed properties to the flowers, fruits and leaves. However, in the last years, in Trás-os-Montes region, the deserved importance is not been given to this species, contrarily to what happens elsewhere in the country, being registered a decline in the area occupied by *A. unedo* in this region. In order to preserve and enhance the production of the species, in a first stage was proceeded the evaluation of the diversity of the 4 natural populations of the *A. unedo* from the North interior and Centre of Portugal, through morphological and molecular characterization. In a second phase, were evaluated the chemical proprieties of 19 genotypes belonging to Bragança district with the aim to select those of superior quality. Thus, we expect to contribute for the multiplication and valorization of the population with major interest.

The diversity of the *A. unedo* natural populations was evaluated through the morphologic and molecular analysis, in a total of 46 genotypes. For such, 40 leaves were randomly collected from each tree, in which were measured the leave length and width, dry weight, peduncle length and leave length/width ratio. In the molecular analysis, were used two molecular markers, RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat). The morphological characteristics, specially the dry-weight and the peduncle length, allowed differentiating the various genotypes evaluated. The results obtained from molecular analysis shown that the population of Bragança presents higher genetic diversity (Na=1.93; Ne=1.71; Ho=0.39; Io=0.5; P=93.02), and Castelo Branco was the one that presents lowest diversity (Na=1.43; Ne=1.29; Ho=0.16; Io=0.24; P=43.02). Each population was characterized as a distinct genetic pool, with low intrapopulational variability and with a high level of differentiation. This result suggests that the populations should be conserved separately. The Mantel test showed the inexistence of correlation between the distances matrices calculated in basis on the RAPD and ISSR markers, as well as between this molecular markers and morphological data.

The evaluation of the antioxidant activity was tested in aqueous extracts of the leaves of the 19 genotypes of *A. unedo*, by the reducing power and scavenging effect on the free radicals of DPPH. Total phenols content was assessed through the Folin-Ciocalteau method. The extracts from Vila Verde and Donai genotypes were those who

reported higher antioxidant activity (EC_{50} values of 0.233 and 0.245 mg/mL for the reducing power method respectively, and 0.088 and 0.090 mg/mL for the DPPH method respectively), while the genotype from Vila Boa 2 was the one that reported higher EC_{50} values (0.378 and 0.142 mg/mL for the reducing power and DPPH methods respectively) and consequently lower antioxidant activity. Concerning total phenols, Bragança 1 genotype reported higher content (215.0 mg GAE/g of extract) and the genotype from Vila Boa 4 reported the lowest values (148.0 mg GAE/g of extract).

Keywords: *Arbutus unedo* L., antioxidant activity, total phenols content, genetic diversity, RAPD, ISSR, conservation.

Capítulo 1

Introdução

1.1 Introdução

O medronheiro (*Arbutus unedo* L.) é uma árvore fruteira pertencente à família Ericacea, e ao género *Arbutus*. Encontra-se distribuída por países da orla mediterrânica, nomeadamente todo o sul da Europa, norte de África e Palestina. Pode ainda encontrar-se distribuída por países da orla atlântica, tais como Irlanda e Macaronésia (Canárias) (Celikel *et al*., 2008). Em Portugal existe preponderantemente a sul do rio Tejo nomeadamente na região das Serras do Caldeirão e Monchique (Algarve), podendo contudo, encontrar-se difundido por todo o país, inclusive em Trás-os-Montes (Pedro, 1994).

É uma árvore que cresce tanto em solos ácidos como calcários e pode ocorrer até aos 1200 metros de altitude (Anónimo, 2004b; Krussmann, 1982), podendo ainda crescer em zonas rochosas (Athanasiadis, 1986). Como planta mediterrânea que é tem a capacidade de regenerar após a ocorrência de fogos florestais (Konstantinidis *et al.*, 2006). Tem normalmente um crescimento arbustivo até uma altura de aproximadamente 5 metros, podendo contudo crescer até aos 12 metros (Tutin *et al*., 1981; Seidemann, 1995; Anónimo, 2004a), com ramos que brotam do tronco a partir de 0,5 metros do solo sendo bem espaçados entre si. Conhecido também por ser sempre verde, a copa do medronheiro é arredondada com folhas persistentes de formato elíptico que assumem uma coloração verde escura semelhante à do sobreiro, apresentando um brilho ceroso na face superior. As flores desta árvore, de cor branca ou levemente rosadas, são muito decorativas o que a torna ornamental. As flores são ainda consideradas de elevada importância para o ecossistema por serem uma fonte de néctar para as abelhas (Floris *et al*., 1992; Soro e Paxton, 1999).

Todas estas propriedades fazem do *Arbutus unedo* L. uma árvore de elevada importância ambiental e ornamental. O medronheiro é hoje em dia considerado uma espécie de elevada importância económica e medicinal. O medronho é transformado em diversos produtos alimentares tais como geleias, doces, compotas, licores (Simonetti *et al*., 2008; Pawlowska *et al*., 2006) e aguardente muito conhecida e apreciada principalmente no sul do país (Alarcão-e-Silva *et al*., 2001; Pallauf *et al*., 2008). Relativamente às propriedades medicinais, o medronho é reconhecido pelo seu poder diurético, anti-séptico das vias urinárias e laxativo. Estas propriedades derivam

sobretudo da sua composição química, rica em compostos com propriedades antioxidantes, como sejam fenóis, vitaminas (C e E), carotenóides e ácidos orgânicos (Alarcão-e-Silva *et al*., 2001; Kivçak e Mert, 2001; Pawlowska *et al.*, 2006; Males *et al*., 2006; Pallauf *et al*., 2008). As folhas de *Arbutus unedo* L. são usadas pelo interesse nas suas propriedades antioxidantes, adstringentes, diuréticas, antidiarreicas, depurativas, e mais recentemente utilizadas no tratamento de doenças como hipertensão, diabetes e problemas inflamatórios (Ziyyat e Boussairi, 1998; Mariotto *et al*., 2008; Afkir *et al*., 2008; Oliveira *et al.*, 2009). Estudos fitoquímicos em folhas revelaram que, grande parte destas propriedades resultam da sua composição em compostos fenólicos como taninos, flavonóides, entre outros (Males *et al*., 2006; Fiorentino *et al*., 2007), assim como de α-tocopherol (Kivçak e Mert, 2001).

1.2 Caracterização de uma espécie

Os recursos genéticos vegetais constituem parte essencial da biodiversidade e são responsáveis pelo desenvolvimento sustentável da agricultura, bem como da agro-indústria. Nos últimos anos têm surgido muitos factores que levam ao declínio de várias espécies vegetais, seja devido a incêndios, desflorestação para obtenção de madeira, ou simplesmente para substituir estas espécies por outras de superior valor económico. Esta perda de variabilidade genética conduz a graves problemas ambientais e económicos, impedindo o desenvolvimento de um ecossistema e agricultura sustentáveis. A conservação destes recursos vegetais e o estudo dos genes neles contidos são assim estratégias fundamentais para manter a biodiversidade.

Existem duas estratégias de conservação, a *ex situ* e a *in situ*, que foram definidas na Convenção sobre Diversidade Biológica (1992). Na *ex situ*, a conservação de um determinado recurso genético é feito fora do seu habitat natural. A *in situ* significa conservação dos ecossistemas e habitats naturais e manutenção e recuperação de populações viáveis de espécies no seu ambiente natural, e no caso de espécies cultivadas ou domesticadas, no ambiente onde elas desenvolveram as suas características distintivas. Em ambas as estratégias é necessário proceder-se à localização e à caracterização morfológica, molecular, biológica e química do material vegetal existente em populações silvestres naturais. O conhecimento e compreensão da variação e estrutura genética entre populações e indivíduos são dados essenciais para a

definição de estratégias de conservação e gestão sustentável (Sun *et al*., 1998). Somente com o material vegetal bem caracterizado e preservado se consegue lutar contra as muitas e variadas ameaças à produtividade agrícola e mesmo à extinção da espécie.

1.2.1 Caracterização morfológica

O primeiro passo para proteger legalmente uma cultivar nova, ou uma espécie em perigo é a sua identificação ou caracterização através de critérios devidamente estabelecidos. Tradicionalmente, os investigadores utilizam características morfológicas para o registo e lançamento de novas variedades, sendo consideradas o "cartão de apresentação" de uma espécie, de uma nova variedade ou de uma cultivar. Contudo, este tipo de caracterização torna-se limitado, quando se pretende distinguir genótipos de superior qualidade, aparentados, ou pertencentes à mesma espécie, podendo a distinção não ser adequada e correcta no caso de culturas de base genética relacionada (Smith e Smith, 1992; Pecchioni *et al*., 1996). Por exemplo, Celikel *et al.* (2008), com o objectivo de seleccionar os genótipos de *A. unedo* produtores de frutos de superior qualidade, procederam à caracterização morfológica de medronhos provenientes da região *Central Black Sea* (Turquia). Os resultados obtidos levaram os mesmos autores a seleccionar 5 genótipos por reunirem as características mais favoráveis. Nestes 5 genótipos procederam ainda à avaliação de diversos parâmetros morfológicos das folhas, nomeadamente peso, largura, comprimento e comprimento do pedúnculo. Verificaram que, nos 5 genótipos seleccionados, apesar de apresentarem como característica comum a produção de bons frutos, exibiam diferenças significativas ao nível da folha. Este resultado reforça a ideia de que a caracterização morfológica não é de todo suficiente para avaliar e diferenciar genótipos de uma mesma espécie.

1.2.2 Caracterização molecular

A caracterização molecular tem vindo a substituir bem como a complementar a caracterização morfológica de espécies. A caracterização molecular de espécies é feita com recurso a marcadores de DNA. Estes permitem avaliar a diversidade genética e a relação filogenética entre diferentes espécies, espécies geneticamente próximas ou indivíduos pertencentes à mesma espécie. De igual modo, este tipo de marcadores têm sido usados com sucesso na avaliação da autenticidade e traceabilidade da

variedade/tipo de composição de certos tipos de alimentos (Martins-Lopes *et al.*, 2008). A principal vantagem dos marcadores de DNA é permitirem um acesso directo ao genoma de um indivíduo, sem a necessidade de restrição a características morfológicas e o efeito que o meio ambiente tem sobre estas. A análise do DNA permite também identificar as mutações que ocorrem em regiões não codificadas dos genes, o que não acontece com a análise morfológica.

O facto de haver um número quase ilimitado de marcadores de DNA disponíveis e por permitirem evidenciar a variabilidade genética em diversas espécies vegetais, faz com que o seu uso na caracterização de indivíduos seja crescente. Em seguida será feita uma breve abordagem a apenas dois tipos de marcadores moleculares, nomeadamente à amplificação aleatória de DNA polimórfico (RAPD) e a sequencias simples repetidas (ISSR), por terem sido os utilizados no presente trabalho.

Na técnica de amplificação aleatória de DNA polimórfico - RAPD (do inglês, *Random Amplified Polymorphic* DNA), utiliza-se um único iniciador oligonucleotídico (*primer*), com cerca de dez nucleótidos, e de sequencia arbitrária para realizar a amplificação. As grandes vantagens desta técnica são a sua simplicidade, a rapidez na obtenção de dados, o custo relativamente reduzido comparativamente a outras técnicas moleculares, e a aplicabilidade imediata a qualquer organismo. Não exige ainda sequenciação de nucleótidos, nem o desenho de *primers* específicos.

Contudo, esta técnica tem a desvantagem de apresentar baixa reprodutibilidade e ser pouco consistente de um laboratório para o outro, o que dificulta a comparação dos dados obtidos em diferentes locais. Assim, devem ser tomados cuidados na padronização da técnica no laboratório para a caracterização de espécies. O nível de polimorfismo obtido com RAPDs varia grandemente com a espécie em questão, e tem sido utilizada com sucesso na caracterização de uma enorme variedade de espécies, incluindo cevada (Tinker *et al.*, 1993; Penner *et al.*, 1998), arroz (MacKill, 1995), videira (Büscher *et al.*, 1994; Ye *et al.*, 1998, Vidal *et al.*, 1999; Regner *et al.*, 2000; Luo e He, 2001), entre outras. A técnica de RAPD usada em simultâneo com ISSR origina resultados mais credíveis na análise da estrutura genética de espécies, uma vez que os marcadores RAPD têm sido descritos como estando associados com *locus* funcionalmente importantes (Penner, 1996) e os marcadores ISSR amplificam regiões não-codificantes hiper-variáveis (Esselman *et al.*, 1999).

A técnica de ISSR (do inglês *inter-simple sequence repeat*), representa uma das classes de marcadores moleculares mais recentes e foi desenvolvida a partir da

necessidade de estudar repetições microssatélites sem a utilização de sequenciação do DNA (Zietkiewicz *et al.*, 1994). Esta técnica baseia-se na amplificação termociclica de fragmentos de DNA presentes entre dois microssatélites, utilizando sequencias simples repetidas como *primers*. Teoricamente, é considerado superior ao marcador RAPD em termos de reprodutibilidade e polimorfismo (Qian *et al.*, 2001; Reddy *et al.*, 2002; Boronnikoval *et al*., 2007; Zhao *et al*., 2007; Han e Wang, 2010). É uma técnica simples, rápida e eficiente. Os marcadores ISSR têm sido utilizados para estimar a diversidade genética a nível inter e intra-especifico numa ampla variedade de espécies (Bornet e Branchard, 2001). Devido à sua abundância e dispersão no genoma, também se utilizam para estudar relações entre duas populações muito relacionadas (Redy *et al.*, 1999a; Huang e Sun, 2000; Deshpande *et al.*, 2001), em estudos de "fingerprinting", selecção assistida por marcadores, filogenia e mapeamento genético (Tautz, 1989; Williams *et al.*, 1990; Zabeau e Vos, 1993; Reddy *et al*., 2002).

Estas duas técnicas têm sido usadas na análise de perfis de DNA (Carriero *et al.*, 2002; Kuznetsova *et al.*, 2005), biologia de conservação (Hao *et al.*, 2006; Li *et al*,. 2005), genética de populações (Mamuris *et al.*, 2002; Zhang *et al.*, 2007) e estudos de filogenia (Kochieva *et al.*, 2006).

Tanto quanto é do nosso conhecimento o único trabalho efectuado com o intuito de estudar a diversidade genética de populações naturais de *A. unedo* foi efectuado na Turquia por Takroni *et al*. (2010). Estes autores, recorrendo a marcadores RAPDs, verificaram a existência de uma maior diversidade genética a nível da espécie face à existente ao nível das populações. Em Portugal, este é o primeiro estudo realizado com vista a estudar a diversidade genética de *Arbutus unedo* L. usando marcadores moleculares RAPD e ISSR.

1.2.3 Actividade antioxidante

A formação de radicais livres ocorre nos seres vivos durante o metabolismo celular, formando-se Espécies Reactivas de Oxigénio (ROS) (Satoh *et al.*, 2005). Um excesso de produção destas espécies reactivas pode ocorrer, no ser humano, devido ao stress oxidativo causado pelo desequilíbrio do sistema de defesa antioxidante do corpo. Estas espécies reactivas podem reagir com as biomoléculas tais como proteínas, hidratos de carbono, ácidos nucleícos e lípidos causando danos e até morte a nível celular (Aruoma, 1996a,b; Pulido *et al.*, 2000). Tal facto pode conduzir ao desenvolvimento de

doenças crónicas como cancro, diabetes, arterioesclerose, doenças neurológicas, e outras que envolvem o sistema cardio e cérebrovascular (Halliwell, 1989), e acredita-se ser um factor importante no envelhecimento precoce (Finkel e Holbrook, 2000). Contudo, as células possuem na sua estrutura defesas intrínsecas, nomeadamente enzimas (superóxido dismutase, catalase, glutationa peroxidase, entre outras) capazes de as proteger contra níveis excessivos de radicais livres. Estas defesas podem ser complementadas pela adição exógena de suplementos alimentares constituídos por compostos tais como vitaminas (A, E, β-caroteno), minerais (selénio, zinco) ou proteínas (transferina, albumina) (Ostrovidov *et al.*, 2000), ácido lipóico e flavonoides (apigenina e luteolina), entre outros (Valko *et al.*, 2007).

A procura por antioxidantes provenientes de fontes naturais tem recebido especial atenção e vários trabalhos têm sido desenvolvidos no sentido de identificar compostos susceptíveis de actuar como antioxidantes para substituir os sintéticos (Møller *et al.*, 1999). Tem sido demonstrado por vários investigadores que diversas plantas possuem na sua constituição antioxidantes naturais, como é o caso dos polifenois e flavonoides, os quais foram identificados como bloqueadores de radicais livres e oxigénio activo (Zheng e Wang, 2001).

Oliveira *et al.* (2009) investigaram a capacidade antioxidante de extractos aquosos, metanólicos, etanólicos e dietileter de folhas de *Arbutus unedo* L. através dos métodos do poder redutor, do efeito bloqueador de radicais de DPPH (2,2-difenil-1-picrilhidrazilo) e do efeito sequestrante de radicais superóxido. Quantificaram ainda os fenóis totais pelo método de Folin-Ciocalteau. Os resultados obtidos mostraram que o teor de fenóis totais foi superior em extractos etanólicos, seguido pelo aquoso, metanólico e por fim dietileter. O extracto etanólico foi o que apresentou maior poder redutor (EC_{50} 232,7 µg/mL) e efeito bloqueador de radicais de DPPH (EC_{50} 63,2 µg/mL), seguido pelo extracto aquoso, com respectivamente um EC_{50} de 287,7µg/mL e 73,7µg/mL. No efeito bloqueador de radicais superóxido foi, nos extractos metanólicos, onde se observaram os melhores resultados (EC_{50} 6,9µg/mL). Os autores concluíram que o *Arbutus unedo* L. é uma potencial fonte de antioxidantes naturais tendo em conta o elevado teor em fenóis totais obtidos em extractos etanólicos e aquosos.

1.3 Justificação e objectivos

Em Trás-os-Montes, ao contrário do verificado nas restantes regiões nacionais (em especial do Algarve) e internacionais, tem sido atribuída pouca importância económica ao medronheiro. Face à sub utilização dada a esta espécie fruteira tem-se assistido, nos últimos anos, à sua substituição por espécies florestais. Esta prática, juntamente com os fogos florestais, a acção antropológica e a desertificação das zonas rurais tem contribuído para a redução da área ocupada pelo medronheiro e, consequentemente a uma perda da variabilidade genética. Esta perda genética com consequências nefastas para os ecossistemas naturais poderá, em casos extremos, resultar na extinção da espécie. Face ao exposto, é urgente que se definam estratégias de conservação e gestão sustentável, sendo necessário para tal um conhecimento prévio das populações de medronheiro actualmente existentes na região e em Portugal.

O presente trabalho teve como objectivo geral seleccionar de entre a população natural de *A. unedo* os genótipos de superior qualidade, quanto a propriedades químicas e biológicas. Os genótipos seleccionados poderão vir a ser submetidos a propagação, tendo em vista o seu cultivo pelos agricultores, evitando a extinção desta espécie fruteira. Adicionalmente, a produção, comercialização e consumo de medronheiro poderá ser potenciado, em virtude das suas propriedades medicinais, assumindo desta forma uma maior relevância como mecanismo dinamizador da economia regional.

Assim sendo, os objectivos específicos foram:

i. Optimizar a extracção de DNA de folhas de medronheiro com o intuito de efectuar a sua caracterização genética (Capítulo 3);
ii. Caracterizar morfologicamente e geneticamente 4 populações naturais de *A. unedo* provenientes dos distritos de Bragança, Castelo Branco, Vila Real e Viseu utilizando marcadores moleculares RAPD e ISSR (Capítulo 4);
iii. Caracterizar quimicamente 19 genótipos da região de Bragança pela avaliação da actividade antioxidante através dos métodos de DPPH e Poder Redutor e pela determinação do teor em fenóis totais pelo método de Folin Ciocateu utilizando extractos aquosos das folhas (Capítulo 5);
iv. Seleccionar os genótipos de superior qualidade, quanto a propriedades químicas e biológicas (Capítulo 6).

Espera-se que associado à caracterização genética, a caracterização fitoquímica e biológica, possam por um lado contribuir para o delineamento de programas de maneio e conservação do medronheiro e, por outro, poderão permitir a multiplicação e valorização das populações de maior interesse.

1.4 Referências Bibliográficas

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Capitulo 2

Optimization of DNA extraction for RAPD and ISSR analysis of *Arbutus unedo* leaves

Optimization of DNA extraction for RAPD and ISSR analysis of *Arbutus unedo* leaves

Submitted

Abstract

Genetic analysis of plants relies on high yields of pure DNA. For the strawberry tree (*Arbutus unedo*) this represents a great challenge since leaves can accumulate large amounts of polysaccharides, polyphenols and secondary metabolites, which co-purify with DNA. For this specie, standard protocols do not produce efficient yields of high-quality amplifiable DNA. Here, we present for the first time an improved leaf-tissue protocol, based on the standard cetyl trimethyl ammonium bromide (CTAB) protocol, that yields large amounts of high-quality amplifiable DNA. Key steps in the optimized protocol are the addition of antioxidants compounds, namely polyvinyl pyrrolidone, 1.4-dithiothreitol and 2-mercaptoethanol, in the extraction buffer; the increasing of CTAB (3%, w/v) and sodium chloride (2M) concentration; and an extraction with organic solvents (phenol and chloroform) with incubation of samples on ice. The increased of the temperature used for cell lyses to 70°C shown also to improve both DNA quality and yield. The yield of DNA extracted was 200.0 ± 78.0 μg/μL and the purity, evaluated by the ratio A_{260}/A_{280}, was 1.80 ± 0.021, indicating minimal levels of contaminating metabolites. The quality of the DNA isolated was confirmed by random amplification polymorphism DNA (RAPD) and by inter-simple sequence repeat (ISSR) amplification, proving that the DNA can be amplified via PCR.

Key words: *Arbutus unedo* L.; strawberry tree; DNA isolation; RAPD; ISSR.

2.1 Introduction

The strawberry tree, *Arbutus unedo* L., is a typical evergreen plant of Mediterranean basin, as well as of other regions with hot summers and mild rainy winters. It is native to Greece, Lebanon, Southern Europe and Anatolia (Celikel *et al*., 2008). In Portugal, this specie appears mainly in the south (Algarve region), although it can be found in sparse distribution throughout all the country (Pedro, 1994). The strawberry tree plays an important role in the economy of the regions where they occurs. The production of alcoholic drinks from its fruits, such as liqueurs, and especially brandies, represents the main income for farmers (Alarcão-e-Silva *et al*., 2001). More recent uses are related with biomass production and floriculture (Mereti *et al*., 2002). The strawberry tree has also social and cultural importance since fruits continue to be used in regional gastronomy. They could be applied in the preparation of jams, jellies and marmalades, and in the confectionary of pies and pastry fillings (Alarcão-e-Silva *et al.*, 2001). Both, fruits and leaves, are also used in folk medicine to treat several diseases due to its recognized phytopharmaceuticals properties (Ziyyat *et al.*, 1997; Mariotto *et al.*, 2008; Afkir *et al.*, 2008; Oliveira *et al.*, 2009). Additionally, this specie has landscape importance especially due to its attractive red fruits in the fall and winter, and pinkish-white flowers in the fall.

During the last years several occurrences have caused strawberry tree decline in Portugal. Since this phenomenon may be put this specie in danger it is extremely urgent to adopt management and conservation strategies for the strawberry tree. It is therefore essential to characterize, both morphological and genetically, the different populations of *A. unedo*, which are considered the keys elements in management programs.

However, and as far as we known, the intraspecific genetic biodiversity are not known. This could be related to the difficult to isolated high-quality DNA from *A. unedo* tissues, a key element in such studies that use various molecular techniques. The difficulties encountered while working with this specie were caused by the presence of high amounts of polyphenols, polysaccharides, tannins and other secondary metabolites (Bryant, 1997; Zamboni *et al.*, 2008). In addition, these contaminants interfere in downstream reactions such as DNA restriction, amplification and cloning (Bryant, 1997).

Several protocols for DNA extraction have been success-fully applied to plant species (Doyle and Doyle, 1987; Reichardt and Rogers, 1994; Bryant, 1997), which

were further modified to extract high-quality DNA from plants containing such contaminants (Porebski *et al.*, 1997; Tel-Zur *et al.*, 1997; Cheng *et al.*, 2003; Cota-Sánchez *et al.*, 2006). However, we have previously tested these protocols as well as other unreported methods, and none of them proved to be suitable for extracting DNA from *A. unedo* leaves. All the protocols described yields low, degraded and impure DNA and unamplified in the polymerase chain reaction (PCR).

Thus, the present study aims to improve Doyle and Doyle (1987) method, by modifying some aspects of procedures and extraction buffer composition, with an attempt to isolate high-quality DNA from *A. unedo* leaves. Random amplified polymorphic DNA (RAPD's) reactions and inter-simple sequence repeat (ISSR) amplification was also performed in order to evaluate the suitability of the extracted DNA for PCR-based techniques. As far as we known this are the first report on DNA extraction from *A. unedo*, and we expected that this optimized protocol can be an incentive to performed studies investigating genetic diversity among this specie.

2.2 Material and methods

2.2.1 Plant Material

In February of 2009, fresh and healthy leaves of *A. unedo* were randomly sampled from 19 individuals of different geographically representative natural populations in the Trás-os-Montes region (Northeast of Portugal). After collection, the leaves were ground to a fine powder in a mortar with a pestle in the presence of liquid nitrogen, and stored at -80ºC until DNA extraction.

2.2.2 DNA isolation

The commonly used DNA isolation method, developed by Doyle and Doyle (1987), using cetyl trimethyl ammonium bromide (CTAB) in the extraction buffer was tried in the beginning. Since results proved unsatisfactory, we have developed and tested three modified CTAB protocol (method A, B and C). In these new protocols we have optimized the composition of the extraction buffer and introduced an additional step for proteins removal (Figure 1).

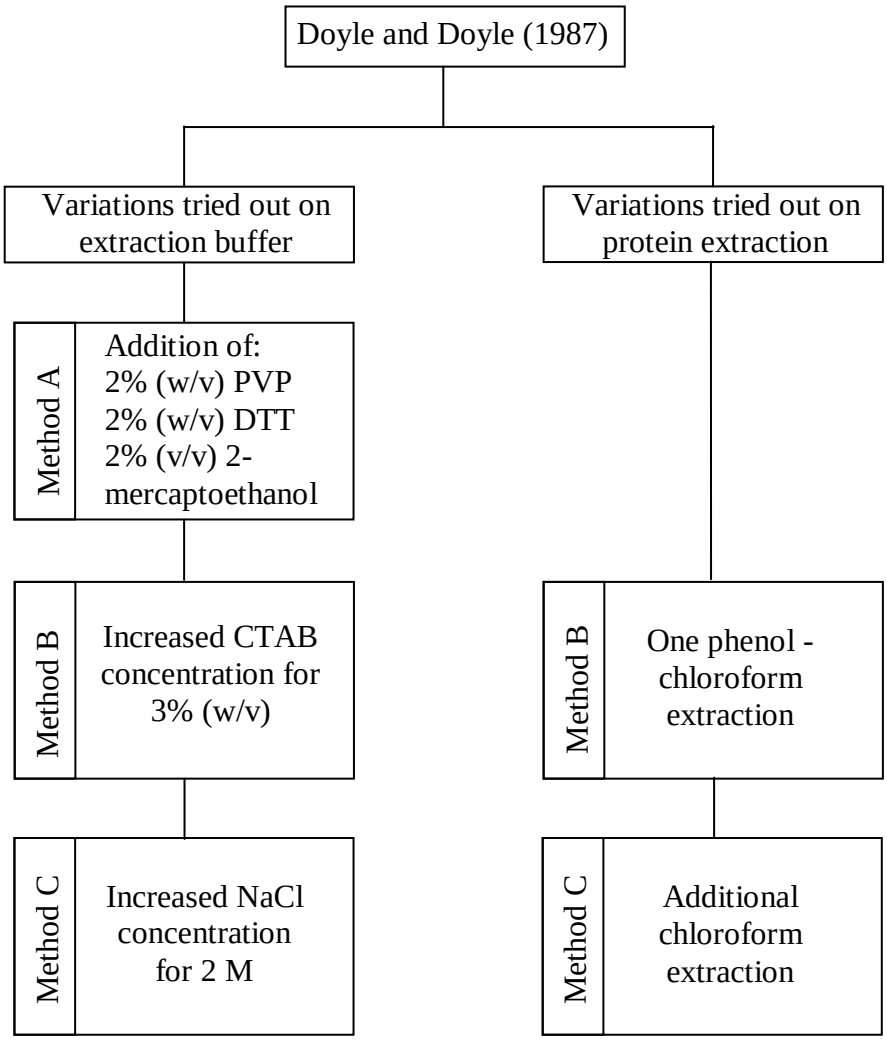

Figure. 1 - Variations tried out for the optimization of DNA extraction from *Arbutus unedo* leaves.

CTAB extraction buffer composition was firstly modified by employing polyvinyl pyrrolidone (PVP), 1.4-Dithiothreitol (DTT) and 2-mercaptoethanol (method A), followed by an increasing of CTAB (method B) and sodium chloride (method C) concentration. To exclude protein impurities we have tested one phenol - chloroform extraction (method B) followed by an additional chloroform extraction (method C). The optimized procedure, which allowed the great improvement on both DNA yield and purity, was described follows.

2.2.3 Reagents and Solutions

The extraction buffer consisted of 3% (w/v) CTAB (Sigma), 100 mM Tris-HCl pH 8.0 (CalBiochem), 20mM EDTA pH 8.0 (Merck) and 2M sodium chloride (NaCl; Merck). After autoclaved for 20 min, 2% (w/v) PVP (mol wt 40.000; Sigma), 2% (w/v)

DTT (Sigma) and 2% (v/v) 2-mercaptoethanol (Merck) were added to the extraction buffer, immediately before used. In addition, phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v, from Fluka), chloroform: isoamyl alcohol (24:1, v/v, from Panreac), TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 70% (v/v) ethanol (Merck), proteinase K (20 mg/ml, from Sigma) and ribonuclease A (10 mg/mL, RNase-A, from Sigma) were prepared. Absolute iso-propanol (Merk) was also needed.

2.2.4 DNA isolation protocol

Ground leaves tissues (approximately 100 mg) were transferred to 2-mL micro tube containing 1.3 mL of pre-heated (70ºC) extraction buffer. The tube was shaken and left to stand for 2 min at room temperature. After that, 10 μL proteinase K (20 mg/mL) was added and the mixture was shaken again for 1 min. The mixture was incubated at 70ºC in a water bath for 30 min with occasional mixing. The tube was centrifuged at 10,000 rpm, for 5 min at 4ºC and the supernatant was transferred to a clean 2-mL micro tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed by using gentle inversion for 5 min, incubated on ice for 10 min and centrifuged at 10,000 rpm for 5 min at 4ºC. The supernatant was transferred to a clean 2-mL micro tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The tube was then gentled inverted for 5 min, incubated on ice for 10 min and centrifuged at 10,000 rpm for 5 min at 4ºC. The upper aqueous phase was transferred to a clean 1.5 mL micro tube and DNA was precipitated by adding one volume of ice-cold iso-propanol (-20ºC), mixed by gentle inversion until the homogeny phase appear, incubated at -20ºC for 1 hour, and centrifuged at 13,000 rpm for 20 min at 4ºC. The obtained pellet was washed with 500 μL of ice-cold 70% ethanol and centrifuged again at 13,000 rpm for 5 min at 4ºC. The supernatant was discarded and the pellet was air-dry for 20 min at room temperature. Finally, the pellet was re-suspended in 50 μL of deionized water or TE buffer and stored at -20ºC. Sometimes DNA could be contaminated with RNA. In this case, it is necessary performed an additional step, by adding 1 μL RNase-A (10 mg/mL) to the sample and incubated it for 30 min at 37ºC.

2.2.5 Concentration, purity and quality of the DNA extracted

The quantity and quality of the DNA obtained were assessed spectrophotometrically at 260 and 280 nm, and the A_{260}/A_{280} ratio was used to assess

contamination with proteins. This spectrophotometric analysis was performed on triplicate samples of extracted DNA, in a PG Instruments Ltd. T70 UV/VIS spectrometer. In order to verify DNA integrity, 3µL DNA was subjected to gel electrophoresis on 1.2% (w/v) agarose gel, stained with ethidium bromide (Sambrook *et al.*, 1989), visualised under UV transilluminator and photographed using the Stratagene Eagle Eye II.

2.2.6 RAPD and ISSR amplifications

RAPD and ISSR analysis was used to test the quality and performance of the DNA extracted from method C, which proved to be the most efficacious compared to others methods tested in the present study (see results).

RAPD reactions were performed in a volume of 25µl containing 20 ng of template DNA, 10x PCR Buffer (10 mM Tris HCl pH 8.3; 50 mM KCl), 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM of each dNTP (Fermentas), 0.4 µM of single primer, 1.0 U of *Taq* DNA polymerase (Thermo Scientific) and ultra pure water up to 25 µL. A total of 20 primers (decamer oligonucleotide purchased from Operon Technologies Inc. - OPA) were used to check the fidelity of amplification. Reactions without DNA were used as negative controls. PCR amplification was performed as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C, and a final extension at 72°C for 10 min.

ISSR reactions were performed in a volume of 25µl containing 10 ng of template DNA, 10x PCR Buffer (10 mM Tris HCl pH 8.3; 50 mM KCl), 2.5 mM $MgCl_2$ (Thermo Scientific), 0.2 mM of each dNTP (Fermentas), 0.4 µM of single primer, 1 U of *Taq* DNA polymerase (Thermo Scientific) and ultra pure water up to 25 µL. A total of 15 primers, designed by Stab Vida (Caparica, Portugal), were screened. Reactions without DNA were used as negative controls. PCR amplification was performed as follows: initial denaturation at 94ºC for 3 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 49ºC, 2 min at 72ºC, and a final 10 min extension at 72ºC.

Amplifications were carried out in a Thermocycler Biometra UNO II (Thermoblock, Biotron). PCR amplifications products were analyzed by electrophoresis at 80 V in 2% (w/v) Wide range/Standard 3:1 Agarose (Sigma) gels in the presence of a 1 *Kb* molecular weight marker (Thermo Scientific). Gel was stained with ethidium bromide (Sambrook *et al.*, 1989), visualised under UV transilluminator and photographed using the Stratagene Eagle Eye II. The experiment was repeated twice.

2.3 Results and Discussion

The strawberry tree is a typical plant specie of the Mediterranean basin. This specie plays diverse roles both in natural environment and as a resource in rural areas, with recognized traditional uses in food industry, phytochemistry, medicine and ornamental plant production. Therefore, it is urgent to preserve the genetic resources of *A. unedo*, integrating the conservation issue with their sustainable utilization. This could only be achieved after known the existing genetic diversity of *A. unedo*, which is practically unknown. Various types of DNA-based molecular techniques are utilized to evaluated genetic variability in plants. These approaches require both high-quality and quantity DNA, which in *A. unedo* presents a great challenge.

In the present study, one standard (Doyle and Doyle, 1987) and three improved methods (method A, B, C) for DNA isolation were applied to *A. unedo* leaves (Figure 1). Firstly, we have tested the CTAB method reported by Doyle and Doyle (1987), which proved to be inadequate. With this method no DNA was extracted (Table 1). This could be probably due to the specific characteristics of this plant like the presence of polyphenols, tannins, polysaccharides, proteins and other secondary metabolites (Males *et al.*, 2006; Fiorentino *et al.*, 2007; Zamboni *et al.*, 2008), which either lead to embedding of DNA into a sticky gelatinous matrix (Do and Adams, 1991) or promote DNA degradation (John, 1992). Taking into consideration the traditional application form of *A. unedo* leaves, the compounds that provide the therapeutic efficacy to the plant could be also a problem in the isolation procedure by binding with the DNA and being precipitated along with it (Pirttila *et al.*, 2001). Accordingly, we have modified Doyle and Doyle (1987) – CTAB protocol to improve DNA yield and quality.

The first yield improvement was achieved by adding antioxidants compounds to the extraction buffer (method A). The addition of PVP, DTT and 2-mercaptoethanol allowed an increased of DNA yield from 0 to 85.4 μg/μL (Table 1).

Table 1 - Yield and purity of DNA extracted from *Arbutus unedo* leaves by different methods. Data represent mean ± SD of 6 replicates. n.d. - Not determined.

Methods	DNA purity (A_{260}/A_{280})	DNA yield (µg/µL)
Doyle and Doyle (1987)	n.d.	n.d.
A	1.48 ± 0.448	85.4 ± 23.2
B	1.51 ± 0.304	112.5 ± 48.2
C	1.80 ± 0.021	200.0 ± 78.0

This procedure proved to be crucial to reduce DNA degradation by oxidized polyphenols, formed during cell lyses. In fact, it is known that *A. unedo* leaves are rich in polyphenolics like flavonoids (Males *et al.*, 2006; Fiorentino *et al.*, 2007; Zamboni *et al.*, 2008), which have been reported to degraded genomic DNA (Peterson *et al.*, 1997). PVP act as adsorbents of polyphenols (John, 1992) while DTT and 2-mercaptoethanol inhibit the oxidation of polyphenols. Although DNA yield was incremented in method A, another problem persist by the presence of contaminating compounds in the DNA samples, particularly proteins and polysaccharides, as visualized in the agarose gel (Figure 2) and confirmed by the low A_{260}/A_{280} ratio obtained (1.48, Table 1). Complete removal of polysaccharides during DNA isolation assumes critical importance due to their well-established interference problems, namely failure of DNA amplifications during PCR due to inhibition of *Taq* polymerase activity (Fang *et al.*, 1992).

Thus, a further optimization was obtained by increasing the concentration of CTAB (method B) and further of NaCl (method C) in the extraction buffer (Figure 1). The combination of high concentration of CTAB (3%, w/v) and NaCl (2M), performed in method C, increased genomic DNA yield by 2.34 and 1.80 times in comparison to methods A and B, respectively (Table 1). This step proved to be very critical for the recovery of pure DNA in the entire isolation process. The use of high concentration of NaCl has been previously pointed to be suitable for the removal of polysaccharides from DNA solutions by increasing their solubility in ethanol, and thus preventing its co-precipitation with DNA (Muhammad *et al.*, 1994; Aljanabi and Martinez, 1997). NaCl combination with the cationic detergent CTAB has also been proved to be beneficial in DNA isolation from polysaccharide-rich plants (Syamkumar *et al.*, 2003). To exclude protein impurities we have tested one phenol - chloroform extraction (method B)

followed by an additional chloroform extraction (method C) (Figure 1). The results obtained showed that the use of organic solvents have substantially removed proteins. In fact, method C has showed to extracted DNA with higher purity (A_{260}/A_{280} ratio equal to 1.80) than method A (A_{260}/A_{280} ratio equal to 1.48) where no organic solvent extraction has been performed (Table 1). In addition, the elimination of proteins was also favoured by the incubation of samples on ice after organic solvent addition. This result suggested that using only DTT and 2- mercaptoethanol in the extraction buffer, as well as of protein-hydrolyzing enzymes like proteinase K, are not sufficient to removed proteins. Thus, the combination of high concentration of CTAB (3%, w/v) and NaCl (2M) in the extraction buffer along with one wash with phenol: chloroform: isoamyl alcohol, followed by another with chloroform: isoamyl alcohol proved very effective to extracted sufficient quantities of high-quality DNA from *A. unedo* leaves. The agarose gel electrophoresis of total genomic DNA showed high molecular weight DNA, with no sign of degradation and contamination (Figure 2).

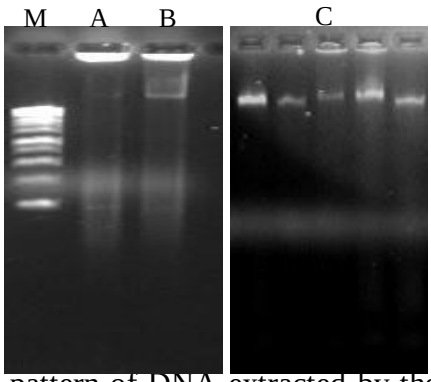

Figure 2 - Electrophoretic pattern of DNA extracted by the different modified CTAB method (method A, B, C) from *Arbutus unedo* leaves. M – *1-Kb* molecular weight marker (Thermo Scientific). The electrophoresis was performed in 1.2% (w/v) agarose gel.

It is also worth mentioning that the increased of the temperature used for cell lyses to 70°C was helpful to improve both DNA quality and yield. With the original incubation temperature (60º) the yield and quality of DNA extracted was lowest (data not shown).

The suitability of isolated DNA from the optimized protocol (method C) in molecular techniques was assessing by RAPD and ISSR analysis, which are useful for assessment of genetic diversity and phylogenetic relationship. The results obtained shown that the DNA extracted from the optimized protocol was of suitable quality to

screen levels of genetic diversity using both RAPD and ISSR and proving that the DNA can be amplified via PCR (Figure 3).

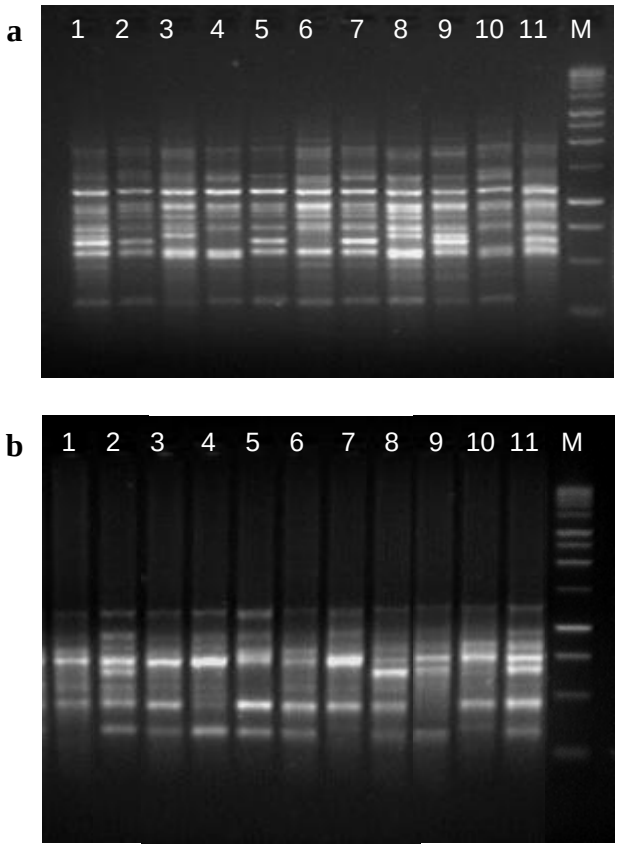

Figure 3 – Amplification of DNA from 11 (Lanes 1-11) *Arbutus unedo* individuals. (a) Random amplified polymorphic DNA (RAPD) using primer OPA-02; (b) and inter-simple sequence repeat (ISSR) amplification using the primer $(CA)_8A$. M: *1-Kb* molecular weight marker (Thermo Scientific). The electrophoresis was performed in 2% (w/v) agarose gel.

The RAPD and ISSR patterns showed considerable genetic variation between *A. unedo* individuals from different geographic origin.

In summary, the Doyle and Doyle (1987) protocol was successfully optimized by adding antioxidants compounds to the extraction buffer, by increasing the incubation temperature and by including an extraction with organic solvents. These changes made possible to obtain high purity DNA from *A. unedo* leaves suitable for further genomic analysis. To our knowledge, no other studies report DNA extraction from this plant. The results obtained will form a strong beginning for future molecular characterization and genetic improvement works in this promising medicinal plant.

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Capitulo 3

Genetic diversity in Portuguese *Arbutus unedo* population using morphological and molecular markers

Genetic diversity in Portuguese *Arbutus unedo* population using morphological and molecular markers

Submitted

Abstract

The genetic variability between 46 *Arbutus unedo* L. genotypes representatives of four natural populations of the North and Centre of Portugal was investigated using morphological characters, inter-simple sequence repeat (ISSR) and random amplified polymorphic (RAPD) for a proper conservation. Five morphological quantitative traits were measured in 40 leaves of each plant, namely, leave length and width, leave length/width ratio, dry-weight and peduncle length. A total of 11 polymorphic primers (5 ISSR and 6 RAPD) were used to assess the genetic variability. The selected RAPD and ISSR primers generated a total of 56 and 45 amplified DNA fragments, respectively. High frequencies of polymorphism, 83.9% for RAPD and 86.7% for ISSR, were detected. Statistical approaches were employed to construct genetic relationships by RAPD, ISSR and morphological analysis. The results obtained for the five morphological traits examined shown variation among *A. unedo* individuals. Cluster analysis by the Unweighted Pair-Group Method Arithmetic Average (UPGMA) of Jaccard's coefficients generated dendrograms for both RAPD and ISSR molecular markers that revealed moderate genetic diversity among the forty six genotypes. ISSR markers were relatively more efficient than the RAPD assay. The Mantel's test was used to correlate the RAPD and ISSR markers, as well as the molecular analysis with morphological characters and the results shown a low correlation (R=0.22 and R=0.09, respectively). The Jaccard's similarity coefficient ranged from 0.53 to 0.98, 0.45 to 0.95, and 0.55 to 0.91 with RAPD, ISSR, and combined dendrogram (RAPD+ISSR), respectively. The analysis for pair-wise shown that the genetic identities follow completely the genetic distance pattern. With POPEGENE analysis was verified that the genetic variation was highest in *A. unedo* population from Bragança and lowest in Castelo Branco population. The results shown that the diversity within populations was lower than between populations. This study revealed the genetic differentiation which

will provide a template for conservation and protection of the populations and species, preserving and maintaining isolated the different genetic pools founded.

Keywords: Genetic Diversity, Random Amplified Polymorphism (RAPD), Inter-Specific Sequence Repeat (ISSR), *Arbutus unedo L.*

3.1 Introduction

The strawberry tree (*Arbutus unedo* L., Ericaceae) thrives on the Iberian Peninsula and in the Mediterranean basin, as well as in other regions with hot summers and mild rainy winters (Celikel *et al.*, 2008). In Portugal, this specie appears mainly in the south (Algarve region), although it can be found in sparse distribution throughout all of the country (Pedro, 1994). This perennial shrub plant with 1.5 to 3 m tall can, occasionally, reach 9 m tall and 8 m wide, and show strong resistance to hard environmental conditions. Its fruits (berries) are spherical, about 2-3 cm in diameter, orange-red when ripe and tasty only when fully ripe in autumn (Anonymous, 2004b). The flowers are small, white or light pink, and assembled in panicles. The leaves are alternate, simple, oblong with toothed margins, with a dark green colour and are up to 4-12 cm long (Males *et al.*, 2006).

In Portugal, the strawberry trees present some importance in local agricultural economies derived, especially from the commerce of jams, marmalades and liquors obtained from arbutus berries, as well as of *A. unedo* based honey (Alarcão-e-Silva *et al.*, 2001). The appearance of both fruits and flowers during the winter months also makes this plant very popular for ornamentation (Males *et al.*, 2006). This specie also present great ecological importance since it avoids erosion of the soils and has the capacity to regenerate rapidly after fires (Gomes and Canhoto, 2009). Another important income is the use of different parts of *A. unedo* in folk medicine. For example, the fruits are frequently used as antiseptic, diuretic and laxative (Ziyyat and Boussairi, 1998; Pallauf *et al.*, 2008). The leaves are used as an infusion, for their astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and in the therapy of some diseases, such as hypertension, diabetes, and in the treatment of inflammatory diseases (Ziyyat *et al.*, 1997; Ziyyat and Boussairi, 1998; Mariotto *et al.*, 2008; Afkir *et al.*, 2008).

During the last years the high anthropogenic pressure on land as well as the occurrence of forest fires, the deforestation and the *A. unedo* replace by other species, have caused the strawberry tree area decline in Portugal. Since this phenomenon may put this specie in danger it is extremely urgent to adopt management and conservation strategies. However, for a successful *A. unedo* conservation is necessary previously known the genetic diversity of native populations; and to date such information is not known.

Traditional methods for testing genetic variability in plant species are based on morphological characters like leaf type, floral morphology and fruit characters, among others (Khurshid *et al.*, 2004; Furat and Uzun, 2010). Studies on use of morphological traits to assess genetic diversity in *A. unedo* have been yet reported in Italy (Mulas *et al.*, 1998) and also in Turkey (Karadeniz *et al.*, 1996; Mulas and Deidda, 1998; Gozlekci *et al.*, 2003; Seker *et al.*, 2004; Celikel *et al.*, 2008). However, the solely used of this method present some disadvantages due to the fact of most morphological traits are highly influenced by environmental conditions or vary with development stage of plant (Kercher and Sytsma, 2000; Ouinsavi and Sokpon, 2010). Consequently, in the last years a number of molecular techniques have been used by researchers to complement morphological traits in assessing plant genetic diversity (Zaefizadeh and Goliev, 2009; Sorkheh *et al.*, 2009). Molecular analyses comprise a large variety of DNA molecular markers, which can be employed in the evaluation of genetic diversity and also in the construction of genetic and physical maps (for a review see Mondini *et al.*, 2009). DNA based markers have many advantages over phenotypic markers in that they are highly heritable, relatively easy to assay and are not affected by the environment (Duran *et al.*, 2009). Among the various molecular markers, PCR-based techniques of random multilocus analysis, namely Random Amplified Polymorphic DNAs (RAPDs) and Inter-Simple Sequence Repeat (ISSR), are commonly use for genetic studies (Korbin *et al.*, 2002; Dangi *et al.*, 2004; Julio *et al.*, 2008) as well as for ecological, evolutionary, taxonomical and phylogeny studies of plant sciences (Raina *et al.*, 2001; Li *et al.*, 2005; Kuznetsova *et al.*, 2005; Hao *et al.*, 2006; Kochieva *et al.*, 2006; Zhang *et al.*, 2007). Both RAPD and ISSR techniques are well established and their advantages and limitations have been documented (Agarwal *et al.*, 2008; Primmer, 2009). The few molecular data regarding genetic variation of *A. unedo* have been recently published by Takroni and Boussaid (2010). In this study, RAPD analysis has been successfully in the evaluation of genetic diversity of nine Tunisian population of *A. unedo*.

Despite its importance, information of genetic diversity of *A. unedo* populations grown in Portugal is not known, which negatively affects its conservation. Therefore, both morphological traits and molecular markers were employed to examine genetic diversity within and among four Portuguese *A. unedo* populations. Since there isn't any information on the *A. unedo* genome we have decided to utilize both RAPD and ISSR

analysis. The results could be useful in guiding future conservation and selection of interesting genotypes for cultivation.

3.2 Material and methods

3.2.1 Study sites and sampling

Forty six genotypes were collected from natural populations of *A. unedo* located in different regions of interior north and center of Portugal (Figure 1), in autumn of 2008. These genotypes were classified into four different populations for analysis, and were designated as Bragança, Vila Real, Viseu and Castelo Branco (Table 1). In each population, fresh branches were randomly harvested from five to twenty two individuals, located in a minimum of 30 m from each other in order to avoid collecting multiple plants from the same parent. The plant material were collected to sterile plastic bags, brought to the laboratory and processed within a few hours. In the laboratory, the leaves were detached from the branches and used for morphological studies and for molecular analysis. For the molecular analysis, young leaves were ground to a fine powder in a mortar with a pestle in the presence of liquid nitrogen, and stored at -80ºC until DNA extraction.

Figure 1 - Map of Portugal showing the geographical distribution of the analysed *Arbutus unedo* individuals. Two *A. unedo* populations were collected in three different regions (Vila Real and Bragança) in the interior north of Portugal; and another two populations were collected in two different regions (Viseu and Castelo Branco) in the interior centre of Portugal.

Table 1 – Samples of *A. unedo* collected in each population.

Population	Samples collected
Bragança	Argoselo
	Bragança 1
	Bragança 2
	Carragosa
	Donai
	Faílde
	Fontes
	Mirandela
	Outeiro
	Pinela 1
	Pinela 2
	Pinela 3
	Vila Boa 1
	Vila Boa 2
	Vila Boa 3
	Vila Boa 4
	Vila Verde
	Vilas Boas 1
	Vilas Boas 2
	Vimioso
	Vinhais 1
	Vinhais 2
Castelo Branco	Fundão 1
	Fundão 2
	Fundão 3
	Fundão 4
	Fundão 5
	Fundão 6
	Fundão 7
Vila Real	Alvações do Tanha 1
	Alvações do Tanha 2
	Alvações do Tanha 3
	Alvações do Tanha 4
	Alvações do Tanha 5
	Bagauste
	Nogueira
	Quintas das Melras
	Vila Real 1
	Vila Real 2
	Vila Real 3
	Cabeceiras de Basto
Viseu	Britiande
	Lamego 1
	Lamego 2
	Lamego 3
	Viseu

3.2.2 Morphological characterization

Five quantitative morphological characters, namely leaf length and width, leaf length/width ratio, leaf peduncle length and leaf dry weigh were measured in forty leaves per genotype (Table 2).

3.2.3 DNA extraction and quantification

Total DNA was extracted following the method described by Doyle and Doyle (1987), using cetyl trimethyl ammonium bromide (CTAB), adapted to *A. unedo* leaves. Briefly, 100 mg of ground leaves tissues were transferred to 2 mL micro tube containing 1.3 mL of pre-heated Lyses buffer [3% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 20mM EDTA pH 8.0, 2M NaCl, 2% (w/v) PVP, 2% (w/v) DTT and 2% (v/v) 2-mercaptoethanol]. The tube was mix by inversion and left to stand for 2 min at room temperature. After that, 10 μL proteinase K (20 mg/mL) was added, mix by inversion for 1 min and incubated at 70ºC for 30 min. Following centrifugation at 10,000 rpm (4 ºC) for 5 min, the supernatant was collected to a tube and one volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added. This mixture was mixed by using gentle inversion for 5 min, incubated on ice for 10 min and centrifuged at 10,000 rpm for 5 min at 4ºC. An equal volume of cold chloroform: isoamyl alcohol (24:1, v/v) is added to the supernatant and the mixture is centrifuged at 10,000 rpm for 5 min at 4ºC. The DNA was precipitated with ice-cold iso-propanol (-20ºC) and the obtained pellet was washed with ice-cold 70% ethanol. The DNA pellet was air-dry for 20 min at room temperature, re-suspended in 50 μL of deionized water and stored at -20ºC until use.

The quantity and quality of the DNA obtained were assessed spectrophotometrically at 260 and 280 nm, and by visualization under UV light, after electrophoresis on 1.2% (w/v) agarose gel. The resuspended DNA was then diluted in sterile distilled water to an appropriated concentration for use in amplification reactions.

3.2.4. Primer screening

Forty 10-mer RAPD primers, corresponding to kits A and H from Operon Technologies (California, USA), and 20 synthesized ISSR primers (STAB Vida, Portugal) were initially screened using three of the *A. unedo* individuals to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified

products. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded.

3.2.5. RAPD and ISSR amplifications

From the 40 decamer oligonucleotide RAPD primers screened (OPA and OPH kit, Operon Technologies Inc.), six primers were selected for analysis (Table 3). An initial optimization of PCR conditions was conducted including the concentration of template DNA, primer, DNA polymerase, $MgCl_2$ and also the annealing temperature. The optimized RAPD reactions were performed in a volume of 25µl containing 10 ng of template DNA, 5µl 5x Green GoTaq® Flexi Buffer (Promega), 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM of each dNTP (Fermentas), 0.4 µM of single primer, 1.0 U of GoTaq® DNA polymerase (Promega) and ultra pure water up to 25 µL. PCR amplification was performed as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C, and a final extension at 72°C for 10 min.

Twenty ISSR primers were screened and five of them (Table 3) were selected for final study because of their strong and reproducible amplification. As for RAPD analysis, an initial optimization of PCR conditions was conducted. This included the concentration of template DNA, primer, DNA polymerase, $MgCl_2$ and the most important the annealing temperature. In the optimized conditions the ISSR reactions were performed in a volume of 25 µl containing 20 ng of template DNA, 5µl 5X Green GoTaq® Flexi Buffer (Promega), 2mM $MgCl_2$ (Thermo Scientific), 0.2 mM of each dNTP (Fermentas), 0.4 µM of single primer, 1.0 U of GoTaq® DNA polymerase (Promega) and ultra pure water up to 25 µL. PCR amplification was performed as follows: initial denaturation at 94ºC for 3 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 49ºC, 2 min at 72ºC, and a final 10 min extension at 72ºC.

Amplifications were carried out in a Thermocycler Biometra UNO II (Thermoblock, Biotron). PCR amplifications products were analyzed by electrophoresis at 80 V in 2% (w/v) Wide range/Standard 3:1 Agarose (Sigma) gels, in 1x TAE (Tris Acetate EDTA) buffer, and in the presence of a 1 *Kb* molecular weight marker (Thermo Scientific). Gel was stained with ethidium bromide, visualised under UV transilluminator and photographed using the Stratagene Eagle Eye II. All the reactions were conducted two times, using DNA of various extractions and different lots of Taq polymerase to verify the reproducibility of results.

3.2.6. Data scoring and analysis

Results for the morphological characters are shown as mean ± standard deviation, maximum and minimum values. Euclidean Distance, based on all morphological characters analyzed, was used to generate a dendrogram by unweighted pair group method analysis (UPGMA). This analysis was carried out using STATISTIC v.9 software. Principal component analysis (PCA) was applied in order to reduce the number of variables (5 variables corresponding to morphological data) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the morphological data of 46 genotypes of *A. unedo* leaves from different locations. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). Statistical analysis was carried out using SPSS software version 17.0 (SPSS Inc.).

In the molecular analysis, only reproducible and well-defined bands were considered as potential polymorphic markers. Diffuse or faint bands as well as bands that occurred in the extremes of the amplified size range were not scored. The comparison of amplified DNA profiles for each RAPD and ISSR primer was performed on the basis of the presence (1) or absence (0) of fragments. These binary data were used to compute the genetic distances of *A. unedo* genotypes according to Jaccard´s coefficient. Combined analysis of the RAPD and ISSR (ISSR+RAPD) data sets were also performed. The coefficients values obtained were used to construct a dendrogram by unweighted pair group method for arithmetic mean (UPGMA) using FreeTree version 0.9.1.50 (Pavlicek *et al.*, 1999). The same software was used for bootstrap analysis (i.e. repeated sampling with replacement) of 1000 permutated datasets, to support dendograms robustness. The software TreeView version 1.6.6 (Page, 1996) was used to draw the dendrograms. A Mantel test was used to determine the correlation between the two marker types (RAPD and ISSR) and also between molecular genetic distances and morphological distance (Mantel, 1967).

For each population, the number of observed alleles (*Na*), number of effective alleles (*Ne*), Nei's (1973) gene diversity (*Ho*), Shannon's information indices (*Io*) and percentage of polymorphic loci (*P*) were calculated. Nei's analysis of gene diversity in subdivided populations (Nei, 1987) was carried out by estimating the total heterozygosity (*Ht*), heterozygosity within populations (*Hs*), diversity coefficient among populations (*Gst*) and gene flow from *Gst* (*Nm*) parameters. All of these

parameters were calculated with the POPGENE software Version 1.31 (Yeh *et al.*, 1999). Nei's (1972) standard genetic identity and standard genetic distance for all pair-wise populations were calculated using the same software. A dendrogram for the four populations was also created using UPGMA analysis and Nei's (1978) unbiased genetic distances.

To analyze the relation between genetic distance and geographic distance between the populations, regression analysis was done using inter-population genetic distance against the geographic distance between them in kilometers. A scatter diagram was prepared and linear regression line was applied.

3.3. Results

3.3.1. Morphological analysis

The mean values of the five characters measured in leaves of 46 *A. unedo* genotypes, their standard deviations and the maximum and minimum values are present in Table 2. The results show, for all the examined characters, morphological variation among *A. unedo* individuals. This result was also confirmed by analysis of variance, which revealed significantly differences between genotypes based on all morphological characters. The morphological characters leaf dry-weight and peduncle length were found to be more variable (Table 2). Among the 46 *A. unedo* genotypes analyzed the leaf dry-weight varied from 0.41 g (genotype Bragança 2) to 0.07 g (genotype Vilas Boas 2) and its mean value was 0.17 g; leaf peduncle length varied from 1.24 cm (genotype Carragosa) to 0.35 cm (genotype Fundão 3) and its mean value was 0.63cm. Leaf length/width ratio, length and width leaf were found to be less variable. Their values varied respectively from 2.9 cm (genotype Vila Boa 4) to 1.9 cm (genotypes Alvações do Tanha 5 and Bagauste); from 9.1 cm (genotype Bragança 2) to 3.8 cm (genotype Vilas Boas 2) and from 3.3 cm (genotypes Bragança 2, Argoselo and Carragosa) to 1.7 cm (genotype Alvações do Tanha 3).

Table 2 - Measurements (mean ± standard deviation, N = 40) of morphological characters determined in leaves of 46 *A. unedo* genotypes. The maximum and minimum values are also shown in parenthesis. In each column different letters mean significant differences ($p < 0.05$). Leaf L/W ratio - Leaf length/width ratio; CV - coefficient of variability.

Genotype	Dry weight (g)	Length (cm)	Width (cm)	Leaf L/W ratio	Peduncle (cm)
Donai	0.20 ± 0.03	6.8 ± 0.7	2.8 ± 0.3	2.5 ± 0.4	0.37 ± 0.05
	(0.14 – 0.24)	(5.5 – 8.0)	(2.1 – 3.4)	(1.7 – 3.3)	(0.26 – 0.47)
Carragosa	0.30 ± 0.07	7.3 ± 0.5	3.3 ± 0.4	2.3 ± 0.3	1.24 ± 0.16
	(0.16 – 0.46)	(6.1 – 8.2)	(2.6 – 4.3)	(1.7 – 2.9)	(0.85 –1.64)
Fontes	0.20 ± 0.03	6.8 ± 0.6	2.6 ± 0.2	2.6 ± 0.3	0.59 ± 0.07
	(0.13 – 0.25)	(5.5 – 7.8)	(2.1 – 3.1)	(2.0 – 3.5)	(0.42 – 0.73)
Vila Verde	0.18 ± 0.03	6.4 ± 0.5	2.3 ± 0.2	2.8 ± 0.3	0.65 ± 0.09
	(0.12 – 0.24)	(5.5 – 7.4)	(1.8 – 2.9)	(2.1 – 3.2)	(0.44 – 0.79)
Vinhais 1	0.17 ± 0.04	5.3 ± 0.4	2.7 ± 0.3	2.0 ± 0.2	0.55 ± 0.05
	(0.11 – 0.25)	(4.5 – 6.0)	(2.1 – 3.5)	(1.5 – 2.5)	(0.42 – 0.65)
Vinhais 2	0.21 ± 0.07	6.4 ± 0.8	2.8 ± 0.6	2.3 ± 0.4	0.67 ± 0.14
	(0.12 – 0.35)	(5.0 – 8.2)	(2.0 – 3.9)	(1.6 – 3.1)	(0.43 – 0.94)
Pinela 1	0.22 ± 0.11	5.8 ± 1.3	2.3 ± 0.6	2.6 ± 0.5	0.46 ± 0.14
	(0.06 – 0.53)	(3.2 – 8.4)	(1.0 – 3.3)	(1.7 – 4.0)	(0.20 – 0.80)
Pinela 2	0.23 ± 0.08	5.9 ± 0.9	2.4 ± 0.6	2.6 ± 0.5	0.75 ± 0.15
	(0.06 – 0.42)	(3.3 – 7.2)	(1.1 – 3.2)	(2.0 – 4.5)	(0.50 – 1.10)
Pinela 3	0.16 ± 0.06	4.8 ± 0.8	2.0 ± 0.5	2.6 ± 0.6	0.50 ± 0.09
	(0.06 – 0.27)	(3.0 – 6.3)	(1.1 – 2.9)	(1.7 – 4.5)	(0.30 – 0.70)
Vila Boa 1	0.16 ± 0.06	5.5 ± 0.9	2.2 ± 0.5	2.5 ± 0.5	0.69 ± 0.14
	(0.07 – 0.31)	(3.9 – 7.0)	(1.1 – 3.2)	(2.0 – 4.5)	(0.30 – 1.10)
Vila Boa 2	0.12 ± 0.04	5.4 ± 1.1	2.4 ± 0.6	2.4 ± 0.5	0.54 ± 0.11
	(0.06 – 0.28)	(3.2 – 8.0)	(1.1 – 3.3)	(1.7 – 3.8)	(0.40 – 0.80)
Vila Boa 3	0.28 ± 0.08	6.0 ± 0.8	2.9 ± 0.6	2.2 ± 0.4	0.74 ± 0.12
	(0.12 – 0.41)	(4.1 – 7.2)	(1.1 – 3.6)	(1.6 – 3.8)	(0.50 – 1.00)
Vila Boa 4	0.14 ± 0.05	4.9 ± 0.7	1.8 ± 0.4	2.9 ± 0.6	0.73 ± 0.12
	(0.05 – 0.29)	(3.1 – 6.6)	(1.0 – 2.6)	(1.8 – 4.7)	(0.40 – 1.00)
Failde	0.14 ± 0.06	4.2 ± 0.8	2.1 ± 0.5	2.0 ± 0.3	0.68 ± 0.13
	(0.05 – 0.28)	(2.9 – 6.1)	(1.1 – 3.4)	(1.5 – 2.8)	(0.40 – 1.00)
Outeiro	0.14 ± 0.06	6.6 ± 1.1	2.9 ± 0.6	2.4 ± 0.5	0.66 ± 0.20
	(0.04 – 0.32)	(4.5 – 9.3)	(1.2 – 4.3)	(1.7 – 3.8)	(0.40 – 1.10)
Argoselo	0.26 ± 0.12	6.9 ± 1.0	3.3 ± 0.7	2.1 ± 0.3	0.96 ± 0.17
	(0.14 – 0.53)	(4.2 – 9.0)	(1.8 – 4.9)	(1.7 – 3.0)	(0.60 - 1.20)

Vimioso	0.12 ± 0.04	4.7 ± 0.7	2.1 ± 0.5	2.2 ± 0.4	0.58 ± 0.12
	(0.07 – 0.21)	(3.5 – 6.2)	(1.2 – 3.1)	(1.6 – 3.2)	(0.40 – 1.10)
Bragança 1	0.11 ± 0.02	4.8 ± 0.6	2.5 ± 0.3	2.0 ± 0.2	0.41 ± 0.08
	(0.08 – 0.19)	(3.2 – 6.4)	(1.9 – 3.3)	(1.4 – 2.3)	(0.20 – 0.60)
Bragança 2	0.41 ± 0.09	9.1 ± 1.1	3.3 ± 0.4	2.7 ± 0.3	0.68 ± 0.13
	(0.27 – 0.62)	(5.7 – 10.7)	(2.3 – 4.2)	(2.1 – 3.7)	(0.50 – 1.00)
Vila Real 1	0.15 ± 0.01	6.5 ± 0.2	2.8 ± 0.1	2.4 ± 0.1	0.53 ± 0.01
	(0.08 – 0.29)	(4.9 – 8.9)	(1.8 – 3.9)	(1.8 – 3.2)	(0.40 – 0.70)
Vila Real 2	0.14 ± 0.01	5.9 ± 0.2	2.4 ± 0.8	2.5 ± 0.1	0.46 ± 0.02
	(0.09 – 0.45)	(1.8 – 8.4)	(1.5 – 3.3)	(1.0 – 3.5)	(0.30 – 0.70)
Vila Real 3	0.12 ± 0.01	5.9 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	0.51 ± 0.01
	(0.07 – 0.18)	(4.7 – 7.3)	(1.4 – 3.1)	(2.1 – 4.1)	(0.40 – 0.70)
Vilas Boas 1	0.18 ± 0.01	6.3 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	0.56 ± 0.03
	(0.07 – 0.31)	(4.1 – 8.4)	(1.5 – 3.7)	(1.8 – 3.3)	(0.30 – 1.00)
Vilas Boas 2	0.07 ± 0.00	3.8 ± 0.1	1.8 ± 0.1	2.2 ± 0.1	0.58 ± 0.01
	(0.04 – 0.13)	(2.5 – 5.1)	(1.2 – 2.6)	(1.5 – 3.1)	(0.40 – 0.70)
Bagauste	0.15 ± 0.01	5.2 ± 0.1	2.8 ± 0.1	1.9 ± 0.0	0.50 ± 0.02
	(0.06 – 0.24)	(3.7 – 6.5)	(1.9 – 3.6)	(1.4 – 2.4)	(0.30 – 0.90)
Alvações do Tanha 1	0.11 ± 0.01	6.1 ± 0.1	2.2 ± 0.1	2.8 ± 0.1	0.61 ± 0.03
	(0.06 – 0.20)	(4.5 – 7.9)	(1.5 – 3.0)	(2.1 – 3.9)	(0.30 – 0.90)
Alvações do Tanha 2	0.09 ± 0.00	4.8 ± 0.1	1.9 ± 0.1	2.6 ± 0.2	0.85 ± 0.02
	(0.05 – 0.16)	(4.0 – 6.2)	(1.1 – 2.6)	(2.1 – 3.9)	(0.60 – 1.20)
Alvações do Tanha 3	0.10 ± 0.00	4.5 ± 0.1	1.7 ± 0.1	2.6 ± 0.1	0.50 ± 0.01
	(0.06 – 0.17)	(3.3 – 5.8)	(1.2 – 2.4)	(1.5 – 3.8)	(0.40 – 0.70)
Alvações do Tanha 4	0.21 ± 0.01	6.1 ± 0.2	3.0 ± 0.1	2.1 ± 0.1	0.56 ± 0.01
	(0.14 – 0.40)	(4.5 – 8.1)	(2.1 – 4.0)	(1.6 – 2.7)	(0.50 – 0.80)
Alvações do Tanha 5	0.24 ± 0.01	5.9 ± 0.1	3.1 ± 0.1	1.9 ± 0.0	0.60 ± 0.01
	(0.13 – 0.38)	(4.7 – 7.1)	(2.4 – 4.0)	(1.4 – 2.4)	(0.40 – 0.70)
Nogueira	0.18 ± 0.02	6.5 ± 0.2	2.6 ± 0.1	2.6 ± 0.1	0.85 ± 0.04
	(0.04 – 0.38)	(4.5 – 9.3)	(1.7 – 3.7)	(1.9 – 3.6)	(0.40 – 1.40)
Britiande	0.14 ± 0.01	5.7 ± 0.2	2.7 ± 0.1	2.2 ± 0.1	0.72 ± 0.04
	(0.06 – 0.23)	(4.0 – 7.8)	(1.7 – 4.1)	(1.6 – 3.1)	(0.40 – 1.20)
Lamego 1	0.24 ± 0.01	7.5 ± 0.1	2.9 ± 0.1	2.6 ± 0.0	0.73 ± 0.02
	(0.11 – 0.43)	(5.3 – 9.8)	(2.1 – 4.0)	(2.2 – 3.2)	(0.40 – 0.90)
Lamego 2	0.12 ± 0.01	6.4 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	0.58 ± 0.03
	(0.05 – 0.22)	(3.3 – 9.2)	(1.6 – 4.2)	(1.5 – 3.3)	(0.30 – 0.90)

Lamego 3	0.16 ± 0.01	6.1 ± 0.1	3.0 ± 0.1	2.1± 0.0	0.68 ± 0.02
	(0.09 – 0.30)	(4.3 – 7.6)	(2.4 – 3.9)	(1.6 – 2.5)	(0.50 – 0.90)
Quinta das Melras	0.14 ± 0.01	5.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	0.62 ± 0.02
	(0.07 – 0.24)	(3.7 – 6.3)	(1.5 – 3.1)	(1.3 – 3.0)	(0.40 – 0.90)
Cabeceiras de Basto	0.11 ± 0.01	5.8 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	0.76 ± 0.02
	(0.06 – 0.18)	(4.4 – 7.3)	(1.7 – 3.2)	(1.8 – 3.1)	(0.50 – 1.00)
Mirandela	0.07 ± 0.00	4.3 ± 0.1	2.0 ± 0.5	2.2 ± 0.1	0.85 ± 0.02
	(0.05 – 0.12)	(3.4 – 5.1)	(1.4 – 2.8)	(1.6 – 3.3)	(0.60 – 1.20)
Viseu	0.14 ± 0.01	6.1 ± 0.1	2.3 ± 0.1	2.7 ± 0.1	0.65 ± 0.02
	(0.08 – 0.22)	(4.2 – 7.8)	(1.6 – 3.0)	(20 – 3.8)	(0.40 – 0.90)
Fundão 1	0.18 ± 0.01	6.1± 0.1	2.7 ± 0.1	2.3 ± 0.0	0.50 ± 0.02
	(0.09 – 0.26)	(4.5– 7.4)	(1.9 – 3.5)	(1.7 – 2.7)	(0.30 – 0.70)
Fundão 2	0.16 ± 0.01	5.7 ± 0.1	2.3 ± 0.1	2.5 ± 0.1	0.71 ± 0.02
	(0.07 – 0.28)	(3.9 – 7.6)	(1.6 – 3.0)	(20 – 3.2)	(0.40 – 1.10)
Fundão 3	0.11± 0.01	4.3 ± 0.1	1.8 ± 0.1	2.4 ± 0.1	0.35 ± 0.01
	(0.06 – 0.20)	(3.2 – 5.3)	(1.3 – 2.4)	(2.0 – 3.2)	(0.30 – 0.50)
Fundão 4	0.17 ± 0.01	5.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.0	0.54 ± 0.02
	(0.07 – 0.30)	(3.7 – 6.9)	(1.6 – 3.1)	(1.8 – 2.8)	(0.20 – 0.80)
Fundão 5	0.12 ± 0.00	4.7 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	0.48 ± 0.01
	(0.08 – 0.19)	(3.8 – 5.8)	(1.4 – 2.9)	(1.8 – 3.1)	(0.40 – 0.70)
Fundão 6	0.15 ± 0.01	5.7 ± 0.2	2.3 ± 0.1	2.5 ± 0.1	0.49 ± 0.02
	(0.07 – 0.26)	(3.8 – 7.9)	(1.5 – 3.4)	(2.0 – 3.1)	(0.30 – 0.70)
Fundão 7	0.27 ± 0.02	6.2 ± 0.2	2.7 ± 0.1	2.3 ± 0.1	1.04 ± 0.02
	(0.13 – 0.46)	(4.3 – 7.9)	(2.0 – 3.7)	(1.8 – 2.8)	(0.70 – 1.30)
Mean ± S. D.	0.17 ± 0.03	5.8 ± 0.3	2.5 ± 0.1	2.4 ± 0.1	0.63±0.04
CV (%)	16.46	4.3	4.7	4.3	6.64

The principal component analysis was applied to the morphological data (leaf length and width, leaf length/width ratio, leaf dry weight and peduncle length) recorded for the 46 *A. unedo* genotypes (Figure 2). PCA shows that 77.7% of the total variance of the obtained data could be explained using only two principal components (PC) factors scores. First PC, explaining about 52.6% of variation, was linked to variables related to leaf length and width, leaf dry weight and peduncle length. Second PC that was responsible for 25.1% of variations was linked to leaf length/width ratio. The ordering of individuals and variable vectors revealed that Bragança 2 genotype was clearly separated from the others mainly due to the effect of leaf length and leaf dry weight. Genotypes belonging to Argoselo and Carragosa are represented in the positive region of the first principal component factor and in the negative region of the second principal component factor due to presenting higher values of leaf width and peduncle length. The values obtained from the leaf length/width ratio were highest in the genotypes from Pinela, Vila Real, Viseu, Fontes and Donai, and lowest for individuals collected in Faílde, Bagauste, Mirandela and Britiande.

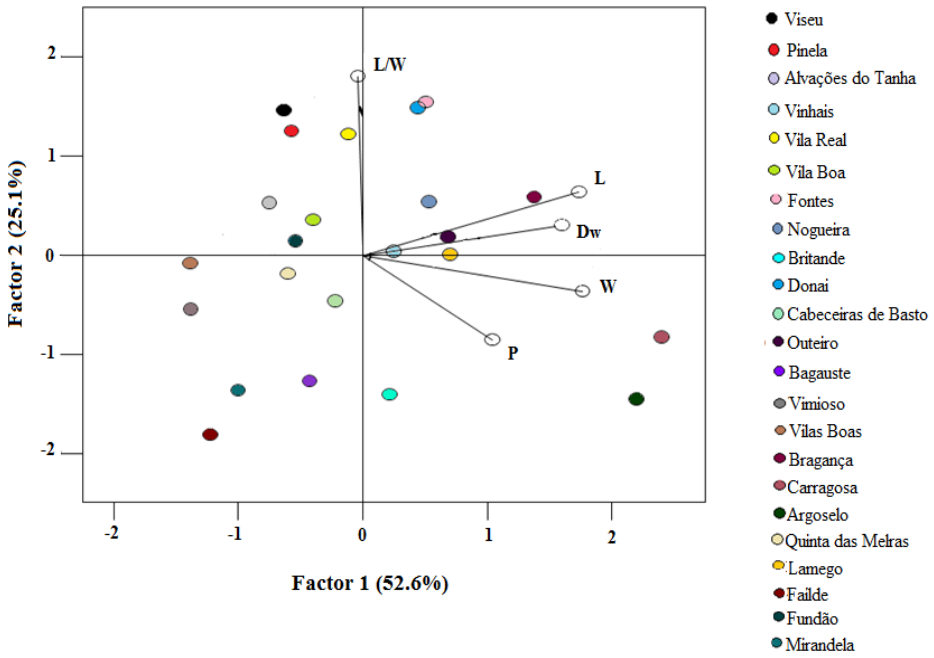

Figure 2 - Principal components analysis using morphological data of the different genotypes of *A. unedo* L. leaves. The PCA factors explain 77.7% of the total variance. Variables: LW - leaf length/width ratio; DW - leaf dry weight; L – leaf length; W – leaf width; P - Peduncle length.

The dendrogram generated based on all the morphological data, for the 46 *A. unedo* genotypes, showed two major clusters, I and II (Figure 4A). The cluster I has

three sub-clusters (Ia, Ib and Ic). In general, the individuals from the same place did not cluster together. This aspect was particularly evident for *A. unedo* genotypes from Bragança and Vila Real which appeared, respectively dispersed throughout all the clusters or in three sub-clusters (Ia, Ib and Ic). Genotypes from Castelo Branco and Viseu are dispersed in two sub-cluster, respectively Ib, Ic and Ia, Ic. Among the population, Vila Boa 4 was the most differentiated genotype whereas Bagaúste and Alvações do Tanha 5 showed the maximum similarity.

3.3.2. RAPD and ISSR band pattern

Among a total of forty arbitrary RAPD decamer primers tested, six revealed polymorphic fingerprint patterns (Table 3). All the chosen primers amplified fragments across the 46 *A.unedo* genotypes, with the number of amplified fragments ranging from six (OPA05) to forty (OPA04) and varying in size from 250 bp to 3,900 bp. Out of a total of 56 bands, 47 were polymorphic, with an average of 7.8 polymorphic bands per primer. The percentage of polymorphic products ranged from 53.9% (OPH08) to 100% (OPA04 and OPA19), with an average of 83.9%. The pattern of RAPD fragments produced by the 10-mer primers OPH08 are shown in Figure 3A.

Of the twenty ISSR primers tested in 46 *A. unedo* genotypes, five generated clear and reproducible variable bands, which size ranged from 200 pb to 3,000 pb (Table 3). The number of amplified fragments per primer ranged from eight for primers 817 and 840 to ten for primers 834 and 858. The five primers produced a total of 45 bands across the 46 *A. unedo* genotypes, of which 39 were polymorphic. The average of total polymorphic bands was 7.8 per primer. The primers that were based on the poly(AG) and poly(TG) motif (834 and 858) produced more percentage polymorphism bands on average (90.0%) than the primers based on the poly(GA) motif (75.0%). An example of ISSR banding profiles are presented in Figure 3B.

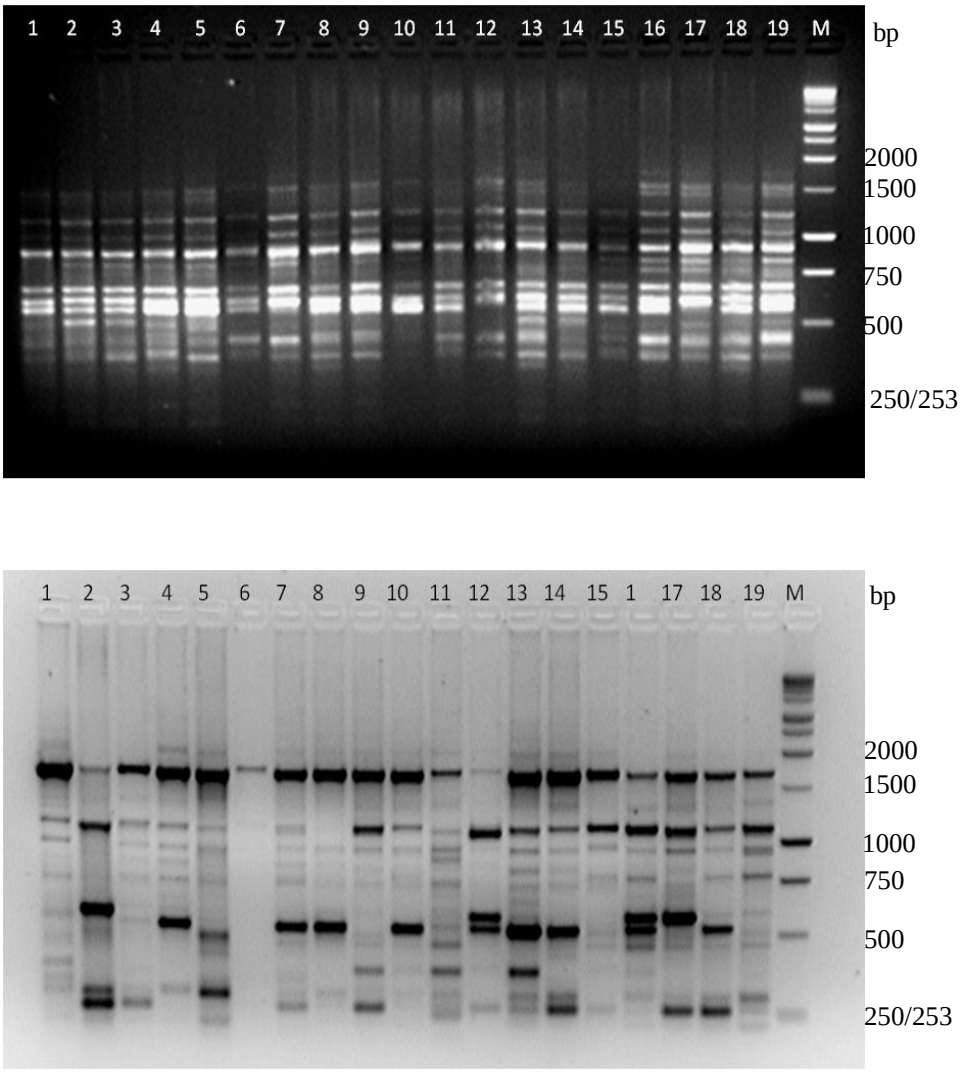

Figure 3 – DNA fingerprinting patterns of 19 *A. unedo* genotypes. A - Random Amplified Polymorphism DNA (RAPD) profile obtained with primer OPH08. B - Inter-Simple Sequence Repeat (ISSR) profile obtained with primer AW17898B. *Lane M* – Molecular mass marker (1 Kb DNA Ladder), *lanes 1* - Outeiro, *2* - Argoselo, *3* - Vimioso, *4* - Bragança 1, *5* - Bragança 2, *6* - Pinela 1, *7* - Pinela 2, *8* - Pinela 3, *9* - Vila Boa 1, *10* - Vila Boa 2, *11* - Vila Boa 3, *12* - Vila Boa 4, *13* - Faílde, *14* - Donai, *15* - Carragosa, *16* - Fontes, *17* - Vila Verde, *18* - Vinhais 1, *19* - Vinhais 2.

Eleven primers including both RAPD and ISSR generated a total of 101 amplified products out of which 86 (84.8%) were polymorphic (Table 3).

Table 3 - Codes and sequences of RAPD and ISSR primers, size of amplified bands, total number of bands and percentage of amplified polymorphic bands generated in *A. unedo*. [a] OP, Operon RAPD primers (Operon Technologies, Inc); [b]STAB VIDA.

Primer	Sequence 5'-3'	Band range (pb)	Total nº bands	Polymorphism (%)
[a]RAPD				
OPA 02	TGCCGAGCTG	300-3,000	09	88.9
OPA 04	AATCGGGCTG	250-2,500	14	100.0
OPA 05	AGGGGTGTTG	300-2,000	06	83.3
OPA 11	CAATCGCCGT	350-3,500	07	85.7
OPA 19	CAAACGTVGG	750-3,900	07	100.0
OPH 08	GAAACSCCCC	400-3,500	13	53.9
Mean			9.3	83.9
[b]ISSR				
817	CACACACACACACACAA	300-1,300	08	87.5
834	AGAGAGAGAGAGAGAGYT	200-1,250	10	90.0
840	GAGAGAGAGAGAGAGAYT	200-1,000	08	75.0
858	TGTGTGTGTGTGTGTGRT	750-3,000	10	90.0
AW17898A	CACACACACACAGT	250-1,750	09	88.9
Mean			09	86.7
RAPD+ISSR			101	
Mean			9.2	84.8

3.3.3. Intra population genetic diversity

A dendrogram based on UPGMA analysis with RAPD data grouped the 46 *A. unedo* genotypes into two main clusters (I and II), with Jaccard's similarity coefficient ranging from 0.53 to 0.98 (Figure 4B). In general, the individuals did not group according to their population affinity. This aspect was particularly evident for genotypes from Vila Real and Viseu. The cluster I has two sub-clusters (Ia and Ib). Sub-cluster Ia was mostly formed by genotypes from Vila Real, Castelo Branco and Viseu, but it contains also genotypes from Bragança. The Ib sub-cluster contains genotypes from Bragança and only one from Viseu. The cluster II includes only genotypes from Bragança. The dendrogram obtained also showed Outeiro as the most differentiated genotype whereas Vila Real 1 and Vilas Boas 2 by the contrary showed strong relationship.

A dendrogram based on UPGMA analysis with ISSR data was produced, with Jaccard's similarity coefficient from 0.45 to 0.95 (Figure 4C). The 46 *A. unedo* genotypes clustered into two main groups. The cluster I have four sub-clusters and comprised genotypes from Vila Real, Castelo Branco and Viseu, and in less number genotypes from Bragança (3 genotypes). The sub-cluster Ia and Ic were mostly formed by genotypes from Vila Real, and the sub-cluster Ib comprised the majority of the genotypes from Castelo Branco (5 genotypes). The genotypes from Viseu did not form a separated cluster and appeared dispersed throughout the sub-clusters Ia, Ib and Id. The cluster II includes only genotypes from Bragança and has two sub-clusters. The dendrogram obtained also showed Argoselo as the most differentiated genotype while Vinhais 1 and Vinhais 2 appeared to be closer to each other. In general, the dendrogram obtained was not in accordance with the geographic distribution of *A. unedo*, but within each group, the genotypes from the same place clustered together with exception of genotypes from Viseu.

The clustering analysis of all individuals based on both RAPD and ISSR data shows more similarity to ISSR based dendrogram (Figure 4D). Jaccard similarity coefficient ranges from 0.55 to 0.91. The dendrogram obtained divided the 46 individuals into two main clusters, I and II. The first sub-cluster Ia was mainly formed by *A. unedo* genotypes from Castelo Branco (7), Vila Real (13) and Viseu (5). In general, these genotypes did not cluster according to its geographic distribution. The sub-cluster Ib, Ic and the cluster II was formed by genotypes from Bragança. In addition it was verified the clustering of the genotypes from the same place. This is evident for genotypes from Vila Boa, Pinela, Vinhais and Bragança.

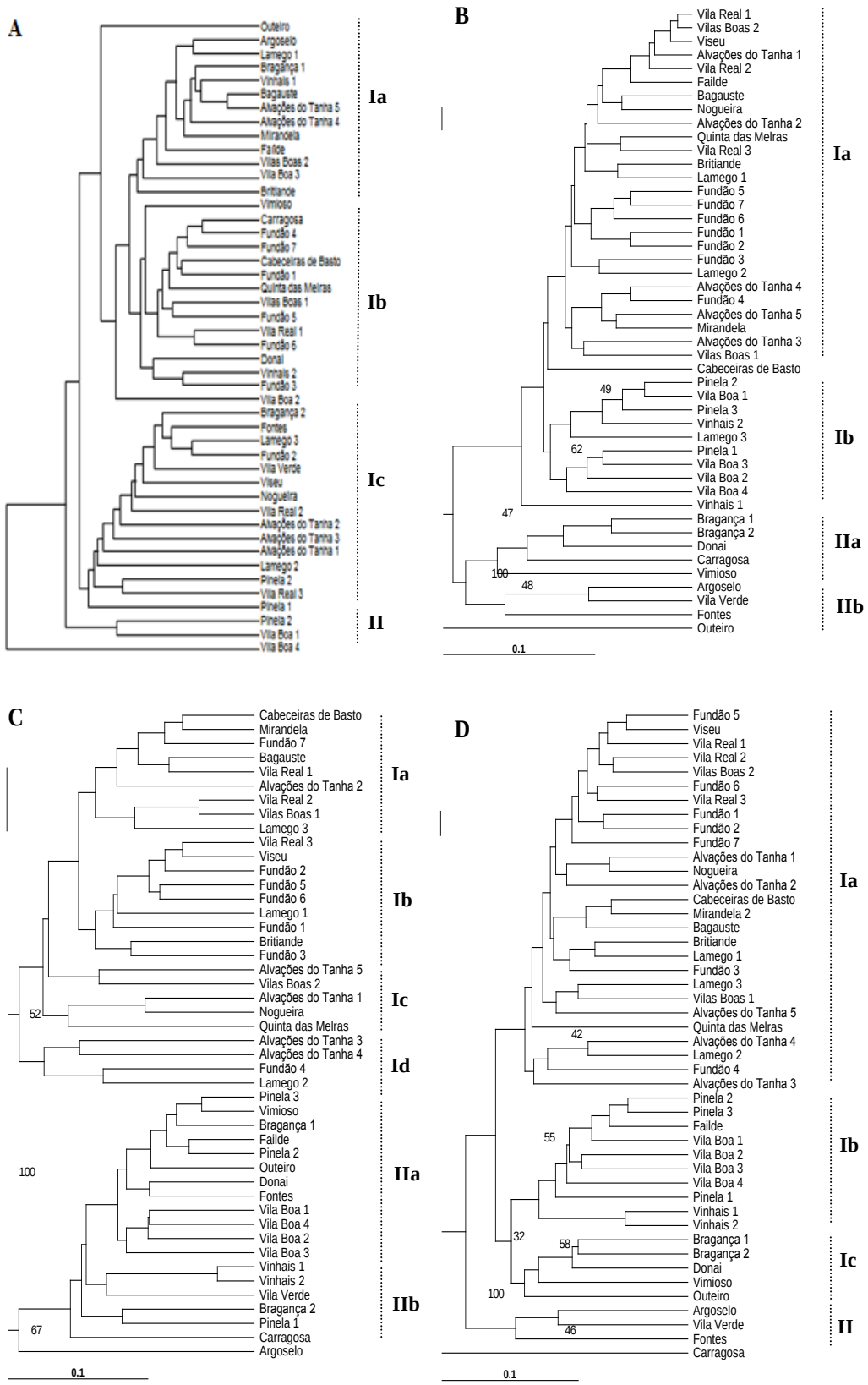

Figure 4 – UPGMA dendrograms of 46 *A. unedo* L. genotypes based on Euclidean Distance, using morphological traits (A) and based on Jaccard's coefficients, using RAPD (B), ISSR (C) and RAPD+ISSR (D).

3.3.4. Inter population genetic diversity

Analysis for pair-wise genetic distance based on both RAPD and ISSR data revealed that genetic distance was minimum between Viseu and Vila Real populations (0.0582) and maximum between Castelo Branco and Bragança (0.2684) populations (Table 4). The genetic identities follow completely the genetic distance pattern. As expected, Viseu and Vila Real would be the most similar (0.9435) whereas Castelo Branco and Bragança were the least similar (0.7646).

Table 4 - Nei's measures of genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *A. unedo*, obtained by RAPD and ISSR markers. Maximum and minimum values are shown in bold face.

Population	Bragança	Castelo Branco	Vila Real	Viseu
Bragança	-----	**0.7646**	0.8060	0.7693
Castelo Branco	**0.2684**	-----	0.9291	0.9106
Vila Real	0.2156	0.0736	-----	**0.9435**
Viseu	0.2623	0.0936	**0.0582**	-----

The dendrogram shown in Figure 5, which resulted from the Nei's (1978) unbiased genetic distances matrix using UPGMA, revealed that clustering of populations are in accordance to its distance geographical positions.

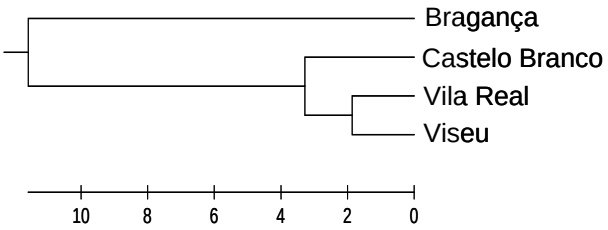

Figure 5 – Dendogram of four *A. unedo* populations obtained by RAPD and ISSR markers using Nei's unbiased genetic distance. Agglomeration method was UPGMA.

For example, Viseu and Vila Real are geographically close populations and group together. Thus, in general there was a positive correlation between the geographical distance and genetic distance, which was confirmed by regression analysis ($R = 0.53$, $p < 0.001$; Figure 6).

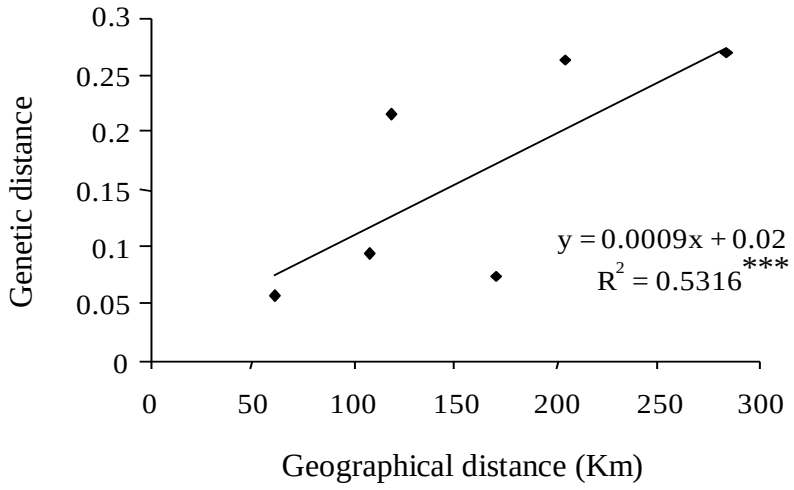

Figure 6 – Regression analysis between genetic distance and geographical distances of four *A. unedo* populations.

3.3.5. Population genetic parameters

The population diversity analysis based on both RAPD and ISSR data and by using POPGENE (Table 5) revealed that among the four *A. unedo* populations, Bragança exhibited the greatest level of variability (Na=1.93; Ne=1.71; Ho=0.39; Io=0.5; P=93.02) while Castelo Branco population exhibited the lowest variability (Na=1.43; Ne=1.29; Ho=0.16; Io=0.24; P=43.02). When all populations were considered, the total genetic diversity (Ht) was 0.329 ± 0.021 and the gene variability within populations (Hs) was 0.247 ± 0.018. The total genetic differentiation coefficient (Gst) among the populations was 0.260. Gene flow (*Nm*) among the populations was 1.499.

Table 5 – Genetic diversity (mean ± SD) in the four populations of *A. unedo* obtained by RAPD and ISSR analysis.

Population	*Na*	*Ne*	*Ho*	*Io*	*P*
Bragança	1.93 ± 0.26	1.71 ± 0.28	0.39 ± 0.13	0.57 ± 0.18	93.02
Cast. Branco	1.43 ± 0.50	1.29 ± 0.38	0.16 ± 0.21	0.24 ± 0.29	43.02
Vila Real	1.58 ± 0.49	1.42 ± 0.40	0.24 ± 0.21	0.35 ± 0.30	58.14
Viseu	1.45 ± 0.50	1.35 ± 0.42	0.19 ± 0.22	0.28 ± 0.31	45.35
Total	2.00 ± 0.00	1.67 ± 0.26	0.39 ± 0.11	0.57 ± 0.13	84.8

3.3.6. Comparison analysis of morphological and molecular markers (RAPD and ISSR)

The matrices for morphological traits and molecular markers (RAPD and ISSR) as well as for RAPD and ISSR markers were compared using Mantel's test for matrix correspondence. The results obtained shown no correlation between the morphological and genetic distance matrices (R = 0.09). Similarly, the correlation between Jaccard's similarity values generated from RAPD and ISSR markers was low (R = 0.22).

3.4. Conclusions

This study is the first attempt to study the genetic diversity of *A. unedo* in Portugal, specifically in the interior north and centre. Such evaluation was based on morphological traits and molecular markers, namely on RAPD and ISSR analysis. The results obtained shown that *A. unedo* genotypes were distinguishable by means of leaf morphology, especially leaf dry-weight and peduncle length, which proved to be the two most discriminating characters. Earlier studies on variation in morphological characters have revealed the existence of considerable variation in leaf characteristics of *A. unedo* grown in the Northwestern of Turkey (Celikel *et al.*, 2008) and also in other forest species (Kramer *et al.*, 2002; Borazan and Babac, 2003; Turna, 2004; Viscosi *et al.*, 2009).

Molecular analysis showed some potential to reveal variations among geographically separated *A. unedo* populations and also among individuals within populations. The genetic diversity estimated by several parameters, by using both RAPD and ISSR analysis, reveals moderate levels of variations within *A. unedo* populations. This result is consistent with a recent analysis of genetic diversity of *A. unedo* in Tunisia, by using RAPD analysis (Takrouni and Boussaid, 2010). As pointed by the same authors, this low level of variation compared to other long-lived plants could be due to genetic drift and selfing. In the present study, it was also observed that the level of variation differed according to populations. The genetic diversity, using both RAPD and ISSR analysis, was highest in *A. unedo* population from Bragança and lowest in Castelo Branco population. There are several reasons that could explain differences in genetic diversity of plant populations. The geographic distribution, mating system, method of seed dispersal and method of reproduction of a species are considered the primary factors influencing the genetic diversity (Hamrick *et al.*, 1992). In our case the reasons for the high level of genetic diversity observed in Bragança population could be related with their distribution area. In fact, from the four populations analysed Bragança is the most widely distributed. As stated previously, species with a wide geographic area generally have more genetic diversity (Hamrick and Godt, 1989; Oliveira *et al.*, 2010).

The analysis based on RAPD and ISSR also shown that the diversity within populations (Hs) was slightly lower than between populations (Gst). The low genetic variation within the individuals of a population points to the fact that the populations are homogenous in nature, whereas the higher genetic variability among the populations indicated that the populations have already differentiated into separate genetic pools. Hence, these different gene pools should be conserved separately and maintained without any inter-mixing. This practice must be adopted with some urgency since the results obtained indicate high movement of genes from one population to another, causing them to become more similar. This is interpreted from the Gst value and high Nm value (1.499) which suggested the occurrence of substantial gene flow among the populations. The long-distance seed dispersal by intervention of forgivers, mainly birds, could be in part be responsible for gene movement between populations.

In a recent study of analysis of genetic diversity of Tunisian *A. unedo* (Takrouni and Boussaid (2010) it was suggested a common origin of *A. unedo* and later succession into different populations by adapting to the varying climatic conditions. This

hypothesis is partly reinforced by our cluster analysis. In both molecular markers, RAPD and ISSR, the different individuals from Vila Real and Viseu did not group according to its geographic distribution. Although this pattern was not observed to all genotypes (genotypes from Bragança as well as from Castelo Branco cluster together according to its geographic distribution), the results suggested that the differentiation among *A. unedo* populations are not related to geographical distance. It could be more related to ecological factors such as altitudes and rainfall influencing flowering time, repining and longevity (Takrouni and Boussaid, 2010).

The comparison of the applicability of ISSR and RAPD as genetic markers to characterize the genetic diversity of *A. unedo* revels that ISSR has more power of discrimination than RAPD. This superiority of ISSR has been reported earlier in other plant species (Qian *et al.,* 2001; Boronnikoval *et al.*, 2007; Zhao *et al.,* 2007; Han and Wang, 2010). Clustering of the 46 *A. unedo* genotypes was not very similar when RAPD- and ISSR-derived dendrograms were compared. This result is similar to other researches (Archak *et al.*, 2003; Souframanien and Gopalakrishna, 2004; Han and Wang, 2010; Tantasawat *et al.*, 2010). This difference can be attributed to marker sampling error or the level of polymorphism detected (Sikdar *et al.*, 2010). Another explanation is related to the fact of RAPD and ISSR target different portions of the genome. The inter-simple sequence repeats are regions lying within the microsatellite, and the amplification loci of RAPD are mainly in the gene expression region (Williams *et al.*, 1990; Zietkiewicz *et al.*, 1994).

An absence of a complete relationship between the morphological and genetic similarities was also found in the present study. A similar finding was observed for wild populations of other plant (Greene *et al.*, 2004; Tantasawat *et al.*, 2010). Several reasons may account for the no correlation between the morphological traits and molecular markers. First, the less number of random primers could not cover vast area of *A. unedo* genome. Second, morphological variation is strongly associated with environmental variation; the morphological similarities observed might be due to different combinations of alleles producing similar phenotypes that might result in morphological similarities or differences that are not proportional to the underlying genetic differences (Pirkhezri *et al.*, 2010).

In conclusion, this is the first assessment on the genetic diversity of *A. unedo* populations in Portugal based on combined analyses of molecular markers (RAPD and ISSR) and morphological data. The low genetic diversity observed within *A. unedo*

populations and the considerable amount of interpopulation differentiation revealed that the four populations should be conserved separately to avoid problems related to maladaptation. However, it is worth stressing that this work needs to be further strengthened with more exhaustive sampling of populations and more advanced molecular techniques.

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Capitulo 4

Arbutus unedo L. leaves as source of phytochemicals with bioactive properties

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Submitted

Abstract

In the last years it is noticed that the strawberry tree (*Arbutus unedo* L.) is being replaced by other species with higher economic value due to the low economic importance attributed to this shrub. As a way to contribute for the selection of superior quality genotypes, the present work intends to study the total phenols content and antioxidant activity of 19 different genotypes of *A. unedo* leaves collected in Trás-os-Montes region in order to encourage their use in the pharmaceutical, chemical and food industrial sectors. Total phenols content was achieved spectrophotometrically using Folin-Ciocalteau reagent with gallic acid as standard. The antioxidant activity was evaluated by two different chemical assays: reducing power of iron (III)/ferricyanide complex assay and the scavenging on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals assay. Bragança 1 genotype contain higher total phenols (215.0 mg GAE/g) and the genotype from Vila Boa 4 reported lower total phenols content (148.0 mg GAE/g). In both methods tested to evaluate antioxidant activity, Vila Verde and Donai genotypes reported higher antioxidant capacity (EC_{50} values of 0.088 and 0.090 mg/mL respectively for DPPH; EC_{50} values of 0.233 and 0.245 mg/mL respectively for reducing power) while Vila Boa 2 reported lower antioxidant potential (EC_{50} values of 0.142 and 0.378 mg/mL respectively in DPPH and reducing power methods). In both methods tested the antioxidant activity was concentration dependent. Linear negative correlations were established between the total phenol contents and the EC_{50} values of the two methods tested in the antioxidant activity. The results showed that *A. unedo* leaves are a potential source of natural compounds with bioactive properties.

Keywords*: Arbutus unedo* L. leaves, antioxidant activity, total phenols.

4.1. Introduction

The *Arbutus unedo* L. (Ericaceae) is a fruit tree species distributed through the Mediterranean-Atlantic area, appearing mainly in the southern European region, northern Africa, Ireland, Palestine and Macaronesia (Canarias) (Celikel *et al.*, 2008). In Portugal, its plantation began in the southern region and it was subsequently broadcast throughout the country, including the region of Trás-os-Montes (Northeast of Portugal) (Peter, 1994). The *Arbutus unedo* L. fruits are edible but are usually consumed after being processed. Among the processed products are the alcoholic beverages like brandy and aromatic distillate. The fruits are also used to produce food products, such as sweets, jams and jellies (Alarcão-e-Silva *et al.*, 2001; Pallauf *et al.*, 2008).

At medicinal level, strawberry tree is widely used in the traditional medicine. It is recognized for having diuretic, antiseptic and laxative effects and by being used to treat cardiovascular pathologies such as arterial hypertension, atherosclerosis and thrombosis (El Haouari *et al.*, 2007; González-Tejero, 1990; Mekhfi *et al.*, 2006; Ziyyat *et al.*, 2002). Their leaves are also used due to their astringent and purgative properties and are also used in the treatment of diabetes and inflammatory conditions (Ziyyat and Boussairi, 1998; Mariotto *et al.*, 2008; Afkir *et al.*, 2008).

In Trás-os-Montes region, contrasting what occurred in other regions of Portugal, no economic importance was attributed to *A. unedo*. Due to such fact, in the last years the areas occupied by *A. unedo* plantations are being replaced by other forest species with higher economic value. Therefore is urgent to act in order to enhance the production, marketing and consumption of leaves and fruits of strawberry tree, contributing to the species preservation and biodiversity.

Recent studies have shown that aqueous extracts of leaves of strawberry tree, collected in Trás-os-Montes region, had a high antioxidant potential (Oliveira *et al.*, 2009). Indeed, phytochemical studies showed that leaf extracts contains several phenolic compounds, like tannins, flavonoids, phenolic glycosides, among others (Males *et al.*, 2006; Fiorentino *et al.*, 2007), as well as α-tocopherol (Kivçak and Mert, 2001). Phenolic compounds are among the natural antioxidants more studied by the scientific community due to possessing biological properties like antioxidant and antimicrobial activity's (Malheiro *et al.*, 2010; Pereira *et al.*, 2006, 2007; Proestos *et al.*, 2005; Sousa *et al.*, 2006, 2008; Zhu *et al.*, 2004). Many of the health problems that are taking special relevance in the modern societies of the industrialized world, like

cardiosvascular diseases, cancer, diabetes, neurological diseases and atherosclerosis, appears to be related with reactive oxygen species (ROS), playing this species as important role in the appearance and prevalence of such diseases. A possible way to decrease and prevent the occurrence of the mentioned health problems is the inclusion of foods containing natural substances with antioxidant activity in our diets. Such fact would allow the incorporation of chemical substances in our organism with the capability to scavenge free radicals and thereby preventing the cellular oxidative stress. At this level, *A. unedo* could be used at the pharmaceutical, chemical and food industry sectors, contributing to a higher economic exploitation of this shrub.

Once known the medicinal properties related to *A. unedo*, our goal was to study the total phenols content and antioxidant activity from leaves of 19 different genotypes from Bragança district. A selection of a superior quality genotype, which could be submitted to spread projects, was also concerned.

4.2. Experimental

4.2.1 Chemicals and reagents

Gallic acid, methanol, 2,2-diphenyl-1-picrylhydrazyl and iron (III) chloride were obtained from Sigma-Aldrich (St. Louis, USA). Sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent, sodium carbonate anhydrous, hydrochloric acid, and di-sodium hydrogen phosphate dihydrate were obtained from Panreac (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

4.2.2 Samples

The leaves of *Arbutus unedo* L. were collected from different municipalities of the district of Bragança. Overall 19 different site samples were selected: Vinhais municipality (Vinhais 1, Vinhais 2, Vila Verde); Bragança municipality (Bragança 1, Bragança 2, Pinela 1, Pinela 2, Pinela 3, Vila Boa 1, Vila Boa 2, Vila Boa 3, Vila Boa 4, Faílde, Donai, Carragosa, Fontes), and Vimioso municipality (Outeiro, Argoselo, Vimioso). Sampling sites are represented in Figure 1.

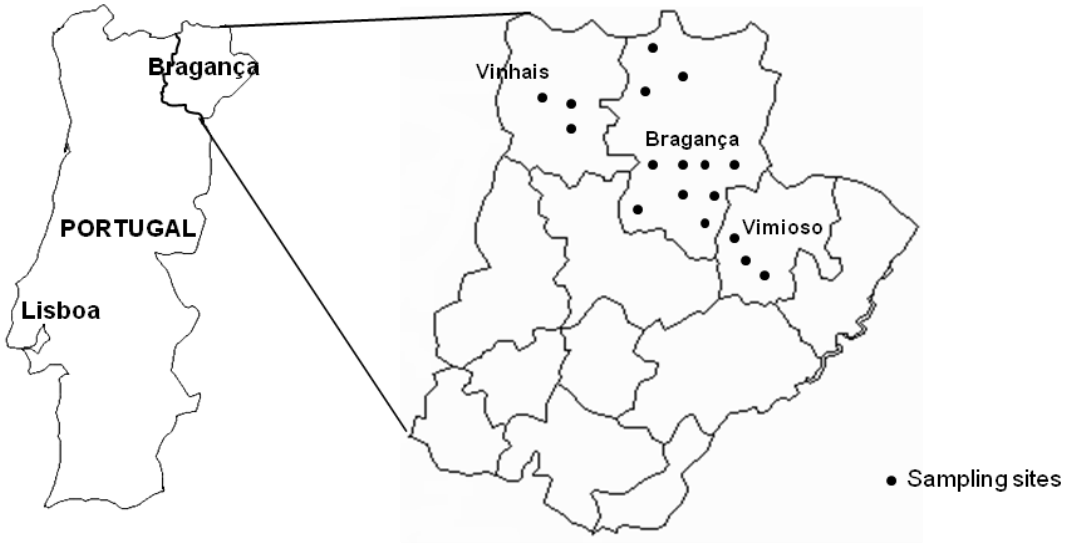

Figure 1 – Representation of the sampling sites in three different municipalities of Bragança district (Northeast of Portugal). Vinhais municipality (Vinhais 1, Vinhais 2, and Vila Verde); Bragança municipality (Bragança 1, Bragança 2, Carragosa, Donai, Faílde, Fontes, Pinela 1, Pinela 2, Pinela 3, Vila Boa 1, Vila Boa 2, Vila Boa 3, and Vila Boa 4), and Vimioso municipality (Argozelo, Outeiro, and Vimioso).

4.2.3 Samples preparation and extraction conditions

For each genotype, four different samples were collected. The leaves were removed from the stem, freeze-dried and then ground. Before any analysis (total phenols determination and antioxidant activity assays), *A. unedo* L. leaves (2 g/sample) were extracted with 250 mL in boiling water for 45 min and filtered through Whatman nº 4 paper. The aqueous extracts were frozen, and lyophilized and redissolved in water to a final concentration of 20 mg/mL and were stored at 4 ºC until analysis.

4.2.4 Determination of total phenols contents

Total phenols quantifications were achieved according to Singleton and Rossi (1965), with some modifications. Thus, 1 mL of the extract solution was mixed with 1 mL of Folin-Ciocalteau's phenol reagent. The mixture was shaken vigorously and left to stand for 3 min. After that, 1 mL of a saturated solution of sodium carbonate was added and the total volume was adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after what the absorbance was read at 725 nm in a Thermo Electron Corporation Genesys 10UV spectrometer. Gallic acid was used as standard, being the results expressed in mg of gallic acid equivalents (GAE)/g of extract.

4.2.5 Antioxidant activity

4.2.5.1 Reducing power assay

The reducing power was determined according to a described procedure (Berker *et al.*, 2007). The extract solution (1 mL) was mixed with 2.5mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance readings indicate higher reducing power). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

4.2.5.2 Scavenging effect on DPPH radicals

The capacity to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano *et al.* (1988). The extract solution (0.3 mL) was mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min at room temperature in dark (until stable absorbance values were obtained). The reduction of the DPPH-radical was measured by continuous monitoring of the absorption decrease at 517 nm.

DPPH scavenging effect was calculated as the percentage of DPPH discoloration using the following equation: % scavenging effect = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

4.2.6 Statistical analysis

All the determinations were carried out in quadruplicate and the results are expressed as mean values and standard deviations. A regression analysis, using Excel for Windows Software, was established between total phenols content of the 19

genotypes and EC_{50} values obtained in the DPPH and reducing power antioxidant assays.

4.3. Results and discussion

4.3.1 Total phenols content

The total phenols content was evaluated using the Folin-Ciocalteau reagent and calculated as Gallic Acid Equivalents (GAE). This reagent has been extensively used to determine the total phenols content of a varied number of matrixes, however some criticisms are appointed to this methodology. The Folin-Ciocalteau also reacts with other non-phenolic reducing compounds such as sugars, amino acids and ascorbic acids that are quantified as phenols.

In table 1 are reported the extractions yield and total phenols content of the 19 different genotypes of *A. unedo* leaves.

The results shown that the genotype Bragança 1 was the one with higher total phenols content (215.0 ± 18.2 mg GAE/g). Within the same region, the genotype Bragança 2 is among the genotypes with lower content of total phenols, reporting 153.1 ± 13.3 mg GAE/g. This fact show us that even within the same region, the total phenols content can change from tree to tree. The same observation is found when we compare the four genotypes from Vila Boa region, being three of the genotypes poorer in total phenols (near 150 mg GAE/g) than Vila Boa 3, being this genotype rich in the same compounds (200.4 ± 14.6 mg GAE/g) (Table 1).

Among all the 19 genotypes studied, Vila Boa 4 was the one with lowest content on total phenols, 148.0 ± 12.4 mg GAE/g. The results obtained in the total phenolics determination are in accordance to those obtained by Oliveira *et al.* (2009).

Table 1: Extraction yield (%) and total phenols content (mg GAE/g) of *Arbutus unedo* L. leaves aqueous extracts from 19 different genotypes.

Genotype	Extraction yield (%)	Total phenols content
Argozelo	34.1 ± 1.81	202.0 ± 9.0
Bragança 1	31.5 ± 8.26	215.0 ± 18.2
Bragança 2	27.8 ± 0.53	153.1 ± 13.3
Carragosa	33.6 ± 1.26	196.4 ± 7.1
Donai	34.8 ± 0.70	180.8 ± 39.7
Failde	41.2 ± 3.76	202.1 ± 17.5
Fontes	40.1 ± 0.41	162.0 ± 11.3
Outeiro	33.4 ± 1.56	166.4 ± 34.7
Pinela 1	37.9 ± 9.15	169.8 ± 14.8
Pinela 2	61.1 ± 7.73	188.0 ± 27.1
Pinela 3	32.4 ± 1.56	184.4 ± 28.8
Vila Boa 1	35.2 ± 0.62	151.3 ± 5.8
Vila Boa 2	38.6 ± 5.23	156.8 ± 3.8
Vila Boa 3	36.46 ± 1.15	200.4 ± 14.6
Vila Boa 4	36.2 ± 0.10	148.0 ± 12.4
Vila Verde	42.2 ± 3.21	199.3 ± 17.1
Vimioso	37.5 ± 1.87	184.8 ± 19.6
Vinhais 1	41.9 ± 6.61	183.3 ± 29.0
Vinhais 2	31.4 ± 4.24	182.6 ± 4.2

The phenolic fraction of *A. unedo* is being studied in the last few years. By HPLC-DAD we tried to identify the phenolic profile of *A. unedo* leaves. Three isomers of kaempferol and four of quercetin were found as preliminary results. In the literature, Males *et al.* (2006) by studying the flavonoidic composition of *A. unedo* leaves identified quercetin, isoquercitrin, hyperoside and rutin in Croatian genotypes. In Turkish genotypes Carcache-Blanco *et al.* (2006) were able to report the identification of (-)-catequin. Fiorino *et al.* (2007) by studying Italian genotypes, identified twelve phenolic compounds, namely arbutin, ethyl gallate, ρ-hydroxybenzoyl arbutin, galloylarbutin, (+)-gallocatechin, catechin, kaempferol 3-*O*-α-L-ramnopyranoside, quercetin 3-*O*-α-L-ramnopyranoside, myricetin 3-*O*-α-L-ramnopyranoside, kaempferol 3-*O*-β-D-arabinofuranoside, quercetin 3-*O*-β-D-arabinofuranoside and myricetin 3-*O*-β-D-arabinofuranoside.

Another preliminary result was the evaluation of the antimicrobial activity of the aqueous extracts against six bacteria [three Gram (+) and three Gram (-)]. The extracts from the leaves of *A. unedo* revealed good antibacterial activity, being observed that *Bacillus cereus* was the most sensitive to the presence of extract (data not shown). The observed antimicrobial activity could be related to the phenolic composition of the extracts allowing inhibiting the microbial development and growth of certain bacteria.

4.3.2 Antioxidant activity

The antioxidant activity of the 19 different genotypes of *Arbutus unedo* L. leaves was assessed by two different chemical assays: reducing power and scavenging effect on DPPH free radicals. The results obtained are expressed as EC_{50} values (mg/mL) and are reported in figures 4 and 5, respectively for reducing power and DPPH chemical assays.

The reducing power chemical assay allow us to observe if the leaves of *A. unedo* L. possesses compounds with reducing capacity, which may indicate their potential antioxidant activity (Meir *et al.*, 1995). The reducing capacity of the extracts of *A. unedo* L. leaves can be monitored through the transition of the solution yellow color to various shades of green and blue depending on the reducing power of each extract tested. Such color transition occurs due to the presence of antioxidant compounds (reducers) that have the capacity to reduce the Fe^{3+}/ferricyanide complex to its ferrous form (Fe^{2+}). So, the formation of Perl's Prussian blue at 700 nm can be used to monitor the concentration of Fe^{2+} (Pereira *et al.*, 2006). The absorbance at 700 nm and the reducing power of the extracts are directly related, so higher absorbance indicates higher reducing power.

In the case of the aqueous extracts of *A. unedo* L. leaves, a concentration-dependent activity for reducing power assay was observed (Figure 2).

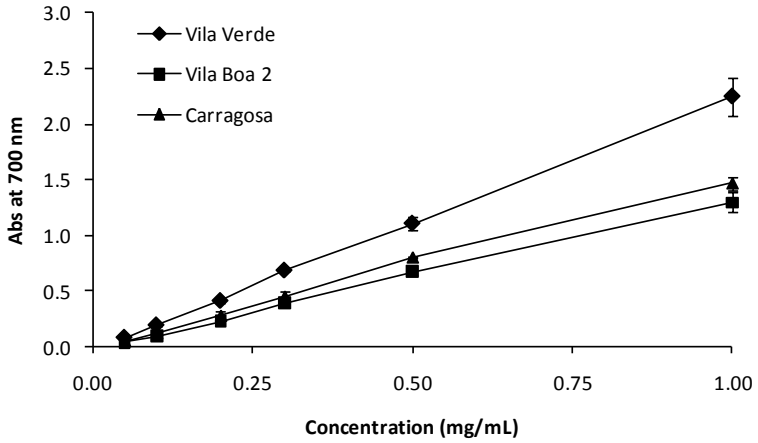

Figure 2 – Reducing power of aqueous extracts of *Arbutus unedo* L. leaves, belonging to Vila Verde, Carragosa and Vila Boa 2 genotypes (mean ± standard deviation, n = 4).

High values of reducing power were obtained at very low concentrations (< 0.4 mg/mL) in the extracts of the 19 genotypes of *A. unedo* L. leaves (Figure 4).

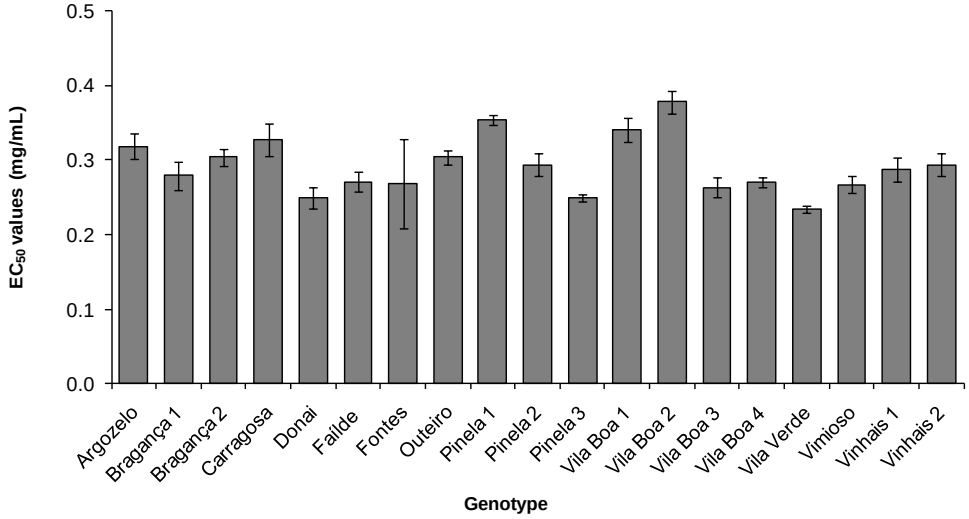

Figure 4 – EC_{50} values (mg/mL)[a] obtained from the reducing power assay tested in the aqueous extracts of the 19 genotypes of *Arbutus unedo* L. leaves (mean ± standard deviation, n = 4). [a]EC_{50} (mg/mL): effective concentration at which the absorbance is 0.5.

Vila Verde and Donai genotypes reported strongest reducing power and consequently lower EC_{50} values (0.233 ± 0.004 and 0.245 ± 0.014 respectively). On the other hand Vila Boa 2 genotype showed higher EC_{50} value (0.378 ± 0.014), which means lower reducing power.

DPPH is known as a stable free radical which possesses a characteristic maximum absorption between 515 and 517 nm. In the presence of antioxidant compounds, the DPPH free radical is reduced to its hydrazine form due to electron donation by the

antioxidant. The DPPH in solution presents a purple color that is diminished to light yellow with the electron donation and scavenging of the free radicals. In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength. This method is an essential tool to access the antioxidant potential, more specifically, the antiradical activity of extracts.

The scavenging effect of *A. unedo* leaves aqueous extracts on DPPH free radicals also showed a concentration-dependent activity (Figure 3).

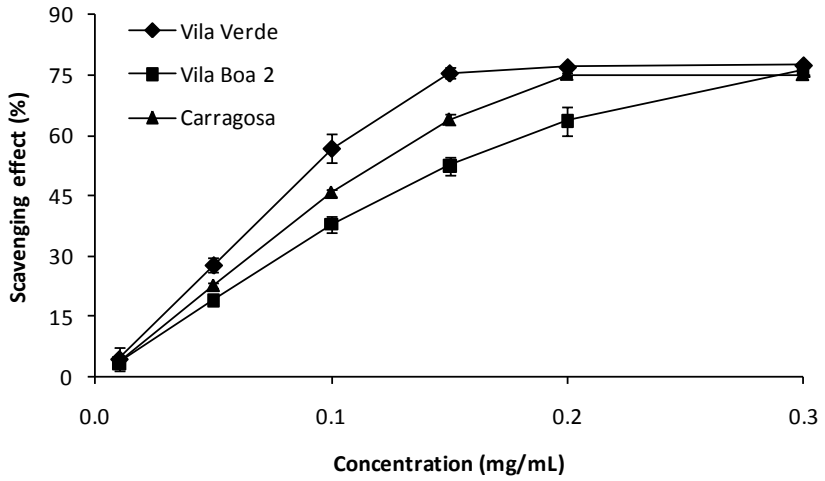

Figure 3 – Scavenging effect on DPPH free radicals of aqueous extracts of *Arbutus unedo* L. leaves, belonging to Vila Verde, Carragosa and Vila Boa 2 genotypes (mean ± standard deviation, n = 4).

A. unedo leaves exhibited strong free radical scavenging activity on DPPH assay at very low concentrations (< 0.15 mg/mL). Once more the extracts from Vila Verde and Donai, and Vila Boa 2 exhibit respectively, higher and lower antioxidant capacity. Vila Verde and Donai genotypes reported lower EC_{50} values (0.088 ± 0.005 and 0.090 ± 0.001 respectively) while Vila Boa 2 genotype reported higher values of EC_{50} (0.142 ± 0.007) (Figure 5).

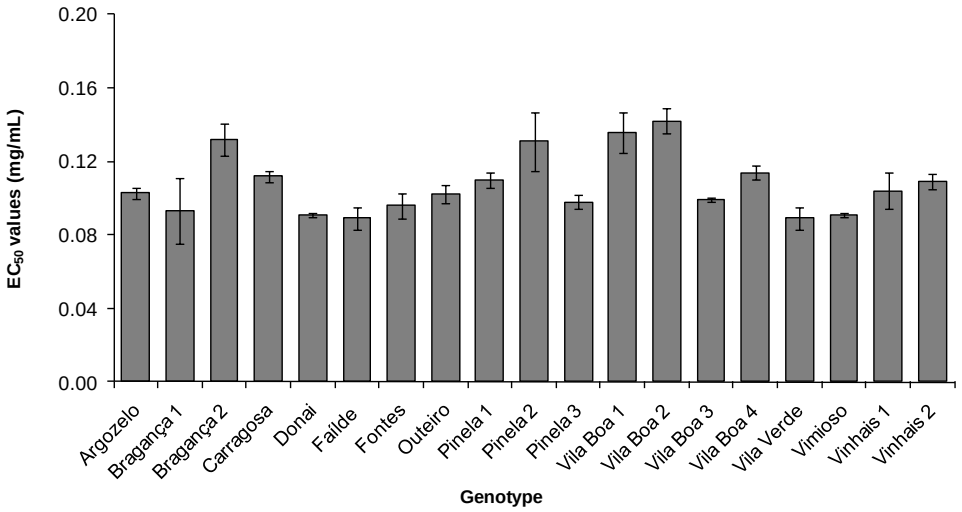

Figure 5 – EC_{50} values (mg/mL)[a] obtained from the DPPH assay tested in the aqueous extracts of the 19 genotypes of *Arbutus unedo* L. leaves (mean ± standard deviation, n = 4). [a]EC_{50} (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.

The results obtained in the antioxidant potential could be related, at least in part, to the content on total phenols compounds found in the different genotypes of *A. unedo* leaves.

Our research group has recently studied the best extraction methodology and its influence on the antioxidant potential of *A. unedo* leaves and the results obtained in the 19 genotypes from Trás-os-Montes are in accordance with those obtained by Oliveira *et al.* (2009). Several other studies corroborates that *A. unedo* leaves and fruits possesses extraordinary antioxidant properties (Andrade *et al.*, 2009; Fortalezas *et al.*, 2010; Pabuçcuoğlu *et al.*, 2003; Tavares *et al.*, 2010).

4.3.3. Correlation between total phenols content and antioxidant activity

The genotypes that exhibit higher antioxidant potential (Vila Verde and Donai) are among those with higher total phenols content. By other hand, Vila Boa 2 genotype reported lower antioxidant activity in both methods tested, and is between the genotypes that contain lower total phenols (Table 1).

When a regression analysis was performed between the values of EC_{50} obtained in the antioxidant evaluation and the total phenols content, very negative correlations (R^2 = 0.094;$P < 0.01$) and extremely negative correlations (R^2 = 0.180 ;$P < 0.001$) were established respectively for DPPH and reducing power methods. Although, some other

minor antioxidant compounds (tocopherols) besides phenolics could influence and contribute to the antioxidant activity observed.

Several works using different samples demonstrated the correlation between total phenolics content and the registered antioxidant activity (Kim *et al.*, 2008; Malheiro *et al.*, 2010; Oliveira *et al.*, 2008, 2009; Paskó *et al.*, 2009; Tawaha *et al.*, 2007).

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Capitulo 5

Discussão de Resultados e Conclusões

Discussão de Resultados e Conclusões

O *Arbutus unedo* L. é uma árvore fruteira de elevado interesse económico, ambiental, ornamental e medicinal. Devido a determinadas ocorrências, como desflorestação, incêndios ou substituição por outras espécies, o cultivo desta árvore, assim como a sua população no seu habitat natural, está em declínio sobretudo no Norte e Centro de Portugal. A avaliação da diversidade genética e a ênfase das principais propriedades desta espécie são etapas importantes no planeamento de estratégias que permitam a sua protecção, conservação e propagação.

Tanto quanto se sabe, este é o primeiro estudo realizado em Portugal, com vista a avaliar a diversidade genética entre e dentro de populações naturais de *Arbutus unedo* L., com o objectivo de obter informação útil para a protecção e conservação da espécie, recorrendo a características morfológicas e marcadores moleculares RAPD e ISSR.

Os resultados obtidos pela análise morfológica mostraram, para todos os parâmetros avaliados, variação entre os indivíduos de *A. unedo*, sendo que o peso seco e o comprimento do pedúnculo foram as características que mais contribuíram para a diferenciação.

Os marcadores moleculares RAPD e ISSR mostraram-se eficientes na avaliação da diversidade genética de *A. unedo*, apresentando valores de percentagem de polimorfismo na ordem dos 83,1% e 86,7%, respectivamente.

Estes dois marcadores moleculares foram igualmente eficientes na avaliação da diversidade genética entre as populações de *A. unedo* separadas geograficamente, bem como entre os indivíduos dentro das populações. Verificou-se que a variação genética intra-populacional é inferior à inter-populacional, e que a localização geográfica das populações não é factor determinante no que concerne ao agrupamento de individuos da mesma população. Esta hipótese é reforçada pela análise dos dendrogramas obtidos tanto para o marcador RAPD como para o marcador ISSR, onde se verifica que os genótipos pertencentes às populações de Vila Real e Viseu não se agrupam segundo a localização geográfica, podendo a diferenciação entre os indivíduos estar relaccionada com outros factores, nomeadamente factores ecológicos como a altitude, e condições climatéricas que influenciam o desenvolvimento da planta.

A nível de diversidade genética inter populacional, verificou-se que as populações mais próximas geneticamente (0,9435), ou seja, com as que revelaram

menor distância genética (0,0582) entre elas foram as de Viseu e Vila Real e as mais distantes geneticamente (0,2684), ou seja menos similares (0,7646) foram as populações de Bragança e Castelo Branco.

Relativamente à diversidade genética intra-populacional, os resultados mostraram que a população de Bragança foi a que apresentou maior diversidade genética entre os indivíduos (Na=1,93; Ne=1,71; Ho=0,39; Io=0,5; P=93,02), e a população de Castelo Branco a que apresentou menor diversidade (Na=1,43; Ne=1,29; Ho=0,16; Io=0,24; P=43,02). O elevado nível de variação genética encontrada na população de Bragança pode ser explicado pelo facto de esta ser composta por um maior número de indivíduos e estes se encontraram distribuídos numa área bastante ampla.

O resultado obtido para o valor do coeficiente de diferenciação genética (0,260), bem como para o fluxo de genes (1,499), apontam para uma significativa troca de genes entre as populações, levando ao cruzamento entre os indivíduos de diferentes populações.

O teste de Mantel evidenciou a inexistência de correlação entre as matrizes de distância calculadas com base nos marcadores RAPD e ISSR, bem como entre estes e os dados morfológicos. As explicações para a não correlação entre os caracteres morfológicos e os marcadores moleculares (R=0,09), podem ser de várias ordens. Por um lado, o número de primers usados na caracterização genética pode não ter sido suficiente para analisar o genoma de *A. unedo* num todo, por outro lado o meio ambiente exerce grande influência nas características morfológicas das plantas. De igual modo, a correlação entre os dois marcadores moleculares RAPD e ISSR revelou-se muito baixa (R=0,22), o que pode ser explicado pelo facto de estes dois marcadores moleculares amplificarem zonas distintas do genoma. O marcador molecular ISSR mostrou-se mais eficiente que o marcador molecular RAPD na análise da diversidade genética entre os 46 genótipos de *Arbutus unedo*.

Com o intuito de salientar algumas características que valorizem esta espécie e permitam a selecção dos melhores genótipos para uma eventual propagação dos mesmos, foi avaliada a actividade antioxidante em extractos de folhas de 19 genótipos de *Arbutus unedo*, pertencentes à região de Trás-os-Montes, pelos métodos do poder redutor e capacidade bloquedora do radical livre DPPH. O teor em fenóis totais foi também avaliado, pelo método de Folin Ciocalteu.

Os resultados obtidos na quantificação do teor em fenóis totais, mostram que dentro da mesma região, o teor destes compostos pode variar significativamente, como é o caso da amostra de Bragança 1 que reportou um teor em fenóis totais superior (215,0 ± 18,2 mg GAE/g) ao da amostra proveniente de Bragança2 (153,1 ± 13,3 mg GAE/g). Também entre as quatro amostras da região de Vila Boa se pode verificar este facto, sendo que entre elas a amostra de Vila Boa 3 apresenta maior valor nestes compostos (200,4 ± 14,6 mg GAE/g) relativamente às outras três, todas elas com valores próximos de 150 mg GAE/g. Globalmente verificou-se que a amostra de Bragança 1 foi a que apresentou o valor mais elevado em teor de fenóis totais com 215,0 ± 18,23 mg GAE/g.

Os extractos aquosos testados revelaram no global potencial actividade antioxidante, apresentando os menores valores de EC_{50} para as amostras de Vila Verde e Donai (0,233 e 0,245 mg/ml para o poder redutor e 0,088 e 0,090 mg/ml para o DPPH), concluindo desta forma, que estas amostras são as que apresentam maior potencial antioxidante. Por outro lado os extractos provenientes da amostra de Vila Boa 2 reportaram menor actividade antioxidante (EC_{50} de 0,378mg/mL para o poder redutor e 0,142 mg/mL para o DPPH).

Ao correlacionar os dados obtidos no teor em fenóis totais com os valores de EC_{50} dos dois métodos testados na actividade antioxidante, verificou-se que o teor em fenóis totais influencia os valores de EC_{50} observados para ambos os métodos, tendo sido estabelecidas correlações extremamente significativas no caso do poder redutor (R^2 = 0,180; P < 0,001) e correlações muito significativas no caso do DPPH (R^2 = 0,094; *P* < 0,01).

Num estudo preliminar, efectuado no sentido de tentar identificar os compostos fenolicos presentes nestes extractos, foram encontrados três derivados de kaempferol e quatro de quertecina. Os extractos aquosos revelaram ainda capacidade em inibir o crescimento de bactérias gram positivo e gram negativo, sendo o *Bacillus cereus* o microrganismo mais sensível na presença dos extractos. A actividade antimicrobiana observada pode estar relacionada com a composição em compostos fenólicos presentes nos extractos que permitem inibir o crescimento de bactérias e o desenvolvimento de outros microrganismos.

Estes resultados servem como base para o incentivo da utilização destes extractos no tratamento de doenças provocadas pela formação de radicais livres, nomeadamente doença de Parkinson, doenças coronárias crónicas e cancro, assim como no tratamento de processos inflamatórios provocados por bactérias.

Um dos objectivos deste trabalho é a selecção dos melhores genótipos do ponto de vista químico e biológico. Tendo em conta que as amostras de Vila Verde e Donai foram, de entre as estudadas, as que apresentaram menores valores de EC_{50} para os dois métodos testados, ou seja, maior actividade antioxidante, e também por se encontraram entre os genótipos que apresentam maior teor em fenóis totais, conclui-se que do ponto de vista biológico estas árvores seriam as escolhidas para conservação e uma eventual propagação.

Contudo, deve-se salientar que este trabalho deve ser complementado com um estudo mais aprofundado no que diz respeito à determinação e quantificação do perfil em compostos fenólicos. Além dos parâmetros estudados, de modo a volorizar a espécie, deveria-se ter em conta também a cacterização da composição química, bem como a sua influência na qualidade de produtos processados a partir de *Arbutus unedo* L.

O trabalho desenvolvido contribuiu para uma primeira avaliação da diversidade genética e morfológica de *Arbutus unedo* L. efectuada em Portugal, com vista à protecção, conservação e incentivo à propagação da espécie. A baixa diversidade genética observada dentro das populações de *Arbutus unedo*, e a considerável diferenciação interpopulacional, revela que as quatro populações devem ser conservadas separadamente. No entanto, é importante realçar que é necessário complementar este estudo através da amostragem de um maior numero de populações e utilizando técnicas moleculares mais avançadas.