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CARACTERIZAÇÃO E ANÁLISE DA DIVERSIDADE GENÉTICA DA COLEÇÃO NUCLEAR DE GERMOPLASMA DE TREVO VERMELHO (*TRIFOLIUM PRATENSE* L) ATRAVÉS DE MARCADORES MORFOLÓGICOS, BIOQUÍMICOS E MOLECULARES

PAULA MENNA BARRETO DIAS
Bióloga/UFRGS
Mestre em Zootecnia/UFRGS

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CARACTERIZAÇÃO E ANÁLISE DA DIVERSIDADE GENÉTICA NA COLEÇÃO NUCLEAR DE TREVO VERMELHO (*TRIFOLIUM PRATENSE* L.) ATRAVÉS DE MARCADORES MORFOLÓGICOS, MOLECULARES E BIOQUÍMICOS[1]

Autora: Paula Menna Barreto Dias
Orientador: Miguel Dall'Agnol
Co-orientadora: Maria Teresa Schifino-Wittmann

RESUMO

O trevo vermelho é uma das leguminosas forrageiras mais utilizadas na agricultura mundial sendo adaptada a um grande número de condições edafo-climáticas. O objetivo deste trabalho foi de avaliar a diversidade genética presente na coleção nuclear de trevo vermelho usando marcadores morfológicos, moleculares e bioquímicos. Os acessos de trevo vermelho da coleção nuclear do NPGS-USDA foram analisados com 21 marcadores morfológicos, 14 *loci* microsatélites (SSR), 114 marcadores RAPD e 15 *loci* isoenzimáticos (esterase). Os marcadores moleculares, utilizados pela primeira vez na coleção nuclear, juntamente com os marcadores morfológicos e bioquímicos evidenciaram a alta diversidade genética presente na espécie, principalmente ao nível intrapopulacional. A análise de agrupamentos realizada com os dados morfológicos revelou cinco grupos distintos em relação ao florescimento precoce, médio e tardio, bem como grupos que mostraram maior persistência e produção nas condições do sul do Brasil. Um total de 78 fragmentos (SSR) foi analisado e um PIC variando de 0,70 a 0,91 com média de 0,86 foi obtido. A distância média de Rogers, baseada nos 78 fragmentos SSR, foi de 0,49. A similaridade média de Jaccard baseada nos 114 fragmentos RAPD foi de 0,21 e permitiu a identificação de todos os acessos. A heterozigosidade média (He) baseada nos dados isoenzimáticos foi de He = 0,238 e a distância média de Rogers foi de 0,14. Os dados isoenzimáticos classificaram os acessos em quatro grupos. Os grupos encontrados não separam os acessos conforme a origem geográfica ou tipo de população (cultivar, selvagem e variedade local). No entanto, algumas concordâncias foram encontradas entre as análises, uma vez que as cultivares do norte da Europa foram agrupadas em ambos os tipos de análise. Os resultados encontrados aqui evidenciaram uma grande e complexa diversidade genética na coleção nuclear de trevo vermelho. Além disso, mostraram algumas populações promissoras que podem ser usadas em programas de melhoramento de trevo vermelho no Brasil.

[1] Tese de Doutorado em Zootecnia – Plantas Forrageiras, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (139p.) Março. 2007.

CHARACTERIZATION AND ANALYSIS OF THE GENETIC DIVERSITY IN THE CORE COLLECTION OF RED CLOVER (*TRIFOLIUM PRATENSE* L.) BY MORPHOLOGICAL, MOLECULAR AND BIOCHEMICAL MARKERS[1]

Author: Paula Menna Barreto Dias
Adviser: Miguel Dall'Agnol
Co-adviser: Maria Teresa Schifino-Wittmann

ABSTRACT

Red clover is one of the most utilized leguminous forage species in the world agriculture being adapted to a great number of edaphic-climatic conditions. The objective of this work was to evaluate the genetic diversity in the core collection of red clover using morphologic, molecular and biochemical traits. The accessions of red clover from the core collection of NPGS-USDA were analyzed with 21 morphological characters, 14 microsatellite *loci* (SSR), with 114 RAPD markers and with 15 *loci* of isozyme (esterase) markers. The molecular markers, used for the first time in the core collection, together with the morphological and biochemical markers pointed out for the high genetic diversity present in the species, mostly at the intrapopulational level. The cluster analysis of the morphological data revealed five distinct clusters that separated early, medium and wild blooming types as well as groups with more persistence and high dry matter production in the Southern Brazilian conditions. A total of 78 fragments (SSR) were scored and the PIC ranged from 0.70 to 0.91 with 0.86 as mean value. The mean Rogers' genetic distance based on the 78 SSR markers was 0.49. The Jaccard's similarity based on 114 RAPD markers was 0.21 and allowed the identification of all accessions. The mean expected heterozygosity (He), based on isozyme data, was He = 0.238 and the mean Rogers' genetic distance was 0.14. Isozyme data clustered the accessions of red clover in four groups. The clusters found with morphological and biochemical markers were not separated according to their geographic origin or type of populations (cultivar, wild or landrace). However, some coincidences between analyses were found once cultivars from north Europe were clustered in both types of analyze. The results found here evidenced the high and complex diversity in red clover core collection. Indeed, they highlighted some promising populations that could be used in the Brazilian breeding programs of red clover.

[1] Doctoral thesis in Forage Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil (139p.) March. 2007.

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LISTA DE ABREVIATURAS

AMOVA	Analysis of Molecular Variation
MS	Matéria Seca
SSR	Simple Sequence Repeat
RAPD	Random Amplified Polymorphic DNA
PIC	Polymorphism Information Content
RFLP	Restriction Length Fragment Polymorphism
NPGS	National Plant Germplasm System
BP	Base Pair

1. CAPÍTULO I

1.1 Introdução

As leguminosas forrageiras têm um importante papel na sustentabilidade de rebanhos para a produção de alimentos derivados da produção animal. Dentre estas, as pastagens temperadas sustentam a maior parte da produção mundial de leite e carne (Humphreys, 2005). No entanto, atualmente tem havido uma mudança de orientação da agricultura nos países desenvolvidos, agora não mais no sentido da produção intensiva, mas no sentido de utilização de sistemas auto-sustentáveis, ecologicamente responsáveis e na direção da vantagem econômica das leguminosas saindo do modelo de altos insumos como os fertilizantes inorgânicos (Humphreys, 2005).

A literatura recente tem mostrado ainda que sistemas baseados em leguminosas têm a habilidade de reduzir problemas ambientais através do aumento da eficiência do uso de nitrogênio, evitando um excesso de N no solo (Rochon et al., 2004). Além disso, projeções em relação ao aquecimento global demonstram o aumento da produção de leguminosas, em relação às gramíneas (Topp & Doyle, 1996), especialmente em áreas de altas latitudes no hemisfério norte (Frame et al., 1998). A busca pela atividade agrícola de alta precisão, economicamente eficiente e orientada no sentido da responsabilidade ambiental pelos impactos de sua atividade, tem colocado em pauta a utilização de sistemas baseados em leguminosas.

O trevo vermelho (*Trifolium pratense* L.) é uma das leguminosas temperadas mais utilizadas no mundo, altamente reconhecida pelo seu grande valor nutritivo, alta capacidade de fixação de nitrogênio, superior ao da alfafa, e pela sua alta produtividade. O trevo vermelho é originário do sudeste da

Europa e Ásia Menor, adapta-se melhor em ambientes com climas temperados, sem extremos de calor ou frio, solos férteis, profundos e bem drenados, com relativamente alto pH, podendo ser usado como silagem, feno, pasto e melhoramento do solo em muitas regiões do mundo (Taylor & Quesenberry, 1996). O trevo vermelho é uma planta alógama, diplóide (2n=2x=14) e auto-incompatível, o que determina uma alta heterozigose e variabilidade genética (Taylor & Smith, 1995).

Na região sul do Brasil é tradicionalmente utilizado nos meses de inverno na tentativa de minimizar as perdas na produção pecuária, uma vez que a base do sistema pecuário no Rio Grande do Sul são as espécies subtropicais de produção estival, que no período de frio cessam seu crescimento e diminuem a qualidade levando à perda de peso dos animais. Apesar de ser considerado perene pode comportar-se como bienal quando em situações de estresse, como ocorre no sul do Brasil, onde esta espécie sobrevive, no máximo por um ou dois anos, apresentando problemas de persistência. As cultivares que são tradicionalmente utilizadas no Rio Grande do Sul (Kenland, Estanzuela 116 e Quiñequeli) não foram desenvolvidas nesta região e, apesar de apresentarem uma boa adaptação, também apresentam alguns problemas principalmente quanto à sua persistência. No entanto, tem sido relatada uma boa produção de forragem em várias regiões do Rio Grande do Sul (Araújo, 1967; Jacques et al., 1995; Fontaneli & Basso, 1995), o que demonstra o potencial produtivo da espécie nesta região.

Montardo et al. (2003b), realizaram a análise genética da persistência de progênies de trevo vermelho em dois locais do sul do Brasil e

constataram que houve interação genótipo ambiente e uma alta herdabilidade da característica. Dentro deste contexto, diversos estudos visando a disponibilização de populações mais adaptadas às condições edafo-climáticas do Rio Grande do Sul, têm sido realizados no programa de melhoramento de trevo vermelho do Departamento de Plantas Forrageiras e Agrometeorologia.

A matéria-prima de um trabalho de melhoramento é a diversidade genética (Allard, 1971). Um programa de melhoramento deve manipular a variação existente entre plantas. Esta variabilidade pode ser obtida tanto por ecótipos locais como por introduções de outras regiões e estas devem ser de regiões as mais variáveis possíveis quanto a solo e clima (Paim, 1994). Uma das principais formas de acesso a variabilidade são as coleções de germoplasma, e estas têm por objetivo representar a variabilidade presente numa espécie. Os curadores são responsáveis pelo desenvolvimento e manutenção de coleções de germoplasma *ex-situ* que servem ao duplo propósito de prover material para o melhoramento no presente e conservar diversidade genética para o futuro (Frankel & Brown, 1995).

A coleção de germoplasma de trevo vermelho do National Plant Germplasm System do United States Departament of Agriculture (NPGS-USDA) é composta de cerca de 800 acessos oriundos de 41 países (Kouamé & Quesenberry, 1993). Para facilitar a conservação e o uso efetivo de coleções de germoplasma Brown (1989), propôs a formação de coleções nucleares que contêm um menor número de acessos e que representam igualmente a variabilidade genética presente numa espécie.

A coleção nuclear de trevo vermelho foi formada por Kouamé & Quesenberry, (1993) através da análise de 463 acessos da coleção de germoplasma de trevo vermelho utilizando dados de 15 medidas morfológicas presentes no banco de dados do NPGS-USDA. No entanto, estas foram avaliadas em épocas e locais diferentes. Esta coleção nuclear é composta de 85 acessos provenientes de 41 países (Kouamé & Quesenberry 1993).

Para a descrição da variabilidade genética em trevo vermelho e avaliação de germoplasma têm-se utilizado listas de descritores morfológicos (IBPGR, 1984), que facilitam a identificação dos acessos com características desejáveis. No entanto, o uso somente de descritores morfológicos para a avaliação e caracterização de germoplasma pode representar um problema para características que sejam de baixa herdabilidade e ocorram com pouca freqüência, uma vez que será necessária a utilização de grande número de indivíduos de cada acesso a ser analisado (Taylor & Quesenberry, 1996). Após a formação da coleção nuclear alguns trabalhos foram realizados para análise da diversidade, no entanto a maioria destes trabalhos utilizam somente caracteres morfológicos ou bioquímicos (Kouamé et al., 1997; Bortnem & Boe, 2002; Bortnem & Boe, 2003; Mosjidis & Klingler, 2006). Apesar da importância da coleção nuclear de trevo vermelho como fonte de variabilidade genética, nenhum trabalho utilizando a tecnologia de marcadores moleculares na análise da diversidade foi realizado.

Os marcadores baseados em polimorfismos de DNA têm sido cada vez mais utilizados na mensuração e descrição da variabilidade genética. Os marcadores moleculares têm sido amplamente utilizados em programas de

melhoramento para a caracterização e estudos da diversidade e distância genética. Dentre esses marcadores, o RAPD (Polimorfismo do DNA Amplificado ao Acaso) (Williams et al., 1990) tem sido amplamente utilizado em diversas espécies de forrageiras (Crochemore et al., 1998; Chtourou-Ghorbel et al., 2002; Johnson et al., 2002). Esta é uma tecnologia bastante acessível, com capacidade de detectar grande polimorfismo e que necessita de quantidade mínima de DNA para análise genotípica.

No entanto, uma limitação do RAPD é a natureza dominante do marcador onde o escore dos dados é baseado na presença ou ausência dos fragmentos amplificados, não permitindo a identificação dos indivíduos heterozigotos. Segundo Jasieniuk & Maxwell, (2001) a inabilidade em detectar heterozigotos reduz a precisão na estimativa de parâmetros genéticos de populações, incluindo a estimativa da freqüência alélica. Como alternativa para minimizar as limitações com relação à dominância do marcador molecular do tipo RAPD, pode-se utilizar concomitantemente marcadores co-dominantes na análise das populações uma vez que estes permitem a identificação dos indivíduos heterozigotos. Além disso, pode-se realizar através destes a estimativa das freqüências alélicas e outros parâmetros genéticos de populações, ou mesmo para a complementação dos estudos de diversidade genética através da análise de um maior número de informação por *locus* analisado.

Outros marcadores, de natureza co-dominante, que têm sido bastante utilizados em estudos de diversidade genética vegetal são os microsatélites ou SSR (Simple Sequence Repeats) e ainda os marcadores

isoenzimáticos. Os marcadores moleculares do tipo microsatélites são rotineiramente utilizados na investigação da estrutura genética e da diversidade de populações naturais (Balloux & Lugon-Moulin, 2002), bem como, em espécies cultivadas como a alfafa (Flajoulot et al., 2005) e o trevo branco (Kölliker et al., 2001). Os marcadores bioquímicos do tipo isoenzimas são co-dominantes, são de baixo custo e tecnicamente acessíveis o que resulta em uma grande quantidade de informação genética obtida em pouco tempo e estes têm sido muito utilizados em análises de diversidade genética também em trevo vermelho (Hagen & Hamrick, 1998; Kongkiatngam et al., 1995; Lange & Schifino-Wittmann, 2000) bem como em outras espécies forrageiras (Bem Brain et al., 2002; Yanaka et al., 2005).

Os marcadores morfológicos e bioquímicos já foram utilizados no estudo da diversidade na coleção nuclear de trevo vermelho do NPGS-USDA (Bortnem & Boe, 2002; Bortnem & Boe, 2003; Mosjidis & Klingler, 2006). No entanto, quando se avalia a diversidade genética utilizando um ou poucos caracteres tem-se menor oportunidade de entender as relações de proximidade entre as populações. Deste modo o uso de diferentes abordagens amplia as oportunidades de evidenciar as diferenças genéticas entre as populações (Brown, 1989). Neste sentido, o estudo da coleção nuclear de trevo vermelho sob diferentes aspectos representa uma abordagem da diversidade genética com ferramentas distintas que podem auxiliar na compreensão e na melhor utilização desta importante fonte de variabilidade para os programas de melhoramento.

1.2. Caracteres morfológicos na análise da diversidade em trevo vermelho

A análise das relações genéticas em espécies cultivadas é um componente importante dos programas de melhoramento e serve para: análise da variabilidade genética dos cultivares, identificação de diversas combinações parentais para criar progênies segregantes com o máximo de variabilidade para a seleção posterior e ainda para a introgressão de genes desejáveis na base genética desejada (Mohammadi & Prasanna, 2003).

O estudo da diversidade genética é um processo pelo qual a variação entre indivíduos ou grupos de indivíduos ou populações, é analisada por um método específico ou ainda pela combinação de métodos (Mohammadi & Prasanna, 2003).

A análise utilizando caracteres morfológicos para o estudo da diversidade, estabelecimentos de grupos, diferenciação de populações naturais e cultivares é uma das ferramentas mais utilizadas na avaliação de germoplasma de diversas espécies cultivadas (Souza & Sorrells, 1991a; Johns et al., 1997), assim como em importantes espécies forrageiras (Steiner & los Santos, 2001; Steiner et al., 2001; Roldan-Ruiz et al., 2001; Johnson et al., 2002). Além disso, o registro e proteção de novas cultivares é ainda baseado na sua maioria em listas de descritores morfológicos.

A grande vantagem dos caracteres morfológicos é que a sua avaliação pode ser feita de uma forma bastante acessível e freqüentemente não necessitam de programas sofisticados para análise. Outra vantagem é que a avaliação baseada em caracteres vegetativos e de morfologia floral está

diretamente relacionado à sobrevivência e reprodução das plantas, e ainda o fato de que muitos caracteres morfológicos são poligênicos, o que faz com que muitos *loci* possam estar implicitamente sendo analisados (Huenneke et al., 1992).

Em trevo vermelho, a análise da diversidade utilizando caracteres morfológicos permitiu a identificação de características que possibilitaram o reconhecimento de grandes grupos nas populações de trevo. Todos os trevos vermelhos podem ser classificados em três grupos, conforme a época de florescimento em: precoce, tardio e selvagem (Bird, 1948). Estes grupos que foram avaliados por Bird (1948) no Canadá, diferem de forma ampla não somente em relação a caracteres botânicos, mas também de uma forma marcante no que diz respeito a suas capacidades de cultivo, persistência e caracteres agronômicos. Os tipos tardios têm hábito de crescimento prostrado, são plantas mais densas, crescem mais lentamente e produzem uma floração esparsa no ano de semeadura, bem como, permitem somente um corte na estação de crescimento. Os tipos precoces, no entanto, possuem um hábito mais ereto, são plantas menos densas, crescem mais rapidamente, têm uma maior intensidade de floração e permitem cortes na estação de crescimento, sob as condições de crescimento do Canadá.

Já os do tipo selvagem apresentam uma ampla variação com relação à época de florescimento, são plantas muito pequenas e menos produtivas no ano de semeadura (Bird, 1948). Taylor & Smith (1995) apontam que a principal diferença entre os tipos precoces e tardios de trevo vermelho está na resposta ao comprimento do dia, uma vez que os tipos precoces

necessitam de um fotoperíodo de 13 a 14 horas e os tipos tardios precisam de 14 horas ou mais para dar início ao florescimento. Segundo esses autores, a maioria dos trevos vermelhos americanos são do tipo precoce com relação ao florescimento, e os autores descrevem ainda que, na Europa, formas distintas de trevo vermelho evoluíram por seleção natural e estão distribuídas de acordo com a latitude.

De acordo com Taylor & Smith (1995) as formas precoces predominam no sul da Europa em latitudes em torno de 50° e as formas tardias estariam distribuídas no norte da Europa em torno de 60° de latitude. Aitiken (1964) avaliando duas cultivares de trevo vermelho contrastantes em relação à época de florescimento (precoce e tardia) aponta também a influência da latitude na época de floração. Segundo Aitiken (1964) os diferentes tipos de trevo vermelho são usados para fins diferentes na Inglaterra e na Nova Zelândia, onde as linhagens precoces tendem a ser usadas para feno e as tardias para pastagens.

Outra característica morfológica que foi estudada em trevo vermelho é a pilosidade. Taylor & Quesenberry (1996) indicam que os caules da maioria dos cultivares americanos são densamente pilosos, enquanto que os caules das populações européias e chilenas de trevo vermelho são na sua maioria glabros. A densa pilosidade dos cultivares americanos pode ter sido resultado da seleção natural para a resistência a insetos ou ainda um mecanismo de tolerância a estresses (Taylor & Quesenberry 1996)

O hábito de crescimento foi estudado por Mirzaie-Nodoushan et al. (1999) através da análise de progênies segregantes para hábito ereto ou

prostrado sendo que os autores verificaram uma dominância parcial do hábito prostrado sobre o hábito ereto e que este caractere seria controlado por poucos genes. Outros trabalhos relacionam ainda o hábito de crescimento e o tipo (precoce ou tardio) com a persistência em trevo vermelho. Choo (1984) avaliou três variedades diplóides e precoces de trevo vermelho e classificou as plantas em cinco grupos de acordo com o desenvolvimento da coroa e do tipo de floração, e evidenciou uma maior persistência nas populações que floresceram menos do que nas populações que tiveram um florescimento intenso no ano de estabelecimento. Christie & Choo (1991) avaliaram variedades tetraplóides de trevo vermelho e demonstraram o mesmo tipo de relação positiva entre o tipo de crescimento mais vegetativo e uma maior sobrevivência.

A relação entre tipos de raízes e persistência em trevo vermelho foi estudada por Christie & Martin (1999) que demonstraram que plantas mais persistentes possuem um grande sistema de raízes com mais raízes adventícias saindo da raiz principal, e os tipos menos persistentes possuem menos raízes adventícias. No entanto, segundo Smith (1989) quando foi realizada a seleção direta para tipo de raízes, os tipos de raiz pivotante apresentaram maior persistência.

Joshi et al. (2001) avaliaram caracteres morfológicos em populações de trevo vermelho realizando o transplante recíproco entre populações, no intuito de verificar o efeito do transplante na adaptação de populações de trevo vermelho a diferentes locais. Os autores verificaram uma forte interação entre efeitos ambientais e genéticos e quando se comparou o desempenho em cada sítio, as populações que foram transplantadas tiveram

uma performance menor em comparação às populações avaliadas em seus sítios de origem. No trevo vermelho, os caracteres reprodutivos como tamanho de inflorescência e num menor grau, o número de inflorescências diferiram entre os sítios havendo uma melhor performance no sítio original, onde ocorreram duas vezes mais inflorescências e estas foram maiores, demonstrando assim uma forte adaptação ao sítio de origem (Joshi et al. 2001). Os autores relatam, no entanto, que os fatores climáticos não foram a maior causa dos efeitos entre os sítios e que a adaptação local pode ser uma importante característica em programas de melhoramento, uma vez que se pode desenvolver variedades para ambientes específicos onde estas serão utilizadas.

Em trevo vermelho, Taylor & Smith (1995) apontaram para o fato de que as cultivares não se adaptam a locais distantes de onde foram desenvolvidas. No entanto, esta adaptação local não implica em restrição da espécie a um local, uma vez que o trevo vermelho é uma espécie alógama, morfologicamente variável e amplamente distribuída (Joshi et al., 2001). Sabe-se que *taxa* que exibem ampla distribuição podem apresentar uma significante diferenciação em ecótipos, podendo ocorrer certas raças geneticamente distintas e adaptadas à condições específicas de solo e clima (Huenneke et al., 1992).

Linhart & Grant (1996) descrevem ainda um fenômeno demonstrado em várias espécies chamado de “depressão alógama” onde progênies resultantes de cruzamentos de indivíduos de diferentes locais tem um valor adaptativo inferior aos pais, uma vez que este processo quebra um

complexo gênico que forma uma importante parte da adaptação a um local específico. Linhart & Grant (1996) chamam ainda atenção para o fato de que a "depressão alógama" homogeiniza o efeito do fluxo gênico entre populações.

Caracteres morfológicos foram utilizados também para medir a produção vegetal e relacionar esta com a persistência em trevo vermelho, utilizado tanto em monocultura (Crusius et al., 1999; Montardo et al., 2003b; Halling et al., 2004; Muntean & Savatti, 2003), quanto em consórcio com outras espécies (Maitre et al., 1985; Mela, 2003). No norte da Europa, Halling et al. (2004) avaliaram dados de produção de trevo vermelho de oito países no período de 1977 a 1997 que apontaram uma produção média de 9.470 Kg MS/ha/ano. Os autores ressaltaram no entanto, a baixa persistência do trevo vermelho quando comparado a outras leguminosas como a alfafa e o trevo branco. O trevo vermelho na América Latina é utilizado em regiões temperadas e subtropicais principalmente no Chile, Argentina, Uruguai e no sul do Brasil (Paim, 1988).

Na região sul do Brasil Montardo et al. (2003b) realizaram a análise genética da persistência em progênies de trevo vermelho em dois locais, avaliando na segunda estação de crescimento e constataram que houve interação genótipo ambiente e que a herdabilidade média da característica foi de 65%. Os autores indicaram ainda que, dentro de certos limites, a seleção para persistência no ambiente com maior estresse mostrou-se mais vantajosa ou, no mínimo, tão eficiente quanto a seleção em ambientes melhores para o cultivo de trevo vermelho. Em avaliação subseqüente, realizada nas 33 progênies selecionadas nos dois locais os autores verificaram que existe

variabilidade para novos progressos com seleção para persistência nas populações selecionadas. Com relação à produção, Montardo et al. (2003b) verificaram uma produção de 7.490 Kg/MS/ha para a região que apresentou a menor persistência. Na mesma região do Brasil Crusius et al. (1999) avaliaram características agronômicas e sua influência na produção e persistência em linhagens melhoradas no Chile (tardias) e em populações naturalizadas (precoces). Os autores encontraram uma alta correlação entre o número de hastes por planta e a produção de matéria seca, indicando que quanto maior o número de hastes na planta, maior a quantidade de matéria seca produzida. Com relação à persistência, Crusius et al. (1999) encontraram uma alta correlação entre o início do florescimento (após transplante para campo experimental) e a produção de matéria seca, indicando que mesmo as plantas mais tardias que floresceram mais tarde, produziram mais matéria seca. No entanto estas foram menos persistentes.

Outro caractere que foi avaliado em trevo vermelho é o efeito de cortes sobre o rebrote e a persistência. Fan et al. (2004) avaliaram numa região subtropical da China (31°33'N, 109°04'E) o efeito de cortes em diferentes estágios reprodutivos de uma cultivar local. O experimento demonstrou que a rebrota diminui com repetidos cortes no estádio reprodutivo e isto refletiu no tamanho da planta e no rendimento. Fan et al. (2004) sugerem ainda que o corte do trevo vermelho para forragem deveria ser feito antes do florescimento. No Chile, Ortega et al. (1991) avaliaram em três estações de crescimento a produção de matéria seca e a persistência de 20 cultivares de trevo vermelho.

Os autores relataram uma produção média de 19,3 ton/MS/ha nas três estações avaliadas.

A produção e a multiplicação de sementes é um dos pontos mais importantes dentro de um programa de melhoramento. E freqüentemente no melhoramento de forrageiras, tem-se que melhorar caracteres contrastantes como alta produção de matéria seca e a alta produção de sementes. Montardo et al. (2003a) avaliaram a influência dos componentes de produção no rendimento de sementes e encontraram uma alta correlação entre número de inflorescências por planta e rendimento de sementes. Dados morfológicos foram utilizados na formação da coleção nuclear de trevo vermelho por Kouamé & Quesenberry (1993). Os autores avaliaram a coleção de germoplasma do NPGS-US (*National Plant Germplasm System – United States*), composta por mais de 800 acessos, utilizando 15 caracteres morfológicos nos 463 acessos de trevo vermelho cujos dados encontravam-se disponíveis nos bancos de dados do NPGS. Foram encontrados três grupos principais que agruparam os trevos em precoces, médios, e tardios em relação ao período de floração. Deste modo, foi sugerida uma amostragem estratificada por país de origem, dentro de cada um dos três grupos encontrados, para a formação da coleção nuclear de trevo vermelho (Kouamé & Quesenberry, 1993). Após a formação da coleção nuclear, esta foi avaliada em relação a alguns caracteres morfológicos como cores de sementes (Bortnem & Boe, 2003) e freqüência de marca branca na folha (Bortnem & Boe, 2002).

Bortnem & Boe (2003) avaliaram as populações em relação à cor de semente e encontraram uma grande variabilidade nos acessos da coleção

nuclear de trevo vermelho. Os autores indicaram que estas foram divididas em cinco classes de cores e que esta característica poderia ser utilizada como um bom descritor morfológico. No entanto, Bortnem & Boe (2003) não relataram diferenças de origem ou tipo de material em relação às classes encontradas.

No trevo vermelho a ausência de mancha branca nas folhas (ou folhas variegadas) é conseqüência de um genótipo homozigoto recessivo (Taylor, 1982). Bortnem & Boe (2002) estudaram a freqüência do genótipo recessivo que determina a ausência de marca foliar em populações da coleção nuclear e evidenciaram que a presença deste caractere foi extremamente rara ocorrendo em menos de 25% dos acessos. No entanto Bortnem & Boe (2002) verificaram que a ausência de mancha foliar foi mais comum entre os acessos das regiões central e sul da Europa.

Apesar de sua grande utilidade na avaliação de germoplasma e melhoramento genético, a diversidade fenotípica encontrada em algumas características pode ser devido à variações ambientais, e muitas vezes torna-se difícil determinar o quanto da variabilidade fenotípica encontrada é devido a diferenças genéticas e o quanto é devido a diferenças entre os ambientes (Huenneke et al., 1992).

Além disso, diversos estudo filogenéticos utilizando tanto caracteres morfológicos (Bulinska-Radomska, 2000) quanto moleculares (Watson et al., 2000; Ansari et al., 2004; Ellison et al., 2006), têm concordado com a taxonomia proposta por Zohary & Heller (1984) e demonstrado que o trevo vermelho, além de diferir em número cromossômico das outras espécies presentes na mesma seção, apresenta um pequeno sucesso no que diz

respeito à hibridação natural com espécies consideradas próximas como o *Trifolium diffusum* e o *Trifolium pallidum*. A hibridação do trevo vermelho com espécies do gênero não tem sido muito utilizada no melhoramento pois muitas vezes é necessário utilizar técnicas como o resgate de embriões para obter híbridos viáveis dos cruzamentos (Taylor & Smith, 1995), ou ainda utilizar plantas poliplóides para os cruzamentos que segundo Repkova et al. (2006), apresentam maior sucesso nos cruzamentos quando comparadas as plantas diplóides.

Deste modo, o melhorista de trevo vermelho tem que buscar variabilidade principalmente dentro da própria espécie, e para isto necessita com freqüência recorrer a trocas de germoplasma entre instituições, coleta de populações naturalizadas ou ainda a bancos de germoplasma.

1.3. Marcadores bioquímicos na análise da diversidade genética em trevo vermelho.

O conhecimento de como a variabilidade genética está estruturada e como as características ambientais e da própria espécie influenciam nesta distribuição é de suma importância para o manejo dos recursos genéticos (Hamrick, 1983). A estimativa da extensão da variabilidade dentre e entre populações de uma espécie é útil para analisar a estrutura genética das populações, monitorar bancos de germoplasma e predizer potenciais de ganhos genéticos em programas de melhoramento (Kongkiatngam et al. 1995).

As isoenzimas foram, por várias décadas, os marcadores bioquímicos mais utilizados em análises genéticas e no melhoramento vegetal. Isoenzimas são diferentes formas moleculares de proteínas que exibem a mesma especificidade enzimática (Cavalli, 2003). No melhoramento de plantas, isoenzimas têm sido utilizadas para a detecção de ligação gênica com caracteres mono e poligênicos, identificação de variedades, na seleção indireta de caracteres agronômicos, introgressão gênica e avaliação de germoplasma (Ferreira & Grattapaglia, 1998). As isoenzimas possuem muitas características que as tornam úteis como marcadores genéticos para estudos de diversidade vegetal. Esta técnica é bastante acessível, barata e um grande número de amostras podem ser processadas ao mesmo tempo, resultando num grande número de dados obtidos numa mesma análise. Além disso, uma das características mais importantes das isoenzimas é a sua expressão co-dominante onde os genótipos homozigotos e heterozigotos podem ser

distinguidos, permitindo a obtenção de análises de parâmetros populacionais (Jasieniuk & Maxwell, 2001). As isoenzimas podem ser usadas para quantificar a diversidade genética e para análise da estrutura genética de populações (Jasieniuk & Maxwell 2001).

Diversas espécies de plantas cultivadas já tiveram seu germoplasma avaliado com o uso das isoenzimas, como o centeio (Matos et al., 2001), a aveia (Souza & Sorrells, 1991b) e o arroz (Buso et al., 1998). Em alfafa, uma das forrageiras mais cultivadas no mundo, Jenczewski et al. (1999b) realizaram um estudo utilizando isoenzimas e caracteres morfológicos e evidenciaram fluxo gênico entre populações naturais e cultivadas da Espanha. Os autores relataram que as populações dividiram-se em três grupos em relação às populações naturais: a) populações naturais com freqüências alélicas dos *loci* isoenzimáticos intermediárias a das populações cultivadas; b) populações naturais com freqüências alélicas distantes das observadas nas cultivadas e finalmente; c) populações naturais com *loci* isoenzimáticos com alta similaridade aos observados nas populações cultivadas. Os autores evidenciaram, a partir destes resultados, a formação de populações híbridas entre populações selvagens e populações cultivadas em função do fluxo gênico.

Num estudo realizado por Balfourier et al. (1998) para verificar a diversidade genética entre duas espécies de azevém (*Lolium perene* e *Lolium rigidum*) utilizando 12 *loci* isoenzimáticos, os autores verificaram uma alta diversidade genética intrapopulacional nas duas espécies e uma maior diversidade nas populações do *Lolium rigidum*.

Em trevo vermelho diversos trabalhos foram realizados utilizando os marcadores isoenzimáticos. Kongkiatngam et al. (1995) analisaram a variação genética dentro e entre duas cultivares de trevo vermelho (Ottawa), de origem canadense, e (Essi) de origem européia. Foram analisados 21 *loci* isoenzimáticos com um total de 43 alelos, utilizando 12 diferentes sistemas enzimáticos. Os autores verificaram uma maior variação na cultivar de origem européia, que apresentou polimorfismo em 13 dos *loci* avaliados e um maior número de alelos por *locus*. Kongkiatngam et al. (1995) verificaram ainda um alto nível de variação genética intrapopulacional nas duas cultivares avaliadas, no entanto as cultivares puderam ser individualmente caracterizadas. Este estudo demonstrou que os marcadores isoenzimáticos são eficientes em estimar a variabilidade genética dentro e entre cultivares de trevo vermelho.

A fim de avaliar a variação genética entre três populações naturais e duas cultivares russas de trevo vermelho, através da análise de 19 *loci* isoenzimáticos, Semerikov & Belyaev (1995) observaram um polimorfismo variando de 47% a 59% para todas as populações. Encontraram uma maior heterozigosidade nas cultivares (15% a 19%) do que nas populações naturais (13% a 17%). Os autores relatam ainda que as diferenças genéticas entre grupos de cultivares e de populações selvagens foram significativamente maiores do que as variações presentes dentro de cada grupo. Segundo Semerikov & Belyaev (1995), estes resultados indicam que a origem das cultivares, de regiões centrais da área de distribuição desta espécie, é determinante da alta variabilidade genética.

Hagen & Hamrick (1998), num estudo com objetivo de determinar a estrutura genética de nove populações naturais de trevo vermelho da região sudoeste dos Estados Unidos, através da análise de 13 *loci* isoenzimáticos, verificaram uma alta diversidade genética dentro das populações e uma baixa divergência entre as populações. Os autores concluíram que esta alta variabilidade intrapopulacional sugere que estas populações estariam estabelecidas há bastante tempo, não sendo populações fundadoras, e que o baixo nível de divergência genética é provavelmente devido a altas taxas de fluxo gênico resultante da dispersão de pólen e sementes.

Utilizando quatro sistemas enzimáticos, na avaliação de 36 acessos de oito espécies de trevo, Lange & Schifino-Wittmann (2000) obtiveram oito grupos, cada um correspondendo a uma espécie e constataram uma alta diversidade intraespecífica para os acessos de trevo vermelho examinados. As autoras indicaram ainda uma concordância dos grupos obtidos com a classificação taxonômica tradicional para o gênero *Trifolium* proposta por Zohary & Heller (1984).

Yu et al. (2001) conduziram um intenso estudo sobre a diversidade de 34 cultivares norte-americanas de trevo vermelho, utilizando 13 *loci* isoenzimáticos com um total de 28 alelos analisados. Detectaram um polimorfismo médio de 73,98%, uma alta variabilidade intracultivar variando de 98,4% a 99,7% e uma baixa diferenciação entre grupos de cultivares. Yu et al. (2001) indicaram ainda que os grupos formados não corresponderam à origem ou ao pedigree das cultivares.

Semerikov et al. (2002) avaliando a relação genética entre cultivares e populações naturais russas, utilizando 10 sistemas isoenzimáticos, constataram uma maior variação intrapopulacional nas cultivares do que nas populações naturais. Segundo Semerikov et al. (2002) o melhoramento teve um pequeno impacto na variação genética nos *loci* isoenzimáticos, uma vez que existe ainda uma grande variação dentro das cultivares para os *loci* examinados.

Mosjidis et al. (2003) mediram a diversidade genética em 15 populações selvagens da Rússia e examinaram a correspondência da diversidade e do fluxo gênico com dados de georeferenciamento. Os autores verificaram que 90% do *loci* foram polimórficos ao menos em uma população. As estimativas de fluxo gênico baseadas em predições dos dados geográficos coincidiram com as estimativas de fluxo gênico dos *loci* isoenzimáticos. Segundo Mosjidis et al. (2003) estes resultados suportam o uso de dados geográficos e de sensoriamento remoto no desenvolvimento de mapas que auxiliem na coleta de germoplasma, na avaliação e no entendimento dos padrões de diversidade genética no germoplasma coletado.

Recentemente, Mosjidis & Klingler (2006) mediram a diversidade genética nos 81 acessos da coleção nuclear de germoplasma de trevo vermelho do NPGS-US (National Plant Germplasm System - United States). Utilizando sete sistemas enzimáticos, os autores detectaram 10 *loci* com 30 alelos evidenciando um alto polimorfismo (77,5%) entre os acessos. Os autores mediram a diversidade genética entre cultivares, populações selvagens e variedades locais presentes na coleção nuclear e verificaram uma maior

diversidade presente nas populações selvagens, apresentando estas, quase o dobro da diversidade presente nos acessos classificados como variedades locais e cultivares. No entanto, segundo Mosjidis & Klingler (2006) a variabilidade isoenzimática não permitiu a discriminação dos acessos em grupos relacionados, uma vez que a análise de agrupamentos resultou em três grupos, o primeiro grupo contendo quatro acessos, o segundo grupo composto por três acessos e um último grupo formado por 73 acessos da coleção nuclear e não houve uma clara relação da distribuição da variabilidade com a distribuição geográfica dos acessos.

Apesar de ser amplamente utilizada, de baixo custo e fácil execução, a técnica de análise de isoenzimas apresenta algumas limitações. Os genes que codificam as isoenzimas representam uma pequena fração do genoma, limitando a análise de polimorfismos pois somente as substituições nucleotídicas que modifiquem a mobilidade eletroforética da enzima são detectadas, e muitas variações alélicas não são detectadas em função da redundância do código genético e de migração similar ao longo do gel (Jasieniuk & Maxwell, 2001). Segundo Jasieniuk & Maxwell (2001) os marcadores isoenzimáticos são inadequados para resolver diferenças genéticas muito pequenas, deste modo estes não são indicados para estudos de paternidade, variação genética entre linhagens extremamente relacionadas ou identificação individual. Outras limitações das isoenzimas como marcadores moleculares dizem respeito a: especificidade de formas isoenzimáticas em alguns tecidos vegetais, o que pode ser uma limitação para a análise; ao polimorfismo isoenzimático em resposta condições ambientais; às diferenças

na atividade isoenzimática associadas a estádios diferentes de desenvolvimento.

No entanto, as isoenzimas têm sido amplamente utilizadas em estudos de diversidade genética e mostram-se extremamente úteis em função da facilidade da análise e rápida interpretação de dados.

1.4. Marcadores moleculares do tipo RAPD na análise da diversidade genética em trevo vermelho.

Com o advento das técnicas de biologia molecular surgiram vários métodos de detecção de polimorfismo genético ao nível de DNA, os chamados marcadores moleculares. O desenvolvimento da técnica de PCR (*Polimerase Chain Reaction* ou Reação em Cadeia da Polimerase) levou ao desenvolvimento de outras técnicas capazes de identificar polimorfismo. O PCR é uma técnica poderosa, utilizada para ampliar pequenas seqüências específicas de nucleotídeos em quantidades acessíveis para análise, a partir de uma quantidade mínima de DNA. Baseia-se na síntese enzimática *in vitro* de um segmento específico de DNA na presença da enzima DNA polimerase e de *primers* (iniciadores) específicos ou não. Estes iniciadores delimitam a seqüência de DNA de fita dupla a ser amplificada, tanto no sentido jusante quanto à montante, cujos resultados são milhões de cópias idênticas.

Os marcadores moleculares permitem gerar uma grande quantidade de informações sobre a diversidade genética do germoplasma utilizado pelo melhorista, auxiliando-o na seleção dos progenitores de populações básicas ao estabelecer programas de melhoramento (Ferreira & Grattapaglia, 1998). Além da grande quantidade de informação disponibilizada, os marcadores moleculares apresentam a vantagem, sobre os marcadores morfológicos e bioquímicos, de serem neutros e não estarem sujeitos a efeitos ambientais e ontogênicos (Ferreira & Grattapaglia, 1998).

Um dos primeiros marcadores moleculares utilizados foi o RAPD (*Random Amplified Polymorphic* DNA ou Polimorfismo de DNA amplificado ao

acaso) (Williams et al., 1990). A técnica de RAPD distingue-se das demais técnicas moleculares pelo fato de utilizar seqüências iniciadoras com 10 pares de bases de extensão, cuja seqüência nucleotídica é arbitrária, ao contrário das outras que requerem informações a respeito da seqüência do DNA alvo para o desenho de seqüências iniciadoras específicas (Williams et al., 1990).

As principais vantagens do RAPD com relação a outras técnicas são a sua facilidade, rapidez, a necessidade de quantidades mínimas de DNA e principalmente a grande quantidade de polimorfismo gerado por um único marcador. O polimorfismo do RAPD é de natureza dominante, ou seja, apenas são detectados os indivíduos homozigotos, uma vez que ao se observar uma banda RAPD em gel de agarose, não é possível distinguir se aquele segmento originou-se a partir de uma ou duas cópias da seqüência amplificada. A natureza molecular do polimorfismo do RAPD ainda não é inteiramente conhecida. Entretanto, segundo Ferreira & Grattapaglia (1998), evidências experimentais indicam que a diferença de apenas um par de bases (mutação de ponto) são suficientes para causar a não complementaridade da seqüência iniciadora com o sítio de iniciação, o que pode impedir a amplificação de um segmento. Outras fontes de polimorfismo podem incluir deleções de sítios de iniciação presentes no DNA alvo ou inserções que colocam dois sítios de iniciação adjacentes a uma distância acima daquela em que a enzima DNA polimerase é capaz de percorrer e amplificar o segmento (Ferreira & Grattapaglia, 1998). Deste modo, o polimorfismo RAPD é detectado somente pela presença de segmentos de DNA que são amplificados em alguns indivíduos e em outros não.

Diversos trabalhos têm sido publicados, com a utilização de marcador molecular do tipo RAPD, na avaliação de polimorfismo e na estruturação da diversidade genética nas mais diversas culturas e populações naturais (Johns et al., 1997; Buso et al., 1998; Gauer & Cavalli-Molina, 2000; Martins-Lopes et al., 2007). Em espécies forrageiras, diversos estudos vêm sendo conduzidos, tanto para a caracterização da variabilidade quanto para o estabelecimento de relações entre as diferentes espécies e cultivares.

Gustine et al. (2002) avaliaram a variação genética dos marcadores RAPD em coleções e cultivares de trevo-branco (*Trifolium repens*) e verificaram similaridades genéticas surpreendentes, segundo os autores, para as oito populações derivadas de diferentes regiões geográficas e climáticas. Segundo os autores, esta similaridade poderia indicar uma origem européia comum para os trevos brancos encontrados nas pastagens norte-americanas.

Mengoni et al. (2000) utilizaram dados de RAPD para verificar as relações genéticas entre populações de alfafa (*Medicago sativa*). Os autores verificaram que foi possível a identificação de indivíduos dentro da população e ainda foram encontrados valores altos de variação genética intrapopulacional e interpopulacional.

Em *Lathyrus*, Chtourou-Ghorbel et al. (2002) caracterizaram a variação genética em cinco espécies e verificaram que a variação está proporcionalmente distribuída entre as espécies e foi ainda possível estabelecer grupos de maior similaridade entre as espécies estudadas.

Em *Poa pratensis*, Johnson et al. (2002) relataram que, com a utilização de RAPD e marcadores morfológicos, foi possível a caracterização

da coleção de germoplasma da USDA (*United States Department of Agriculture*) com 228 acessos de 26 diferentes países. Os autores verificaram que os grupos formados não foram associados fortemente com regiões particulares de origem. No entanto, os autores ressaltaram que a correlação entre os dados morfológicos e os de RAPD foi altamente significativa e indicaram ainda que, tanto os dados morfológicos quanto os de RAPD são importantes para acessar a variabilidade nesta espécie.

Em trevo-vermelho, num dos primeiros trabalhos realizados com marcadores RAPD, estes foram utilizados por Kongkiatngam et al. (1995) para a caracterização da variabilidade genética em duas cultivares e foram observados um alto nível de diversidade dentro de cada cultivar e uma menor variação entre as diferentes cultivares.

Campos-de-Quiroz & Ortega-Klose (2001) num estudo utilizando RAPD para avaliar o relacionamento genético e os níveis de variabilidade existente entre um grupo de 16 progenitores elite de trevo vermelho, evidenciaram uma alta variabilidade genética presente no material de melhoramento chileno, sendo que 75% desta variabilidade foi devido à variabilidade intrapopulacional. Resultados semelhantes foram encontrados por Ulloa et al. (2003) na análise de 20 populações e cultivares do Chile, Argentina, Uruguai e Suíça, onde 80% da diversidade foi devido à variabilidade intrapopulacional.

Em análise realizada com dados morfológicos e de RAPD em 33 populações russas de trevo vermelho Greene et al. (2004) verificaram que, apesar da alta variabilidade encontrada com os marcadores RAPD estes não

foram correlacionados significativamente com os marcadores morfológicos, no entanto, houve correlação entre a variação encontrada com os marcadores RAPD e a variação ambiental verificada em populações em locais isolados com menor fluxo gênico entre elas. Embora técnicas moleculares como RAPD representem uma análise bastante rápida do genoma vegetal, a necessidade de germinação de sementes para obtenção de material foliar para a extração de DNA, pode representar uma dificuldade logística e um custo elevado quando se precisa analisar um grande número de populações.

Greene et al. (1999) analisaram a qualidade de fragmentos RAPD gerados a partir da amplificação do DNA extraído de sementes de milho, algodão, soja, trigo e trevo vermelho e concluíram que o DNA extraído de sementes apresentava uma boa qualidade e que fragmentos RAPD gerados podem ser empregados com sucesso na análise de diversidade genética destas espécies. Sweeney et al. (1996) analisando os fragmentos RAPD gerados a partir do DNA extraído de *bulks* (mistura) de sementes de oito diferentes espécies (*Cynodon dactylon, Festuca rubra, Poa annua, Poa supina, Agrostis stolonifera, Poa pratensis, Lolium perenne* e *Festuca arundinacea*), verificaram que em todas as espécies avaliadas o DNA extraído foi amplificado com sucesso e os fragmentos apresentaram boa qualidade, sendo indicados para análise RAPD dos cultivares dessas espécies. O uso de DNA extraído de *bulks* de sementes mostra-se bastante eficiente uma vez que elimina a necessidade de germinação para a obtenção de tecido foliar, o que dependendo do número de cultivares a ser avaliado pode ser caro e consumir

bastante tempo em rotinas de laboratório como extração e quantificação de DNA (Sweeney & Danneberger, 1997).

Kongkiatngam et al. (1996) avaliaram o uso de marcadores RAPD de uma mistura de amostras de DNA genômico (*bulk*) para identificar cultivares de trevo vermelho, e testando o efeito dos diferentes números de indivíduos na mistura, concluíram que os padrões de *bulks* obtidos de 20 indivíduos na mistura são representantes da variabilidade dos cultivares. Os autores sugeriram a análise dos RAPD em *bulks* como um procedimento efetivo na avaliação de cultivares de trevo vermelho uma vez que esta removeu a grande variabilidade intracultivar que pode muitas vezes interferir na análise, tornando difícil evidenciar a variabilidade presente entre grupos. A análise RAPD com a utilização de amostras de DNA em *bulk* tem sido utilizada tanto para quantificar a variabilidade presente em coleções de germoplasma quanto para avaliar a estruturação desta variabilidade.

Bortolini et al. (2006) através do uso de DNA extraído de *bulk* de 20 indivíduos analisaram com a técnica de RAPD a coleção nuclear de trevo branco (*Trifolium repens*) e evidenciaram uma alta variabilidade genética presente nos 78 acessos da coleção, permitindo ainda a identificação individual de cada acesso. A análise em *bulk*s foi utilizada também na análise de germoplasma de outras importantes espécies forrageiras em conjunto com outros caracteres.

Steiner & los Santos (2001) em um estudo para caracterizar e comparar classificações de marcadores morfológicos e RAPD de 28 genótipos ecologicamente variados de cornichão (*Lotus corniculatus*) do NPGS (National

Plant Germplasm System) e determinar o relacionamento destes descritores com características ecogeográficas dos locais de coleta dos genótipos, concluíram que a utilidade de combinar características genéticas, morfológicas e ecológicas revela combinações entre vários genótipos, as quais não seriam evidenciadas com a utilização de somente uma medida.

Apesar do grande polimorfismo gerado pelos marcadores RAPD, estes apresentam no entanto algumas limitações. Segundo Jasieniuk & Maxwell (2001) a inabilidade do RAPD em detectar heterozigotos (dominância) reduz a precisão na estimativa de parâmetros genéticos de populações, incluindo a estimativa da freqüência alélica. Como resultado disso, deve-se amostrar de 2 a 10 vezes mais indivíduos por *locus* para assegurar a mesma exatidão obtida com marcadores codominantes como o RFLP e o microssatélite (Lynch & Milligan, 1994). Embora existam técnicas matemáticas como a proposta por Lynch & Milligan (1994) que visam estimar a frequência alélica em populações utilizando os marcadores RAPD, os resultados obtidos da utilização destas estimativas têm se mostrado tendenciosos (Jenczewski et al., 1999a).

Apesar destas limitações a técnica de RAPD tem sido amplamente utilizada e com sucesso, na análise da diversidade genética e avaliação de cultivares das mais diversas espécies.

1.5. Marcadores moleculares microsatélites (SSR) na análise da diversidade genética em trevo vermelho.

Atualmente um dos marcadores moleculares que mais tem sido utilizado para a análise da diversidade genética é o microsatélite ou SSR (*Simple Sequence Repeat* ou Seqüências Simples Repetidas) (Litt & Luty, 1989). Microsatélites consistem de pequenas sequências repetidas, em geral com um a quatro nucleotídeos em *tandem*, como, por exemplo, $(AC)_n$, onde n indica o número de vezes que a seqüência se repete. A amplificação desses fragmentos é feita com um par de iniciadores específicos, de vinte a trinta bases, complementares a seqüências únicas que flanqueiam o microsatélite (Cavalli, 2003). Os segmentos amplificados a partir destes sítios quase que invariavelmente apresentam um extenso polimorfismo resultante da presença de diferentes números de elementos simples repetidos (Ferreira & Grattapaglia, 1998).

Tendo em vista a expressão co-dominante e o multialelismo, os marcadores SSR são os que possuem o mais elevado conteúdo de informação de polimorfismo ou seja, “PIC” (*Polymorphism Information Content*) na terminologia de marcadores moleculares (Ferreira & Grattapaglia, 1998). A natureza altamente mutável dos *loci* SSR aumenta a possibilidade da existência de muitas variantes alélicas em cada *locus* e mais de 30 alelos têm sido reportados em alguns casos (Karp et al., 1996). As principais vantagens da análise de microsatélites são: a) possuem expressão co-dominante, o que permite o cálculo de medidas de diversidades genéticas populacionais; b) apresentam alto polimorfismo; c) são muito freqüentes e distribuídos ao acaso

ao longo de todo o genoma; d) a conservação de sítios de microsatélites entre espécies relacionadas permite, em alguns casos, a utilização de iniciadores de uma espécie para outras espécies afins (Cavalli, 2003). Estes marcadores têm sido amplamente empregados na análise de germoplasma de importantes culturas vegetais como a soja (Kuroda et al., 2006; Wang & Takahata, 2007), e em plantas modelo como o arroz, *Arabidopsis thaliana* (Lawson & Zhang, 2006) e *Medicago truncatula* (Ellwood et al., 2006).

Em forrageiras, os marcadores do tipo microsatélites têm se mostrado uma importante ferramenta tanto para análise de diversidade de germoplasma quanto para o estudo de relações genéticas entre cultivares. Em estudo realizado utilizando oito *loci* SSR em sete cultivares de alfafa, Flajoulot et al. (2005) evidenciaram uma alta variabilidade intracultivar. Os autores ressaltaram ainda que a técnica de SSR mostrou-se bastante eficiente ao evidenciar pequenas mas significativas diferenças genéticas entre as cultivares.

Uptmoor et al. (2003) conduziram um estudo utilizando os SSR, em conjunto com outras técnicas, para verificar as relações genéticas entre variedades locais africanas e cultivares de sorgo (*Sorghum bicolor*), os autores evidenciaram uma alta variabilidade genética intrapopulacional e uma clara separação genética entre cultivares e variedades locais.

Kölliker et al. (2001) desenvolveram marcadores SSR para o trevo branco (*Trifolium repens*) e verificaram um nível de polimorfismo de 88% quando testados em sete genótipos de trevo branco. Quando testada a amplificação destes *loci* em outras espécies aparentadas o maior sucesso

detectado foi em *Trifolium nigrescens* (75%) e *Trifolium ambiguum* (70%), espécies reconhecidamente próximas do trevo branco. Em trevo vermelho, no entanto, o sucesso de amplificação destes *loci* foi de apenas 45%.

Barrett et al. (2004) desenvolveram um mapa genético com marcadores microsatélites para trevo branco utilizando SSR genômicos (neutros) e SSR ligados a seqüências expressas do genoma ou EST (Expressed Sequence Tag) e compararam os dois tipos de marcadores em relação ao seu polimorfismo. Os autores relataram que, embora os EST-SSR apresentem maior transferabilidade para outras espécies, estes apresentaram menor polimorfismo quando comparados aos SSR genômicos.

Nas leguminosas a análise genômica e a genética molecular avançaram rapidamente nos últimos anos com uma atenção especial nas duas espécies de leguminosas modelo, *Lotus japonicus* e *Medicago truncatula* (Kölliker et al., 2006)

Apesar do trevo vermelho ser uma das leguminosas forrageiras mais importantes na agricultura mundial, a tecnologia de marcadores SSR só foi desenvolvida recentemente para esta espécie. Kölliker et al. (2006) desenvolveram 27 marcadores SSR para o trevo vermelho e verificaram para estes níveis de polimorfismo que variaram de 71% a 88% na análise de 24 genótipos.

Recentemente, um consórcio de pesquisadores japoneses e russos (Sato et al., 2006) realizaram uma extensa análise do genoma do trevo vermelho, neste estudo foi investigada a estrutura do genoma utilizando uma variedade de tecnologias genômicas incluindo *FISH* (*Fluorescence In Situ*

Hibridization), construção de bibliotecas de *cDNA*, seqüenciamento e notação de seqüências. Os autores disponibilizaram um mapa genético altamente saturado do genoma de trevo vermelho utilizando 1305 *loci EST-SSR* originados no estudo e 167 *loci* RFLP disponibilizados por Isobe et al. (2003) em um estudo anterior.

A transferência destes marcadores a outros genomas foi examinada utilizando 11 variedades de trevo vermelho (88 indivíduos) de modo a facilitar a utilização destes marcadores no melhoramento. Foi evidenciado um número médio de alelos por *locus* de 6,5 e um alto valor de PIC variando de 0,05 a 0,89 com média de 0,60. O tamanho do genoma do trevo foi estimado em 440Mb e segundo Sato et al. (2006) é comparável ao de *Medicago truncatula* e *Lotus japonicus* e, uma vez que 78% das seqüências EST examinadas mostraram similaridade significante a seqüências de genes registrados, o espaço gênico do trevo pode ser em torno de 65% do genoma total (Sato et al., 2006).

Os autores indicaram ainda a presença de microsintenia entre o genoma de trevo vermelho e das leguminosas modelo, o que é de suma importância, uma vez que o entendimento do funcionamento de genes de interesse agronômico nestas espécies, pode vir a ser aplicado prontamente no melhoramento de trevo vermelho. Uma das intenções deste estudo foi a de conectar a genômica ao melhoramento, disponibilizando marcadores mapeados e ligados a genes e informações que podem ser úteis ao programas de trevo vermelho (Sato et al., 2006). Outras importantes ferramentas genômicas têm sido aplicadas em trevo vermelho.

Sullivan & Thoma (2006) compararam uma extensa base de cDNAs de trevo vermelho, incluindo vários genes ligados à fotossíntese, com seqüências de *Medicago truncatula* verificando uma identidade de 92% com os genes da espécie modelo. Este alto nível de similaridade indica que ferramentas genômicas como a análise de expressão gênica (micro e macroarranjos) realizadas na espécie modelo podem ser úteis na análise da expressão genômica em trevo vermelho. Além disso, alguns genes de interesse farmacêutico e agronômico já foram clonados no trevo vermelho.

Segundo Morris & Greene (2001) um dos principais usos futuros do trevo vermelho está na indústria farmacêutica uma vez que estudos vêm indicando que as isoflanovas presentes no trevo vermelho desempenham um importante papel na prevenção de doenças como a osteoporose, câncer, doenças cardíacas e no tratamento dos sintomas da menopausa. As isoflavonas são metabólitos secundários encontrados na maioria das leguminosas, e em grande quantidade no trevo vermelho, e muitas destas isoflavonas agem como fitohormônio (Humphreys, 2005). Novogen, uma empresa farmacêutica australiana, já disponibiliza no mercado um fitoterápico com isoflavonas derivadas de trevo vermelho para o tratamento dos sintomas da menopausa (Morris & Greene, 2001).

Kim et al. (2003) clonaram e expressaram o gene da isoflavona sintetase de trevo vermelho, os autores evidenciaram uma alta similaridade deste com isoflavonas sintetase de outras espécies como *Medicago truncatula*, trevo branco, soja e alfafa. No entanto, análises filogenéticas revelaram uma

maior proximidade do gene isoflavona sintetase de trevo vermelho com o mesmo gene em *Medicago truncatula* (Kim et al., 2003).

Outro importante gene que foi clonado em trevo vermelho é o gene da polifenol oxidase (PPO), uma vez que o trevo vermelho apresenta importantes quantidades deste composto (Sullivan et al., 2004). Polifenol oxidases são uma classe de enzimas que têm despertado um grande interesse na pesquisa de processos de ensilagens de alfafa e outras leguminosas, pois foi demonstrado que estas impedem a quebra das proteínas, atuando como inibidoras das proteases vegetais, e diminuem a perda de proteínas que pode chegar a 87% nos processos de ensilagem (Sullivan et al., 2004). Outros trabalhos têm demonstrado outra importante característica das PPO, que é o papel que estas exercem na proteção da proteína vegetal contra a degradação que ocorre no rúmen dos animais, e que causa perdas importantes de nitrogênio durante o processo de conversão de proteína vegetal para a proteína animal (Kingston-Smith et al., 2003; Broderick et al., 2004; Sullivan et al., 2004).

O uso industrial de plantas cultivadas, como as forrageiras, vem se expandindo e diversificando. Dentro deste contexto o melhoramento de trevo vermelho deve adaptar-se a essas novas demandas por variedades que apresentem determinadas características e uma maior concentração de produtos de interesse industrial. Para isso é fundamental a utilização de novas tecnologias da biologia molecular e da genômica que auxiliam na manipulação mais precisa de genótipo e fenótipo (Humphreys, 2005). Atualmente um dos estudos que tem sido realizado é a análise de desequilíbrio de ligação entre

caracteres de interesse agronômico e marcadores moleculares, o que pode facilitar o uso efetivo dos marcadores na seleção destes caracteres (Breseghello & Sorrells, 2006).

Rubenstein et al. (2006) avaliaram dados de nove anos de requisição de germoplasma das dez principais plantas cultivadas no mundo, num dos maiores bancos mundias de germoplasma, o National Plant Germplasm System (NPGS) dos Estados Unidos, e verificaram que 62% das requisições buscam por acessos com características específicas relativas a estresses abióticos e tolerância a patógenos. Isso aponta para novas demandas, mais especializadas, nos bancos de germoplasma. Deste modo, os bancos de germoplasma devem incorporar de médio a longo prazo nos passaportes dos acessos, além das informações de avaliação tradicionais, dados mais precisos relativos à coleta, como descritores ecológicos (Steiner & Greene, 1996) e informações de georeferenciamento (Greene et al., 1999).

Acessos com essa qualidade de passaporte são particularmente importantes para identificar adaptações específicas (Greene et al., 1999), e a necessidade de caracterizar a adaptação ambiental dos acessos torna-se cada vez maior, uma vez que os recursos genéticos são cada vez mais utilizados em programas de melhoramento que visam sistemas agriculturais com baixos níveis de insumo e para propósitos especializados (Steiner & Greene, 1996).

1.6. Hipóteses e Objetivos do estudo

Em função de que o modo de reprodução, os mecanismos de dispersão de sementes e pólen, aliados às características edafo-climáticas de diferentes regiões geográficas, influenciam na estrutura genética das populações, espera-se evidenciar diferenças no padrão e na estruturação da diversidade genética nas populações que compõem a coleção nuclear de trevo vermelho, populações essas que são oriundas de diferentes países e continentes;

Espera-se caracterizar a diversidade genética do trevo vermelho independente da ferramenta de análise;

Espera-se que as diferentes ferramentas (marcadores morfológicos, bioquímicos e diferentes classes de marcadores moleculares) sejam igualmente capazes de verificar a distribuição e estruturação da diversidade nos níveis intrapopulacionais e entre grupos, de acordo com a origem e a natureza das populações;

O objetivo geral deste estudo foi o de quantificar e classificar a diversidade genética presente na coleção nuclear de trevo vermelho, utilizando para isto, diferentes ferramentas de análise como os marcadores morfológicos, bioquímicos e diferentes classes de marcadores moleculares, bem como comparar as diferentes ferramentas de análise.

Os objetivos específicos foram:

- quantificar a diversidade genética presente na coleção nuclear de trevo vermelho;

- verificar a distribuição e estruturação da diversidade nos níveis intrapopulacionais, interpopulacionais e entre grupos de acordo com a origem e natureza (selvagem ou cultivada) das populações;
- verificar correlações entre a variabilidade medida através das diferentes ferramentas de avaliação;

O presente trabalho está apresentado na forma de capítulos. Os capítulos II e III, cada um referente a um artigo, são apresentados na língua inglesa e conforme as regras das revistas as quais serão submetidos (apêndices 7 e 8). O Capítulo I é composto pela introdução e a revisão da literatura, seguidos da hipótese e objetivos do presente estudo. O Capítulo II é relativo a análise da diversidade utilizando marcadores morfológicos e moleculares do tipo microsatélites. O Capítulo III é composto pela análise da diversidade utilizando os marcadores bioquímicos e moleculares do tipo RAPD. Finalizando o trabalho, o Capítulo IV é composto das considerações finais relativas ao conjunto de resultados obtidos. Logo após, seguem as referências bibliográficas relativas a introdução e a revisão bibliográfica do Capítulo I.

2. CAPÍTULO II

GENETIC DIVERSITY IN THE CORE COLLECTION OF RED CLOVER (*Trifolium pratense* L.) REVEALED BY MORPHOLOGICAL AND MICROSATELLITES (SSR) MARKERS

Diversidade genética na coleção nuclear de trevo vermelho (*Trifolium pratense* L.) revelada por marcadores morfológicos e microsatélites (SSR) [2]

Paula Menna Barreto Dias[3] ; Bernadette Julier[4]; Jean Paul Sampoux[3]; Philippe Barre[3]; Miguel Dall'Agnol[5]

RESUMO

O trevo vermelho é uma importante leguminosa forrageira, no entanto, muito pouco é conhecido ainda sobre a diversidade genética presente na coleção nuclear de germoplasma do USDA. Marcadores morfológicos e moleculares (SSR) foram usados pela primeira vez juntos para verificar a diversidade genética e a distribuição da variação em 58 acessos da coleção nuclear e uma população cultivada no sul do Brasil. Os acessos de trevo vermelho apresentaram uma grande variação para a maioria dos 21 caracteres avaliados. Um total de 78 fragmentos (SSR) foi analisado, o número de alelos observados variou de 6 a 18 e 11,1 foi o número médio de alelos por *locus*. A diversidade genética medida com os marcadores SSR foi alta variando de 0,70 (RCS1868) a 0,91 (RCS0033) com uma média de 0,86. A análise de variância molecular (AMOVA) revelou que a maior parte da variação (83,6%), foi devido à variação intrapopulacional. A análise de agrupamentos dos marcadores morfológicos revelou cinco classes que separaram em grupos os tipos de comportamento de florescimento precoce, médio e tardio bem como, grupos que apresentaram maior persistência e maior produção de matéria seca nas condições do sul do Brasil. Embora os marcadores moleculares tenham separado os acessos também em cinco grupos, não houve coincidência entre a composição dos grupos encontrados pelos marcadores morfológicos e moleculares. Os marcadores morfológicos e moleculares evidenciaram a alta diversidade genética presente na coleção nuclear de trevo vermelho e mostraram grupos mais persistentes e com alta produção de matéria seca que poderiam ser utilizados nos programas de melhoramento de trevo vermelho para esta região do Brasil.

Palavras chaves: trevo vermelho, *Trifolium pratense*, microsatélites, marcadores morfológicos, diversidade genética

[2] Artigo redigido nas normas da revista Euphytica

[3] Aluna de Doutorado PPG/Zootecnia/Plantas Forrageiras/UFRGS, Bolsista CAPES
[4] Unité de Génétique et d'Amélioration des Plantes Fourragères, INRA, BP6, 86600, Lusignan, France
[5] Professor adjunto da Faculdade de Agronomia/Departamento de Plantas Forrageiras e Agrometeorologia/UFRGS

Genetic diversity in the core collection of red clover (*Trifolium pratense* L.) revealed by morphological and microsatellites (SSR) markers.

ABSTRACT

Red clover is an important forage legume species however very little is know about the genetic diversity in the USDA core collection. Morphologic and molecular (SSR) markers were used for the first time together to assess the genetic diversity and the distribution of variation in 58 accessions from core collection and one population cultivated in Southern Brazil. Accessions of red clover presented large variations for most of the 21 morphological traits evaluated. A total of 78 fragments (SSR) were scored, the number of alleles detected ranged from 6 to 18 and 11.1 was the mean number of alleles per *locus*. Genetic diversity measured with SSR markers was high and ranged from 0.70 (RCS1868) to 0.91 (RCS0033) with 0.86 as mean value. The analysis of molecular variance (AMOVA) revealed that the largest proportion of variation (83.6%) was due to intrapopulational variability. The cluster analysis of the morphological markers revealed five distinct clusters that separated early, medium and late blooming types as well as groups that showed more persistent and high dry matter production in the Southern Brazilian conditions. Although the molecular markers separated accessions also into five clusters there was no coincidence between the composition of groups found by morphological and molecular markers. Morphological and molecular markers evidenced the high genetic diversity present in the core subset of red clover and showed groups of accessions more persistent and with high forage yield that could be used in the Brazilian red clover breeding programs for this region.

KEY WORDS: red clover, *Trifolium pratense*, microsatellite, morphologic markers, genetic diversity

INTRODUCTION

Although its Mediterranean origin, red clover (*Trifolium pratense* L.) is a cosmopolitan species adapted to many edaphic-climatic conditions (Taylor and Smith 1979). The broad range of adaptation in common plants can be due by the existence of a high number of local adapted genotypes rather than by the existence of one single general-purpose genotype (Joshi et al. 2001). In red clover, this was evidenced by the fact that most of cultivars are not adapted to areas far from where they were developed (Taylor and Smith 1995). Red clover is one of the most utilized legume forage in the world agriculture (Bowley et al. 1984). In South America red clover has an important cultivated area, with 200,000 ha in Argentina (Rosso and Pagano 2005), 100,000 ha in Chile (Ortega et al. 2003). In Brazil, red clover is utilized to lessen the lack of forage during the critical fall-winter period, especially in the southern state of "Rio Grande do Sul". However the low persistency of the cultivars historically utilized in Brazil (Kenland, Quiñequeli, Estanzuela) has denied high productions of red clover. A breeding program aimed to creating adapted cultivars to this region, and the development of a local seed production are thus needed, and is currently underway by the Federal University of Rio Grande do Sul (UFRGS).

Many works using different approaches, such as morphologic (Kouamé and Quesenberry 1993; Choo 1984; Bulinska-Radomska 2000), molecular (Kongkiatngam et al. 1995; Campos-de-Quiroz and Ortega-Klose 2001; Kölliker et al. 2003; Greene et al. 2004; Sato et al. 2006; Herrmann et al. 2006) and biochemical (Hagen and Hamrick 1998; Yu et al. 2001; Mosjidis and Klingler 2006) have pointed out to the large genetic diversity in red clover, both among and within populations. However, this large variability represents at the same time an advantage and a challenge, since the genetic variability is needed in a breeding program, but genetic conservation and evaluation of this variability in a germplasm collection is not an easy task. Thus the large size, heterogeneous structure and the lack of passport information in many germplasm collections has often hindered the efficient maintenance and utilization of gene bank materials (Kouamé and Quesenberry 1993; Greene et al. 1999; Steiner et al. 2001).The germplasm collection of red clover of National Plant Germplasm System of United

States Department of Agricultural (NPGS-USDA) is composed by 800 accessions from 41 different countries (Kouamé and Quesenberry 1993).

The concept of "core collection" was considered as a form to facilitate the use of germplasm in the breeding programs (Brown 1989) and since then many core collections have been formed in the diverse species of cultivated species (Souza and Sorrells 1991; Steiner et al. 2001). Nowadays, the red clover core collection contains 85 accessions originating from 41 countries (Mosjidis and Klingler 2006). It is important to note that this core collection was based on morphologic data already present in the Germplasm Resource and Information Network (GRIN) databank, deriving from tests in different environments and seasons, using different criteria for measurement and not for all the populations at the same time (Kouamé and Quesenberry 1993). No traits related to molecular markers were used. After the formation of the core collection, few works were published and all using only morphologic traits (Bortnem and Boe 2002; Bortnem and Boe 2003). However, when one or a few traits are used to characterize collections, there is less opportunity to understand relationships among different traits within collections. Use of many traits extends the possibilities to evidence genetic differences. Microsatellites markers or tandem simple sequence repeats (SSR) are routinely used to investigate the genetic structure and the genetic diversity of natural populations (Balloux and Lugon-Moulin 2002) and to measure the genetic diversity in other cultivated legume species like alfalfa (Flajoulot et al. 2005) and white clover (Kölliker et al. 2001). Although the importance of red clover as an cultivated plant, no works using SSR markers were done in the core collection of this species.

The objective of this work was to evaluate the genetic diversity in a part of the USDA-NPGS core collection of red clover using morphologic traits together with SSR molecular markers, and to make this information more accessible for red clover improvement programs allowing the development of cultivars more adapted to the Brazilian conditions.

MATERIAL AND METHODS

Plant material

From the red clover core collection of NPGS-USDA, a subset of 57 populations from 35 different countries (Table 1), and one population cultivated in Brazil were selected. The accessions of red clover in the GRIN were classified in terms of genetic improvement as cultivar, wild, landrace, cultivated material, breeding material and uncertain (Table 1). The 58 populations were planted in a experimental field and in greenhouse, and were evaluated for different morphological traits, with a total of 21 variables (10 traits in the field and 11 in the greenhouse). The scales of variables in this study were used as suggested in IBPGR (1984).

Table 1. Passport data of red clover populations from the USDA-GRIN core collection and results from cluster morphological classification.

Population	Morphological Cluster	Origin	Latitude	Longitude	Elevation (m)	Name	Type
PI 237705	I	Denmark				Hinderupgaard III	cultivar
PI 217507	I	Denmark				Early otofte III	cultivar
PI 314840	I	Norway				JRIPO F81	cultivar
PI 188905	I	Sweden				Merkur	cultivar
PI 235847	II	Sweden				HEBY	cultivar
PI 229799	I	Finland				Tammisto	cultivar
PI 236455	I	Finland				Mynskyla	cultivar
PI 310459	II	Switzerland				Dettenbuhl	cultivar
PI 179146	I	Switzerland				Perennial Bernois	cultivated
PI 266047	I	Poland				Wielkolistna	cultivar
PI 384058	I	Poland				Viola	cultivar
PI 225119	I	Germany				NSL 060146	cultivar
PI 187008	I	Germany	49.32 N	09.21E	400	N° 1687	cultivated
PI 294481	I	Austria				Reichersbersger	cultivar
PI 318887	I	Hungary				Bakonyi	landrace
PI 318888	I	Hungary				Cigandi	landrace
PI 315522	III	Italy				G 17210	uncertain
PI 418889	V	Italy	44.04 N	11.52 E	700	296	wild
PI 249870	V	Greece	40.37 N	23.47 E	610	N 17109	wild
PI 419294	I	Greece	39.12 N	21.01 E	1400	122	wild
PI 220856	I	Portugal				NSL 060141	uncertain
PI 307948	V	Spain	40.55 N	05.18 W	795	N° 942	wild
PI 253583	V	Spain	40.03 N	02.26 W	643	K 2086	wild
PI 188680	III	France				G 13945	landrace
PI 207972	III	France				NSL 060134	uncertain
PI 189174	III	Netherland				Groninger	cultivar
PI 201191	I	Netherland				NSL 060106	uncertain
PI 187224	I	Belgium				Oudenaerdse	cultivar

PI 234448	I	Belgium	50.58 N	03.46 E		N° 16104	cultivar
PI 204506	IV	Turkey	39.45 N	37.02 E	1270	N° 292	uncertain
PI 204507	IV	Turkey	38.21 N	38.19 E	862	N° 382	uncertain
PI 371959	IV	Bulgaria				SOFIA 52	cultivar
PI 315533	III	Bulgaria				Sofia 66	cultivar
PI 251564	III	Yugoslavia				19660	cultivated
PI 207520	II	Afghanistan				N° 12623	uncertain
PI 228365	II	Iran	35.40 N	51.26 E	1463	N 14849	uncertain
PI 250899	V	Iran	36.16 N	59.28 E	979	K 1689	wild
PI 401469	III	Romania				Perena	cultivar
PI 232941	III	Hungary				Fertodi	cultivar
PI 345673	III	Ukraine				Gloria ulucsennaja	cultivar
PI 440737	II	Russia	45.03 N	42.06 E	300	D-1600	wild
PI 345675	I	Russia				Kirovskij 159	cultivated
PI 419565	II	Japan	43.10 N	141.1 E	30	SV-0116	Breeding material
PI 419550	III	Japan	43.10 N	141.1 E	30	TENDA-Selection	Breeding material
PI 184960	III	Australia				NSL 060086	Uncertain
PI 376880	III	New Zealand				Grasslands Hamua	cultivar
PI 306188	III	United Kingdon				Drewitts	cultivar
PI 306185	III	United Kingdon				Berrys	cultivar
PI 315534	I	Canada				Memmos	cultivar
PI 286116	III	Canada				Redon	cultivar
PI 295355	II	USA	40.47 N	89.36 W	240	Rahn Redclover	landrace
PI 230229	IV	USA	41.06 N	78.53 W	110	N° 55-7	landrace
PI 302421	IV	Colombia				Trebol rojo	cultivar
PI 304842	IV	Chile				Quiñequeli	cultivar
PI 449326	IV	Chile				Trebol rosado	landrace
PI 271627	IV	India	31.40 N	76.59 E	1870	N° 1214	uncertain
PI 226952	IV	Ethiopia	07.33 N	37.51 E	2440	N° 363	wild
-	I	Brazil	30.06 S	51.39 E	46	EEA /UFRGS	cultivated

Morphological evaluation

From each population, 20 seeds were germinated in Petri dishes with watered filter paper and after 15 days were planted in plastic pots (750 ml) in a greenhouse (May 2004). Each plant was inoculated with 1 mL of a solution of *Rhizobium leguminosarum bv. trifolii* (SEMIA 222 and SEMIA 2082 the recommendation from Laboratório de Fixação Biológica do Nitrogênio/ Fundação Estadual de Pesquisa Agropecuária /LFBN/ FEPAGRO/Porto Alegre/Brazil) in aqueous solution (concentration of 10^9 cells.mL^{-1}). In August 2004, 10 plants for each population were planted in the field at Eldorado do Sul, Rio Grande do Sul, Brazil (30°05'02" S, 51°36'58" W and elevation 40 m). Irrigation was applied to maintain soil moisture through the growing season. Weeds were controlled through weeding and herbicides. The experimental field was arranged

as a complete randomized design with 58 populations and 10 repetitions (plants). Ten other plants were transferred to pots of 5 kg soil capacity into a greenhouse located at Porto Alegre, Rio Grande do Sul, Brazil (30º 01'59" S, 51º13'48" W and elevation 10 m). There was no randomization in the greenhouse but all plants of each population were changed of place in the greenhouse, once per month, in order to provide the similar luminous intensity to each plant.

The phyllochron was measured in the greenhouse in September 2004, one secondary stem was marked for each plant and the leaf emission was verified once a week during four weeks. The phyllochron was calculated as the inverse of difference between the number of leaves in the first day and the last day, divided by the total number of days. For each stage of the foliar emission, a note was given based on the scale of Maitre et al. (1985) . The growth habit was scored using a nominal scale 1) erect, 2) medium and 3) prostrate in October 2004, on each individual in the field and in the greenhouse. The height of the plants was measured, in centimeters, as the natural length (without stretching) of the longest branch of each plant in October 2004 in the field and in the greenhouse. The diameter of inflorescence was scored for each population in field and in the greenhouse, on ten random inflorescence per plant in October 2004.

The number of inflorescences of each population was counted in the greenhouse and in the field, one month after the first inflorescence appeared in each population (in October and November 2004). This time of one month was considered sufficient for full blooming of each plant. The inflorescence color was scored with a table of colors standard (Royal Horticultural Society – FANZ- Purple Group 74 and 75) on one inflorescence for each plant. The blooming date of populations was scored, as the date on which 50% of plants of each population were flowered in the field and in the greenhouse (in October and November, 2004). In December 2004, the disease susceptibility was scored in plants in the field and in the greenhouse describing the intensity of the symptoms using a nominal scale (0: no symptoms, 1: less than 25% of the plant area affected, 2: 25% affected, 3: 50% affected, 4: 75% affected, 5: 100% affected and 6: died). Samples of died plants were taken to laboratory to identify with precision the pathogens. In both field and greenhouse, the disease was identified as powdery mildew caused by *Erysiphe trifolii*. The leaf area was measured in January

2005 in the field and in the greenhouse on three random leaves from each plant (total of 9 leaflets for each individual), using a LiCor-3100 leaf area meter. In January 2005, plants in the field and in the greenhouse were harvested to measure aerial dry matter per plant. The plants were placed in individual paper bags and dried at 60°C for one week and weighted. The persistency of each population in the field and in the greenhouse was measured as the number of individuals alive in each population at the end of the experiment before the harvest (January 2005).

Statistical Analysis

Analysis of variance were carried out, to identify traits that significantly differ among populations, using PROC GLM of SAS (SAS Institute 2001) on single data from field and greenhouse and in the combining data from the two environments to test interactions between condition (field or greenhouse) and populations. Non-significant traits in all analysis were excluded from further procedures. A Person product – moment correlation matrix was calculated for the combined data (field and greenhouse) using PROC CORR (SAS Institute 2001). If two traits measured in the field and in the greenhouse were significantly correlated with an *r*-value higher than 0.65, only one trait was included for further analysis. Because different scales were used for the traits measurement, data were first standardized to a mean of 0 and a variance of 1 using PROC STANDARD (SAS Institute 2001).

A principal component analysis was carried out on the set of 58 populations using the standardized mean values of traits exhibiting significant population effect and the traits for which only population data were available (blooming date and persistency). The correlation between variables and axis was calculated. A cluster analysis was then performed using these populations using PROC FASTCLUS (SAS Institute 2001), based on Euclidean distances between populations calculated from their coordinates on the principal axes referring to eigenvalues equal or above 1 (Thompson and Nelson 1998; Rosso and Pagano 2005) and this classification process included:

1. Five independent runs of disjoint cluster analysis requesting a maximum of 6 clusters and using different sets of initial cluster seeds. The maximum number of clusters was defined according to the number of axes with eigenvalues equal or above 1 resulting from principal component analysis.

2. A top clustering obtained by crossing the clustering of the five independent runs of disjoint cluster analysis: the populations were gathered in the same top cluster if they were clustereding together in the same cluster in each of the 5 runs from the disjointed cluster analysis.
3. A hierarchical cluster analysis performed with the above top clusters as individuals and using Ward's minimum variance method.

Molecular evaluation

Two levels of analysis were conducted. Firstly, the within-population diversity was evaluated on three populations (PI 237705, PI 249870 and PI 204506) each represented by 11 plants. Secondly, the clustering of populations obtained with morphological traits was tested using one plant of each of the 58 populations. One plant (PI 250899) was repeated to control of PCR quality and reproducibility. So 92 plants of red clover were germinated (17/03/2006), then transferred to the greenhouse (20/03/2006) and installed in pots with 5.49 cm^3 soil capacity, pH= 5.8 and composition: 90% of turf and 10% of sand. Young leaflets (50 mg) of the 92 plants were harvested and DNA was extracted following the method described by Cheung et al. (1993). DNA concentration and quality were measured in 1.2% concentration agarose gel using as a comparison four standard concentrations of phage λ DNA. The DNA was diluted in working solutions of 15ng/µl.

Fourteen primer pairs of microsatellite markers were tested (Table 2). These primers were chosen from the study of Sato et al. (2006), in which they were highly polymorphic, and based on their position on the genetic linkage map, in order to have two markers on each linkage group and to have markers distributed throughout the genome. These primers were generated through mapped microsatellite markers adjacent to EST sequences and are gene-associated markers (Sato et al. 2006). After the test, seven primers were selected according to the quality of fragment amplification (Table 2). Reactions were carried out in 96 well plates of 20µl final volume solution containing 1 X buffer, 0.2 mM of each dNTP, 0.8 µM of each SSR primer (forward, reverse), 1.5 mM of $MgCl_2$, 2 µl of genomic DNA (15 ng/µl) and 0.6 units of Platinum Taq Polymerase (Invitrogen Inc.). Reactions were run using a "touchdown PCR" program: denaturation period of 5 min at 95°C, followed by 8 cycles of 30s at 95°C, 30 s at 62°C

and 30s at 72°C in which the annealing temperature was decreased by 1°C every cycle, followed by 28 cycles of 30s at 95°C, 30s at 55°C, 30s at 72°C and a final extension for 5 min at 72°C. PCR products were separated on 6% denaturing polyacrylamide gel, with a 0.5 X TBE buffer. Electrophoresis was performed at 70W, for approximately 2h 10min, on a 38 x 32.5 cm gel apparatus (Biorad) and revealed using silver staining method of Tixier et al. (1997).

Molecular data collection and analysis

All SSR fragments for the 92 individuals were scored and the analyses of microsatellite diversity were conducted at the locus level. The 58 individuals and the three populations were analyzed separately. In each case, the total number of alleles per locus (A), the allelic frequencies and the polymorphism information content ($PICi=1-\sum_i p_{ij}^2$, where P_{ij} is the frequency of the *j*th allele for the *i*th marker) were calculated.

For the three populations, the observed (Ho) and expected (He) heterozygosities, based on Nei's unbiased estimate were calculated using Genetix version 4.05 (Belkhir 2004) and the fixation index (Fis) was estimate according to Weir and Cockerham (1984). The deviations from Hardy Weinberg Equilibrium (HWE) at individual loci and for all loci were tested with Markov chain method, with default parameters using GENEPOP software (Raymond and Rousset 1995). The Rogers' distances were calculated between the 33 individuals from the three populations and cluster analysis was performed on Rogers' distances using the unweighted pair-group procedure with an arithmetic mean (UPGMA) and reliability of the clustering was tested by computing Mantel test statistics for the correlation of the distance matrix and the cophenetic matrix using the NTSYSPC 2.10 software (Rohlf 2000). Analysis of molecular variance (AMOVA) was realized using Arlequin version 3.1 (Excoffier and Schneider 2005) to test the parts of among-population and within-population diversities.

For the 58 individuals, the Roger's distances were calculated then clustering and Mantel test were performed as above. An AMOVA was carried out to evaluate the part of variation explained by the clusters found with the morphological markers. To infer the structure and genetic relationships among the 58 individuals, the dataset was analyzed using Structure version 2.1 (Pritchard and Donnelly 2003), running Structure to search the number appropriate of groups and to test the consistency of the groups

found with morphological markers with the molecular data. Structure uses a Bayesian clustering method algorithm that assumes the existence of K subpopulations (where K may be unknown) and each of these subpopulations is characterized by a set of allele frequencies at each locus and assigns portions of individual genome to these clusters or subpopulations. The ancestry model with no-admixture was chosen because it is cited by Falush et al. (2003) as appropriate for studying very discrete populations and it may be more successful than the admixture model at detecting subtle structure in populations. Using correlated allele frequency option, multiple runs of Structure were performed by setting K from 1 to 10 groups. The burn-in time and replication number was set to 10,000 and 100,000 respectively, which is considered appropriate by (Pritchard and Donnelly 2003) and each run was replicated ten times. For each run output, individuals were assigned into a group according to their highest proportion of membership into this group. Correlation between distance matrices generated from morphologic and SSR markers was compared using the product moment correlations (r) derived from the normalized Mantel Z (Mantel 1967). Matrices comparisons were carried out using the MXCOMP module of the NTSYS-pc version 2.10 (Rohlf 2000).

Table 2. Sequences and linkage group of 14 primers tested in 58 populations of red clover.

Primer	Linkage Group	Motif	Fw_primer (5' to 3')	Rv_primer (5' to 3')
RCS0089	LG 1	ATC	CAAACCAATGCCAACAACAG	AATGATGATTCCCGTGATGG
RCS0907	LG 1	AAC	ATTTGAGCACAAGGCCTCAC	TGGGGAAGTGAAGGATGTTC
RCS3095*	LG 2	AAAT	GTGTTCCATTAGAGGCGGAA	AGCGGCTCGTTTTAATGCTA
RCS3102*	LG 2	AAAT	AGCGGCTCGTTTTAATGCTA	CTTTTAGTGACAGGTTTGTATGTCA
RCS3015	LG 3	AAC	GAGGTCGTCCCCCTAAGC	TGTGGGAGGAGGAGGTACAG
RCS0033*	LG 3	AAT	AAATTATCATTTTGCAAATTTTA	GCAGATTATGAGGAATAACATTG
RCS1307	LG 4	GGGA	CCCTTCTAGCCTAGCAACCA	GCGGAAAAGATTCAGCCTAA
RCS1920	LG 4	AAG	GAGAAAAGAAAGAAGTCTCTGAAGGA	CCCCCAAAATACAAAACCCT
RCS1518*	LG 5	ATC	GCACGAGGCACACACTACTT	CGAAGCAGGTTGGAAAACAT
RCS1737*	LG 5	ATC	GGCACGAGGCACACTACTTC	AGCTCAAGCTCAACGGACAT
RCS1868*	LG 6	ATC	CCACCTTAGACCACAGCCAT	GCTCACCTTCAGAATCCTCG
RCS1255	LG 6	AAG	TCAGTGATGAATCGATTTTGTTT	CGTCAACGGTGACTGCATAG
RCS2185	LG 7	AAG	AAACAATCAAAAACCGACAACA	TGCTGTTCCATCACCAATTT
RCS0793*	LG 7	AAG	CGCAATCTTTCTTCTCATTTCA	TTCAACATGCAGGCTAAGAAAA

*Primers used in this work.

RESULTS

Classification with morphological traits

Analysis of variance (not shown) indicated significant differences among populations for six traits measured in field (leaf area, growth habit, weight, size of plant, size of inflorescence and number of inflorescences) and for seven traits measured in greenhouse (leaf area, growth habit, weight, size of plant, size of inflorescence disease, number of inflorescences and phyllochron). The disease scored in the field and the color of inflorescences in both environments were not significantly different among populations and were not utilized. Over the data from field and greenhouse, analysis of variance indicated that, for all traits, the condition (field or greenhouse) had a major effect on the traits. All the condition x population interactions were significant but low (Table 3).

Table 3. Mean and analysis of variance for morphological traits of 58 populations of red clover evaluated at Eldorado do Sul (field) and Porto Alegre (greenhouse), Rio Grande do Sul, Brazil in 2004 and 2005. All mean squares were significant at $p \leq 0.05$.

			Mean Squares		
Trait	Mean	Condition	Population	Population x Condition	Error
Leaf area (LA)	9.17	1827.01	80.60	15.83	11.23
Growth Habit (GH)	1.38	9.52	5.04	0.96	0.09
Weight (WT)	25.90	270150.00	1114.17	885.44	445.58
Size of plant (SP)	17.48	1809.72	390.07	92.38	34.65
Size of inflorescence (SI)	2.21	17.83	0.68	0.31	0.20
Disease (DI)	1.24	364.22	8.61	6.39	1.84
Color of inflorescence	75.09	0.003	0.29	0.002	0.00
Number of inflorescences (NI)	9.54	14342.33	445.80	436.51	80.99

The analysis of correlation on the data from field and greenhouse (Table 4) indicated that the growth habit measured in the field and in the greenhouse had a high coefficient of correlation (r^2= 0.66 p=0.05). This suggests that evaluation of this trait in one location would be sufficient for population description, so only growth habit scored

in field was utilized. Leaf area, size of plant and size of inflorescences of greenhouse and field were correlated, but weight, number of inflorescences, blooming date and persistency showed low correlation between greenhouse and field (Table 4). Blooming date (BLG) was correlated with size of inflorescence (SIG) and negatively correlated with leaf area (LAG) (Table 4). Weight (WTG) was correlated with number of inflorescences (NIG) and negatively correlated with phyllochron (PHY). Persistency was very weakly correlated to the traits evaluated in both greenhouse and field, even if persistency in the greenhouse (PEG) had a significant negative correlation with size of plant (SPG). Finally, all traits with exception of growth habit (greenhouse) and color of inflorescence (field and greenhouse) were utilized for further analysis.

Table 4. Correlations between traits analyzed in 58 populations of red clover in the field and in the greenhouse. All correlations were tested at $p \leq 0.05$.

	LAG*	WTG	SPG	SIG	NIG	PEG	BLG	GHG	PHY	DIG	LAF	WTF	SPF	SIF	NIF	PEF	BLF	GHF
LAG	1.00																	
WTG	0.31	1.00																
SPG	0.51	0.43	1.00															
SIG	-0.35	-0.03	-0.08	1.00														
NIG	-0.03	0.41	0.16	0.28	1.00													
PEG	0.28	0.05	-0.33	-0.09	-0.25	1.00												
BLG	0.35	0.08	0.04	-0.66	-0.18	0.10	1.00											
GHG	-0.28	-0.19	-0.68	0.20	0.03	0.22	-0.03	1.00										
PHY	-0.15	-0.39	-0.19	0.02	-0.09	-0.01	0.04	0.17	1.00									
DIG	-0.04	0.14	0.28	0.16	0.33	-0.51	-0.03	-0.26	-0.04	1.00								
LAF	0.56	0.34	0.48	-0.36	-0.03	-0.14	0.33	-0.34	-0.11	0.20	1.00							
WTF	-0.18	0.25	0.35	0.39	0.15	-0.36	-0.30	-0.24	-0.06	0.41	-0.02	1.00						
SPF	0.09	0.08	0.65	0.04	0.05	-0.40	-0.18	-0.69	-0.05	0.27	0.35	0.49	1.00					
SIF	-0.26	0.02	0.02	0.51	0.41	-0.26	-0.32	0.06	0.03	0.26	-0.11	0.24	0.02	1.00				
NIF	-0.02	-0.12	-0.25	0.33	0.04	0.20	-0.28	0.25	0.12	-0.09	-0.03	-0.09	-0.18	0.21	1.00			
PEF	-0.17	0.15	-0.02	0.08	0.14	0.06	0.03	0.02	-0.08	0.01	-0.04	0.22	0.01	0.11	-0.31	1.00		
BLF	-0.10	-0.05	-0.19	-0.22	-0.04	0.03	0.15	0.24	0.09	-0.07	-0.17	-0.05	-0.26	0.13	-0.12	0.07	1.00	
GHF	-0.48	-0.24	-0.71	0.15	-0.02	0.15	0.02	0.66	0.22	-0.03	-0.52	-0.12	-0.61	0.03	0.30	-0.05	0.12	1.00

*Morphologic traits abbreviations as indicated in the Table 3. G: greenhouse, F: field.

The principal component analysis was realized with the means of 17 traits from field (eight traits) and greenhouse (nine traits) and indicated that the first five principal component axes were responsible for 68% of the variance (Table 5). The first axis was explained by the size of plants (greenhouse and field), growth habit (field) and leaf area (greenhouse). In the second axis, size of inflorescence (greenhouse and field), blooming date (greenhouse), weight (field) and specific leaf area (field) were the most representative characters. The third principal axis was explained by persistency,

blooming date and number of inflorescences in field. The weight (greenhouse), phyllochron (greenhouse) and number of inflorescences (greenhouse) contributed to the fourth axis. The last component was related to disease (greenhouse).

Table 5. Principal component analysis (PCA) made with 17 morphological traits measured on 58 populations of red clover, showing eigenvalues and proportion of variation associated with the first five PC axes and the contribution of the traits to the axes.

	PC axis				
	1	**2**	**3**	**4**	**5**
Eigenvalues	3.77	3.2	1.65	1.58	1.31
Cumulative proportion of variation	0.22	0.41	0.51	0.60	0.68
	Eigenvectors				
Trait					
LAF*	0.49	-0.59	-0.35	0.22	0.14
LAG	0.66	-0.38	-0.16	-0.01	0.29
WTG	0.53	-0.02	0.04	0.68	0.03
WTF	0.42	0.60	0.16	-0.08	-0.20
SPG	0.90	-0.02	-0.09	-0.06	-0.03
SPF	0.72	0.22	-0.10	-0.43	-0.23
DIG	0.42	0.43	0.22	-0.10	0.48
SIG	-0.13	0.80	-0.32	0.13	-0.17
SIF	0.01	0.67	0.01	0.21	0.27
PHY	-0.32	0.04	0.02	-0.48	0.27
GHF	-0.78	0.17	0.13	0.10	0.25
PEG	-0.40	-0.46	-0.30	0.43	-0.31
PEF	0.08	0.15	0.53	0.33	-0.43
BLG	0.08	-0.69	0.39	0.03	0.34
BLF	-0.24	-0.10	0.53	0.10	0.19
NIG	0.25	0.48	0.07	0.50	0.33
NIF	-0.34	0.20	-0.70	0.11	0.31

*Morphologic traits abbreviations as defined in Table 3. G: greenhouse, F: field.

The hierarchical cluster analysis on the Euclidean distance matrix produced five classes (Figure 1). The membership of each population to a cluster is indicated in Table 1 and mean and standard deviation for each trait was synthesized for each cluster group (Table 6). Cluster I contained the largest number of populations (22) and was characterized by erect growth habit, low number of inflorescences (field and greenhouse), high leaf area (greenhouse and field) and had the latest blooming date (greenhouse). This cluster was formed principally by cultivars, and from these, the

majority were from northern Europe high latitudes from countries such as Denmark, Norway, Finland and Sweden.

The Cluster II was composed by seven populations, with medium blooming date (field and greenhouse), the highest number and lowest size of inflorescences and by the remarkable low persistency measured in the field. Another characteristic of this group is the large leaf area measured both in the field and in the greenhouse.

Cluster III contained 15 populations characterized by the remarkable early blooming date in the greenhouse, erect growth habit and the highest size and weight of plant in field. This group was mainly composed most by cultivars from Central or South European countries from lower latitudes such as Italy, France, Romania, Hungary and Ukraine.

Cluster IV is composed by nine populations which were characterized by the highest weight (field and greenhouse), the slowest phyllochron, the highest occurrence of disease, the lowest persistency in the greenhouse and a medium blooming date. In this cluster, all cultivars from South America and populations of Africa considered by GRIN as a naturalized material were grouped.

Cluster V was the smallest with only five populations, which were characterized by a prostrate growth habit, the highest phyllochron, the latest blooming date (field), the lowest leaf area in field and greenhouse, the lowest occurrence of disease, the lowest weight and size of plants in the field and in the greenhouse. This group presented high persistency in the greenhouse and in the field. This group was totally composed by wild populations, from South European countries (Italy, Greece, Spain) and Iran.

Table 6. Means and standard deviations of morphological traits in each cluster obtained in a subset of populations of the red clover core collection

Traits	Clusters				
	I	**II**	**III**	**IV**	**V**
	Mean ± SD				
Leaf area Greenhouse	9.9 ± 1.9	9.5 ± 0.9	8.1 ± 1.0	8.2 ± 1.7	4.9 ± 1.9
Leaf area Field	12.2 ± 1.8	12.9 ± 2.2	10.8 ± 1.7	11.6 ± 2.1	6.3 ± 0.9
Growth Habit Field	1.1 ± 0.2	1.5 ± 0.3	1.2 ± 0.2	1.4 ± 0.3	2.6 ± 0.3
Weight Greenhouse	10.7 ± 3.9	8.4 ± 2.5	7.7 ± 1.4	14.3 ± 4.9	6.3 ± 2.3
Weight Field	37.7 ± 10.3	32.0 ± 7.1	47.7 ± 12.4	60.0 ± 9.7	28.7 ± 13.5
Size of plant Greenhouse	18.0 ± 2.7	15.4 ± 4.7	17.3 ± 2.6	19.3 ± 4.0	5.8 ± 2.2
Size of plant Field	19.3 ± 3.1	16.6 ± 3.5	22.9 ± 2.5	21.4 ± 5.7	9.1 ± 4.7
Size of inflorescence Greenhouse	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.1	3.3 ± 0.1	2.9 ± 0.1
Size of inflorescence Field	2.7 ± 0.4	2.6 ± 0.2	2.7 ± 0.3	2.9 ± 0.2	2.5 ± 0.3
Disease Greenhouse	1.7 ± 0.9	1.4 ± 0.9	1.5 ± 0.7	3.2 ± 1.4	1.1 ± 0.5
Number of inflorescences Greenhouse	3.3 ± 1.0	3.4 ± 0.3	3.4 ± 1.0	5.5 ± 1.0	3.4 ± 0.9
Number of inflorescences Field	7.8 ± 6.5	39.2 ± 11.1	13.5 ± 8.8	15.0 ± 10.8	21.3 ± 13.0
Phyllochron	8.1 ± 1.5	7.9 ± 1.34	7.9 ± 1.1	7.6 ± 1.0	9.3 ± 2.7
Blooming Greenhouse	133.4 ± 20.7	112.7 ± 15.6	98.4 ± 2.9	108.4 ± 15.6	119.6 ± 21.5
Blooming Field	207.9 ± 16.4	208.0 ± 0.0	208.0 ± 0.0	208.0 ± 0.0	213.4 ± 12.1
Persistency of Plants Greenhouse	76.8 ± 19.4	78.5 ± 12.1	71.3 ± 17.2	48.9 ± 38.2	90.0 ± 10.0
Persistency of Plants Field	27.3 ± 15.8	10.0 ± 10.0	31.3 ± 13.5	35.6 ± 18.1	30.0 ± 25.5

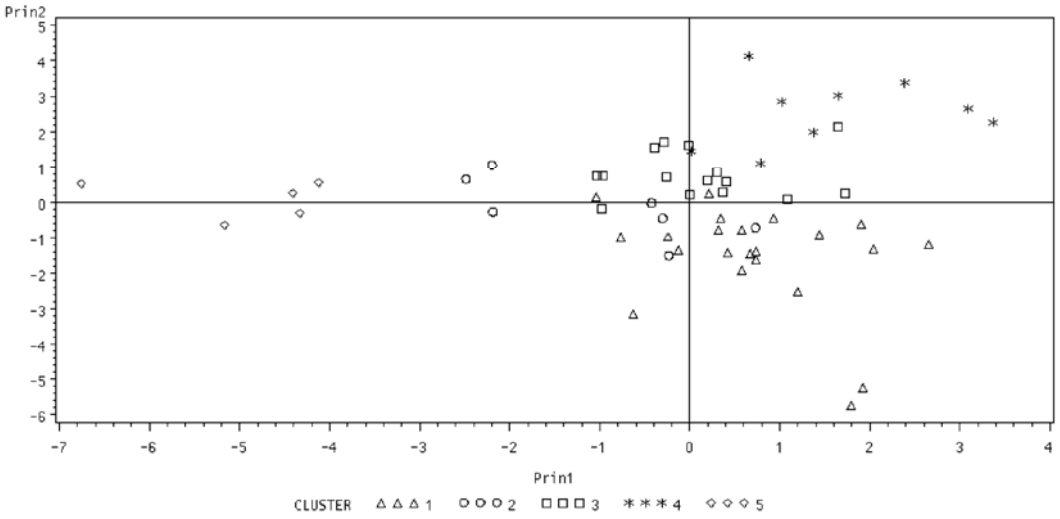

Figure 1. Two dimensional representation of genetic relationships among 56 red clover populations determined on the basis of principal component analysis of morphological data.

Molecular classification

Population genetic diversity – analysis of three populations

All loci were polymorphic in at least one population or individual of the populations analyzed. A total of 63 fragments were scored and the number of alleles observed ranged from 7 to 12, with size ranging from 78 to 234 bp (base pair) and the mean number of alleles per locus was 9.0 (Table 7). The PIC values for the seven markers ranged from 0.64 (RCS1518) to 0.85 (RCS0033) with a mean value of 0.75 (Table 7).

Table 7. Diversity statistics for 7 SSR markers analyzed in 3 populations and in 56 individuals of red clover.

	3 populations														56 individuals		
				PI				PI				PI					
Loci	Allele Sizes	A	PIC	237705				249870				204506				Allele size	A	PIC
				He	Ho	Fis	P value	He	Ho	Fis	P value	He	Ho	Fis	P value			
RCS3095	196-228	8	0.75	0.814	0.818	-0.006	0.475	0.592	0.500	0.164	0.552	0.659	0.571	0.143	0.851	196-236	11	0.86
RCS1737	78-132	9	0.68	0.837	0.700	0.171	0.415	0.515	0.182	0.658	0.014	0.209	0.222	-0.067	1.000	72-111	11	0.88
RCS1518	155-210	7	0.64	0.726	0.300	0.600	0.004*	0.395	0.100	0.757	0.047	0.216	0.111	0.500	0.061	155-189	10	0.89
RCS0033	162-222	12	0.85	0.895	0.400	0.566	0.000*	0.840	0.727	0.140	0.122	0.492	0.250	0.509	0.073	135-222	18	0.91
RCS3102	152-184	8	0.75	0.784	0.727	0.075	0.759	0.520	0.455	0.130	0.532	0.663	0.500	0.256	0.520	152-192	11	0.87
RCS0793	180-234	12	0.83	0.896	0.727	0.196	0.390	0.623	0.636	-0.022	0.904	0.884	1.000	-0.139	0.723	177-234	11	0.88
RCS1868	156-174	7	0.73	0.732	1.000	-0.398	0.452	0.732	0.727	0.006	0.294	0.616	0.400	0.363	0.002*	159-174	6	0.70
Total		63															78	
Mean		9.00	0.75	0.812	0.668	0.172	1.000	0.602	0.475	0.262	0.998	0.534	0.436	0.224	0.889		11.1	0.86

* Significant at P<0.05 after Bonferroni correction.

The average genetic diversity (He) calculated for the three populations was 0.65 and ranged from 0.534 (PI 204506) to 0.812 (PI 237705) indicating high within population variability. Over the seven loci, the Fis was not significant suggesting that the populations as a whole were in Hardy-Weinberg equilibrium (Table 7). However, all values of Fis for all loci were tested for each population and the loci that were not in H-W equilibrium were RCS1518 in PI 237705, RCS0033 in PI 237705 and RCS1868 in PI 204506. The mean observed heterozygosity tended to be lower than expected heterozygosity suggesting that there is a deficiency of heterozygotes for these loci. One reason for the excess of homozygotes for these loci in these populations may be due to the presence of null alleles. In that case, heterozygous plants carrying one null allele were scored as homozygous for the readable allele. Interestingly, the genetic diversity in

the population PI 237705 (cultivar) was higher than in the other two populations considered by GRIN as wild and uncertain.

The averaged Rogers' genetic distance calculated between the three populations was 0.28 and ranged from 0.26 (PI 249870 and PI 204506) to 0.32 (PI 237705 and PI 249870). The UPGMA clustering of genetic distances resulted in three clusters, one with the individuals from population PI 237705, the second jointed individuals from population PI 204506 and the third jointed the individuals from population PI 249870. The correlation between cophenetic values derived from the dendrogram and the Rogers' coefficient was r = 0.91 (P< 0.001), indicating a good fit of the clustering based on the original distance matrix. The AMOVA results showed that there is an important component of variance among populations (16.4%), but the major component of total variability (83.6%) was due to variation within populations (Table 8).

Table 8. Analysis of molecular variance for three red clover populations and 56 individuals based on 7 SSR loci.

Source of variation	d.f.	Sum of squares	Variance components*	% Total variance
3 populations				
Among populations	2	25.264	0.47833	16.4
Within populations	61	148.705	2.43778	83.6
total	63	173.969	2.91611	
56 individuals				
Among clusters	4	16727	0.058	1.9
Within clusters	107	316576	2.959	98.1
total	111	333304	3.017	

* Components were significant at P< 0.001; the probability of obtaining a more extreme random value computed from non-parametric procedures (1000 data permutations)

Structure 2.1 identified correctly the three populations in the data set. The Pr (K) estimates for K=1 to K=10 and corresponding values of estimates of the posterior probabilities of K confirmed the diversity was structured in three populations (K=3) (Table 9). Each individual was correctly assigned to its population, except three plants of population PI 204506 that were clustered to population PI 249870 (Figure 2).

Table 9. Posterior probabilities of K from inferred number of clusters (K) for three populations of red clover and for the 56 individuals of red clover with no prior five groups morphological information.

	3 populations		56 individuals	
K	Ln P(K)[a]	P(K)[b]	Ln P(K)[a]	P(K)[b]
1	-747.04	7.97E-40	-1574.06	8.22E-73
2	-687.1	8.57E-14	-1502.72	7.90E-42
3	-657.52	0.6024	-1466.16	5.96E-26
4	-672.32	2.25E-07	-1435.96	7.78E-13
5	-658.58	0.2087	-1408.08	0.998
6	-658.68	0.1888	-1425.72	2.17E-08
7	-682.44	9.06E-12	-1414.66	0.001
8	-757.04	3.62E-44	-1436.36	5.21E-13
9	-737.4	1.22E-35	-1453.16	2.63E-20
10	-723.2	1.80E-29	-1525.32	1.20E-51

[a] Natural logarithm of estimated probability of data
[b] Posterior probability of K

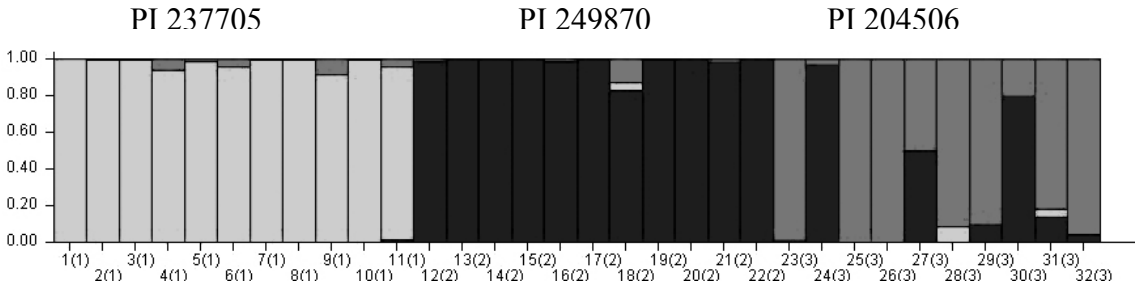

Figure 2. Assignment of 32 individuals of red clover in 3 populations. Each individual is represented by a thin vertical line, which is partitioned into three colored segments that represent the individual proportion of membership to the three clusters.

Population genetic diversity and structure – analysis of 58 individuals

For the 58 individuals, only 56 were analyzed because populations PI 314840 and PI 266047 were confirmed as tetraploid (database from GRIN) and were excluded from analysis. A total of 78 fragments were scored, the number of alleles detected ranged from 6 to 18 and the mean number of alleles per locus was 11.1 (Table 7). The PIC values for the seven markers ranged from 0.70 (RCS1868) to 0.91 (RCS0033) with 0.86 as mean value. The averaged Rogers' genetic distance calculated between the 56 individuals was 0.49 and ranged from 0.11 (PI 376880 and PI 419550) to 0.65 (PI 204507 and PI 187224). However, the correlation between cophenetic values and the Rogers' coefficient was r = 0.63 ($P< 0.001$), indicating no good fit for the clustering based on the original distance matrix . When testing the five clusters found with the

morphological analysis with the molecular SSR data, the AMOVA results showed that the major component of total variability (98.1%) was due to variation within groups and the component of variation among the five groups was significant but low (1.9 %) (Table 8).

Structure 2.1 was used with the molecular SSR data to search the correct number of clusters with no prior information about the five clusters found with morphological data and the posterior probabilities of K assigned the populations into five groups (Table 9). The structuration was not related to geographic origin of the populations (Figure 3). There was no correspondence between the composition of groups found by morphological and molecular analysis : the correlation between the matrices distance based on morphologic data and SSR marker data was not significant ($r = 0.02$; $P \leq 0.64$).

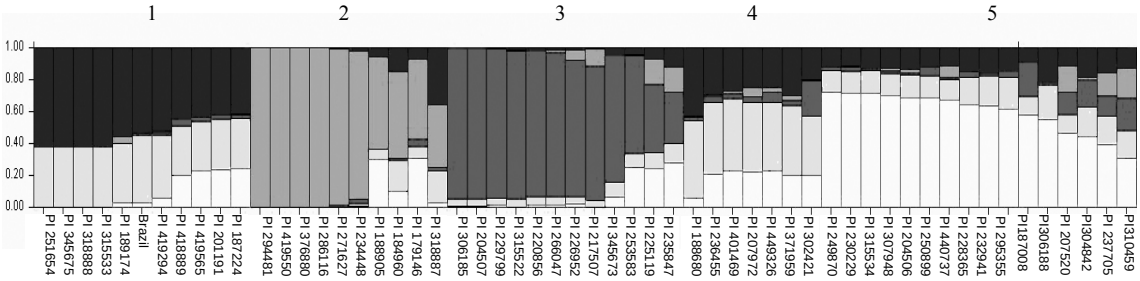

Figure 3. Assignment of 56 individuals of red clover into five clusters. Each individual is represented by a thin vertical line, which is partitioned into five colored segments that represent the individual proportion of membership to the five clusters (See Table 1 for PI code information).

The differences found between the morphologic averages in the five molecular clusters were not clear as those found with the morphological markers (data not show). Although the low correlations found between the distance matrices and the composition of clusters, there where some concordances between populations that were clustered together in both analyses. The populations present in cluster I of morphological classification (Table 1) seems to be distributed in the first three groups of the molecular classification (Figure 3).

Interestingly the group 1 of the molecular classification (Figure 3) was formed mostly by populations that were also grouped together in the group I of the morphological analysis such as PI 187224, PI 419294, Brazil, PI 189174, PI 318888 and PI 345675. The group 2 was formed by populations that were also clustered in the

group I of morphological classification such as PI 318887, PI 179146, PI 294481, PI 188905, PI 234448 and populations that were clustered in the morphological cluster III such as PI 419550, PI 184960, PI 286116 and PI 376880.

The group 3 was formed mostly by populations from morphological clusters I (PI 235847, PI 225119, PI 217507, PI 266047, PI 220856 and PI 229799) and III (PI 345673, PI 315522 and PI 306185). Group 4 was composed by populations that were also clustered in the group III (PI 188680, PI 207972 and PI 401469) and IV (PI 371959, PI 302421 and PI 449326) of morphological clusters. Finally group 5 was composed by populations of cluster I, II, III, IV, V of morphological clusters. Interestingly, there was a correspondence between the cluster V and 5 found by morphological and molecular markers where the populations PI 249870, PI 307948 and PI 250899, wild populations of red clover, were also clustered.

DISCUSSION

Morphological analysis

Diversity

In our study, we analyzed 58 populations of red clover core collection and evidenced the high significant variation for some morphological traits evaluated in greenhouse and field. The major effect in the variation was the growing condition. Although low, interactions between population and condition were identified. It is interesting to note that these interactions may indicate that genotypes respond differently at different conditions and this may be linked to local specialization. Joshi et al. (2001) analyzed with reciprocal transplants in Europe the local adaptation in red clover and found that the reproductive characters like size and number of inflorescences were higher in the origin site than elsewhere.

Montardo et al. (2003b) in an evaluation made in the Southern region of Brazil also pointed out for importance of the local adaptation of red clover populations and cultivars. In breeding programs, this may be taken in account to drive the direction of selection for specialized genotypes that may have greater production and stability in the selected environment or genotypes with good capacity to the different environments and that could be indicated for a broad region (Montardo et al. 2003b). Plant breeding activity may take advantage of local adaptation making high performance varieties developed for the specific environments in which they will be used (Joshi et al. 2001).

The interactions between growing conditions and populations were generally significant. But leaf area, size of plant, size of inflorescences and growth habit measured in field and greenhouse were highly correlated. Such traits can be measured in one environment without loss of information. Traits such as weight, number of inflorescences, blooming date and persistency had lower correlations between the measures made in the field and in the greenhouse. The low correlation and the high difference between persistency measured in the field and in the greenhouse indicated that the analysis of such traits may be highly sensitive to the environmental conditions. The measures made in the field may be more appropriate to select populations more adapted to the local conditions.

Correlations

The study of correlations is important in the breeding activity in order to associate all the possible valuable features in the newly created genotypes (Muntean and Savatti 2003) and may be a useful tool for indirect breeding of complex traits. In this study, correlations between important agronomic traits were evidenced. The correlation analysis revealed links between variables related to blooming, production and persistency. The correlation found between blooming and leaf area was also described by Rosso and Pagano (2005) when analyzing wild populations and cultivars of red clover, suggesting that plants with large leaves may have late blooming. The high correlation found between blooming and size of inflorescence indicated that plants with late blooming date had the smallest flowers when compared to early blooming date plants which had the largest flowers. This type of information could be used during selection of valuables genotypes in breeding programs. Several works have demonstrated the importance of number of stems and number of inflorescences per plant as important components for prediction of seed production in red clover (Crusius et al. 1999; Montardo et al. 2003a; Herrmann et al. 2006) although no work was done using the size of inflorescence as a potential seed production indicator.

The relationship between production of dry matter (weight) and number of inflorescences was also found by Rosso and Pagano (2005) when analyzing naturalized populations and cultivars of red clover in Argentina, a region with climatic conditions similar to that of southern Brazil . The negative correlation found between weight and phyllochron may indicate that plants with low phyllochron may have a superior herbage yield and this can be used as a indirect tool for yield prediction in breeding programs. The negative correlation found between size of plant and persistency measured in the greenhouse may indicate that taller plants of red clover were less persistent. However, the persistence measured in the field had a negative correlation with number of inflorescences. This may also indicate that plants with high blooming were less persistent.

This relation between high intensity of blooming and low persistence was also found in others works with diploid (Bird 1948; Choo 1984) and tetraploid (Christie and Choo 1991) red clover cultivars. The lack of persistency had been related in several studies (Choo 1984; Mela 2003; Montardo et al. 2003b; Fan et al. 2004; Rosso and Pagano 2005) in different countries where red clover is cultivated. One strategy in the

breeding of complex traits like persistency is the indirect selection using highly correlated traits. In this study, although the selected traits were highly important for the formation of groups, they were weakly correlated with persistency. Thus, the indirect selection for breeding might not be possible Levels of variability for all traits analyzed in this study were comparable to those found in the entire germplasm collection of red clover by Kouamé and Quesenberry (1993). In this study we confirmed the high variation for all traits, analyzing the same populations for the first time using different environments (field and greenhouse).

Structuration with morphological markers

All the traits that showed genetic variation (size of plants, growth habit, leaf area, size of inflorescence, number of inflorescences, blooming, weight, persistency, phyllochron and disease susceptibility) contributed to structure the diversity in the core collection. Greene et al. (2004) analyzed Russian wild populations of red clover using 15 morphological traits and suggested flower length and plant height as attributes that would contribute to an unbiased estimate of morphological distances. Rosso and Pagano (2005) analyzed cultivars and naturalized populations using 14 morphological traits and suggested forage yield as one of the most important traits to explain variation in these populations.

Morphological data separated the 58 populations into five distinct clusters of germplasm. All red clovers may be grouped into three divisions correspond to early, late and wild flowering types (Taylor and Smith 1995). The clusters found here corresponding somewhat to this classification. The cluster I presented populations with late blooming date and were almost all red clover cultivars from Europe high latitudes. Taylor and Smith (1995) describes that distinct plant types of red clover have evolved through natural selection and in Europe these types are distributed largely according to latitude. Late blooming populations of red clover are more common to north of 60°N latitudes. Populations of cluster II presented the lowest persistency in the field and a high intensity of blooming, two traits that were found as linked by Choo (1984) in populations and cultivars of red clover. The populations of cluster III that included populations from southern Europe, were characterized by early blooming. Taylor and Smith (1995) also described that the early-blooming populations of red clover predominate in European south 50° latitudes. The cluster IV clustered the populations

with the highest dry matter production in both environments of Brazil used here, and included populations originating from South America. These populations are probably more adapted to the Brazilian conditions than the other populations. The high persistency presented by the populations of cluster V may be linked to the more prostrate growth habit and late blooming. This link between growth habit and persistency was also found by Smith and Bishop (1993) where plants with prostate growth habit were more persistent. The populations present in this cluster may be used in the breeding activity of red clover as source of persistency, once they proved to be more adapted to southern Brazilian conditions. Mirzaie-Nodoushan et al. (1999) suggest that these more persistent types may be useful in crossings to increase the persistence of erect commercial cultivars.

This type of structuration was based on a contribution of many morphological traits. Contrastingly, Kouamé and Quesenberry (1993) found only three groups, characterized by early, medium or late blooming when they analyzed the entire USDA Germplasm collection of red clover with 15 morphological descriptors from the computer database of GRIN. Similar pattern of morphological structuration was also described for wild populations of red clover from North Europe by (Greene et al. 2004) that found five morphologic classes, but recognized three classes (once two classes were composed only for one population) corresponding to late, medium and late blooming groups. When naturalized populations collected in Argentina and 14 non-improved populations of red clover (from different origins) from USDA germplasm bank were analyzed for 14 morphological traits, in a region with similar latitude and climatic conditions to Southern Brazil, by Rosso and Pagano (2005), they found morphological division in two clusters according to early and late blooming types, different growth types and geographic origin for populations.

When comparing the morphological clusters in terms of persistency, the cluster II presented the lowest persistency and the cluster V the highest persistency. Although similar levels of production of dry matter (weight of plant) were showed by both clusters, differences in the persistency may be linked to the high intensity of blooming presented (number of inflorescences) by the cluster II and the prostrate growth habit presented by the cluster V. Leaf area was another trait that was contrasted when comparing the clusters. All these traits were reported as related to persistency in the

analysis of populations and cultivars of red clover (Choo 1984; Christie and Choo 1991). Among the clusters with intermediate persistency, the cluster IV had the highest production of dry matter and may be used for the selection of populations more adapted to the southern Brazilian conditions.

Structuration with molecular markers

A large genetic diversity was observed with the SSR markers. The number of alleles observed and the polymorphism information content (PIC) for each marker were similar to those found by (Sato et al. 2006), in which the number of alleles ranged from 6 (RCS3095) to 13 (RCS0033) and the PIC value for the same loci ranged from 0.67 (RCS1868) to 0.86 (RCS0033). Similar results for populations of the core collection were found by Mosjidis and Klingler (2006) with 10 isozyme loci. The populations were at Hardy-Weinberg equilibrium, which is compatible to the multiplication in panmitic conditions of the populations.

Contrastingly, Mosjidis and Klingler (2006) found a Hardy-Weinberg disequilibrium, perhaps because of the presence of null alleles in the isozyme loci. The high degree of genetic diversity was expected by its allogamous nature. We found higher genetic diversity than other studies based on RAPD markers (Kongkiatngam et al. 1995; Kongkiatngam et al. 1996; Ulloa et al. 2003) and isozyme markers (Mosjidis and Klingler 2006), probably because microsatellite markers display high mutation rates, and are thus expected to reveal fairly high amounts of polymorphisms especially when used at the species level (Thuillet et al. 2005). But Kölliker et al. (2006) found higher mean values of diversity than those reported here, which is probably related to the nature of the populations, the use of more SSR loci and the analysis of more individuals per population. However, the values reported here were in agreement with the results reported for allogamous legume species like alfalfa (Flajoulot et al. 2005) and white clover (Kölliker et al. 2001).

A high within-population diversity (83.6% of the total variation in AMOVA) was observed with SSR markers. Indeed, red clover is an outcrossing species with a high degree of gametophytic self-incompability (Taylor and Quesenberry 1996). The cultivars are synthetic, corresponding to heterogeneous populations consisting of heterozygous individuals (Bowley et al. 1984; Taylor and Quesenberry 1996). Landraces or natural populations also are heterogeneous populations obtained after

cross-pollination of many plants. Consequently, high levels of within-population variability are expected (Kölliker et al. 2003). Other studies in red clover (Kölliker et al. 2003) and in other forage crops (Kölliker et al. 2001) found an extensive within population genotypic diversity also when using different types of molecular markers (Campos-de-Quiroz and Ortega-Klose 2001; Ulloa et al. 2003; Kölliker et al. 2003). The variation for within-population diversity ranged from 68.3 to 99.5% in studies realized with SSR markers in other allogamous legume species such as *Medicago sativa* (Mengoni et al. 2000) and in red clover using AFLPs (Herrmann et al. 2005) and RAPDs (Ulloa et al. 2003). Phenotypic diversity among populations was also observed with phenotypic traits (Rosso and Pagano 2005). The structuration of the diversity among three populations obtained with AMOVA indicated that population effect explained only 16.4% of the total variation for SSR markers. When analyzing the genetic distance between three populations, the two populations from southern Europe (PI 249870 and PI 204506) were more similar than they were from the Danish cultivar (PI 237705). Others studies have found high within-cultivar variation and low variation among cultivars (Kongkiatngam et al. 1995; Campos-de-Quiroz and Ortega-Klose 2001; Kölliker et al. 2003).

The structuration of the core collection was studied with the analysis of a single individual from 56 populations. Each individual is not considered as representative of the population which it comes from, but the clustering of populations can be realized (Muller et al. 2003). The AMOVA made with the five morphological clusters using the molecular data failed to confirm this clustering. The SSR data gave a structure also with five groups, that were not related to evident geographic origin nor directly to morphological characteristics. There was no direct correspondence between the composition of groups found by morphological and molecular analyses. However, some concordances between the morphologic and the SSR structuration were highlighted, when was verified that the clusters found in the SSR analysis were composed somewhat by the distribution of the morphological clusters. Mosjidis and Klingler (2006) analyzed the core subset using isozyme markers and did not found major discriminant groups of populations. The absence of a relationship between the morphology and genetic similarities was also found for wild populations of red clover (Greene et al. 2004) and for other cross-pollinating species as *Lotus corniculatus* (Steiner and los Santos 2001).

However, in alfalfa, the subspecies structuration was obtained with RAPD markers, even if within-subspecies structuration was different between RAPD and morphological traits (Crochemore et al. 1998). Several reasons may account for the discordance between the morphologic and SSR markers. Firstly, morphological variation is strongly associated with environmental variation, the morphological similarities observed may 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 (Johns et al. 1997). Secondly, specific traits with adaptive value may have accumulated in habitats subjected to similar ecologic conditions, independently of the genetic origins of the populations (Steiner and los Santos 2001). Thirdly, the evolutionary rates of morphologic characters having adaptative value and those originating from selectively neutral DNA can be different (Linhart and Grant 1996). Although the SSRs used here were isolated from ESTs, their allelic frequencies did not seemed related to morphological traits so they can be considered as neutral markers. Fourthly, the high levels of within-population variation can hinder the separation of related cultivars of outbreeding species (Kölliker et al. 2003).

The information found here evidenced the high genetic diversity and also pointed out the high within-population diversity in the red clover core collection. Several works pointed that the analysis of diversity in outcrossing species requires different approaches like molecular markers of different nature, morphological and biochemical markers (Steiner et al. 2001; Budak et al. 2004). It can be assumed that the structuration obtained with non neutral markers (i.e. defined in genes involved in traits) would give another structure, more similar to that obtained with morphological traits.

CONCLUSIONS

This study showed the broad range of genetic diversity in a subset of the USDA core collection of red clover measured with morphological and molecular markers. The genetic diversity revealed through the morphological markers was structured around five main types, depending on plant size, growth habit, blooming date and inflorescence size. Specific morphologic traits were found to be related to collecting site ecogeographic conditions. Depending on the breeding objectives, diversity is available for all traits of agronomic interest in the core collection. But persistency, a major breeding objective, especially under Brazilian conditions, was correlated to contrasting traits like small plant size and prostrate growth habit. Molecular diversity measured for the first time in the core collection with SSR markers was high and pointed out the high variability available at the within-population level.

The absence of correlation between distances measured with morphological and molecular markers revealed the complexity of the genetic diversity present in red clover, indicating that other types of strategies for a comprehensive analysis of this diversity are needed.

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3. CAPITULO III

ANALYSIS OF GENETIC DIVERSITY IN THE CORE COLLECTION GERMPLASM OF RED CLOVER (*TRIFOLIUM PRATENSE* L.) WITH ISOZYMES AND RAPD MARKERS

Análise da diversidade genética na coleção nuclear de germoplasma de trevo vermelho (*Trifolium pratense* L.) com marcadores isoenzimáticos e RAPD[1]

Dias, P.M.B[2]; Pretz, V.F[3]; Dall'Agnol, M.[4]; Schifino-Wittmann[4], M.T; Zuanazzi, J.A[5].

RESUMO

O trevo vermelho (*Trifolium pratense*) é uma leguminosa forrageira com considerável importância na agricultura mundial. A diversidade genética medida com marcadores RAPD juntamente com marcadores bioquímicos é desconhecida na coleção nuclear de germoplasma dos Estados Unidos. Os objetivos foram: (i) acessar a diversidade genética na coleção nuclear por meio de marcadores bioquímicos e RAPD; (ii) comparar diferentes abordagens na análise de dados isoenzimáticos; (iii) comparar os resultados dos diferentes tipos de marcadores. Um total de 15 loci isoenzimáticos com 30 alelos foram detectados e 114 fragmentos RAPD foram analisados. Ambos marcadores evidenciaram altos níveis de diversidade genética. A análise das isoenzimas na forma de dados binários foi altamente correlacionada com a abordagem dos dados como freqüência alélica. Não houve correlação entre a análise de agrupamentos realizada com marcadores bioquímicos e moleculares. Os dados isoenzimáticos classificaram os acessos de trevo vermelho em quatro grupos. A análise de variância molecular evidenciou alta variação dentro dos grupos (44,11%), entre os grupos (40,77%) e uma baixa variação (15,12%) intrapopulacional. O padrão da alta diversidade genética presente na coleção nuclear é complexo. Deste modo, dados de passaporte mais precisos e o uso de diferentes marcadores de diferentes naturezas pode ser útil para o melhor entendimento e utilização desta rica diversidade natural.

PALAVRAS CHAVES: trevo vermelho–isoenzimas–RAPD–diversidade genética–coleção nuclear

[1] Artigo redigido nas normas da revista Plant Breeding

[2] Aluna de Doutorado PPG/Zootecnia/UFRGS Bolsista CAPES

[3] Aluna do curso de graduação em Agronomia/UFRGS

[4] Professor adjunto da Faculdade de Agronomia/Departamento de Plantas Forrageiras e Agrometeorologia/UFRGS

[5] Professor adjunto da Faculdade de Farmácia /UFRGS

Analysis of genetic diversity in the Core Collection Germplasm of red clover (*Trifolium pratense* L.) with isozymes and RAPD markers

ABSTRACT

Red clover (*Trifolium pratense*) is a forage legume with considerable economic importance in the world agriculture. The genetic diversity measured with RAPD and biochemical markers in the United States germplasm core collection is unknown. The objectives were to: (i) assess the genetic diversity in the core collection by means of isozymes and RAPD markers; (ii) compare different approaches for isozyme data analyses; (iii) compare the results found with the different types of markers. A total of 15 esterase isozyme loci with 30 alleles were detected and 114 RAPD fragments were analyzed. Both markers evidenced high levels of genetic diversity. The analysis of isozymes as binary data proved to be highly correlated with the allelic frequencies approach. There was no correlation between the cluster analyses with biochemical and molecular markers. Isozyme data clustered the accessions of red clover in four groups. Analysis of molecular variance evidenced high variation placed within groups (44.11%), among the groups (40.77%) and low variation palced at the intrapopulational level (15.12%). The pattern of the high genetic diversity present in the red clover core collection is complex. Therefore, more accurate passport data for the accessions and the use of different types of markers from different natures may be helpful for the knowledge and better utilization of this rich natural diversity.
KEY WORDS: Red clover–isozymes–RAPD–genetic diversity–core collection

Introduction

Red clover is a short-lived herbaceous forage crop thought to have originated in southeastern Europe and Asia Minor near the Mediterranean Sea (Taylor and Quesenberry 1996). Widely used in the world agriculture, red clover is second to alfalfa in economic importance among forage legumes, and is grown for hay, silage, forage and as a soil conditioner (Greene et al. 2004). Although its Mediterranean origin (Taylor and Smith 1979), red clover is a cosmopolitan species adapted to a great number of climatic conditions. Red clover is highly self-incompatible and sets seed almost entirely by cross-fertilization (Taylor and Smith 1995). As a result, individual plants are heterozygous and populations are heterogeneous (Mirzaie-Nodoushan et al. 1999). Many works using morphologic (Kouamé and Quesenberry 1993; Christie and Choo 1991; Bulinska-Radomska 2000), molecular (Kongkiatngam et al. 1995; Campos-de-Quiroz and Ortega-Klose 2001; Kölliker et al. 2003; Greene et al. 2004; Herrmann et al. 2005; Sato et al. 2006) and biochemical traits (Hagen and Hamrick 1998; Yu et al. 2001; Mosjidis et al. 2003; Mosjidis and Klingler 2006) have showed this high genetic diversity, both among and within populations.

Nowadays, the current interest in the sustainable agriculture where leguminous such as red clover play an important role in the atmospheric N fixation and in the reduction of atmospheric and water pollution (Rochon, et al. 2004), promises to broaden the use of red clover. Indeed recent studies of utilization of red clover by the pharmaceutical industry have pointed that dietary phytoestrogens play in important role in the prevention of menopausal symptoms (Beck et al. 2003), cancer (Atkinson et al. 2004a), osteoporosis (Atkinson et al. 2004b) and heart disease (Dixon 2004). The industrial use of red clover is expanding and diversifying (Humphreys 2005; Morris and

Greene 2001) and future breeding activities have to be able to exploit a wider range of diversity in red clover (Greene et al. 2004) making new varieties that supply the demand of these new market.

This diversity is represented in germplasm collections all over the world and curators are responsible for providing useful breeding material in the present and conserving crop gene pools for the future (Frankel and Brown 1995). However, the large size and heterogeneous structure of many collections has often hindered the efficient maintenance and utilization of gene bank materials (Kouamé and Quesenberry 1993; Steiner et al. 2001), as well as the lack of geographic and ecological descriptors in the passport data of many accessions of germplasm collections (Steiner and Greene 1996; Greene et al. 1999). The germplasm collection of red clover of the National Plant Germplasm System of United States Department of Agricultural (NPGS-USDA) is composed by 800 accessions from 41 different countries (Kouamé and Quesenberry 1993).

The concept of "core collections" was considered as a form to facilitate the use of germplasm in the breeding programs (Brown 1989) and then many core collections have been formed in the diverse species of cultivated plants (Souza and Sorrells 1991; Steiner et al. 2001). A core collection was developed for red clover using morphological and physiological descriptors (Kouamé and Quesenberry 1993). After the formation of the red clover core collection only few works using other traits were published and all only using morphologic or biochemical characters (Bortnem and Boe 2002; Bortnem and Boe 2003; Mosjidis and Klingler 2006). Knowledge of the amount and distribution of genetic variability within a species is vital to plant breeders because it is an important consideration when selecting germplasm to be included in a breeding program (Yu et al.

2001). However, when one or a few traits are used to characterize collections, there is less opportunity to understand relationships among different traits within collections (Brown 1989). In this way the use of many traits extends the possibilities to evidence genetic differences in a broad range. Molecular markers such as RAPD (random amplified polymorphic DNAs), have been used for the analysis of genetic diversity in red clover (Kongkiatngam et al. 1996; Campos-de-Quiroz and Ortega-Klose 2001; Greene et al. 2004; Ulloa et al. 2003; Kongkiatngam et al. 1996) and in other forage crops such as alfalfa (Mengoni et al. 2000), Birdsfoot trefoil (Steiner and de los Santos 2001) and white clover (Gustine et al. 2002; Dolanska and Curn 2004). The RAPD procedure does not require previous genomic knowledge, is a simple and low cost technique and markers usually are very polymorphic. Despite all these advantages, RAPD markers suffer from a major limitation for the analysis of population structure because complete genotypic determination is largely hampered by their dominant nature (Lynch and Milligan 1994). On the other hand, isozymes as codominant markers are useful for estimating the allelic frequencies and population genetic parameters and have proven to be reliable in genetic studies with natural populations (Hagen and Hamrick 1998; Lange and Schifino-Wittmann 2000; Semerikov et al. 2002; Mosjidis et al. 2003) and cultivars of red clover (Kongkiatngam et al. 1995; Yu et al. 2001). The use of molecular and biochemical markers in such way represents an analysis of different aspects of the diversity of traits that can be influenced by the environment and ontogeny, as in the case of the biochemical markers, and also the variability in neutral characters as in the case of the molecular markers. The objective of this work is to evaluate the genetic diversity in the core collection of the USA red clover germplasm using isozyme and RAPD markers for the first time together.

Materials and Methods

Plant Material

Seeds of 79 red clover accessions from the core collection of NPGS-USDA were obtained from the USDA-ARS Plant Introduction Station at Pullman (W6), Washington and one population cultivated in Brazil were selected. Therefore, a total of 80 populations were analyzed (Table 1). The accessions from the core collection were classified by the NPGS on the basis of improvement status into cultivars, landraces, breeding material and wild populations when the data were available (Mosjidis and Klingler 2006).

Isozymes markers

From each accession, 10 seeds were germinated in Petri dishes with watered filter paper and after 15 days were planted in plastic pots (750 ml soil capacity) in a greenhouse. Each plant was inoculated with 1 mL of a solution of *Rhizobium leguminosarum bv. trifolii* (SEMIA 222 and SEMIA 2082 the recommendation from Laboratório de Fixação Biológica do Nitrogênio/ Fundação Estadual de Pesquisa Agropecuária /LFBN/ FEPAGRO/Porto Alegre/Brazil) in aqueous solution (concentration of 10^9 cél. mL^{-1}). After one month the plants were transferred to pots of 5 kg soil capacity. The greenhouse was located at Porto Alegre, Rio Grande do Sul, Brazil (30º 01'59" S, 51º13'48" W and elevation 10 m). Five to ten plants per accession, with a total of 535 individuals were examined for isozyme patterns. The enzymatic system selected was esterase α and β (EST, EC, 3.1.1.-). Preliminary tests were performed to identify the best gel and buffer systems, buffer concentrations and tissue amount in the samples. Isozymes were analyzed in about 100mg of young leaves taken from mature plants ground in 170 μL of extraction buffer (Collins et al. 1984). One plant of *Adesmia*

bicolor was used as control in all gels. Electrophoresis was conducted in 10% polyacrylamide gel in horizontal migration at constant current of 40mA. Migration was performed at approximate 4°C and was stopped when the front line reached 8 cm away from the application point, which occurred after 2h30. Gels were stained with staining solution specific for EST of Scandalios (1969). The gels were fixed in Ayala solution, photographed and analyzed. All monomorphic and polymorphic anodic and cathodic bands were analyzed. Bands were characterized by their rates of migration and also by their colour (α dark brownish to blackish and β light to dark reddish). The rate of migration was calculated as ratio of the migration point of the band from the origin to the migration point of the front line.

Table 1. Summary of isozyme variation within 80 populations of *Trifolium pratense*. Parameters calculated on the basis of 15 loci were percentage of polymorphic loci (P), mean number of alleles per locus (A), observed heterozygosity (Ho) and expected heterozygosity (He).

N°	Accessions	Origin	Type	P(0.95)	A	Hobs.	Hexp.
1	PI 237705	Denmark	Cultivar	1.000	2.00	0.500	0.516
2	PI 196424	Denmark	Cultivar	0.333	1.33	0.111	0.101
3	PI 217507	Denmark	Cultivar	0.500	1.50	0.500	0.263
4	PI 237714	Denmark	Cultivar	0.333	1.33	0.056	0.177
5	PI 314840	Norway	Cultivar	0.333	1.33	0.333	0.175
6	PI 188905	Sweden	Cultivar	0.333	1.33	0.333	0.175
7	PI 235847	Sweden	Cultivar	0.333	1.33	0.300	0.174
8	PI 235854	Sweden	Landrace	0.200	1.20	0.200	0.111
9	PI 235870	Sweden	Landrace	0.500	1.50	0.250	0.212
10	PI 235867	Sweden	Landrace	0.750	1.75	0.400	0.393
11	PI 229799	Finland	Cultivar	0.667	1.67	0.533	0.344
12	PI 236455	Finland	Cultivar	0.667	1.67	0.333	0.351
13	PI 310459	Suíça	Cultivar	1.000	2.00	0.458	0.486
14	PI 310465	Suíça	Uncertain	1.000	2.00	0.313	0.392
15	PI 179146	Suiça	Cultivar	0.500	1.50	0.292	0.178
16	PI 234925	Switzerland	Wild	0.500	1.50	0.250	0.206
17	PI 239696	Switzerland	Unceratain	0.750	1.75	0.213	0.384
18	PI 266047	Poland	Cultivar	0.250	1.25	0.250	0.132
19	PI 632214	G 21245	Uncertain	0.500	1.50	0.458	0.269
20	PI 293591	Poland	Uncertain	0.250	1.25	0.250	0.143
21	PI 384058	Poland	Cultivar	1.000	2.00	1.000	0.526
22	PI 255894	Poland	Cultivar	0.500	1.50	0.500	0.278
23	PI 225119	Germany	Cultivar	0.333	1.33	0.067	0.063
24	PI 187008	Germany	Cultivar	1.000	2.00	0.000	0.394
25	PI 234836	Germany	Wild	1.000	2.00	0.000	0.394
26	PI 294481	Austria	Cultivar	1.000	2.00	0.389	0.406
27	PI 318887	Hungary	Landrace	1.000	2.00	0.905	0.524

28	PI 318888	Hungary	landrace	1.000	2.00	0.905	0.524
29	PI 315522	Italia	Wild	1.000	2.00	0.250	0.417
30	PI 418889	Italia	Wild	1.000	2.00	0.467	0.456
31	PI 249870	Greece	wild	0.333	1.33	0.148	0.122
32	PI 419294	Greece	Wild	0.667	1.67	0.222	0.202
33	PI 220856	Portugal	Uncertain	0.667	1.67	0.200	0.328
34	PI 311492	Spain	Wild	0.333	1.33	0.200	0.156
35	PI 307948	Spain	Wild	0.333	1.33	0.000	0.063
36	PI 253583	Spain	Wild	0.667	1.67	0.067	0.211
37	PI 188680	France	Landrace	0.500	1.50	0.500	0.263
38	PI 207972	France	Uncertain	0.500	1.50	0.500	0.263
39	PI 189174	Netherland	Cultivar	0.333	1.33	0.200	0.156
40	PI 201191	Netherland	Uncertain	0.333	1.33	0.074	0.122
41	PI 187224	Belgium	Cultivar	0.333	1.33	0.200	0.156
42	PI 234448	Belgium	Cultivar	0.333	1.33	0.067	0.067
43	PI 205313	Turkey	Uncertain	0.333	1.33	0.200	0.156
44	PI 120105	Turkey	Landrace	0.333	1.33	0.000	0.119
45	PI 171870	Turkey	Uncertain	0.333	1.33	0.111	0.101
46	PI 204506	Turkey	Uncertain	0.333	1.33	0.333	0.185
47	PI 204507	Turkey	Uncertain	0.333	1.33	0.333	0.182
48	PI 314487	Georgia	Wild	0.667	1.67	0.267	0.296
49	PI 371959	Bulgaria	Cultivar	0.333	1.33	0.333	0.182
50	PI 294797	Bulgaria	Landrace	0.333	1.33	0.167	0.136
51	PI 315533	Bulgaria	Cultivar	0.333	1.33	0.167	0.143
52	PI 251564	Yugoslavia	Cultivar	0.500	1.50	0.357	0.269
53	PI 207520	Afghanistan	Uncertain	0.333	1.33	0.083	0.083
54	PI 228365	Iran	Uncertain	0.333	1.33	0.100	0.090
55	PI 250899	Iran	Wild	0.333	1.33	0.000	0.147
56	PI 401469	Romania	Cultivar	0.500	1.50	0.250	0.214
57	PI 232941	Hungary	Cultivar	0.500	1.50	0.125	0.125
58	PI 345673	Ukraine	Cultivar	0.250	1.25	0.250	0.143
59	PI 228160	Russia	Cultivar	0.250	1.25	0.000	0.121
60	PI 440737	Russia	Wild	0.333	1.33	0.333	0.191
61	PI 345675	Russia	Cultivar	0.667	1.67	0.500	0.318
62	PI 419565	Japan	Breeding material	0.667	1.67	0.667	0.381
63	PI 419550	Japan	Breeding material	0.667	1.67	0.267	0.252
64	PI 184960	Australia	Uncertain	0.500	1.50	0.250	0.212
65	PI 376880	New Zealand	Cultivar	0.250	1.25	0.125	0.102
66	PI 187284	UK	Cultivar	0.333	1.33	0.333	0.180
67	PI 306188	UK	Cultivar	0.667	1.67	0.571	0.344
68	PI 306185	UK	Cultivar	0.667	1.67	0.583	0.360
69	PI 315534	Canada	Cultivar	0.333	1.33	0.333	0.185
70	PI 286116	Canada	Cultivar	0.250	1.25	0.100	0.089
71	PI 286222	Canada	Cultivar	0.500	1.50	0.100	0.178
72	PI 295355	USA	Landrace	0.500	1.50	0.250	0.279
73	PI 230229	USA	Landrace	0.250	1.25	0.250	0.136
74	PI 306677	Ecuador	Landrace	1.000	2.00	0.333	0.448
75	PI 302421	Colombia	Cultivar	0.250	1.25	0.000	0.133
76	PI 304842	Chile	Cultivar	0.250	1.25	0.083	0.076
77	PI 449326	Chile	Cultivar	1.000	2.00	0.208	0.375
78	PI 271627	India	Wild	1.000	2.00	0.875	0.525
79	PI 226952	Ethiopia	Wild	0.500	1.50	0.200	0.178
80	Brazil	Brazil	Cultivar	0.250	1.25	0.125	0.102
Mean				0.525	1.525	0.284	0.238
SD				0.260	0.260	0.216	0.131

Isozyme Data Analysis

Two types of analysis were conducted in order to compare the accuracy of estimate methods. Firstly were noted the mobility values of the each band from all gels and these bands were scored as binary characteristics, giving value 1 when the band is present and 0 when band is absent for each individual. Data were transformed into a binary matrix and Jaccard's similarity index was calculated. The resulting similarity matrix was utilized to construct a dendrogram by cluster analysis with the UPGMA (Unweighted Pair-Group procedure with an Arithmetic Mean). The reliability of the clustering was tested by computing Mantel test statistics for the correlation of the distance matrix and the cophenetic matrix. All these computations are performed using the NTSYS-PC 2.10 software (Rohlf 2000).

Secondly the number of alleles for each isozyme locus were scored and population genetic parameters calculated on a population basis. The genetic parameters calculated were percentage of polymorphic loci (P), mean number of alleles per locus (A), observed heterozygosity (Ho) and expected heterozygosity (He) based on Nei's unbiased estimate were calculated using Genetix version 4.05 (Belkhir 2004) and the fixation index (Fis) was estimated according to Weir and Cockerham (1984). The deviations from Hardy Weinberg Equilibrium (HWE) at individual loci and for all loci were tested with Markov chain method, with default parameters using GENEPOP software (Raymond and Rousset 1995). The Rogers' distances were calculated between the 80 populations and cluster analysis was performed on Rogers' distances using the unweighted pair-group procedure with an arithmetic mean (UPGMA) and reliability of the clustering was tested by computing Mantel test statistics for the correlation of the distance matrix and the cophenetic matrix using the NTSYSPC 2.10 software (Rohlf

2000). Furthermore, the NTSYS program was also used in the ordination of populations by principal component analysis (PCA) on the matrix of allele frequencies. Analysis of molecular variance (AMOVA) was realized using Arlequin version 3.1 (Excoffier and Schneider 2005) based on the allele frequencies to test the parts of among-population and within-population diversities. Finally the distances matrices generated by binary data and allelic frequencies data, were compared computing the correlation using the product moment correlations (r) derived from the normalized Mantel Z (Mantel 1967). Matrices comparison were carried out using the MAXCOMP module of the NTSYS-PC program (Rohlf 2000).

RAPD markers

From the same 80 populations 20 seeds per population were bulked and utilized for DNA extraction using the method of Doyle and Doyle (1987). DNA was quantified by comparison with the ethidium-bromide stained standard concentrations in 1% agarose gels. DNA was diluted in TE (10mM Tris-HCl, pH 8.0, 1mM EDTA) to achieve a final concentration of 3ng/ μL. A total of eight random primers (Operon Technologies, Alameda, CA, USA) were used for RAPD analysis (Table 2). The amplifications reactions conditions were performed following Ferreira and Grattapaglia (1998). The final volume reaction was 15 μL containing 15ng of genomic DNA, 15ng of primer, 2.5mM of each dNTP, 50mM of $MgCl_2$, 1x PCR Buffer (20mM Tris-HCl (pH 8.4), 50mM KCl) and 1U of Taq Polymerase (Invitrogen Inc.). The DNA amplification was conducted on a PTC-100 (Programmable Thermal Controller MJ Research, INC.) thermal cycler programmed for 40 cycles of: 1 min at 93^{o}C, 1 min at 36^{o}C, 2 min at 72^{o}C and a final extension of 5 min at 72^{o}C. Reaction products were separated by electrophoresis in 1.5% agarose gels using a Tris-Borate-EDTA (TBE)

buffer system. Gels were stained with ethidium bromide, visualized under UV light and images captured with a Kodak DC 290 digital camera. Gel images were analyzed with Kodak 1D Image Analysis software (Version 2.0.2, Eastman Kodak Co., Scientific Imaging Systems, Rochester, N.Y.).

Table 2. Primers, sequences and summary of RAPD markers used to characterize red clover populations.

Primers	Sequence 5' to 3'	N° of markers	Size (bp) min-max
OPA01	CAGGCCTTC	13	520-1800
OPA09	GGTCCCTGAC	10	350-1280
OPA18	AGGTGACCGT	17	196-2000
UBC2	CCTGGGCTTC	13	230-1600
UBC4	CCTGGGCTGG	13	220-1870
OPB14	TCCGCTCTGG	15	260-1850
OPG04	AGCGTGTCTG	17	270-1800
OPG06	GTGCCTAACC	16	280-1784
	Total	114	

RAPD data analysis

RAPD data were transformed into a binary matrix and Jaccard's similarity index was calculated using the module SIMQUAL from the NTSYS-PC computer package (Rohlf 2000). The resulting similarity matrix was utilized to construct a dendrogram by cluster analysis with the UPGMA (Unweighted Pair-Group procedure with an Arithmetic Mean) using the module SAHN from the NTSYS-PC computer package (Rohlf 2000). The reliability of the clustering was tested by computing Mantel test statistics for the correlation of the distance matrix and the cophenetic matrix using the module MAXCOMP of the NTSYS-PC 2.10 software (Rohlf 2000).

Comparison between RAPD and Isozymes

The Jaccard's distances matrices generated by RAPD data and by isozyme binary data were compared by computing Mantel test statistics for the correlation using the module MAXCOMP of the NTSYS-PC 2.10 software (Rohlf 2000).

Results

Isozyme binary data

A total of 14 bands EST were detected of these eight were α and six were β. The mean Jaccard's similarity between the 80 populations was 0.30 and ranged from 0 to 1. The cophenetic correlation was r = 0.86, which indicated the dendrogram produced from the cluster analysis gave a good fit when compared with the distance matrix. The dendrogram was cut at the average similarity point and resulted in five groups (Figure 1). We recognize four groups, once one of the five groups contain only two populations.

Isozyme allelic frequencies data

A total of 15 isozyme loci with 30 alleles were detected by means of esterase (EST) enzyme system in the 80 red clover populations. All loci were polymorphic in at least one population. The percent polymorphic loci (P) within populations (Table 1) ranged from 0.20 in PI 235854 to 1.00 in PI 237705, PI 234925, PI 239696, PI 266047, PI 632214, PI 293591, PI 384058, PI 179146, PI 310465, PI 255894, PI 225119, PI 187008, PI 234836, PI 294481, PI 318887, PI 318888, PI 315522, PI 306677, PI 449326, PI 271627 and PI 418889. The average percent polymorphic loci (P) was 0.52 for all the populations. The effective number of alleles per locus (A) ranged from 1.2 to 2.0 and the average within populations was 1.52. The expected heterozygosity (He) ranged from 0.063 in PI 307948 to 0.526 in PI 304058 and the mean value for the populations was He = 0.238. Over the 15 loci, the Fis was not significant suggesting that the populations as a whole were in Hardy-Weinberg equilibrium, furthermore the mean observed heterozygosity within accessions Ho = 0.284 was close to He = 0.238 (Table 1). The range in Rogers genetic distance was high and varied from 0 to 0.26 and the average Rogers genetic distance was 0.14. The correlation between the

cophenetic matrix and the matrix based on Rogers distance was r = 0.79, indicating a good fit to the dendrogram derived from the cluster analysis. The dendrogram of Rogers genetic distance (Figure 2) was cut at the point of average distance and indicated that all 80 populations of red clover were clustered in five groups. Similar to the classification with binary data we recognize four groups since one group of the five was composed only by two populations. The principal component analysis (PCA) (Figure 3) indicated that 74.7 % of the variation was accounted by the first three dimensions. Plotting of those three dimensions indicated also that were four groups (Figure 3).

The AMOVA results (Table 3) indicated highly significant genetic differences ($P< 0.000$) between the four groups as well as between the populations of red clover. Of the total genetic diversity, 44.11% was attributed to populations within groups, 40.77% to groups and only 15.12% to within populations difference.

Comparison between isozyme binary and allelic frequencies data

The coefficient of correlation between the matrix of Jaccard's similarity and Rogers' distance of isozyme data was r = 0.70.

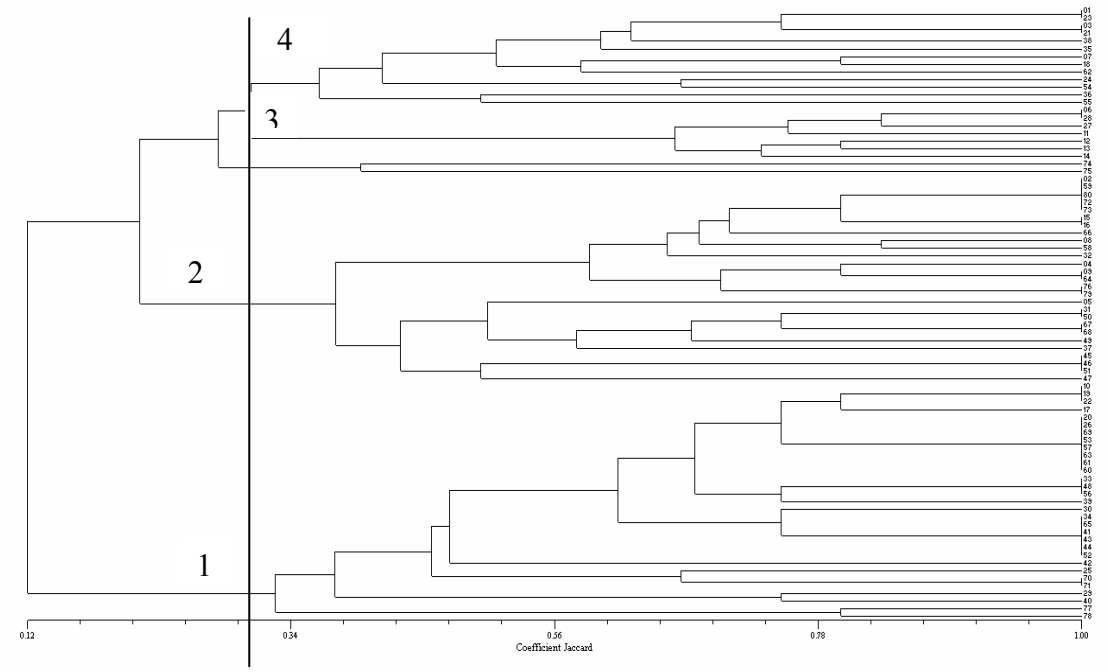

Figure 1. Dendrogram of isozyme binary data based on Jaccard's similarity for 80 accessions of red clover. The thin line indicates the average similarity and the cutting point of the dendrogram.

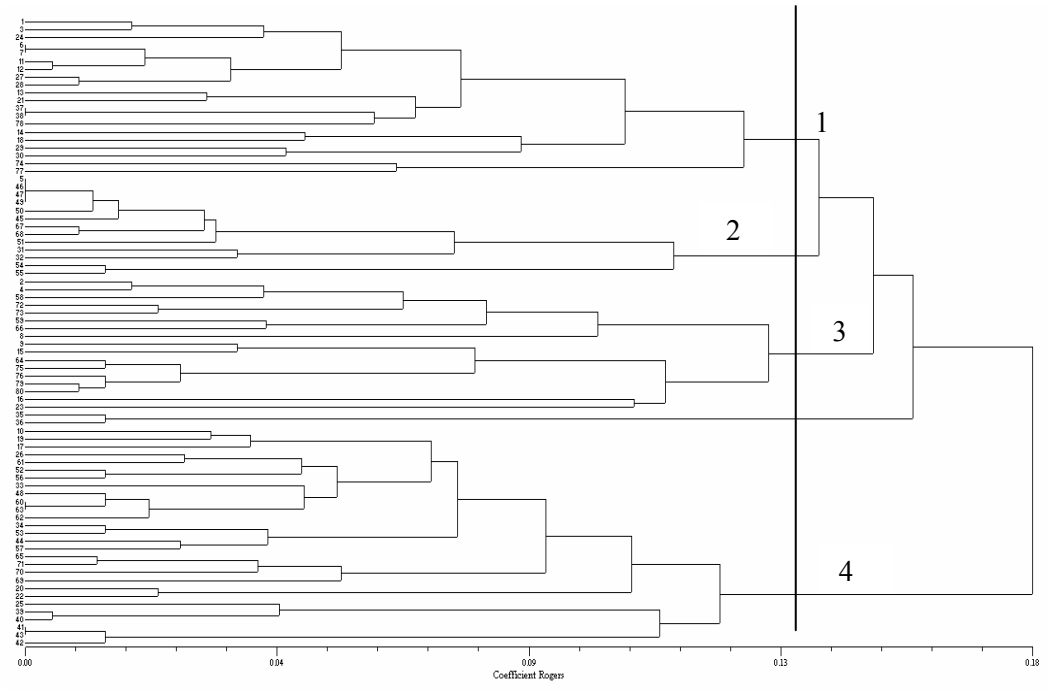

Figure 2. Dendrogram of isozyme allelic frequencies data based on Rogers distance for 80 populations of red clover. The thin line indicates the average similarity and the cutting point of the dendrogram.

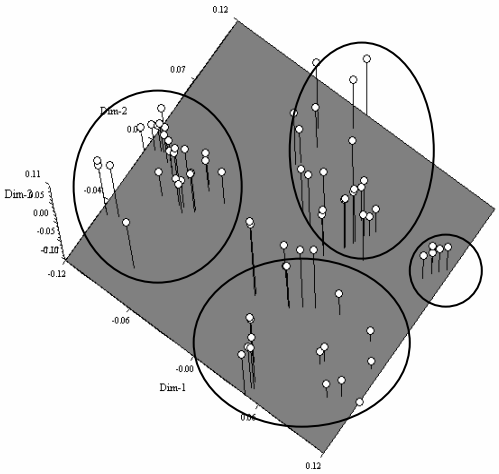

Figure 3. Three dimensional representation of the principal component analysis based on allele frequencies at the 15 isozyme loci. The three axes explain 74.7% of the total variation.

Table 3. Analysis of Molecular variance (AMOVA) for 80 populations of red clover

Source of variation	d.f.	SSD	MSD	% Total Variance	P-value*
Among groups	3	878.846	105.246	40.77	<0.000
Among populations/Groups	76	1.182.251	113.868	44.11	<0.000
Within populations	990	386.335	0.39024	15.12	<0.000

*nonparametric randomization test (1000 permutations).

RAPD data

All the eight primers used were polymorphic in all populations examined showing a high degree of molecular variation. The total number of fragments detected was 114 and the number of fragments per primer ranged from 13 to 17 (Table 2). The fragments generated ranged from 196 to 2000bp (base pairs). The Jaccard's distance based on 114 RAPD markers ranged from 0.00 to 0.68 and averaged 0.21. The coefficient of correlation between the matrix of Jaccard's and the cophenetic matrix was low (r = 0.66) what indicated that the dendrogram (Figure 4) produced from the cluster

analysis gave a poor fit when compared with the distance matrix. Indeed the scatter plots of principle component scores did not support the cluster analysis and ACP indicated that only 28.9% of the RAPD variation was accounted for in the first three components. RAPD data did not separated the populations in clusters that corresponded to the same found with biochemical markers.

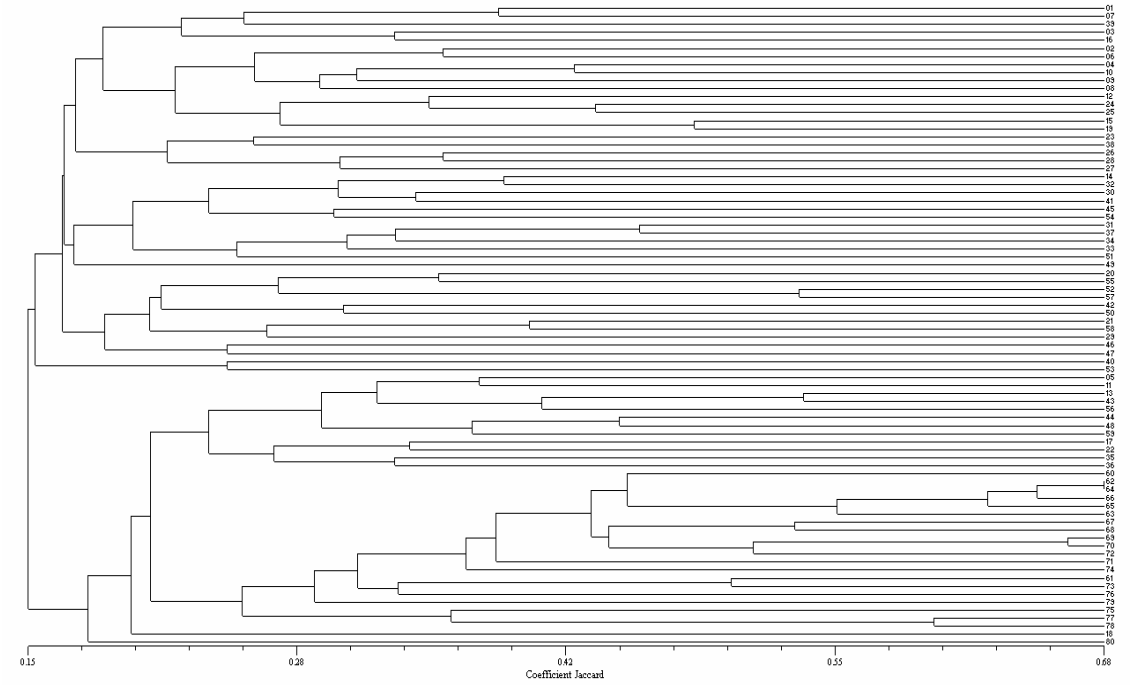

Figure 4. Dendrogram of RAPD data based on 114 markers for 80 accessions of red clover.

Comparison between RAPD and Isozymes classification

There was no correspondence between the Jaccard's matrices based on isozyme data and RAPD marker data. When compared using a Mantel test the matrices were not correlated (r = 0.04). Both markers found high genetic diversity in red clover, but RAPD markers revealed more genetic diversity (mean Jaccard similarity = 0.21) than the biochemical marker (mean Jaccard similarity = 0.30).

Discussion

In this study we found a number of bands and levels of genetic diversity that were in agreement with previous results that scored esterase isozymes patterns as binary data. Lange and Schifino-Wittmann (2000) found 17 bands in four populations of red clover composed by commercial cultivars, locally-developed cultivars and naturalized populations and Malaviya et al. (2005) reported 18 esterase bands for six different species of *Trifolium*. Similar levels of genetic diversity for esterase loci were found by Lange and Schifino-Wittmann (2000) ranging from 0.24 to 0.96 with an average of 0.30 when analyzing eight species of *Trifolium* with four enzymatic systems using binary data. When isozymes were analyzed by allelic frequencies data, we found high levels of polymorphism that were in agreement with other works (Table 1). Although the mean value of percent polymorphic loci was lower than in other previous reports, these results agree with reports for wild as well as to cultivated red clover populations and also indicated that the diversity in red clover is high. Hagen and Hamrick (1998) found for naturalized red clover populations P = 0.68, A = 1.45 and He = 0.250. For wild populations of red clover Semerikov et al. (2002) found He that ranged from 0.1277 to 0.1958 with an P ranging from 0.41 to 0.52 and Mosjidis et al. (2003) reported P = 74, A = 1.7 and He = 0.323. For cultivated red clover Kongkiatngam et al. (1995) reported values of He ranging from 0.10 to 0.12 when comparing two different cultivars and Yu et al. (2001) measured for 34 cultivars of red clover average values of P = 74, A = 1.59 and He = 0.285. For the same core collection Mosjidis and Klingler (2006) reported average values of P = 77.5, A = 1.66 and He = 0.316. We found in this study a lower number of polymorphic loci than the reported by Mosjidis and Klingler (2006) but proportionally the same level of heterozygosity, once with one enzyme system we found

the same number of alleles. Furthermore, esterases are complex and heterogeneous group of enzymes with multiple substrate specificity (Scandalios 1969) and are one of the enzymatic systems with higher polymorphism in plants (Weeden and Wendel 1990). The results found here were in agreement with Mosjidis and Klingler (2006) and indicated that the core collection has a large amount of genetic diversity measured with isozymes. When comparing the distances matrices generated by isozyme binary and allelic frequency data we found a high correlation between these two different types of approach. This type of information may become the interpretation of isozyme patterns less laborious especially when dealing with a high number of individuals, populations and enzymatic systems. There were also concordances between the number of clusters found between isozyme binary and allelic frequency data, as in both dendrograms the populations of red clover were clustered in four groups (Figure 1 and 2).

The principal component analysis made with the allelic frequencies (PCA) (Figure 3) evidenced the clustering of red clover populations in four groups (Figure 3). Globally the clusters found here grouped the populations from the same country together in each of four clusters, this indicate that there is a low genetic variation for the loci examined between the populations from the same country. The clusters were not separated according to their geographic origin or type of populations (cultivar, wild or landrace). However, for group 1 (Figure 2) there was a remarkable presence of cultivars of red clover from Europe high latitudes from countries such as Denmark, Finland, Sweden and Switzerland. Similar results were found by Dias et al. (unpublished) for the same core collection when analyzed using molecular (microsatellites) and morphological markers and these populations were also clustered. According to Taylor and Smith (1995) distinct plant types of red clover have evolved

through natural selection and in Europe these types are distributed largely according to latitude.

The AMOVA (Table 3) evidenced the high percentage of variation placed at the groups and the low percentage of variation attributed to the intrapopulational level. Many works have pointed to the high intrapopulational diversity present in red clover (Mosjidis and Klingler 2006; Yu et al. 2001) examined with different types of isozyme markers. However, other works that compared natural and cultivar populations of red clover have also found important fraction of the genetic diversity placed at the groups and within groups variation (Semerikov and Belyaev 1995; Semerikov et al. 2002). This low genetic diversity within populations found here may be explained by the fact that esterases are enzymes that use several different substrates and respond directly to the environmental diversity (Robinson 1998). Thus, populations of different geographic origins may be more differentiated than individuals placed at the same population. The high genetic diversity found with the RAPD markers were in agreement with other previous reports for red clover when cultivated (Kongkiatngam et al. 1996; Campos-de-Quiroz and Ortega-Klose 2001; Ulloa et al. 2003) or wild populations (Kölliker et al. 2003; Herrmann et al. 2005) of red clover were analyzed.

Although the RAPD data showed a higher degree of molecular variation between the red clover population than isozyme marker, RAPD data did not separate the populations in clusters distinct as those formed by the biochemical data. Although smaller, the genetic diversity found with the isozyme marker was more structured and allowed a more accurate formation of groups than the genetic diversity found with the RAPD markers. Several reasons may explain this absence of correlation between the biochemical and molecular markers. Many works that compared RAPD and isozyme

markers in red clover and in other species have found correlation (Kongkiatngam et al. 1995; Jenczewski et al. 1999) while others found no correlation (Matos et al. 2001; Benchacho et al. 2002; Yanaka et al. 2005) between these two types of markers. The absence of correlation may be linked to the nature of the markers, since RAPD reveals high neutral polymorphism from non-coding regions of the genome. On the other hand, isozymes polymorphism were estimated from coding-regions that may be conserved to maintain the function of the enzymes (Gottlieb 1982). By using biochemical and RAPD marker we showed the high genetic diversity present in the core collection of red clover. The genetic diversity revealed through the biochemical marker showed structuration that clearly separated the populations of Europe high latitudes in the core collection. The other groups had no difference for geographic origin either for type of population according to the classification of USDA-NPGS in cultivars, landrace or wild accessions of red clover. Molecular diversity measured for the first time in the core collection with the RAPD markers was high and in agreement with other works. The high genetic diversity present in the red clover core collection was complex and more accurate passport data for the accessions and the use of different types of markers from different natures may be helpful for the knowledge and better utilization of this rich natural diversity.

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4. CAPITULO IV

4.1 CONSIDERAÇÕES FINAIS

A análise utilizando diferentes ferramentas como os marcadores morfológicos, bioquímicos e moleculares permitiu evidenciar e quantificar a alta diversidade genética presente nas populações da coleção nuclear de trevo vermelho.

Os marcadores moleculares, utilizados pela primeira vez na coleção nuclear, revelaram uma alta variação estruturada principalmente ao nível intrapopulacional.

Os marcadores morfológicos e bioquímicos evidenciaram padrões de distribuição da diversidade de acordo com a origem, evidenciando as similaridades presentes entre algumas cultivares de origem nórdica de trevo vermelho.

As baixas correlações encontradas entre os marcadores refletem a complexidade da diversidade presente na espécie e sugerem a necessidade do uso de diferentes ferramentas e dados de passaporte que auxiliem efetivamente na compreensão global dos padrões de diversidade.

Através da análise com marcadores morfológicos foram evidenciados grupos de populações com maior persistência como as populações presentes no grupo morfológico V bem como maior produção de matéria seca nas condições do sul do Brasil como as populações presentes no grupo morfológico IV, e que poderiam ser utilizadas em programas de melhoramento desta espécie para esta região.

Os caracteres morfológicos que mais contribuíram para a classificação das populações da coleção nuclear de trevo vermelho foram: altura das plantas, hábito de crescimento, área foliar, tamanho de inflorescência, peso das plantas, data de florescimento e persistência.

Os marcadores morfológicos e bioquímicos revelam-se mais eficientes na estruturação da diversidade sugerindo que caracteres ligados à expressão gênica podem auxiliar no entendimento das relações de diversidade dentro da coleção nuclear.

Sugere-se que uso conjunto de diferentes abordagens de análise pode ajudar no esclarecimento da estrutura da diversidade genética na coleção nuclear de trevo vermelho.

A utilização de novas tecnologias, como os marcadores moleculares, pode auxiliar na formação de uma coleção nuclear que represente efetivamente a diversidade genética presente em trevo vermelho.

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4.3 APÊNDICES

Apêndice 1. Análise de Variância dos dados morfológicos de 58 acessos de trevo vermelho

The GLM Procedure
Class Level Information

Class	Levels	Values
Condition	2	Campo Experimental Casa de Vegetação
Population	58	1 3 5 6 7 11 12 13 15 18 21 23 24 26 27 28 29 30 31 32 33 35 36 37 38 39 40 41 42 46 47 49 51 52 53 54 55 56 57 58 60 61 62 63 64 65 67 68 69 70 72 73 75 76 77 78 79 80

Number of observations 1160
Dependent Variables With Equivalent Missing Value Patterns

Pattern	Obs	Dependent Variables
1	1059	IAF
2	1108	Hábito
3	1057	Peso Planta
4	1127	Altura Planta
5	879	Tamanho inflorescência
6	1160	Doenças Cor inflorescência
7	726	Número de flores

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

The GLM Procedure

Dependent Variable: IAF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	7457.77235	64.85019	5.77	<.0001
Error	943	10593.74322	11.23409		
Corrected Total	1058	18051.51558			

R-Square	Coeff Var	Root MSE	IAF Mean
0.413138	33.49951	3.351729	10.00531

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	1827.011818	1827.011818	162.63	<.0001
Population	57	4594.092728	80.598118	7.17	<.0001
Condition*Population	57	902.245241	15.828864	1.41	0.02

The GLM Procedure

Dependent Variable: Hábito

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	360.0432841	3.1308112	32.49	<.0001
Error	992	95.5984127	0.0963694		
Corrected Total	1107	455.6416968			

R-Square	Coeff Var	Root MSE	Habito Mean
0.790189	21.48414	0.310434	1.444946

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	9.5150874	9.5150874	98.74	<.0001
Population	57	287.5341205	5.0444583	52.35	<.0001
Condition*Population	57	54.9816694	0.9645907	10.01	<.0001

Apêndice 1. Continuação… Análise de Variância dos dados morfológicos de 58 acessos trevo vermelho

The GLM Procedure

Dependent Variable: Peso planta

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	384756.5180	3345.7089	7.51	<.0001
Error	941	419295.0942	445.5846		
Corrected Total	1056	804051.6122			

R-Square	Coeff Var	Root MSE	Poid Mean
0.478522	82.04132	21.10887	25.72956

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	270149.9960	270149.9960	606.28	<.0001
Population	57	63507.5218	1114.1670	2.50	<.0001
Condition*Population	57	50470.0242	885.4390	1.99	<.0001

The GLM Procedure

Dependent Variable: Altura Planta

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	29229.71151	254.17140	7.34	<.0001
Error	1011	35031.87500	34.65072		
Corrected Total	1126	64261.58651			

R-Square	Coeff Var	Root MSE	TaillePlant Mean
0.454855	32.86959	5.886486	17.90861

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	1809.72476	1809.72476	52.23	<.0001
Population	57	22234.03414	390.07077	11.26	<.0001
Condition*Population	57	5265.84449	92.38324	2.67	<.0001

The GLM Procedure

Dependent Variable: Tamanho inflorescência

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	71.0100900	0.6174790	3.02	<.0001
Error	763	155.7872024	0.2041772		
Corrected Total	878	226.7972924			

R-Square	Coeff Var	Root MSE	TailleFluer Mean
0.313099	15.51321	0.451860	2.912742

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	17.83432678	17.83432678	87.35	<.0001
Population	57	38.79830516	0.68067202	3.33	<.0001
Condition*Population	57	17.93176031	0.31459229	1.54	0.0077

Apêndice 1. Continuação… Análise de Variância dos dados morfológicos de 58 acessos trevo vermelho

The GLM Procedure

Dependent Variable: Doenças

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	1219.131034	10.601139	5.75	<.0001
Error	1044	1923.200000	1.842146		
Corrected Total	1159	3142.331034			

R-Square	Coeff Var	Root MSE	Maladie Mean
0.387970	109.6391	1.357257	1.237931

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	364.2241379	364.2241379	197.72	<.0001
Population	57	490.9310345	8.6128252	4.68	<.0001
Condition*Population	57	363.9758621	6.3855414	3.47	<.0001

The GLM Procedure

Dependent Variable: Cor inflorescência

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	16.91379310	0.14707646	Infty	<.0001
Error	1044	0.00000000	0.00000000		
Corrected Total	1159	16.91379310			

R-Square	Coeff Var	Root MSE	Coulefleur Mean
1.000000	0	0	75.09138

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	0.00344828	0.00344828	Infty	<.0001
Population	57	16.81379310	0.29497883	Infty	<.0001
Condition*Population	57	0.09655172	0.00169389	Infty	<.0001

The GLM Procedure

Dependent Variable: Número de flores

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	115	66910.9914	581.8347	7.18	<.0001
Error	610	49406.7841	80.9947		
Corrected Total	725	116317.7755			

R-Square	Coeff Var	Root MSE	Fleurs Mean
0.575243	118.8610	8.999707	7.571625

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	14342.33421	14342.33421	177.08	<.0001
Population	57	25410.55088	445.79914	5.50	<.0001
Condition*Population	57	24880.89623	436.50695	5.39	<.0001

Apêndice 2. Planilha de entrada de dados para análise de componentes principais de dados morfológicos de 56 populações de trevo vermelho. Variáveis avaliadas : X1=peso planta CV, X2= peso planta CE, X3= altura planta Cv, X4= altura planta CE, X5= tamanho flor CV, X6= tamanho flor CE , X7= Doenças , X8= N° de flores CV , X9 = N° de flores CE , X10= Filocrono CV, X11=hádito CE, X12= persistência CV, X13= persistência CE, X14= floração CV, X15= floração CE. CE= Campo experimental, CV= Casa de vegetação.

Acesso	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15
1	8.15	41.10	18.1	20.4	2.68	1.83	0.4	3.13	2.00	11.38	1	40	10	139	208
3	8.22	40.65	20.9	18.9	2.53	0.5	2	4.10	15.50	8.57	1.2	80	0	131	208
5	13.31	18.02	18.5	17.5	1.25	0.85	1.2	2.75	1.00	6.98	1	100	30	186	208
6	11.11	37.00	16.9	15.6	2.75	1.15	1.4	4.67	5.20	7.19	0.8	90	30	131	208
7	12.02	18.46	17.4	14	3.2	2.08	0.7	3.22	32.50	5.99	1.2	90	10	131	208
11	13.10	27.75	21.4	15.5	0.9	0.55	1.1	4.25	15.00	7.69	1.1	80	40	186	208
12	11.04	34.74	17	15.6	2.1	1.8	1.7	4.60	4.40	6.74	1	60	20	131	235
13	9.44	31.02	23.5	18.3	2.43	1.65	2.1	3.00	37.00	7.53	1	70	0	104	208
15	13.31	38.59	21.1	22.8	2.8	0.2	2.1	3.50	21.00	7.27	1.1	90	0	104	165
18	12.43	38.06	19.9	15.8	2.43	1.23	1.7	2.11	2.00	8.49	1.1	90	30	131	208
21	11.13	49.90	18.5	19.2	2.25	1.45	4	2.33	2.80	10.37	1	100	0	131	235
23	10.60	38.89	17.7	21.1	2.3	1.65	2.8	3.88	1.00	7.67	1	70	30	131	168
24	10.82	46.58	21.5	15.8	2.98	1.88	1.6	2.89	14.00	8.36	1.2	90	40	131	208
26	7.70	38.83	17.7	19.5	2.1	1.5	0.7	2.33	2.17	10.47	1.1	60	20	131	208
27	8.85	47.74	18.2	25	2.55	0.71	0.1	2.20	6.00	7.97	1	100	50	131	208
28	13.23	46.18	17.3	19.2	2.9	0.9	1.7	4.00	8.50	9.33	1.1	80	30	131	208
29	9.27	59.44	15.9	21.3	3	0.83	1.2	2.70	13.75	9.63	1.4	80	30	104	208
30	4.77	40.29	8.8	14.8	2.9	1	1	2.89	34.80	13.36	2	80	30	104	208
31	4.64	14.06	2.9	6.4	2.85	1.59	1	4.00	29.71	10.71	2.9	100	10	104	208
32	6.70	36.81	15.2	17.6	3	1.16	0.3	2.80	6.40	9.21	0.9	80	40	104	208
33	6.61	27.35	12.1	13.9	3.3	2.21	2.1	3.30	16.00	8.05	1.1	80	60	131	208
35	5.05	31.84	6	9.5	2.95	1.41	2	3.57	15.83	8.18	2.3	100	60	139	208
36	10.12	42.33	4.9	4.4	2.25	1.8	0.7	2.00	1.75	6.87	2.5	90	50	147	235
37	6.50	23.94	17	19.7	2.75	1.6	2.8	3.00	12.67	8.10	0.9	70	40	97	208
38	7.95	42.79	16.2	25.7	2.95	1.23	1.9	4.78	24.00	6.83	1	90	10	97	208
39	6.30	70.95	20.5	22.3	2.6	0.2	2.5	1.63	1.00	7.02	1.3	50	40	104	208
40	21.08	30.56	23.6	17.9	2.65	0.5	1.6	4.78	1.50	4.58	0.8	70	30	104	208
41	5.28	40.12	16.5	26.6	2.35	0.9	2.8	1.75	7.25	6.44	1	30	10	125	208

Apêndice 2. Continuação... Planilha de entrada de dados para Análise de componentes principais de dados morfológicos de 56 populações de trevo vermelho.

Acesso	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15
42	18.92	30.03	18.3	18.4	2.35	1.83	2	3.38	4.33	7.11	0.9	70	30	125	208
46	20.00	71.45	16.1	16.9	3.4	2.71	3.2	3.88	40.83	7.21	1.4	90	30	97	208
47	22.07	72.76	16.4	17.5	3.27	2.08	1.9	6.50	17.86	6.43	1.5	70	40	97	208
49	17.05	54.03	18.9	17	3.05	1.8	0.5	4.50	15.50	6.98	0.7	80	60	97	208
51	6.02	42.40	18.5	23.1	2.8	2.27	0.3	3.71	10.00	8.75	0.8	70	30	97	208
52	8.55	47.55	16.3	24.8	2.65	1.6	1.5	2.70	2.67	6.12	1.1	80	40	97	208
53	7.63	30.83	10.2	20.9	3.05	2.45	0.1	3.70	53.90	7.54	1.7	100	10	97	208
54	6.87	31.31	10.1	11.8	3.05	1.63	0.5	3.50	29.71	7.64	1.7	70	0	104	208
55	7.13	15.03	6.3	6	2.85	1.08	1	4.30	24.40	7.56	1.9	80	0	104	208
56	8.10	55.21	15.7	19.1	3.03	1.78	1.2	3.67	15.00	7.09	1.2	60	30	97	208
57	6.99	32.21	14	15.3	2.5	2.26	0.9	2.90	12.00	6.16	0.9	80	20	97	208
58	7.98	34.81	14.9	17.4	3.2	2.44	0.8	3.50	4.33	9.03	0.8	90	20	97	208
60	5.67	34.12	13.7	9.9	3.15	2.36	2.2	3.80	36.50	7.75	1.5	80	10	97	208
61	9.19	13.75	15.2	14.9	1.3	1.35	0.9	4.60	4.67	9.37	1	80	30	131	235
62	6.19	41.17	16.2	18	2.73	1.68	2	3.13	56.00	9.77	0.9	70	10	131	208
63	8.70	47.38	18.5	23.5	2.38	1.62	1.6	4.00	13.00	7.34	0.7	60	60	97	208
64	6.58	48.12	17.7	21.4	3.15	2.61	1.7	4.90	6.25	9.57	1.1	100	40	97	208
65	7.21	43.97	17.1	22.6	3.05	1.94	1.7	3.50	10.20	7.21	1.1	70	40	104	208
67	8.85	49.16	17.2	22.2	3	2.37	1.3	4.50	30.50	8.64	1.1	70	40	97	208
68	10.70	55.77	24.6	24.3	3.05	2.54	1.1	3.90	18.71	8.30	0.9	30	20	97	208
69	5.78	60.29	16.4	18	2.55	1.55	3.1	2.29	10.50	7.35	1.5	50	30	131	208
70	5.29	56.70	15.3	20	2.8	2.19	2.5	1.71	28.50	8.14	1.2	70	10	97	208
72	11.71	37.52	17.3	15.6	2.45	1.88	2.4	4.00	29.00	9.72	1.7	70	30	125	208
73	15.49	68.94	18.8	22.5	3.39	2.74	3.8	6.40	15.00	7.66	1.1	70	60	97	208
75	14.17	52.59	28.6	26.3	3.3	2.77	3.6	5.78	17.29	8.73	1.2	50	30	104	208
76	12.20	63.02	18.3	29.2	2.35	2.03	4.7	5.86	5.00	7.87	1.1	0	10	131	208
77	8.61	57.13	21.9	23.7	3.28	2.87	3.6	4.33	6.50	8.26	1.4	0	20	131	208
78	7.26	43.95	15.5	15.4	3.43	2.82	5.1	6.70	8.17	9.33	1.4	0	50	97	208
79	11.55	56.10	19.5	13.2	3.05	2.53	2.2	5.30	9.00	6.17	1.4	80	20	125	208
80	9.44	32.36	14.1	17.8	2.67	1.91	1.7	4.00	21.00	8.48	1.2	100	40	158	208

Apêndice 3. Tabela entrada de dados para cálculo de PIC de 7 loci SSR em 56 populações de trevo vermelho

Calculo PIC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
RCS3095	0.5	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0	0.5	0	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5
RCS1737	0	0	0.5	1	0.5	1	0.5	1	0	0.5	0	0	0.5	0	0	0.5	0	0.5	0	0	0	0.5	0	0	0	0	0.5	1
RCS1518	0	0	0.5	1	0	1	0	1	0	0.5	0	0.5	0.5	0	0	0	0	0.5	0	0	1	0	0	0	0	0	0.5	0
RCS0033	0.5	1	0	0	0	0	0	0	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	1	0.5	0.5	0.5	0	0	0	0	0	0.5	0
RCS3102	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0	0.5	0	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5
RCS0793	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
RCS1868	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0	0.5	0.5	0	0.5	0.5	0.5	0.5	0	0.5	0.5	0	0.5	0.5	0.5	0	0	0.5	0

Calculo PIC	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
RCS3095	0.5	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0
RCS1737	0	0	0	0	0	0.5	0	0	0.5	1	0.5	0	0.5	0.5	0	0.5	0	0	0.5	0.5	0	0	1	0	0	1	0	0
RCS1518	0	1	0	0.5	1	0.5	0	1	0.5	0.5	1	0.5	0.5	0	0.5	0	0	0.5	0.5	0	0	0	1	0	0	1	0	0.5
RCS0033	0	0.5	0	0.5	0	0.5	0.5	0.5	0.5	0.5	0	0	0.5	0	0	0	0	1	0.5	0	0	0	0	0.5	0	0	0.5	0.5
RCS3102	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	1	0.5	0.5	0	0	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0
RCS0793	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0	0.5
RCS1868	0	0	0	0.5	0.5	0.5	0.5	0.5	0	0	0.5	0.5	0.5	0.5	0	0.5	0	0	0.5	0.5	0	0	0.5	0	0.5	0.5	0.5	0

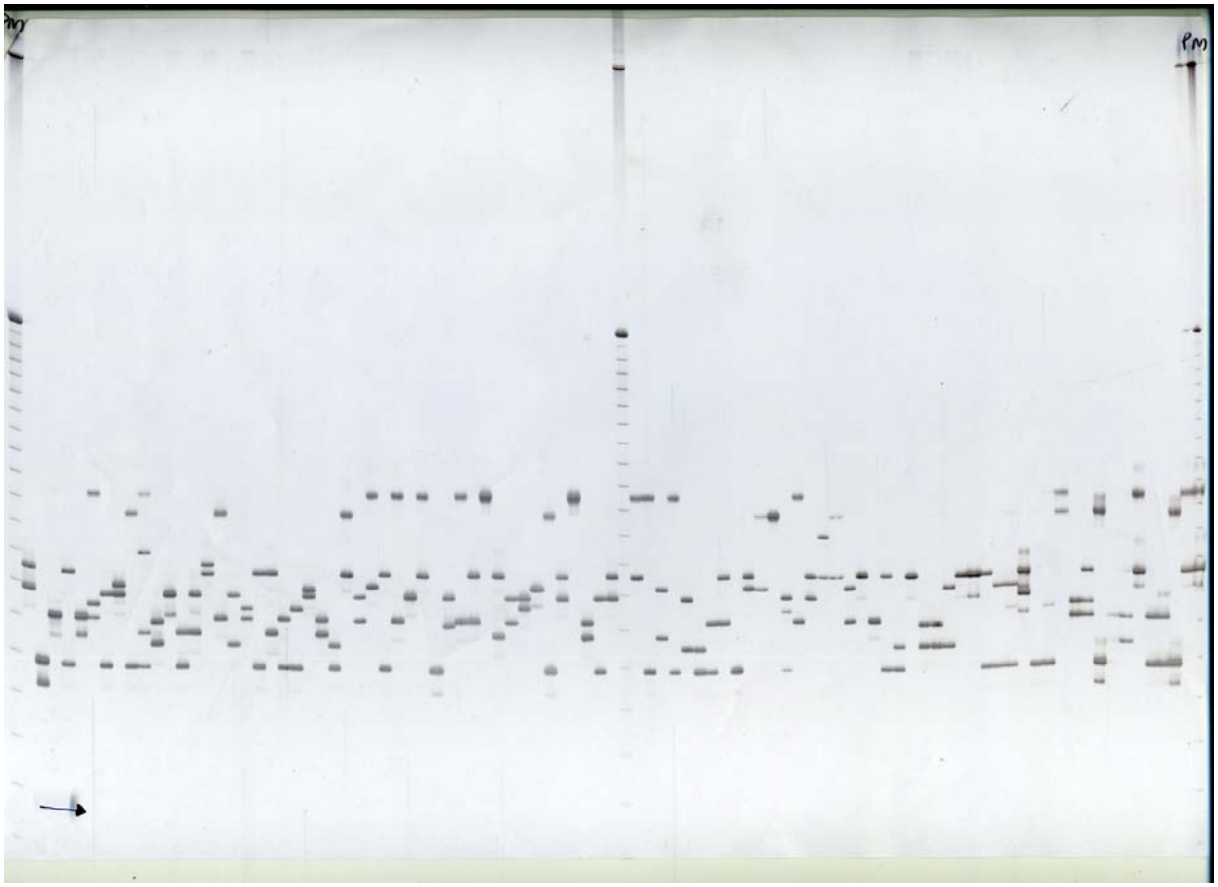
Apêndice 4. Parâmetros populacionais de 7 loci SSR em 56 populações de trevo vermelho He= Heterozigosidade esperada, Ho= Heterozigosidade observada, P= polimorfismo, número de alelos /Locus.

	Hexp.	Hobs.	P	Alelos/Locus
1	0.71	0.71	0.71	1.71
2	0.57	0.57	0.57	1.57
3	0.80	0.80	0.80	1.80
4	0.71	0.71	0.71	1.71
5	0.60	0.60	0.60	1.60
6	0.71	0.71	0.71	1.71
7	0.86	0.86	0.86	1.86
8	0.71	0.71	0.71	1.71
9	0.57	0.57	0.57	1.57
10	0.57	0.57	0.57	1.57
11	0.43	0.43	0.43	1.43
12	0.57	0.57	0.57	1.57
13	0.67	0.67	0.67	1.67
14	0.57	0.57	0.57	1.57
15	0.71	0.71	0.71	1.71
16	0.83	0.83	0.83	1.83
17	0.43	0.43	0.43	1.43
18	0.71	0.71	0.71	1.71
19	0.67	0.67	0.67	1.67
20	0.71	0.71	0.71	1.71
21	0.57	0.57	0.57	1.57
22	0.57	0.57	0.57	1.57
23	0.43	0.43	0.43	1.43
24	0.14	0.14	0.14	1.14
25	1.00	1.00	1.00	2.00
26	0.50	0.50	0.50	1.50
27	0.43	0.43	0.43	1.43
28	0.67	0.67	0.67	1.67
29	0.29	0.29	0.29	1.29
30	0.57	0.57	0.57	1.57
31	0.67	0.67	0.67	1.67
32	1.00	1.00	1.00	2.00
33	0.71	0.71	0.71	1.71
34	0.83	0.83	0.83	1.83
35	0.83	0.83	0.83	1.83
36	0.67	0.67	0.67	1.67
37	0.86	0.86	0.86	1.86
38	0.71	0.71	0.71	1.71
39	0.33	0.33	0.33	1.33
40	0.71	0.71	0.71	1.71
41	0.71	0.71	0.71	1.71
42	0.43	0.43	0.43	1.43
43	0.67	0.67	0.67	1.67
44	0.43	0.43	0.43	1.43
45	0.50	0.50	0.50	1.50
46	1.00	1.00	1.00	2.00
47	0.40	0.40	0.40	1.40
48	0.86	0.86	0.86	1.86
49	0.43	0.43	0.43	1.43
50	0.29	0.29	0.29	1.29
51	0.80	0.80	0.80	1.80
52	0.57	0.57	0.57	1.57
53	0.57	0.57	0.57	1.57
54	0.40	0.40	0.40	1.40
55	0.57	0.57	0.57	1.57
56	0.43	0.43	0.43	1.43
Mean	0.62			

Apêndice 5. Dados de 7 loci SSR de 56 populações de trevo vermelho

SSR Loci	Allele Sizes	Alleles observed	PIC value	H exp.	H obs.
RCS3095	196-236	11	0.86	0.872	0.786
RCS1737	72-111	11	0.88	0.856	0.347
RCS1518	155-189	10	0.89	0.851	0.348
RCS0033	135-222	18	0.91	0.905	0.453
RCS3102	152-192	11	0.87	0.872	0.796
RCS0793	177-234	11	0.88	0.886	0.875
RCS1868	159-174	6	0.70	0.710	0.643
Total		78			
Mean (SD)		11.1		0.85 (0.06)	0.606 (0.21)

Apêndice 6. Gel de SSR *locus* RCS 0793 de 56 indivíduos de trevo vermelho.



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References
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Saunders DS (1976) The biological clock of insects. Sci Am 234(2):114–121
3. Journal issue with issue editor:

Smith J (ed) (1998) Rodent genes. Mod Genomics J 14(6):126–233
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Mod Genomics J (1998) Rodent genes. Mod Genomics J 14(6):126–233
5. Book chapter:

Brown B, Aaron M (2001) The politics of nature. In: Smith J. The rise of modern genomics, 3rd edn. Wiley, New York

6. Book, authored:
South J, Blass B (2001) The future of modern genomics. Blackwell, London

7. Book, edited:
Smith J, Brown B (eds) (2001) The demise of modern genomics. Blackwell, London

8. Chapter in a book in a series without volume titles:
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Smith SE (1976) Neuromuscular blocking drugs in man. In: Zaimis E (ed) Neuromuscular junction. Handbook of experimental pharmacology, vol 42. Springer, Berlin Heidelberg New York, pp593–660

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Chung S-T, Morris RL (1978) Isolation and characterization of plasmid deoxyribonucleic acid from Streptomyces fradiae. In: Abstracts of the 3rd international symposium on the genetics of industrial microorganisms, University of Wisconsin, Madison, 4–9 June 1978

13. Paper presented at a conference:

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14. Patent:

Name and date of patent are optional Norman LO (1998) Lightning rods. US Patent 4,379,752, 9 Sept 1998

15. Dissertation:

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California
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20.1. Online database
Healthwise Knowledgebase (1998) US Pharmacopeia, Rockville. http://www.healthwise.org. Cited 21 Sept 1998

Supplementary material/private homepage
Doe J (2000) Title of supplementary material. http://www.privatehomepage.com. Cited 22 Feb 2000
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Schnurbusch, T., C. Möllers, and H. C. Becker, 2000: A mutant of Brassica napus with increased palmitic acid content. Plant Breeding 119, 141-144.

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4.4 VITA

Paula Menna Barreto Dias, nasceu em São Borja em 01 de janeiro de 1975 é filha de Clemar Dias e Maria da Graça Menna Barreto Dias. Realizou o ensino fundamental em São Borja e mudou-se para Porto Alegre onde cursou o ensino médio. Formou-se em Ciências Biológicas em 1998 pela Universidade Federal do Rio Grande do Sul.

De 1995 a 1998 realizou trabalho como bolsista de iniciação científica da FAPERGS e CNPq no Departamento de Genética da UFRGS, sob orientação da professora Nance Nardi.

Ingressou em 1999 como bolsista de Apoio técnico do CNPQ para prestação de serviço no auxílio da montagem e manutenção do Laboratório de Análises Genéticas do Departamento de Plantas Forrageiras e Agrometeorologia da Faculdade de Agronomia da UFRGS.

Em 2000 ingressou como aluna do curso de Mestrado em Zootecnia da Faculdade de Agronomia da UFRGS. Conclui o curso de mestrado em março 2003.

Ingressou em 2003 no curso de Doutorado em Zootecnia da mesma instituição. Em 2005 realizou estágio de doutorado sanduíche como bolsista do programa CAPES-COFECUB, no Institut National de la Recherche Agronomique (INRA) de Lusignan na equipe de melhoramento genético de plantas forrageiras.