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Swiss Mattenklee landraces, a distinct and diverse genetic resource of red clover (*Trifolium pratense* L.)

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Abstract Genetic variability within and among 19 landraces and cultivars of red clover (*Trifolium pratense* L.) was investigated by means of amplified fragment length polymorphism (AFLP) analysis in order to assess the potential value of Swiss Mattenklee landraces as genetic resources for plant breeding and the preservation of biodiversity. Populations were classified into three groups according to their origin and agronomic features: Mattenklee landraces (8), Mattenklee cultivars (8) and field clover cultivars (3). Analysis of molecular variance based on 276 polymorphic AFLP markers revealed 80% of total variability to be due to variability within populations while 12% were attributed to variability among groups. Stepwise discriminant analysis identified a subset of 126 AFLP markers which best separated individual plants into the three respective groups. Genetic distances between populations were considerably larger among groups than among populations within the same group, providing further evidence for the genetic distinction between Mattenklee landraces, Mattenklee cultivars and field clover cultivars. AFLP markers identified two landrace clusters, containing three and four populations respectively, which, together with one additional landrace, may sufficiently represent the genetic variability of all eight landraces investigated. The results of this study strongly suggest that Swiss Mattenklee landraces form a genetically distinct group of red clover. The data obtained provide criteria on how to efficiently manage, preserve and exploit Mattenklee germplasm.

Keywords Mattenklee · *Trifolium pratense* L. · Landraces · Genetic variability · AFLP

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Introduction

To efficiently preserve, manage and exploit genetic resources, detailed knowledge on the genetic variability within a germplasms collection is indispensable. Such information may assist plant breeders in deciding which germplasms to include in breeding programs and may also allow the identification of accessions that substantially contribute to the overall diversity of the species (Grenier et al. 2000).

Landraces, also known as local populations, traditional cultivars or farmers varieties (Zeven 1998), provide a valuable resource for plant breeding as well as for the preservation of genetic diversity. In various crops such as wheat (Skovmand et al. 2001), barley (Lakew et al. 1997), rice (Yang et al. 1994), maize (Zeven 2000) and pearl millet (Ouendeba et al. 1995), numerous landraces have been collected, characterised and exploited for several purposes. Landraces may be used as starting populations for cultivar development (Lakew et al. 1997) or as sources for the introgression of genes and QTLs conferring resistance to biotic (Huang et al. 1997) and abiotic stresses (Forster et al. 2000). In some forage legumes (e.g. red clover, *Trifolium pratense* L.), landraces may be of particular value since modern cultivars are genetically not as far advanced as compared to other crops such as grain cereals (Hill et al. 1988; Woodfield and Caradus 1994). Although a number of landraces have been described and utilised in forage crops such as alfalfa (*Medicago sativa* L.; Julier 1996), white clover (*Trifolium repens* L.; Annicchiarico and Piano 1997) and red clover (*T. pratense* L.; Kouamé and Quesenberry 1993), there is little information available on the genetic variability of such populations and their relationship to modern cultivars.

Molecular markers allow for a rapid assessment of genetic diversity directly at the genome level and have been extensively used to characterise genetic resources in various plant species (Fahima et al. 1999; Crouch et al. 2000; Grenier et al. 2000; Semagn et al. 2000). Amplified fragment length polymorphism (AFLP; Vos et al. 1995)

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markers are particularly useful for diversity studies. The technique is based on generic PCR primers and allows the detection of a large number of loci in a single assay (Powell et al. 1996).

Red clover, *T. pratense* L., is one of the most important forage legumes of temperate climates. Due to its ability to fix atmospheric nitrogen and its high nutritive value (Taylor and Quesenberry 1996), it is widely used for grass-clover leys in crop rotation and also is an important component of permanent pastures and meadows. Red clover is an extremely polymorphic, diploid species (2n = 14). Zohary and Heller (1984) identified more than 40 forms or varieties, but no detailed taxonomy of this species has yet been established (Taylor and Quesenberry 1996). However, this extensive variability is only partly exploited. Of 4,233 classified red clover accessions, only 42% are advanced cultivars, 12% are landraces and the remaining 45% consist of wild ecotypes (Taylor and Quesenberry 1996). Landraces and ecotypes of red clover not only form a valuable source for breeding, they substantially contribute to the biodiversity of natural and managed grasslands. Changes in land-use patterns and the increased use of elite cultivars threaten the genetic diversity of native and naturalised ecotypes and emphasises the need for an efficient management of genetic resources (Morris and Greene 2001).

Red clover cultivation in Europe dates back to the third century and reached economic importance in Flanders around 1600. From there, cultivation spread rapidly to most temperate regions of the world (Taylor and Quesenberry 1996). In Switzerland, farmers probably started red clover seed production in the 18th century based on Flemish plant material (Merkenschlager 1934; Koblet and Nüesch 1960). Seed was traditionally harvested in the third year after sowing or later. Thus, a certain selection for persistent genotypes was practiced. Over time, a Swiss form of red clover, known as Mattenklee, was developed, which is characterised by increased persistency and early flowering (Koblet and Nüesch 1960; Nüesch 1976). Mattenklee landraces specifically adapted to local climates and conditions were developed and maintained on individual farms. Germplasm from Mattenklee landraces was also integrated in the Swiss clover breeding program and today a broad range of Mattenklee cultivars is available and widely used throughout Europe (Boller 2000a). Mattenklee cultivars and landraces are distinguished from other forms of red clover mainly by their improved persistency which makes them particularly useful for ley farming systems. Less persistent forms of red clover are generally used in shorter crop rotation and are, in this paper, referred to as field clover.

Seed from improved red clover cultivars became widely available in the late 20th century. Thus, the use of Mattenklee landraces declined dramatically. Today only small seed lots of approximately 100 old landraces are still available, which were collected on Swiss farms in 1971/72 (Nüesch 1976). These landraces may provide a valuable genetic resource for the further advancement of red clover cultivars as well as for the preservation of genetic diversity in less intensively managed pastures and meadows. However, there is no information available on their genetic compositions or structures. Although isozyme and RAPD markers have been used to characterise cultivars from North America, Europe, Japan and Chile (Kongkiatngam et al. 1996; Campos-De-Quiroz and Ortega-Klose 2001; Yu et al. 2001), there is a general lack of studies on the molecular characterisation of ecotypes and landraces of red clover.

In the present study we used AFLP markers to assess the potential value of landraces of red clover as genetic resources. The objectives were: (1) to characterise genetic variability within and among Mattenklee landraces and cultivars; (2) to determine whether Mattenklee landraces form a distinct group of red clover; and (3) to compare genetic diversity between Mattenklee populations and field clover cultivars.

Materials and methods

Plant material

The 19 populations of red clover (*T. pratense* L.) analysed in this study represented three groups according to their origin and their agronomic features (Table 1). Mattenklee landraces (group I) consisted of old landraces collected in 1971/72 from Swiss farms where they were maintained for many decades (Nüesch 1976). Mattenklee cultivars (group II) are Swiss cultivars of red clover which are distinguished from field clover cultivars (group III) mainly through their improved persistence. In addition, the white clover (*T. repens*) cultivar Bombus was included in the analysis as a reference for the comparison of genetic distances. Plant material from 24 randomly selected individual plants of each landrace and each cultivar was collected for AFLP analysis. Individual plants will be referred to as genotypes, while the term population will be used for landraces and cultivars and the term group refers to the three types of populations, i.e. Mattenklee landraces, Mattenklee cultivars and field clover cultivars.

AFLP analysis

DNA was extracted from fresh plant material using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) and quantified using PicoGreen (Molecular Probes, Eugene, Ore., USA) and a fluorimeter (LS-30; Perkin Elmer Instruments, Shelton, Conn., USA) as well as by visual inspection on a 1% (w/v) agarose gel.

AFLP analysis was performed following the method of Vos et al. (1995). AFLP templates were prepared by restriction digestion and adaptor ligation of 1 μg genomic DNA. Adaptors and primers (see Table 2) were synthesised by Microsynth, Balgach, Switzerland. *Eco*RI + C and *Mse*I + A primers were used to amplify double-digested, adaptor ligated DNA (20 ng) in a 20-μl reaction containing 1 × PCR buffer, 5 pmol of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP and 0.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif., USA). PCR was performed in a MJ PT-200 (MJ Research, Waltham, Mass., USA) thermocycler using an initial denaturation step of 2 min at 94 °C, 26 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. Amplification products were diluted by adding 100 μl of H_2O. For subsequent selective amplification, *Eco*RI and *Mse*I primers with three additional nucleotides each were used (see Table 2). Selective amplification reactions contained 1 μl of diluted pre-amplified AFLP template, 5 pmol of 6-FAM, HEX or TET labelled*Eco*RI + CNN primer, 6 pmol of *Mse*I

Table 1 Red clover (*T. pratense* L.) landraces and cultivars and white clover (*T. repens* L.) cultivar used for investigation

Name	Origin[a]	Elevation (m asl)	Latitude (N)	Longitude (E)	Last year of selection
Group I: Mattenklee landraces (ML)					
LR8	Bubikon	510	8°49'	47°16'	
LR127	Zäziwil	700	7°40'	46°54'	
LR189	Sumiswald	700	7°45'	47°02'	
LR239	Köniz	680	7°25'	46°56'	
LR292	Lanzenhäusern	810	7°21'	46°51'	
LR300	Burgistein	830	7°30'	46°47'	
LR325	Affoltern i.E.	800	7°44'	47°04'	
LRDet	Dettenbühl	508	7°38'	47°15'	
Group II: Mattenklee cultivars (MC)					
Corvus	Zurich				1991
Formica	Zurich				1989
Merula	Zurich				1994
Milvus	Zurich				1990
Pavo	Zurich				1995
Pica	Zurich				1991
Renova	Zurich				1964
Rüttinova	Zurich				1980
Group III: Field clover cultivars (FC)					
Lucrum	Germany				n.a.[b]
Merviot	Belgium				n.a.
Mont Calme	Changins				1970
White clover					
Bombus	Zurich				1994

[a] Location of collection (landraces) or cultivar development (cultivars). Unless otherwise mentioned, all localities are situated in Switzerland
[b] Information not available

+ CNN primer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP and 0.4 U of *Taq* DNA polymerase in a total volume of 20 μl. PCR was performed using a touchdown PCR protocol with an initial denaturation of 2 min at 94 °C, 12 cycles of 1 min at 94 °C, 30 s at 65 °C (–0.7 °C per cycle) and 1 min at 72 °C, followed by 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C with a final extension of 5 min at 72 °C. AFLP fragments were analysed on an ABI PRISM 310 Genetic Analyzer using a POP 4 polymer and a 47 cm × 50 μm capillary (Applied Biosystems, Foster City, Calif., USA).

Data analyses

AFLP patterns were analysed using Genescan 3.1 and Genotyper 3.7 software (Applied Biosystems). AFLP markers were visually scored for presence (1) or absence (0) and entered into a binary matrix containing the AFLP profile of each genotype. Each AFLP pattern was independently scored by two different persons and only polymorphic bands which could be scored unequivocally were included in the analysis. Five genotypes were excluded from further analysis due to repeatedly unscoreable AFLP patterns.

The binary data matrix with no missing values was subjected to multivariate analysis procedures as described by Semagn et al. (2000) using the SAS v. 8.0 statistical package (SAS Institute, Cary, N.C., USA). (1) Principle component analysis was carried out using the SAS procedure PROC PRINCOMP and the entire set of polymorphic markers obtained through AFLP analysis; (2) PROC STEPDISC was employed to identify a subset of AFLP markers which were the best discriminating factors among the 19 red clover populations (P = 0.15 for adding and retaining variables); and (3) canonical discriminant functions were calculated using the previously identified subset of AFLP markers and the SAS procedure PROC CANDISC.

Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to calculate variance components within and among groups and populations. Pairwise genetic distances between populations were calculated using the coancestry coefficient of Reynolds et al. (1983). Significance of variance components and differentiation between populations was tested using a non-parametric procedure based on 1,000 permutations according to Excoffier et al. (1992). AMOVA and coancestry coefficients were computed using the Arlequin 2.0 software (Schneider et al. 2000). Cluster analysis was performed on coancestry coefficients using the unweighted pair-group procedure with an arithmetic mean (UPGMA) of the NTSYS-pc 2.1 software (Rohlf 2000). Reliability of the clustering was tested by computing Mantel test statistics for the correlation of the distance matrix and the cophenetic matrix. Bootstrap analysis was performed on marker frequencies per population using the software package PHYLIP (Felsenstein 2002) with 10,000 replicated datasets and Reynold's genetic distance. Bootstrap values were transferred to the dendrogram obtained by cluster analysis of coancestry coefficients which showed identical topology as the dendrogram obtained through bootstrap analysis. Genetic variability within populations was estimated by calculating the average pairwise Euclidean distance (Excoffier et al. 1992; Huff et al. 1993) among genotypes.

A model-based approach as described by Pritchard et al. (2000) was used to infer population structure on the data set and to assign individuals to a pre-defined number of populations. This method is suitable to demonstrate the presence of population structure and to identify a meaningful number of populations present in the data. Calculations were performed using the Structure 2.0 software (http://pritch.bsd.uchicago.edu; Pritchard and Wen 2002). Independent runs for K (the number of populations) between 1 and 20 were performed with two different models (no admixture and the admixture model) based on 100,000 iterations.

Results

Characteristics of red clover AFLP markers analysed

The six AFLP primer combinations generated a total of 276 polymorphic markers, ranging in size from 60 to 345 base pairs (Table 2). The number of polymorphic markers detected with each primer combination ranged from 36 to 58 with an average of 46. Each of the 451 genotypes was characterised through a unique AFLP phenotype (data not shown). The number of polymorphic markers within each red clover group ranged from 215 for field clover cultivars to 267 for Mattenklee landraces. Within individual populations, the lowest number of polymorphic markers was found in the Mattenklee cultivar Pica with 151 markers, while the Mattenklee landrace LR239 showed with 229 the highest number of polymorphic markers (data not shown). Although marker frequencies varied considerably among groups and populations, no group- or population-specific markers were identified.

Distinction of groups

Principle component analysis based on all 276 polymorphic markers resulted in a moderate separation of the 451 genotypes into the three respective groups (Fig. 1). While the first three principle components (PCs) explained 17% of the total variation among samples, each of the following PCs explained less than 2%. The relatively poor separation of the three groups is also illustrated by the low level of genetic variation attributed to variation among groups (12%) as revealed by analysis of molecular variance (AMOVA; Table 3A).

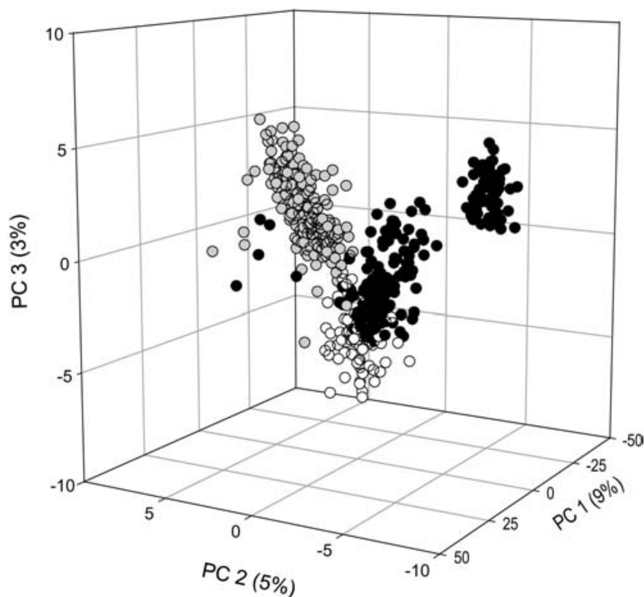

Fig. 1 Principle component analysis of 451 *T. pratense* L. plants from 19 populations and three groups using 276 AFLP markers (*black* = Mattenklee landraces; *grey* = Mattenklee cultivars; *white* = field clover cultivars)

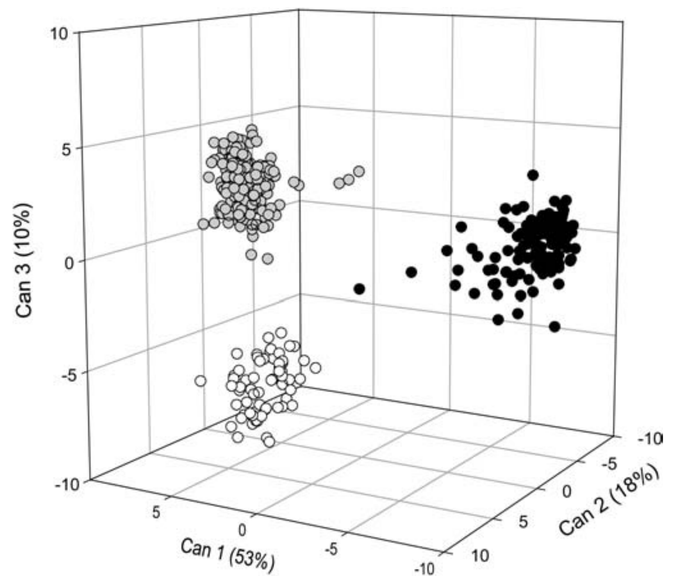

Fig. 2 Canonical discriminant analysis of 451 *T. pratense* L. plants from 19 populations and three groups using 126 AFLP markers which differentiated best among the 19 populations as determined by stepwise discriminant selection (*black* = Mattenklee landraces; *grey* = Mattenklee cultivars; *white* = field clover cultivars)

Table 2 AFLP primer combinations used for selective amplification and number of polymorphic markers detected within each group of 19 *T. pratense* L. landraces and cultivars

Primer pairs (5' → 3')	Number of polymorphic markers across			
	Mattenklee landraces[a]	Mattenklee cultivars[b]	Field clover cultivars[c]	All populations
E[d]+ACT/M[e]+CAC	41	36	32	41
E+ACA/M+CAC	40	40	43	43
E+ACT/M+CTA	50	47	36	51
E+AGA/M+CTA	54	54	39	58
E+AGG/M+CAC	35	33	27	36
E+AGT/M+CTA	47	44	38	47
Total	267	254	215	276

[a] Eight landraces, 24 individuals each
[b] Eight cultivars, 24 individuals each
[c] Three cultivars, 24 individuals each
[d] Primer core specific for *Eco*RI site (Vos et al. 1995)
[e] Primer core specific for *Mse*I site (Vos et al. 1995)

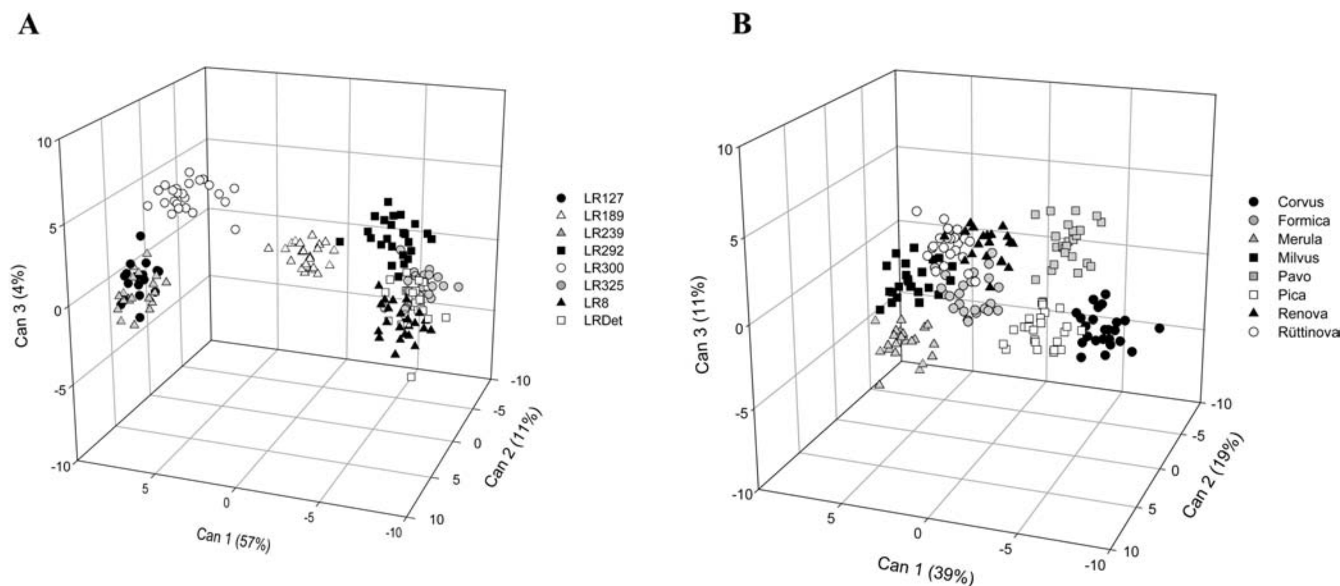

Fig. 3 Canonical discriminant analysis of eight Mattenklee landraces (**A**) and eight Mattenklee cultivars (**B**) using 126 AFLP markers which differentiated best among the 19 populations as determined by stepwise discriminant selection

Table 3 Analysis of molecular variance (AMOVA) for eight landraces and 11 cultivars of *T. pratense* L. using 276 polymorphic AFLP markers (A) and a subset of 126 markers which are the best differentiating factors among the 19 populations as determined using stepwise discriminant selection (B)

Source of variation	*df*	Sum of squares	Variance component[a]	% Total variance
A				
Variance among groups[b]	2	1,282.0	4.0	12.0
Variance among landraces and cultivars	16	1,451.0	2.7	8.1
Variance within landraces and cultivars	431	11,454.7	26.6	79.9
B				
Variance among groups	2	987.2	3.1	17.9
Variance among landraces and cultivars	16	934.8	1.9	11.1
Variance within landraces and cultivars	431	5,359.3	12.4	71.0

[a] Components were significant at $P < 0.001$, the probability of obtaining a more extreme random value computed from non-parametric procedures (1,000 data permutations)
[b] Three groups consisting of eight Mattenklee landraces, eight Mattenklee cultivars and three field clover cultivars, respectively (Table 1)

Stepwise discriminant analysis using the 19 populations as class variables identified a set of 126 AFLP markers which discriminated best among the 19 populations analysed. The first three discriminant functions (Can) of a canonical discriminant analysis based on these 126 AFLP markers explained 81% of the total variation observed among genotypes. A plot of Can 1 (53%), Can 2 (18%) and Can 3 (10%) revealed a clear separation of the three red-clover groups (Fig. 2). Can 1 mainly separated Mattenklee landraces (group mean = –8.7; Fig. 2, black symbols) from Mattenklee cultivars (group mean = 6.9; Fig. 2, grey symbols) and field clover cultivars (group mean = 6.1; Fig. 2, white symbols), while Can 3 was the most powerful discriminant between Mattenklee cultivars (group mean = 2.4) and field clover cultivars (group mean = –1.4). The better discrimination of the three groups achieved with the set of 126 AFLP markers was also reflected in AMOVA where the proportion of genetic variability among groups was increased to 18% of the total variability detected (Table 3B). Stepwise discriminant analysis using the three groups as class variables identified 97 AFLP markers as best discriminating factors and resulted in a similar separation of the three groups where Can 1 explained 84% and Can 2, 16% of the variation (data not shown). In order to optimally separate populations, only the set of 126 markers as determined with stepwise discriminant analysis and populations as class variables was considered for further analysis.

Comparison of landraces and cultivars

In order to elucidate population relationships based on individual genotypes, canonical discriminant analysis was carried out separately for Mattenklee landraces and

Mattenklee cultivars (Fig. 3). Due to the small sample size of only three populations no meaningful result was obtained with canonical discriminant analysis for field clover cultivars and these cultivars were therefore excluded from this analysis. For Mattenklee landraces, the first three canonical discriminant functions explained 72% of the variation and allowed to clearly separate all individuals of LR300 and LR189 (Fig. 3A). The landraces LR127 and LR239 formed a distinct cluster. The same was true for LR8, LRDet and LR325 which formed a cluster close to LR292. The Mattenklee cultivars Corvus, Pavo, Pica and Merula were separated by the first three canonical discriminant functions which explained 69% of the variation in this data set (Fig. 3B). The remaining four Mattenklee cultivars formed one cluster with Milvus and Formica being slightly separated from the rest. Discrimination of populations based on individual plants was confirmed by plotting class means of the first three canonical discriminant functions for each population (data not shown).

Genetic distances between red clover populations, expressed as coancestry coefficients, ranged from 0 (LRDet/LR325) to 0.61 (Milvus/LR127; Table 4). Comparisons of red clover populations with the white clover cultivar Bombus resulted in coancestry coefficients which ranged from 0.86 to 1.13 (Table 4). All F_{st} values were significant at $P < 0.05$ except for the LRDet/LR325 and the LR127/LR239 comparison. The average coancestry coefficient within groups was 0.12 for Mattenklee cultivars and field clover cultivars, and 0.17 for Mattenklee landraces. The average coancestry coefficient between groups was 0.36 for Mattenklee landraces and Mattenklee cultivars, 0.30 for Mattenklee landraces and field clover cultivars, and 0.34 for Mattenklee cultivars and field clover cultivars. UPGMA clustering of genetic distances (coancestry coefficients) resulted in two major clusters, one comprising all Mattenklee cultivars, the other including all Mattenklee landraces as well as the field clover cultivars (Fig. 4). Cluster analysis including white clover as an outgroup (UPGMA as well as Neighbour joining) did result in the same topology of the red clover cluster (data not shown). Mattenklee landraces LR239, LR127 and LR300 formed a tight cluster (Fig. 4, ML I) and were clearly separated from the Mattenklee landrace LR189, which formed a separate cluster (ML II), the landraces LR325, LRDet, LR292 (ML III) and the field clover cultivars (FC I). The Mattenklee cultivars also formed a distinct cluster and were subdivided into two additional clusters (MC I + II; Fig. 4). The correlation coefficient between cophenetic values derived from the dendrogram and the coancestry coefficients was $r = 0.81$ ($P < 0.0001$), indicating a good fit of the clustering with the original distance matrix. UPGMA clustering based on individual plants and Euclidean squared distance was largely congruent with the clustering based on coancestry coefficients of populations. Eighty nine percent of all individual plants were correctly assigned to one of the four major clusters (ML I-III, MC I+II, FC I; data not shown).

Table 4 Genetic distance for eight landraces and 11 cultivars of *T. pratense* L. and one cultivar of *T. repens* L. based on 126 AFLP markers. Coancestry coefficient (Reynolds et al. 1983) calculated using 24 individuals per population (below diagonal) and significance of F_{st} values [above diagonal; + = significant difference ($P < 0.05$) determined using a non-parametric permutation approach and 1,000 permutations (Excoffier et al. 1992), T = no significant difference]. For description of populations refer to Table 1

Type	Mattenklee cultivars								Mattenklee landraces								Field clover cultivars			White clover
	REN	RüT	MIL	FOR	COR	PIC	MER	PAV	239	127	300	189	325	DET	8	292	MON	MER	LUC	BOM
REN		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RüT	0.03		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MIL	0.10	0.04		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FOR	0.08	0.06	0.06		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
COR	0.10	0.15	0.25	0.20		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PIC	0.08	0.11	0.23	0.19	0.07		+	+	+	+	+	+	+	+	+	+	+	+	+	+
MER	0.11	0.11	0.17	0.19	0.17	0.11		+	+	+	+	+	+	+	+	+	+	+	+	+
PAV	0.06	0.09	0.20	0.16	0.09	0.04	0.09		+	+	+	+	+	+	+	+	+	+	+	+
239	0.37	0.4	0.54	0.51	0.43	0.43	0.49	0.35		T	+	+	+	+	+	+	+	+	+	+
127	0.42	0.46	0.61	0.59	0.5	0.50	0.55	0.40	0.01		+	+	+	+	+	+	+	+	+	+
300	0.34	0.36	0.48	0.45	0.39	0.40	0.44	0.33	0.03	0.05		+	+	+	+	+	+	+	+	+
189	0.26	0.28	0.40	0.36	0.34	0.33	0.38	0.29	0.20	0.25	0.13		+	+	+	+	+	+	+	+
325	0.18	0.22	0.37	0.35	0.25	0.23	0.32	0.22	0.25	0.31	0.25	0.17		T	+	+	+	+	+	+
DET	0.22	0.25	0.41	0.39	0.29	0.26	0.36	0.24	0.24	0.28	0.24	0.20	0.00		+	+	+	+	+	+
8	0.22	0.24	0.41	0.40	0.30	0.27	0.35	0.24	0.29	0.34	0.28	0.21	0.03	0.02		+	+	+	+	+
292	0.22	0.28	0.42	0.41	0.31	0.29	0.36	0.26	0.24	0.29	0.21	0.17	0.03	0.03	0.03		+	+	+	+
MON	0.23	0.26	0.40	0.38	0.32	0.29	0.33	0.24	0.34	0.39	0.32	0.22	0.16	0.17	0.16	0.17		+	+	+
MER	0.26	0.30	0.45	0.44	0.33	0.30	0.37	0.27	0.41	0.48	0.42	0.32	0.21	0.22	0.22	0.26	0.10		+	+
LUC	0.28	0.34	0.46	0.47	0.37	0.33	0.39	0.28	0.40	0.48	0.41	0.33	0.25	0.25	0.27	0.29	0.16	0.09		+
BOM	0.86	0.97	1.14	1.16	1.09	1.08	1.13	0.98	0.92	1.07	1.02	0.97	0.85	0.89	0.97	0.99	1.00	1.03	1.01	

The proportions of individual populations assigned to inferred clusters were used to identify meaningful groups based on the model-based clustering method of Pritchard et al. (2000). Although proportions assigned to individual clusters were smaller for the admixture model when compared to the non-admixture model, the population structure revealed by both models was largely congruent (data not shown). Since the use of the non-admixture model is recommended for dominant markers (Pritchard and Wen 2002) and yielded more pronounced clusters when compared to the admixture model, only results obtained with the non-admixture model are presented. For each number of populations (K), meaningful clusters were identified which contained at least one population represented with a proportion of 50% or more. For $K = 3$ and $K = 4$, all populations were assigned to the respective number of meaningful clusters. The obtained clusters reflected the topology of the dendrogram obtained through cluster analysis at coancestry coefficient values of 0.3 and 0.2 respectively (Fig. 4). For larger values of K, the number of clusters containing at least one population represented with a proportion larger than 50% increased to a value of nine for $K = 12$, but remained constant for any larger value of K tested. The clusters obtained with values of K ranging from 12 to 20 were congruent with the topology of the UPGMA dendrogram at coancestry coefficient values of 0.1 (Fig. 4).

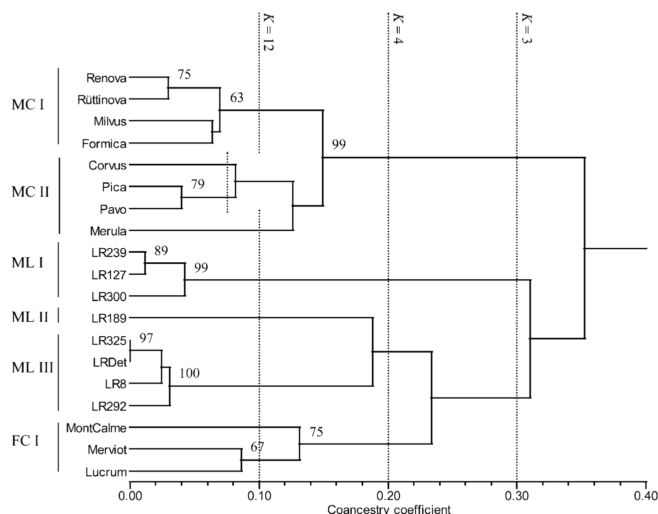

Fig. 4 UPGMA clustering of 19 *T. pratense* L. populations from three groups (*MC* = Mattenklee cultivars; *ML* = Mattenklee landraces; *FC* = field clover cultivars) based on coancestry coefficients (Reynolds et al. 1983) derived from 126 AFLP markers and 24 individual plants per population. *Numbers* above branches indicate bootstrap values derived from 10,000 re-sampling cycles (only values above 60% are shown). *Dotted lines* indicate the tree topology which corresponds to the grouping obtained with a model-based clustering method (Pritchard et al. 2000) assuming three different numbers of populations (K)

Variability within landraces and cultivars

The total extent of genetic variability detected with the set of 126 selected AFLP markers (see above) was primarily due to variation between genotypes within populations (71%; Table 3). AMOVA performed on each individual group separately revealed the variance component for within-population variation to be higher for Mattenklee landraces (12.8%) when compared to Mattenklee cultivars (12.4%) and field clover cultivars. (11.4%, data not shown). Within individual populations, genetic diversity expressed as Euclidean squared distance ranged from 21.2 for the field clover cultivar Lucrum to 31.1 for the Mattenklee cultivar Renova (Table 5). Average Euclidean distance across all populations was 24.8. While the Euclidean distance was higher than the average within five Mattenklee landraces, the same was true for only three Mattenklee cultivars and one field clover cultivar. The percentage of polymorphic markers observed within individual populations ranged from 56.3 for the Mattenklee cultivar Corvus to 81.0 for the Mattenklee landrace LR239, with an average of 66.9. A comparison of the three red clover groups revealed the largest genetic diversity within Mattenklee landraces, followed by Mattenklee cultivars and field clover cultivars which showed the least diversity (Table 5).

Table 5 Genetic diversity within populations and groups for eight landraces and 11 cultivars of *T. pratense* L. based on 126 AFLP markers

Item	Percentage of polymorphic markers[a]	Euclidean squared distance[b]
Within populations[c]		
LR8	63.5	24.6
LR127	72.2	23.4
LR189	69.0	24.1
LR239	81.0	28.5
LR292	64.3	23.9
LR300	65.1	25.5
LR325	77.0	28.0
LRDet	65.9	26.4
Corvus	56.3	22.2
Formica	60.3	23.0
Merula	61.1	23.2
Milvus	61.1	22.6
Pavo	71.4	26.1
Pica	58.7	21.5
Renova	80.1	31.1
Rüttinova	72.2	28.7
Lucrum	57.9	21.2
Merviot	62.7	21.6
Mont Calme	72.2	25.3
Average within groups		
Mattenklee landraces	69.8	25.8
Mattenklee cultivars	65.2	24.8
Field clover cultivars	64.3	22.7

[a] A total of 126 markers which are the best differentiating factors among the 19 populations as determined by stepwise discriminant selection was scored

[b] Average distances for pairwise comparisons of genotypes

[c] See Table 1 for description of populations

Discussion

Genetic variability within germplasm collections may strongly affect their value as genetic resources for breeding. AFLP analysis of 19 red clover populations from different groups (Mattenklee landraces, Mattenklee cultivars and field clover cultivars) revealed a substantial amount of genetic variability within this germplasm collection. The largest proportion of the total variability detected was attributed to variability between genotypes within populations (Table 3). Red clover is an outcrossing species with a high degree of gametophytic self-incompatibility (Taylor and Quesenberry 1996) and populations are therefore composed of heterogeneous individuals. Consequently, high levels of within-population variability are expected. The amount of genetic variability detected within Mattenklee cultivars was slightly higher than the variability within Mattenklee cultivars or field clover cultivars, and comparable to values previously reported for red clover (Campos-De-Quiroz and Ortega-Klose 2001) and other forage species such as white clover (*T. repens*; Kölliker et al. 2001) and meadow fescue (*Festuca pratensis*; Kölliker et al. 1998).

Principle component analysis (PCA) based on all 276 polymorphic AFLP markers moderately separated individual red clover plants into the respective groups (Fig. 1). The main reason for the incomplete separation apparently is the high variability observed within populations. However, PCA, where no class information is used in order to calculate principle components, already indicates a clear distinction between the three groups. The separation was greatly improved using canonical discriminant analysis and a subset of 126 AFLP markers which best discriminated among populations. Discriminant analysis proved powerful to sort individual plants of strongly heterogeneous populations into biologically meaningful groups. Similarly, discriminant analysis was successfully employed to separate lowland and central-highland ecotypes of *Phytolacca dodecandra* which were previously not separated by principle component analysis (Semagn et al. 2000).

Despite the clear separation of the three groups, no clear-cut distinction between Mattenklee and field clover was possible based on AFLP analysis. While discriminant analysis revealed a slightly closer relationship between Mattenklee cultivars and field clover cultivars when compared to Mattenklee landraces (Fig. 2), cluster analysis based on coancestry coefficients clearly separated Mattenklee landraces from Mattenklee cultivars but placed field clover cultivars into a subcluster within Mattenklee landraces (Fig. 4). While bootstrap values for the cluster containing all Mattenklee cultivars were higher than 60% and therefore considered relevant, only lower values were observed for the cluster containing Mattenklee landraces and field clover cultivars. However, using a model-based clustering method based on three inferred populations revealed the same close relationship between Mattenklee landraces and field clover cultivars (Fig. 4). This, together with the fact that the latter method does not rely on prior population information when compared to discriminant analysis, is a clear indication for a closer relationship of Mattenklee landraces to field clover cultivars when compared to Mattenklee cultivars.

A similar situation was observed for Indian wheat genotypes where *Triticum durum* landraces formed a cluster with *Triticum dicoccum* cultivars and were clearly separated from *T. durum* cultivars (Pujar et al. 1999). The authors speculated a limited number of domestication events during the evolution of *T. dicoccum* cultivars to be one reason for the observed clustering. This is unlikely to be the case for red clover, where a similar overall selection intensity can be assumed for Mattenklee cultivars and field clover cultivars. However, it can not be excluded that field clover cultivars have been used to improve pastures and meadows where Mattenklee landraces were maintained, or that Swiss red clover landraces have been used in the development of field clover cultivars as is known for Mont Calme (Boller 2000a). Another possible explanation for the clear separation of Mattenklee cultivars could be strong selection targeting mainly one trait, i.e. persistence. Nevertheless, Mattenklee landraces, Mattenklee cultivars and field clover cultivars form three genetically distinct groups. To further elucidate genetic relationships in the *T. pratense* complex, more detailed studies involving wild clover populations as well as a larger number of field clover cultivars are necessary.

Genetic distances (coancestry coefficients) between populations were considerably larger among groups than among populations within the same group, providing further evidence for the genetic distinction between Mattenklee landraces, Mattenklee cultivars and field clover cultivars (Table 4). Coancestry coefficients between red clover populations reached up to 50% of the values obtained from comparisons between red clover populations and the white clover cultivar Bombus. White clover (*T. repens*) is not a close relative of red clover and hybrids between both species have only been obtained by means of embryo rescue (Taylor and Quesenberry 1996). Hence, the variability observed among Mattenklee and field clover populations is quite remarkable.

Due to high levels of intra-population variation, separation of closely related cultivars of outbreeding species can be difficult (Yu and Pauls 1993; Kongkiatngam et al. 1995; Guthridge et al. 2001). However, we observed almost complete separation of five out of eight Mattenklee cultivars using discriminant analysis on individual plants (Fig. 3B). This separation was also largely congruent to cluster analysis based on coancestry coefficients and partially reflected the breeding history of the cultivars. Rüttinova was directly selected from Renova while Pica and Pavo were developed using Mattenklee germplasm as well as wild clover ecotypes collected in Croatia (Boller 2000b). Cluster analysis also separated older cultivars (last selection before 1991) from newer cultivars (Table 1, Fig. 4). In general, there was a substantial amount of variability observed among Mattenklee cultivars with coancestry coefficients comparable

to values observed among cultivars of alfalfa (Mengoni et al. 2000) and white clover (Gustine et al. 2002). For red clover, two studies report similarly high levels of variability among cultivars (Kongkiatngam et al. 1996; Campos-De-Quiroz and Ortega-Klose 2001) while relatively little genetic diversity was found among 34 North American red clover cultivars by Yu et al. (2001).

One major concern in maintaining and exploiting germplasm collections is the identification of populations which truly contribute to the genetic diversity of a collection and are not simply duplicates of populations already represented (Gilbert et al. 1999). The Mattenklee landraces analysed in this study were not only distinct from Mattenklee cultivars and field clover cultivars, but most landraces were also clearly separated from each other. Although variability among landraces was larger than variability among populations of the two other groups, there were two pairs of landraces where no significant population differentiation was observed (Table 4, Fig. 4). The Swiss Mattenklee landrace collection at FAL-Reckenholz, Zurich, consists of populations collected from farms where landraces were traditionally maintained for many decades. However, the exact origin of some landraces is difficult to determine (Nüesch 1976) and it can not be excluded that some landraces were supplemented with external germplasm in times of seed scarcity. This could be one possible explanation for the high genetic similarity of LR239/LR127 and LR325/LRDet. For the latter pair this is particularly likely since LRDet was officially recommended for cultivation for many years (Badoux et al. 1967). Based on genetic distances, it appears appropriate to summarise the eight Mattenklee landraces in two major germplasm pools (MR I, MR III; Fig. 4). The Mattenklee landraces analysed in this study may be sufficiently represented through one population of each pool and the population LR189, which displayed high genetic distance to all landraces. However, considering the low genetic variability among some of the morphologically distinct Mattenklee cultivars, closely related landraces such as LR8 and LR292 may still represent valuable genetic resources.

The clustering of Mattenklee landraces based on coancestry coefficients did not reflect the geographic location where the populations were sampled from. For example, the genetic distance between LR8 and LR292 was quite low although the two populations were located 155 km apart, while LR189 and LR325 showed considerable genetic distance but originated from locations only 5 km apart. No significant correlation between geographic separation and genetic distance was also observed in wild emmer wheat (*Triticum dicoccoides*; Fahima et al. 2002) and white clover (Kölliker et al. 2001). Such a lack of correlation may be explained by a sharp local differentiation as opposed to a gradual change in allele frequencies across the geographic range of a species (Fahima et al. 2002). In addition to local climatic conditions, varying sources of initial red clover germplasm as well as differences in selection targets and intensities, may be plausible explanations for a strong local differentiation of Mattenklee landraces.

The results of this study strongly suggest that Swiss Mattenklee landraces form a distinct genetic resource of red clover and are genetically different from Mattenklee cultivars and field clover cultivars. Due to the high genetic variability, Mattenklee landraces form a valuable gene pool for red clover breeding as well as for the preservation of biodiversity. AFLP analysis combined with AMOVA and canonical discriminant analysis proved highly effective for identifying putative duplicates and for determination of genetic variability within and among Mattenklee landraces. Such information complements morpho-physiological evaluations and allows for an efficient management and exploitation of Mattenklee germplasm collections.

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