Level and distribution of genetic diversity in the European species *Nardus stricta* L. (Poaceae) inferred from chloroplast DNA and nuclear amplified fragment length polymorphism markers

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Education and Culture

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Content

ABSTRACT

The present level and distribution of genetic variation is defined by the interplay between gene flow, drift, inbreeding, mutations and selection and reflects both contemporary and historical processes (e.g. Pleistocene glaciations). Especially, the breeding strategy of a species is a dominant factor influencing the pattern of genetic diversity. In this study, using AFLP molecular markers and cpDNA sequences, I investigate neutral genetic variation of nuclear and chloroplast genomes of European populations of the grass *Nardus stricta*.

The results of the present study indicate a lack of genetic variation in European *N. stricta* populations; there were no cpDNA variation detected and only the Austrian population had different AFLP pattern from other populations analyzed. In earlier studies, it has been suggested that *N. stricta* reproduces via agamospermy, and the observed lack of genetic variation strengthens this hypothesis about asexual reproduction in *N. stricta*. Also, given the lack of variation in both nuclear and chloroplast DNA it is likely that the present European populations of *N. stricta* come from one refugium only, but it is impossible to say which based on our data. However, nucleotide variability is not the only source of variation that results in phenotypic variation. Epigenetic variation might enable the wide geographical distribution of *N. stricta* and explain the observed lack of nucleotide variation. This might be interesting to investigate further.

Even though AFLP is a high resolution technique, the resolving power of the techniques used should be carefully investigated. I strongly emphasize that these results are preliminary as they are only based on screening of a few populations. More populations need to be screened and possibly also more primer/restriction enzymes combinations before any conclusions can be drawn.

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INTRODUCTION

FACTORS INFLUENCING GENETIC VARIATION

The present distribution of genetic diversity depends on contemporary and historical factors; some contemporary forces increase genetic diversity (gene flow, mutations), some decrease (genetic drift, inbreeding), while some can have either effect (natural selection). The neutral genetic variation of a population is mainly influenced by the interplay between gene flow and genetic drift (Eckert *et al.*, 2008). In large populations of outcrossing species gene flow dominates over genetic drift, homogenizing populations (Hedrick, 2011). In small populations of inbreeding species, genetic structure is mainly dominated by genetic drift, which leads towards differentiation of populations (Allendorf, 1983; Loveless & Hamrick, 1984; Hedrick, 2011). Besides the population size, the size fluctuations and spatial isolation, breeding system (dispersal pattern) is also an important factor influencing the amount of gene flow (Hedrick, 2011). Sexual reproduction is the dominant breeding system in flowering plants, however, vegetative reproduction also exist to some extent. Furthermore, in some species asexual, apomictic reproduction is the only type of reproduction (Eckert, 2002). Still, this is extremely rare (Eckert, 2002) since sexual reproduction is an important evolutionary strategy for maintaining genetic variation of the species.

HISTORICAL EVENTS

Historical events can still play a part in determining spatial structuring of genetic variation, even though they have ceased to work. In European flora and fauna, a well-documented example is the Pleistocene glaciations where repeated expansions from and retraction to glacial refugia still leaves marks in the present day genetic structure of populations (Taberlet *et al.,* 1998; Hewitt, 2000; Stewart *et al.,* 2010). Established refugia are in the Iberian Peninsula, Italy and the Balkans, but smaller, cryptic refugia have been identified further north and east (Provan & Bennett, 2008). The genetic diversity in peripheral populations has been hypothesized to be depleted due to repeated founder events during interglacial recolonization from

the southern regions (Hewitt, 2004). Also, Wright's isolation by distance model suggests that genetic diversity gradually decreases with increasing the distance from the core population (Wright, 1942). Therefore, populations are expected to have highest degree of genetic diversity at the geographical centre of the species range (southern refugia), while at the periphery of species range they become smaller, more isolated and with depleted genetic variation due to increased drift and decreased gene flow (Brussard, 1984; Lawton, 1993; Lesica & Allendorf, 1995; Vucetich & Waite, 2003). This widely accepted model has been challenged and some studies support the hypothesis (Sagarin & Gaines, 2002a; Husak & Linder, 2004; Sorte & Hofmann 2005), but many do not (Ribeiro & Fernandes, 2000; Brewer & Gaston, 2002; Sagarin & Gaines, 2002b; Kluth & Bruelheide, 2005; Sagarin *et al.* 2006; Samis & Eckert 2007). Thus, analysis of present spatial distribution of genetic diversity is useful in testing whether peripheral populations are always with depleted genetic diversity and in determining evolutionary forces that shaped the populations resulting in the present pattern of genetic diversity.

STUDY SPECIES

Nardus stricta. (Fig. 1) is a perennial, diploid (2n=30) grass (Bowden, 1960). Its flowers are protogynous and hermaphrodite (Chadwich, 1960a), which is a common reproductive strategy favoring outcrossing (Richards, 1997). Still, the breeding strategy of *N. stricta* is not fully investigated. Some embryology and microsporogenesis studies reported agamospermous seed production in at least some European and New Zealand populations, but outcrossing has also been observed (Coulon, 1923; Chadwich; 1960a, Rychlewski, 1961; Conert *et al.,* 1998; Kissling, 2004; Kissling, 2006). Description of the species morphology and ecology are provided in Hubbard (1992), Conert *et al.* (1998) Edgar & Connor (2000). Geographically, *N. stricta* is distributed throughout Europe (Meusel *et al.,* 1965; Conert *et al.,* 1998) with limits of its distribution including Russian Larelia and

Russian Lapland and on the east the Arctic European Russia and the Volga-Kama region. It has been introduced to New Zealand and other parts of

the world where it is invasive (Conert *et al.,* 1998, Kissling, 2004, Kissling, 2006, Fig. 2). *N. stricta* plays a central part in grassland communities in western and central Europe. It is not preferred forage and in mixed vegetation stock it tends to dominate under heavy grazing, eliminating certain plants like *Deschampsia flexuosa,* while outcompeted under lower grazing pressure (Austrheim *et al.,* 2007). Despite *N. stricta* being an ecologically important and much studied species, there are no genetic studies of structure or breeding system of *N. stricta* populations.

Figure 1.
Photograph from Roman, Enrico. *Nardus stricta* L. uirig.altervista.org. Web. 25. July 2013.

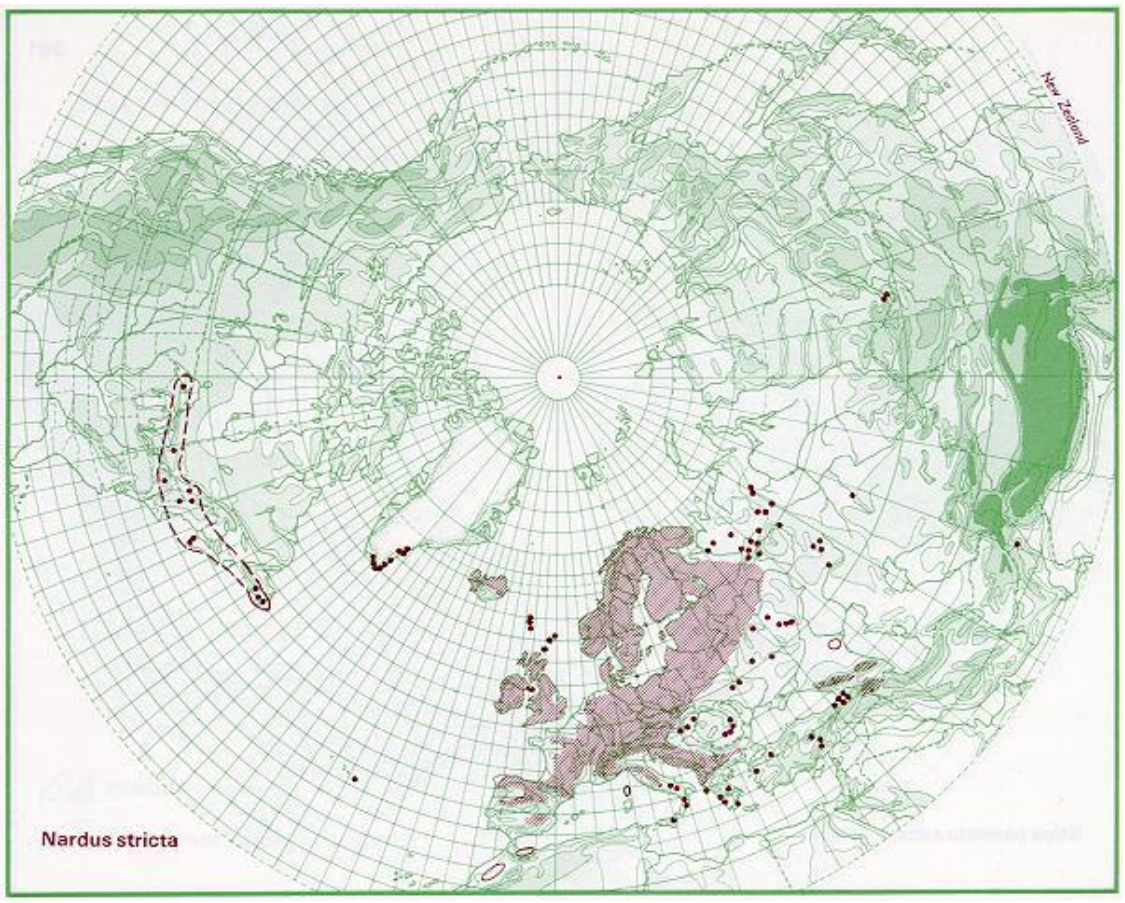

Figure 2.
Geographical distribution of *Nardus stricta* in North Europe (Hultén, E. & Fries, M. 1986)

STUDY APPROACH

The genetic structure of matgrass was investigated combining amplified fragment length polymorphism (AFLP: Vos *et al.,* 1995) and sequencing of chloroplast DNA (cpDNA) regions. AFLP is a commonly used method for assessing genetic diversity in grasses (Balfourier *et al.,* 1998; Roldán-Ruiz *et al.,* 2000; Fjellheim & Rognli 2005a, b). AFLP enables dominant, multilocus and genome wide DNA profiling and thus is suitable for molecular characterization of any genome (Azhaguvel *et al.,* 2006). In addition, large number of polymorphic markers provides a high resolution for studying variation between and within populations (Karp *et al.,* 1996). AFLP is reproducible and requires very small amounts of DNA (Vos *et al.,* 1995). This method does not require any knowledge about the sequence, which is very convenient in this study as no genetic data have been published for *N. stricta* up to date. Still, AFLPs have their particular problems. This relates to scoring reliability

(Bonin *et al.* 2007). An additional drawback is that AFLP markers show dominant inheritance, which makes it difficult to estimate some genetic parameters such as heterozygosity. However, solutions to this problem have been proposed (Excoffier *et al.,* 1992; Lynch & Milligan, 1994; Stewart & Excoffier, 1996; Zhivotovsky, 1999). Sequencing of cpDNA regions has also show to be very useful in studying natural populations of grasses (Doyle *et al.,* 1992; Saltonstall, 2001; Fjellheim *et al.,* 2006). Phylogeographic studies show that cpDNA variation of some species is geographically structured (McLachlan *et al.,* 2005; Petit *et al.,* 2008). These large scale genetic diversity studies were mainly focused on tree species (McLachlan *et al.,* 2005; Petit *et al.,* 2008), alpine species (Alsos *et al.,* 2005; Fujii & Senni 2006) and agricultural grass species (Guthridge *et al.,* 2001; Fjellheim & Rognli, 2005a, b; Tanhuanpää & Manninen, 2012). These species are in the focus of research because they either have a long life span with a wide distribution range that is strongly defined by the climatic conditions (tree species) or they are economically important species (*Lolium perenne* L., *Festuca pratensis* Huds., *Phleum pratense* L.). However, few studies can be found of common, widespread, and ecologically important grasses. In this study are used AFLP to investigate polymorphisms in nuclear genome, and sequencing to determine variation in neutral regions of the chloroplast genome. The mutation rate of the nuclear genome is higher than of the organellar (chloroplast) genome (Wolfe *et al.,* 1989), and following this, the nuclear genome is convenient for capturing ongoing and recent evolutionary processes, while the highly conserved cpDNA is convenient for revealing historical evolutionary events. Phylogeographic studies of cpDNA have been already used to reconstruct migration routes and historical colonization process in different world regions (Avise 2000; Fjellheim *et al.* 2006; Ahmed *et al.* 2013; Kim *et al.* 2013; Shimono *et al.* 2013). Finally, cpDNA is maternally inherited, while with nuclear genome we can capture both maternal and paternal gene flow. Both methods capture neutral variation, thus relationship between genetic drift and gene flow is expected to be revealed.

AIM In the present study neutral genetic variation was assessed using nuclear AFLP method and cpDNA sequencing of European *N. stricta* populations on a broad geographical scale to (i) describe genetic diversity in relation to different geographic regions (ii) to investigate if any genetic structure is present in the gene pools (iii) to trace migration routes and reveal colonization history in the postglacial period.

MATERIALS AND METHODS

PLANT MATERIAL AND LOCALITIES

European populations of *N. stricta* covering a broad geographical range were included in the study: from Portugal in the west to Romania in the east, and from Italy in the south to Norway in the north. Representatives of all three glacial refugia in the southern Europe were encompassed.

The plants were raised from seeds either sampled in the field or obtained from gene banks. Two populations from gene banks (Germany and Portugal) were initially included in the study, but they turned out to be misclassified and thus were left out of further work. Fresh leaf material from 10 individuals from each population was collected and stored at -80^{0}C. For AFLP an initial screen of primer combinations was done on one individual per population to find primer combinations giving high quality fingerprints with appropriate level of variation. Subsequently, all individuals would be tested for variation in the primer combinations chosen. For cpDNA, one individual from geographically distinct populations were screened for variation. Geographically distinct populations, including potential glacial refugia, were expected to be genetically most different. Five populations were screened for cpDNA and six for AFLP. Information about populations and type of analysis in each are provided in Table 1.

Table 1. *Nardus stricta* L. populations included in the study and details about their localities.

	Country of origin	AFLP[1]	cpDNA[2]	Locality	Latitude	Longitude	Altitude [m]
1	France	x	x	France, Hautes-Pyrénées, Bagnères de Bigorre, La Mongie, elevation 1800 m.	42.90° N	0.16° W	1800
2	Greece		x	Greece, Metsovo, Anilio, ca. 4.6 km east of Anilio, ski resort Zigos 500m northeast from lift station, mountain pasture used for sheep grazing	39.75° N	21.23° E	1696
3	Italy	x	x	Monte Rosa, Alps	46.57° N	11.03° W	1610
4	Norway	x	x	Norway, Kvikne, ca. 6.5 km north east of Kvikne Fjellhotell, shore of Falningsjøen	62.60° N	10.42° W	887
5	Poland	x	x	Poland, Siemianowka reservoir, ca. 4 km west of the reservoir, pinus forest next to small road leading from road 687 to Tarnopol, ca. 400 m before the first houses of Tarnopol	52.90° N	23.76° E	162
6	Austria	x		Torfau, Ulrichsberg	48.40° N	13.54° E	626
7	Romania	x		Romania, Apuseni mountains, mountain meadow north of the road 764A, ca. 3 km west of Stâna de Vale, east exposition	46.69° N	22.58° E	1127

[1] Samples screened for AFLP
[2] Samples screened for cpDNA variation

Figure 3.

Each number represents one population included in the study. Color shows type of analysis conducted in the population (orange for cpDNA and AFLP, yellow AFLP only and red cpDNA only)

ANALYSIS OF cpDNA VARIATION

The five populations screened for variation in cpDNA non-coding regions were from France, Greece, Italy, Norway and Poland (Table 1). The selected populations were geographically distant and therefore expected to be most differentiated. 21 non-coding cpDNA regions were screened for variation. Primer pairs were chosen from the literature. Some primers were universal chloroplast primers, while some were grass specific. Details about the primers' sequence and the source are given in Table 2.

Fresh leaf material was collected and stored on -80°C until DNA extraction. Prior to DNA isolation, plant material was disrupted using TissueLyser (QIAGEN®, Valencia, CA, USA) (38 Hz for 30 seconds for two times). DNA was isolated using QIAGEN® (Valencia, CA) kit according to the manufacturer's description. DNA quality and quantity was measured using spectrophotometer (NanoDrop8000, Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was also checked with gel electrophoresis.

Polymerase chain reactions (PCR) were performed in volumes of 25µL containing 1 µL DNA template, 0.4 µM each primer (Invitrogen, Carlsbad, CA, USA) 0.2 mM of each of nucleotides dNTP (Invitrogen), 0.75 U HotStartTaq polymerase (Invitrogen) and 1x PCR buffer (Invitrogen). Four different PCR programs were used depending on the primers' length, nucleotide composition and the size of amplification product. For primer pairs rps16F:trnQR, trnS(GCU)F:psbDR, trnTF:trnER, trnCF:rpoBR, psaAF:ORF170R, rbcLF:trnT2R, trnTF:trnLR, trnCF:ycf6R, psbMF:trnDR, trnS:trnG the PCR program consisted of 95°C for 10 minutes, followed by 35 cycles of 95°C for 60 s, 53°C for 60 s and 72°C for 2 min, and completed at 72°C for 5 minutes. For the primer combinations rbcLF:psalR, trnQF:trnRr, rpoB:trnCR the annealing temperature was 48°C and for the primer pairs trnHF:trnKR, psbHF:petBR, rpl16F:rps3R, petBF:petDR, petNF:trnCR, pbsMF:petNR, trnD (GUC)F:psbMR, trnS(GCU)F:psbDR, trnCF:probR the annealing temperature was 50°C. Because

optimal PCR conditions can vary according to the thermocycler, the temperatures given are only indicative. A Mastercycler (Eppendorf, Hamburg, Germany) thermocycler was used. The PCR products were sent to GATC Biotech: Sequencing and Bioinformatics AG (http://www.gatc-biotech.com), where the single read sequencing in plates was performed. Sequences were edited and assembled using Sequencher 4.0.5 (Gene Codes Corporation, AnnArbor, MI, USA).

Table 2. PCR primers for amplifying and sequencing chloroplast DNA

Location	Forward primer 5'-3'	Location	Reverse primer 5'-3'	Author
rps16F	GTT GCT TTC TAC CAC ATC G	trnQR	GTTCGAATCCTTYCGTCCC	Saltonstall *et al.,* 2001
trnS(GCU)F	GCC GCT TTA GTC CAC TCA GC	psbDR	CTATGGGGTCACARCCSAGG	Saltonstall *et al.,* 2001
trnTF	ACT CAG TGG TAG AGT AAT GCC	trnER	CTCCTTGAAAGAGAGATGTCC	Saltonstall *et al.,* 2001
trnCF	GRT AAA GGA TTT GCA GTC CC	rpoBR	TTCTMGGGAACTCCACATACG	Saltonstall *et al.,* 2001
psaAF	TCG AAA TCG TGA GCA TCA GC	ORF170R	TCTCAAGTACGGTTCTAGG	Saltonstall *et al.,* 2001
trnTF	CAT TAC AAA TGC GAT GCT CT	trnLR	TCT ACC GAT TTC GCC ATA TC	Small *et al.*, 1998
trnCF	CCA GTT CRA ATC YGG GTG	ycf6R	GCC CAA GCR AGA CTT ACT ATA TCC	Shaw *et al.*, 2007
psbMF	AGC AAT AAA TGC RAG AAT ATT TAC	trnDR	GGG ATT GTA GYT CAA TTG GT	Shaw *et al.*, 2007
trnS	AGA TAG GGA TTC GAA CCC TCG GT	trnG	GTA GCG GGA ATC GAA CCC GCA TC	Shaw *et al.*, 2007
rbcLF	TGT ACA AGC TCG TAA CGA AGG	psaIR	CTAAGCCTACTAAAGGYACG	Saltonstall *et al.,* 2001
trnQF	GGG ACG GAA GGA TTC GAA CC	trnRr	ATTGCG TCC AAT AGG ATT TGA A	Dumolin *et al.*, 1998
rpoB	CKA CAA AAY CCY TCR AAT TG	trnCR	CAC CCR GAT TYG AAC TGG GG	Shaw *et al.*, 2007
trnH	ACG GGA ATT GAA CCC GCG CA	trnKR	CCGACTAGTTCCGGGTTCGA	Demesure *et al.*, 1995
psbHF[1]	TCT TCT GTT TTA CTG GAC GGA AT	petBR[1]	TGC AAT TGC CTG AAT CTG AA	Fjellheim, unpublished
rpl16F[1]	TGA AAT GAG AAA GCG TGC AA	rps3R[1]	CTT CGC ATT CTG TCC AGT GA	Fjellheim, unpublished
petBF[1]	GTA CCT GAC GCC ATT CCA GT	petDR[1]	GGC TCG AGC AAG AAT GAA AG	Fjellheim, unpublished
petNF[1]	CAG CCC AAG CGA GAC TTA CT	trnCR[1]	CAG GGG ACT GCA AAT CCT T	Fjellheim, unpublished
psbMF[1]	AAA AAC AGC CAG CCA AAA TG	petNR[1]	AGT ATG GGG GAG GAG TGG AC	Fjellheim, unpublished
trnD(GUC)F[1]	CTG TCA AGG CGG AAG CTG	psbMR[1]	CAT TTT GGC TGG CTG TTT TT	Fjellheim, unpublished
trnS(GCU)F[1]	GCC GCT TTA GTC CAC TCA GC	psbDR[1]	CTA TGG GGT CAC ARC CSA GG	Fjellheim, unpublished
trnCF[1]	GRT AAA GGA TTT GCA GTC CC	rpoBR[1]	TTC TMG GGA ACT CCA CAT ACG	Fjellheim, unpublished

[1] Grass specific primers

ANALYSIS OF NUCLEAR GENOME VARIATION USING AFLP

Variation in nuclear genome was investigated using AFLP. The individuals screened were from populations from Austria, France, Norway, Italy, Poland and Romania. The AFLP protocol (Vos *et al.,* 1995) optimized for *N. stricta* was used. Detailed protocol modifications are given in the Appendix 1.

Genomic DNA restriction and ligation of restriction site-specific adapter oligonucleotides were done separately. Firstly, genomic DNA (0.2 ng) was digested with 15U of EcoRI and 10U of MseI enzymes (New England BioLabs, Ipswich, MA), in 10 x RL buffer. RL buffer was made from 100mM trisHAc, 100 mM MgAc, 500 mM KAc, 50 mM DTT. Subsequently, restriction site-specific oligonucleotide adapters (Invitrogen, Table 3) were ligated with 1U T4 DNA ligase (New England BioLabs), 100 mM ATP (Sigma Aldrich, St. Louis, MO) and 10x RL buffer and then the mix was incubated on 37°C for 3 hours. Restriction fragments with annealed adaptors were used as a template DNA for preselective PCR amplification. The selective PCR preamplification [30 cycles (94°C 30 s, 56°C 1 min, 72°C 1 min)] was performed in a 50 µl reaction mixture containing 5 µl template DNA, 10 µM EcoRI- primer+1 (E01, Invitrogen), 10 µM MseI-primer+1 (M01, Invitrogen), 0.2 mM of each dNTP (Invitrogen), 5U of AmpliTaq DNA polymerase (Invitrogen), 10x RL buffer and 25 mM $MgCl_2$ (Invitrogen).

After selective preamplification, the templates were diluted with MQ water (1:10). Selective PCR amplification [95°C 10 min, then lowering the annealing temperature each cycle for 0.7°C 11 cycles (94°C 30 s, 65°C 30 s, 72°C 60 s), followed by 24 cycles (94°C 30 s, 56°C 30 s, 72°C 30 s), and finally 72°C 10 min)] was performed in a 15 µl reaction mixture containing 5 µl of diluted template (preamplified +1/+1), 10 µM fluorescently labeled EcoRI-primer+3 (Invitrogen), 10 µM MseI-primer+3 (Invitrogen), 0.2 mM of each dNTP, 2 µl 10x RL buffer, 25 mM $MgCl_2$ and 5U of HotStart Taq polymerase (Qiagene, Hilden, Germany). All PCR amplifications were performed with Tetrad 2 thermal cycler (Bio-Rad, Hercules, CA, USA).

For selective PCR amplification two EcoRI+3 primers (Invitrogen) fluorescently labeled (E32: AAC-3; E33: AAG-3) and 14 MseI+3 primers (Invitrogen) were used (M31: AAA-3; M32: AAC-3; M33: AAG-3; M34: AAT-3; M35: ACA-3; M36: ACC-3; M37: ACG-3; M38: ACT-3; M39: AGA-3; M40: AGC-3; M41: AGG-3; M42: AGT-3; M43: ATA-3; M44: ATC-3). 17 EcoRI+3 and MseI+3 primer pairs for selective amplification were tested in order to determine combinations that generate an appropriate level of polymorphism. GeneScan-500 LIZ size standard (Applied Biosystems, USA) and ABI 3730 automated sequencer (ABI-3100, Applied Biosystems, USA) were used for separation of amplified fragment. Data were analyzed using the software GeneMapper® Software (ABI Applied Biosystems, Foster City, CA, USA). AFLP profiles of each sample were scored for absence (0) or presence (1) into a binary matrix.

Table 3. Adapter and primer oligonucleotides used in AFLP

Primer	Oligonucleotide
AFLP adapters	
MseI-Adapter	5'GAC GAT GAG TCC TGA G3' 3'TA CTC AGG ACT CAT5'
EcoRI-Adapter	5'CTC GTA GAC TGC GTA CC3' 3'CTG ACG CAT GGT TAA5'
Core sequences of AFLP primers without selective bases	
MseI-primer	5'GAT GAG TCC TGA GTA A3'
EcoRI-primer	5'GAC TGC GTA CCA ATT C3'
Preamplification primers +1/+1	
MseI-primer+1 (M01)	5'GAT GAG TCC TGA GTA A**A**3'
EcoRI-primer+1 (E01)	5'GAC TGC GTA CCA ATT C**A**3'
Selective amplification +3/+3	
MseI-primer+3 (M31)	5'GAT GAG TCC TGA GTA **AAA**3'
MseI-primer+3 (M32)	5'GAT GAG TCC TGA GTA **AAC**3'
MseI-primer+3 (M33)	5'GAT GAG TCC TGA GTA **AAG**3'
MseI-primer+3 (M34)	5'GAT GAG TCC TGA GTA **AAT**3'
MseI-primer+3 (M35)	5'GAT GAG TCC TGA GTA **ACA**3'
MseI-primer+3 (M36)	5'GAT GAG TCC TGA GTA **ACC**3'
MseI-primer+3 (M37)	5'GAT GAG TCC TGA GTA **ACG**3'
MseI-primer+3 (M38)	5'GAT GAG TCC TGA GTA **ACT**3'
MseI-primer+3 (M39)	5'GAT GAG TCC TGA GTA **AGA**3'
MseI-primer+3 (M40)	5'GAT GAG TCC TGA GTA **AGC**3'
MseI-primer+3 (M41)	5'GAT GAG TCC TGA GTA **AGG**3'
MseI-primer+3 (M42)	5'GAT GAG TCC TGA GTA **AGT**3'
MseI-primer+3 (M43)	5'GAT GAG TCC TGA GTA **ATA**3'
MseI-primer+3 (M44)	5'GAT GAG TCC TGA GTA **ATC**3'
EcoRI-primer+1 (E32)[1]	5'GAC TGC GTA CCA ATT C**AA C**3'
EcoRI-primer+1 (E33)[2]	5'GAC TGC GTA CCA ATT C**AA G**3'

[1, 2] fluorescently labeled primers

RESULTS

cpDNA VARIATION

None of 21 primer pairs revealed any variation in cpDNA among 5 geographically distinct *N. stricta* populations. In total 13826 bp was analysed. Table 4 shows lengths of aligned sequences for each primer combination.

Table 4. Lengths of aligned sequences and number of variations for primer combinations tested

Chloroplast region	Aligned length	No. of variations
rps16F-trnQR	678	0
trnS(GCU)F-psbDR	760	0
trnTF-trnER	521	0
trnCF-rpoBR	709	0
psaAF-ORF170R	868	0
trnTF-trnLR	787	0
trnCF-ycf6R	603	0
psbMF-trnDR	580	0
trnS-trnG	770	0
rbcLF-psaIR	410	0
trnQF-trnRr	134	0
rpoB-trnCR	790	0
trnH-trnKR	804	0
psbHF-petBR	1100	0
rpl16F-rps3R	866	0
petBF-petDR	667	0
petNF-trnCR	845	0
psbMF-petNR	237	0
trnD(GUC)F[1]	389	0
trnS(GCU)F-psbDR	693	0
trnCF-rpoBR	615	0

AFLPs

Several AFLP protocols were tested and the quality of output data differed among them. In all protocols one individual from six geographicaly distinct populations was analysed. In Appendix are provided details of the used protocol variants.

Among 17 primer combinations which were tested 6 yielded informative data in all studied individuals. In total, the six primer combinations generated 224 scorable AFLP bands. Scoring matrices for the 6 primer combinations are given in the Appendix. Fragments of length ranging from 35 to 458 bp were identified. Furthermore, individuals from France, Norway, Poland and Romania had identical phenotype. In comparison with those samples, in Italian sample 2 (0.89%), while in Austrian 33 bands were missing (14.73%). EcoRI-primer+3(E32) fluorescent primer was found to be superior over EcoRI-primer+3(E33) in the generation of scorable fragments. More precisely, some EcoRI-primer+3(E33)/MseI-primer+3 combinations did not yield any fragments in selective amplification and thus scorable data were not obtained. Considering that substantial amount of variation in AFLP patterns was not detected, more individuals from each population were not screened for the variation at this initial stage of research.

DISCUSSION

Using 21 cpDNA and 6 AFLP primer combinations, very low levels of genetic variation was detected among European *N. stricta* populations. cpDNA did not reveal any variation, while the AFLP profiles of only one population (Austria) differed from profiles of the other populations. The AFLP method is robust, reproducible and commonly used in population genetics studies for inspecting genetic variation (DeHaan *et al.,* 2003; Chikmawati *et al.,* 2012; Kimball *et al.,* 2012; Zhu *et al.* 2013) and detecting species history (Gaudeul *et al.,* 2004; Schonswetter *et al.,* 2004, Schorr, 2009) thus indicating that the results derived from the present study are reliable.

The extremely low level of variation in the nuclear genome despite the wide geographical distribution of *N. stricta* across different climates is remarkable. Widely distributed species that survived Holocene climate changes, such as *N. stricta*, are expected to have at least modest level of genetic differentiation (e.g. to show different genetic variation patterns) due to genetic drift (founder effect) and inbreeding (Hedrick, 2011). Furthermore, outcrossing, wind pollinated species are expected to show a high degree of diversity as shown in the grasses *Festuca pratensis* Huds., *Melica nutans*, *Lolium perenne* L., *Phleum pratense* L. (Guthridge *et al*., 2001; Tyler, 2002; Fjellheim *et al.,* 2005a; Fjellheim *et al.,* 2006; Tanhuanpaa & Manninen 2012). The observed absence of variation in cpDNA is less surprising than the absence of variation in nuclear genome. This is because the rate of nucleotide substitution in cpDNA is lower, it is more conserved and therefore evolution of chloroplast genome is slower (Wolfe *et al.,* 1987). However, many studies have found intraspecific variation in chloroplast DNA (Fjellheim *et al.,* 2006; Diekmann *et al.,* 2009) with fewer regions screened. Furthermore, some of the regions sequenced are amongst the most variable in the grasses (Saski *et al.,* 2007). Hence, I would have expected some variation also in the dataset of *N. stricta*.

The Austrian and to a lesser extent Italian populations differed in nuclear AFLP patterns from other populations, while there was not any variation in cpDNA detected. The chloroplast DNA of the Austrian population was unfortunately not analysed, thus it is not possible to say whether Austrian population belongs to another refugium. Still, given the overall lack of variation in both nuclear and chloroplast DNA it is likely that the present European populations of *N. stricta* comes from one refugium only and it is impossible to say which based on our data. Furthermore, given the low level of nuclear DNA variation it is likely that the founding population was severely bottlenecked.

Even with one, severely bottlenecked founding population, I would have expected differentiation between contemporary populations due to genetic drift and lack of gene flow. One explanation for the genetic uniformity of the populations could be that *N. stricta* has an asexual reproduction mode and agamospermous seed production. AFLP has been shown to allowing us to distinguish between sexual and asexual mode of reproduction by detecting the absence or presence of recombinations. Sexual reproduction due to the recombinations should result in heterogenous AFLP patterns, while clonar reproduction and selfing in the lack of variation in the AFLP patterns (Rosendahl *et al.,* 1997).

The agamospermous breeding strategy has been suggested as the predominant breeding strategy for at least some *N. stricta* populations from Europe and New Zealand (Coulon, 1923; Rychlewski, 1961; Chadwic, 1960a; Richards, 1997; Kissling *et al.,* 2006). Agamospermous seed production results in genetically identical offspring, developed parthenogenetically, from an unreduced egg cell. The main source of novel genetic variation is recombination during meiosis in sexually reproducing species generating mutations. During asexual reproduction meiosis does not occur, thus reduced level of genetic diversity is expected in agamospermous species populations (Pfeiffer *et al.,* 2011). Also, molecular analysis

in asexual species (including apomictic taxa) revealed little to no genotypic diversity (e.g. Lynch *et al.,* 1998). Thus, extremely low level of variation observed in the present study strengthens hypothesis about agamospermous reproduction in *N. stricta,* as already suggested in some earlier non genetic studies (Coulon, 1923; Rychlewski, 1961; Bunce & Barr, 1988; Chadwic, 1960a; Richards, 1997; Kissling *et al.,* 2006). Despite the fact that 'clonal' species are expected to be genetically identical, molecular techniques such as AFLP have revealed at least low level of genetic divergence in some taxa, for example in sterile *Grevillea infecunda* (Kimpton *et al.,* 2002) or *Posidonia oceanica* (Rozenfeld *et al.,* 2007). Therefore, similar to these examples, detected variation in AFLP in Austrian and Italian populations does not weakenes the hypothesis about the clonal reproduction in *N. stricta*.

The variation in the Austrian and Italian samples might be explained by the combined sexual and clonal reproduction in these populations. Factors such as environmental conditions or stress might influence the ratio of sexual to clonal reproduction within a population, resulting in variation in AFLP patterns.

It is puzzling that *N. stricta* has such a wide geographic and climatic distribution as the basis for selection and adaptation is genetic variation. However, nucleotide variability is not the only source of variation that results in phenotypic variation. Epigenetic mechanisms may also play an important role in adaptation to local environmental conditions. Different methylation patterns can influence access to genetic information (Verhoeven *et al.,* 2010) allowing individuals with the same genotype to express their genes differently. DNA methylation pattern determines biological processes like genomic imprinting, transcription and gene silencing (Jost & Saluz, 1993; Finnegan *et al*., 2000; Hafiz *et al.,* 2001; Martienssen & Colot, 2001; Paszkowski & Whitham, 2001). For instance temperature during embryo development has influenced expression of adaptive traits in the offsprings of *Picea abies* (Kvaalen & Johnsen, 2008) and in genetically identical apomictic *Taraxacum*

officinale, epigenetic variation between individuals was induced by various stress treatments (Verhoeven *et al.,* 2010). In a pioneering paper, Herrera and Bazaga (2011) did one of the first studies of epigenetic variation in an ecological context and found that stress induced by browsing was related to variation in multilocus epigenotypes in *Viola cazorlensis*. Thus, wide geographical distribution across the range of climates in the studied species might be enabled because of the methylation differences among individuals from different populations. To investigate if the populations can be regulated through differences in methylation I suggest an additional experiment using methylation-sensitive restriction enzymes in the AFLP protocol, as have been used for plants earlier (Xiong *et al.,* 1999; Liu *et al.,* 2001; Verhoeven *et al.,* 2010; Herrera & Bazaga, 2011). To ensure reliable results, the whole experiment should be re-done, as sampling at the exact same developmental stage is important to acquire comparable results between individuals (Yakovlev *et al.*, 2010).

Even though AFLP is a high resolution technique, in some cases it failed to detect genotypic diversity (Fay *et al.,* 1999;). Therefore, in studies revealing low diversity it has to be carefully investigated whether the results are the result of low discriminating power of the primercombinations chosen (Arnaud-Haond *et al.,* 2005). It could be interesting to repeat the diversity analyses of these populations applying a genotyping by sequencing approach, which in recent years have been available also for non-model species (e.g. reduced representation genome sequencing, commonly referred to as RAD-tags. Peterson *et al.,* 2012). Using these methods, it is possible to identify and study variation in tens of thousands of SNP's (Baird *et al.*, 2008), giving a greatly enhanced resolution compared to classical molecular markers. However, given the low levels of variation in both cpDNA and nuclear DNA analyses in combinations with previous studies showing that *N. stricta*

predominantly has agamospermous seed production, the low level of variation detected seems reasonable.

CONCLUSION

Based on the obtained results, I conclude that *N. stricta* populations within the investigated region in Europe have an extremely low level of genetic variation. An Austrian and to a lesser extent and Italian population differed in nuclear AFLP patterns from other populations, while there was not any variation in cpDNA detected. cpDNA of Austrian population was not analysed, thus it is not possible to assert whether Austrian population originates from another refugium. Still, due to the lack of variation in AFLP and cpDNA in all other populations it is more likely there is combined sexual and clonal reproduction in Austrian population. Environmental conditions, stress response or some other factor might influence the ratio of sexual to clonal reproduction within a population, resulting in different AFLP patterns. On the basis of these results, it is difficult to track the postglacial history of this species, and also the level of gene flow and genetic drift, but it seems as the basis for the European populations is one single, bottlenecked population. The lack of variability could also be attributed to the asexual mode of reproduction (agamospermy), which results in genetically identical offspring. These are the first molecular genetic data supporting agamospermous seed production in *N. stricta,* which has already been reported in some previous studies (Coulon, 1923; Chadwic, 1960a; Rychlewski, 1961; Richards, 1997; Kissling *et al.,* 2006).

Still, it remains unclear how *N. stricta* populations have been adapted to different climate conditions without variation among populations. Nucleotide variability is not the only source of variability that results in phenotypic variation. Epigenetic mechanisms may also play an important role in the adaptations to local environmental conditions and enable wide distribution range. This might be interesting to investigate further and I suggest to apply a methylation-sensitive AFLP marker protocol that have been developed for testing methylation pattern in plants.

I strongly emphasize that these results are preliminary as they are only based on screening of a few populations. More populations need to be screened and possibly also more primer combinations before any conclusions can be drawn.

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APPENDIX

The AFLP were carried out essentially as described in Vos *et al.* (1995). Several modifications were used in order to optimize the procedure for *N. stricta*..

Protocol Version 1.

Steps included were: (i) Annealing of adaptor pairs (ii) Restriction Ligation (iii) Preselective amplification (iv) Selective amplification.

For each of the adaptor pairs the mix was made from 100 µM of forward and reverse primers. For 5 min on 94°C mixtures were incubated at a heating block after which mixes were slowly cooled down.

Adapter sequences were:

Eco-F: 5'- CTC GTA GAC TGC GTA CC -3'

Eco-R: 5'- AAT TGG TAC GCA GTC TAC -3'

Mse-F: 5'- GAC GAT GAG TCC TGA G -3'

Mse-R: 5'- TAC TCA GGA CTC AT -3'

(ii) Restriction and ligation (RL) were done in one reaction. 0.2 µg of genomic DNA was digested with 40 U of EcoRI and 50U of MseI restriction enzymes and restriction site-specific oligonucleotide adapters were ligated with 5U of T4 DAN ligase, 10x T4 ligase buffer, 0.5 M NaCl, 1mg/ml BSA and 10µM of each adaptor pair. Incubation was performed for 3 hours at 37°C. Products (restriction fragments with adaptors annealed) were stored at -80°C unless they were used at once. Products were

diluted with sterile, distilled deionised MQ water (1:10) and used as a template for (iii) preselective PCR amplification.

(iii) Preselective amplification was carried in a mix containing 10x PCR Buffer I (buffer contains 15mM $MgCl_2$), 10mM dNTP, 10µM of each Eco-RI-A and MseI-A primers, and AmpliTaq and diluted RL product.

PCR program for preselective amplification was set on 72°C for 2 min, 30 cycles (94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min), 72°C for 10 min.

Selective amplification (iv) was performed in 12.5 µl reaction mixture containing diluted (1:10) preselective amplification product, 10x PCR buffer I, 25mM of $MgCl_2$, 1mg/ml BSA, 10mM of dNTP and 10 µM of each EcoRI-primer+3 and MseI-primer +3 (Table 1).

PCR program for selective amplification was set on 95°C for 10 min, 1 cycle (94°C for 30 sec, 65°C for 1 min, 72°C for 1 min), then decreasing the annealing temperature by 0.7°C each cycle to 56°C (13 cycles), followed by an additional 23 cycles (94°C for 30 sec, 56°C for 1 min and 72°C for 1 min) and final extension at 72°C for 10 min.

Successful amplifications after preselective and selective amplification of target sequences were verified using gel electrophoresis (5 µl of preselective product on a 1% gel, 80V for 30 min). Smears appeared.

Table 1. Primer combinations (EcoRI-primer+3 and MseI-primer+3) used in Protocol Version 1. to identify optimal primer sets for AFLP characterization of *N. stricta*

Primers specific to rare-cutting restriction site sequence	Primers specific to frequent-cutting restriction site sequence
E32	M36
E33	M37
	M38
	M39
	M40

Protocol Version 1. yielded no distinct fragments in any of primer combinations used. The whole procedure was repeater in the same manner, but the results were the same.

Because bands on gel electrophoresis pictures after restriction-ligation seemed not enough smeared, it was assumed that restriction-ligation (ii) step should be improved. Thus, Protocol Version 2, with separated restriction and ligation steps was used.

Protocol Version 2.

In this protocol variant, Becker *et al.* (1995) was mainly followed.

Steps included: (i) Annealing of adaptor pairs (ii) Restriction (iii) Ligation (iv) Preselective amplification (v) Selective amplification.

Procedure for the step (i) was the same as described in Protocol Version 1. Genomic DNA was (ii) restricted by mixing 0.2 µg of genomic DNA, 10U of EcoRI and 15U of MseI restriction enzymes and 10x RL buffer. The mix was incubated for 2 hours at 37°C.

For (iii) Ligation of adapters 5 pmol of EcoRI adapter and 50 pmol of MseI adapter, 100 mMol of ATP, 10x RL buffer and 1 U of T4 DNA ligase were added to 10 µl of restricted DN. Ligation mix was incubated for 3 hours at 37°C.

Preselective amplification of adapter ligated DNA was performed with EcoRI and MseI enzymes [with one selective nucleotide (EcoRI-primer+1 and MseI-primer+1)], 0.2 mMol of each dNTP, 5U of AmpliTaq polymerase, 10x PCR buffer, 25 mMol $MgCl_2$and 5 µl of undiluted restriction-ligation template DNA.

PCR amplification was done under the following conditions: 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min.

Final, selective PCR amplification with +3/+3 primer combinations was performed in a mix containing 10 µM EcoRI-primer+3 (fluorescently labeled), 10 µM of MseI-primer+3, 0.2 mM of each dNTP, 10x PCR buffer, 25 mM $MgCl_2$ and 0.8 µl AmpliTaq DNA polymerase.

Selective amplification PCR program used was: 1 cycle (94°C for 30 sec, 65°C for 30 sec, 72°C for 60 sec), then 1 cycle (94°C for 30 sec, 65°C for 30 sec, 72°C for 60 sec) with decreasing the annealing temperature by 0.7°C during 11 cycles, followed by

an additional 24 cycles (94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec) and final extension at 72°C for 10 min.

Gel electrophoresis procedure was the same as in Protocol Variant 1. and it was applied after ligation, preselective and selective PCR amplification.

+3/+3 primer combinations used in Protocol Variant 2. are shown in Table 2.

Table 2. 10 primer combinations screened used in protocol Version 2 to identify optimal primer sets for AFLP characterization of *N. stricta*.

Primers specific to rare-cutting restriction site sequence	Primers specific to frequent-cutting restriction site sequence
E32	M36
E33	M37
	M38
	M39
	M40

Amplifications with E33 primer did not yield any fragments. The number of fragments yielded using E32 is presented in Table 3.

Since it seems that selective PCR amplification in Protocol variant 2. was not very successful, it was suggested to use HotStart polymerase in this last step (v). Thus, Protocol Variant 3. differs from Protocol Variant 2. because HotStart DNA polymerase was used for selective amplification and in PCR program applied.
Primer combinations used for Protocol Variant 3. are presented in Table 3. E33 primer combinations did not yield any fragments.

Table 3. Primer combinations screened used in Protocol Version 3 to identify optimal primer sets for AFLP characterization of *N. stricta*.

Primers specific to rare-cutting restriction site sequence	Primers specific to frequent-cutting restriction site sequence		
E32	M31	M36	M41
E33	M32	M37	M42
	M33	M38	M43
	M34	M39	M44
	M35	M40	

In order HotStart DNA polymerase to be activated, it requires incubation on 95°C for 10 minutes. Therefore, PCR program used was: 95°C for 10 min, 1 cycle (94°C for 30 sec, 65°C for 1 min, 72°C for 1 min), then decreasing the annealing temperature by 0.7°C each cycle to 56°C (13 cycles), followed by an additional 23 cycles (94°C for 30 sec, 56°C for 1 min and 72°C for 1 min) and final extension at 72°C for 10 min.

Finally, in the Table 4. are shown results obtained using the last protocol, for primer combinations that yielded satisfactory number of fragments in samples from all sic populations.

Table 4. AFLP fragments sizes yielded with 6 primer combinations for all six populations included in the analysis

E32_M34[1]	35	44	50	53	58	65	68	78	85	97	101	107	113	123	135	137	140	145
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

152	171	189	200	210	245	254	268	280	284	297	307	321	328
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1

E32_M35	35	41	45	47	54	58	60	65	69	71	77	85	89	94	102	108	119	126
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

129	132	146	170	175	181	187	190	193	197	211	217	221	248	255	258	284	294	310

1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	0	1	1	0	1	1	1	0	1	1	0	0	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

E32_M36	35	41	43	45	49	55	59	65	69	71	73	83	88	90	93	96	104	111
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

113	118	125	136	139	143	147	149	151	135	157	168	171	179	187	194	198	208	215
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

225	233	239	246	252	264	274	294	313	371	385	402
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1

E32_37	59	68	70	78	84	89	95	105	112	116	123	129	131	134	155	157	160	165
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

176	181	200	208	218	223	231	241	267	276	284	295	300	321	332	348	354	360	458
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

E32_M38	78	80	88	98	107	113	116	120	124	132	144	147	154	156	158	164	166	170
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

174	179	192	216	243	146	155	163	274	284	288	295	321	335
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1

E32_M39	88	113	130	135	137	143	145	153	160	175	187	197	213	219	226	235	250	252
FRA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ROM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUS	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0
POL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ITA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

254	258	274	287	292	298	303	310	316	324	338	346	359	392	403	417	423	427	442
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

AFLP kits usually exploit enzyme combinations of EcoRI and MseI. The choice of restriction enzyme and primer combination is crucial to the outcome of AFLP analysis, I suggest some other restriction enzyme combinations to be tested in order to increase the resolution of the analysis.