High SSR diversity but little differentiation between accessions of Nordic timothy (*Phleum pratense* L.)

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A large collection of genebank accessions of the hexaploid outcrossing forage grass species timothy (*Phleum pratense* L.) was for the first time analysed for SSR diversity on individual, population and regional level. Timothy is the most important forage grass species in the Nordic countries. Eighty-eight timothy accessions from Nordic countries and eight accessions around Europe were analysed with recently developed simple sequence repeat (SSR) markers. Timothy proved to be very polymorphic: the 13 selected SSRs amplified a total of 499 polymorphic alleles, the number of alleles per SSR locus varying from 15 to 74. Taking all SSR alleles together, the observed number in each accession ranged from 95 to 203. Levels of diversity were found to be significantly different between countries, vegetation zones and different cultivar types. However, the differentiation between accessions was low: most of the variation (94%) in the studied timothy material was due to variation within accessions and only 5% was between accessions and 1% between countries. Lack of geographical differentiation may reflect the outcrossing and hexaploid nature of timothy. Our results showed that neutral SSR markers are suitable for demonstrating levels of diversity but not alone adequate to resolve population structure in timothy. Nordic timothy material seems to be diverse enough for breeding purposes and no decline in the level of diversity was observed in varieties compared to wild timothy populations. Challenges in analysing SSR marker data in a hexaploid outcrosser were discussed.

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Timothy (*Phleum pratense* L.) is a cool-season perennial grass species distributed naturally throughout Europe and parts of North Africa and Asia. Wild populations of *P. pratense* represent a polyploid series from diploids to octoploids. The cultivated form of timothy is hexaploid. The uniformity of the molecular profile in agricultural *P. pratense* suggests that the formation of this hexaploid is probably post-glacial (Stewart et al. 2011). The genomic composition of hexaploid timothy has not been fully resolved yet, but there is some evidence that the genome contains four doses of *bertolonii* genome and two doses of *rhaeticum*. Both of these genomes derive from the same progenitor and are not very differentiated which explains that both hexasomic and tetradisomic inheritance has been reported in timothy (Stewart et al. 2011). Timothy is cultivated for hay, silage and pasture across the Northern Hemisphere. In Nordic countries timothy is the most important forage grass species due to adaptation to the cool and relatively humid northern climate. The main goal in timothy breeding for this region is to combine high yield, good winter survival, and high feeding quality. Timothy breeding relies on broad genetic variation and utilisation of heterosis, which can be achieved by combining genetically distant individuals with good combining ability in a synthetic variety. Therefore, plant breeders have to be sure that they have sufficient genetic variation available for their breeding programmes.

Genetic diversity within a plant species reflects both the life history traits and distribution of the species. Perennial, outcrossing species are known to have higher genetic diversity and less differentiation among populations than annual self pollinators (Hamrick and Godt 1996). Timothy is a perennial wind pollinating species where hexaploidy is expected to further rise the level of diversity. The abundant centre model (Brown 1984) presumes reduced neutral genetic diversity within peripheral compared to more central populations (Eckert et al. 2008). Although the distribution of hexaploid timothy covers most of Europe (Conert 1998), at the northern margin, namely northern boreal and alpine vegetation zones, harsh winter conditions may limit survival of timothy. This may be reflected in the levels of diversity. Previous studies have also shown that genetic diversity of plant populations may either increase or decrease with increasing altitude (Yan et al. 2009).

NordGen, the Nordic Genetic Resource Center, has a collection of 716 accessions of timothy, originating mostly from Nordic countries. Sixty-four of these are cultivars, others represent natural populations or old landraces. Nordic countries represent a wide geographical region

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with varying growth conditions from southern nemoral zone in Denmark to northern alpine vegetation zone in northern Norway. In addition to latitude, growth conditions are also affected by longitude since conditions in western Norway are maritime and in eastern Finland more continental. Three hundred and seventy-three timothy accessions in the NordGen collection have been previously characterised for morphological and agronomic traits in Finland, Norway, Iceland and Sweden during 1995–1996 (<www.nordgen.org/index.php/skand/content/view/full/344>). Characterisations were mostly made on coarse, relative scale and variation within each accession was not taken into account. This data gives an overall picture of the phenotypic variation present in the collection. However, it doesn't fully describe the levels of genetic diversity, the genetic structure of variation between and among populations nor the genetic distances between individuals or populations.

There are many molecular marker systems available for diversity analyses, from which we chose simple sequence repeats (SSRs) for studying diversity in timothy. Primers for 355 SSR loci in timothy have been developed (Cai et al. 2003), and some of the loci have been located on a diploid timothy map (Cai et al. 2009). SSRs are mostly codominantly inherited, very polymorphic, and with the use of different fluorescent labels, can be multiplexed in PCR. The information content per locus is bigger in SSRs compared to dominant markers because homo- and heterozygotes are detected. However, in polyploid species interpretation of exact marker genotypes is not straightforward and SSR alleles are usually analysed as presence/absence markers.

This study is part of a wider Nordic collaborative research project, where the variation of NordGen timothy collection was evaluated both on phenotypic and genotypic level. Here we report the results of the assessment of genetic diversity using SSR markers. Our aim was to study whether geographical location (vegetation zone, latitude, longitude, altitude) affects the level of genetic diversity. In addition, we studied if genetic markers could find a population structure in the Nordic timothy material and thus help finding heterotic groups among the collection of timothy to be used in variety breeding.

MATERIAL AND METHODS

Plant material

Eighty-eight timothy accessions from Nordic countries (Table 1, Fig. 1) were selected from NordGen collection based on geographical distribution and previous phenotyping data to represent as wide geographical and trait variation as possible, and 15–20 randomly selected individuals per accession were analysed. Most of the accessions, namely 59, were classified as wild accessions, 17 as landraces and 11 as varieties or breeders material. Accessions were divided to six groups according to country of origin: Norway (26), Finland (25), Sweden (25), Denmark (10), Iceland (2), and exotic (8) including all origins outside Nordic countries. Exotic accessions were obtained from different genebank collections. Accessions with known geographical coordinates were divided to six vegetation zones (Moen 1999): 1 = nemoral (11), 2 = boreonemoral (15), 3 = southern boreal (14), 4 = middle boreal (22), 5 = northern boreal (11), and 6 = alpine (2).

DNAs were extracted using the method of Tinker et al. (1993) with the following modifications: lyophilised leaves were crushed with a FastPrep FP120 Cell Disrupter (BIO 101, Thermo Savant, Waltham, MA, USA), in 1 ml CTAB (hexadecyltrimethyl-ammonium bromide) buffer supplied with 70 U of ribonuclease A (Omega Bio-tek, Norcross, GA, USA) and 0.05 mg of proteinase K (Finnzymes, Espoo, Finland). Extractions were first done with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform. DNA concentrations were measured using the GeneQuant II RNA/DNA Calculator (Pharmacia Biotech Ltd., Cambridge, UK).

SSR analyses

SSRs developed for timothy (Cai et al. 2003) were used for assessing diversity in the selected accessions. At the beginning of the study, 35 timothy SSRs were selected using the following criteria: strong amplification (Cai et al. 2003), preferably SSRs with trinucleotide repeats (SSRs containing trinucleotide or higher order repeats have less stuttering: Holton 2001), and some SSRs which have been localised on one position on the diploid timothy map (Cai et al. 2009). The SSRs were optimised and tested for their polymorphism, multiplexing possibilities, and easiness of interpretation. One primer of each primer pair was labelled with a fluorescent dye, FAM (5-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein) or TET (6-carboxytetrachloro-fluorescein) to enable separation and visualisation of amplification products with a MegaBACE 500 Sequencer (GE Healthcare, Buckinghamshire, UK) using MegaBACE ET400-R Size Standard. Thirteen best SSRs (Table 2) were selected for final analyses and were amplified using two different PCR programs in a PTC-220 DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) or a Bio-Rad DNA Engine Tetrad 2 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The first five SSRs in Table 2 were amplified with five cycles of 15 s at 94°C, 15 s at 65°C, and 30 s at 72°C, followed by 30 similar cycles except that the annealing temperature was 60°C. The program started with an initial denaturation step of 5 min at 94°C and was

Table 1. *Ninety-six accessions of Phleum pratense ssp. pratense analysed in the study with 499 SSR markers (each SSR allele treated as a separate marker).*

Number code	Accession no.	Genebank	Name	Country	Cultivar type[1]	Latitude	Longitude	Altitude[2]	Veg. zone[3]	No. of ind.	Observed no. of markers	No. of private markers	A_A[4]	A_I[5]	PWD[6]
1	NGB10828	Nordgen	VA88108	Denmark	W				1	19	120	0	113.1	28.6	31.0
2	NGB10829	Nordgen	VA88112	Denmark	W				1	19	141	2	131.9	32.9	38.7
3	NGB10830	Nordgen	VA88119	Denmark	W				1	19	124	0	116.7	28.8	30.7
4	NGB10831	Nordgen	HF88266	Denmark	W				1	19	148	2	137.9	28.4	32.5
5	NGB15461	Nordgen	Vildbjerg AC0103	Denmark	W	56°11′ N	8°49′58″ E	40	1	19	188	0	171.5	33.3	44.0
6	NGB16650	Nordgen	Ejsing	Denmark	W	56°31′21″ N	8°47′11″ E		1	19	142	0	132.9	31.2	36.2
7	NGB1672	Nordgen	BILBO	Denmark	CV					19	162	1	149.7	31.7	40.1
8	NGB1675	Nordgen	POTA	Denmark	CV					20	119	0	110.1	30.0	32.5
9	NGB4053	Nordgen	SR SALTUM MH0202	Denmark	W	57°14′ N	9°46′ E	6	1	19	145	0	135.2	30.6	36.2
10	NGB4548	Nordgen	NR FARUP MH0202	Denmark	W	55°21′ N	8°41′ E	2	1	20	154	0	139.4	30.3	35.1
11	NGB132	Nordgen	LIPINLAHTI ME0901 SEP A	Finland	L	63°28′ N	29°18′ E	100	4	19	186	0	167.5	31.1	39.1
12	NGB9285	Nordgen	OTTO	Finland	CV					18	188	1	175.9	35.9	46.8
13	NGB14394	Nordgen	KÄRKÖLÄ HM0102	Finland	W	60°55′16″ N	25°17′27″ E		3	19	175	0	162.2	32.7	42.6
14	NGB14399	Nordgen	MAHLAMÄKI MH0104	Finland	W	61°22′00″ N	22°56′19″ E	70	3	20	179	0	162.5	32.9	41.7
15	NGB14403	Nordgen	NÄREKUMPU MH0103	Finland	L	61°57′14″ N	28°26′05″ E		3	20	203	2	180.8	35.0	46.3
16	NGB14404	Nordgen	PAATTINEN MH0201	Finland	L	60°35′11″ N	22°22′16″ E		2	20	176	0	158.4	33.6	40.5
17	NGB14415	Nordgen	HUOLILA MH0204	Finland	L	60°41′00″ N	21°45′06″ E		2	20	141	0	128.0	30.6	35.0
18	NGB14417	Nordgen	MEDVASTÖ MH0101	Finland	W	60°06′02″ N	24°37′36″ E	25	2	18	121	0	115.2	28.4	32.5
19	NGB14419	Nordgen	KIIKAOJA MH0201	Finland	W	61°30′33″ N	22°32′10″ E		3	20	194	1	174.4	32.3	42.3
20	NGB747	Nordgen	NUVVUS AK0401	Finland	W	69°50′ N	26°19′ E	160	6	20	144	1	131.4	30.3	34.7
21	NGB748	Nordgen	UTSJOKI AK0602	Finland	W	69°55′ N	27°03′ E	70	6	20	169	1	153.6	33.7	39.8
22	NGB754	Nordgen	HALOSENRANTA EH0101	Finland	W	66°41′ N	27°30′ E	145	4	19	183	0	168.4	34.7	43.9
23	NGB757	Nordgen	PEKKALA EH0703	Finland	W	66°21′ N	26°52′ E	145	4	19	189	0	173.5	36.2	44.7
24	NGB1095	Nordgen	LAITASAARI ME0201	Finland	L	64°51′ N	25°56′ E		4	20	192	1	170.2	34.2	43.1
25	NGB1096	Nordgen	TUOMIOJA ME0201	Finland	L	64°36′ N	25°02′ E		4	18	191	1	177.5	33.6	44.0
26	NGB1107	Nordgen	JYRINKI ME0101	Finland	L	63°55′ N	24°26′ E		4	19	188	0	171.5	33.6	43.5
27	NGB1111	Nordgen	MÄLÄSKÄ ME0101	Finland	L	64°24′ N	26°19′ E		4	20	194	1	172.7	33.6	44.3
28	NGB151	Nordgen	VARISLAHTI ME0102	Finland	L	62°42′ N	28°42′ E	115	3	18	190	0	176.5	33.9	41.1
29	NGB1115	Nordgen	KILPAU ME0101	Finland	L	64°20′ N	25°07′E		4	19	194	0	178.8	35.1	44.6
30	NGB1119	Nordgen	KATERMA ME0401	Finland	L	64°03′N	29°09′ E		4	19	189	0	170.9	31.9	40.5

(Continued)

Table 1. (Continued).

Number code	Accession no.	Genebank	Name	Country	Cultivar type[1]	Latitude	Longitude	Altitude[2]	Veg. zone[3]	No. of ind.	Observed no. of markers	No. of private markers	A_A[4]	A_I[5]	PWD[6]
31	NGB1122	Nordgen	NÄÄDÄNMAA ME0202	Finland	L	62°30′ N	28°14′ E		3	19	188	0	174.7	35.1	45.5
32	NGB2791	Nordgen	NORRGÅRD AP0101	Finland	L	63°32′ N	22°31′ E		3	19	186	2	169.8	33.3	42.5
33	NGB2798	Nordgen	LÅNGÅMINNE AP0201	Finland	L	62°54′ N	21°43′ E		3	20	186	1	164.9	33.6	41.2
34	NGB2836	Nordgen	LANKAMAA AP0202	Finland	L	62°23′ N	26°14′ E		3	19	184	0	166.8	32.1	41.5
35	NGB4066	Nordgen	TAMMISTO	Finland	CV					19	180	1	164.5	32.7	38.6
36	NGB4140	Nordgen	KORPA	Iceland	L					19	193	1	176.2	33.3	43.9
37	NGB4141	Nordgen	ADDA	Iceland	CV					19	117	0	111.2	31.3	34.6
38	NGB7557	Nordgen	KLEVELAND 01-5-43-2	Norway	W	63°13′ N	11°03′ E	300	4	20	178	0	160.9	33.7	41.7
39	NGB7559	Nordgen	SVENDGÅRD 01-5-43-4	Norway	W	63°13′ N	11°03′ E	100	4	15	152	0	152.0	33.9	42.9
40	NGB7573	Nordgen	ØVRE HERSTAD 01-5-44-3	Norway	W	63°52′ N	11°16′ E	200	3	20	164	1	146.6	32.2	39.2
41	NGB7577	Nordgen	VOLDEN 01-5-44-8	Norway	W	63°22′ N	9°56′ E	20	3	17	179	2	170.4	33.8	42.8
42	NGB7592	Nordgen	SKJØLSVIK 01-5-46-5	Norway	W	62°57′ N	7°48′ E	20	3	19	182	0	168.4	35.3	44.9
43	NGB7597	Nordgen	MÅNA 01-6-48-13	Norway	W	62°06′ N	10°37′ E	600	5	20	155	0	141.4	32.8	40.3
44	NGB7709	Nordgen	NORDSKOT 01-2-13-6	Norway	W	67°50′ N	14°50′ E	15	4	19	186	1	169.9	32.6	42.7
45	NGB10785	Nordgen	SANDBU 01-6-49-4	Norway	W	61°52′ N	9°07′ E	420	5	19	95	0	86.8	32.6	28.9
46	NGB13647	Nordgen	LEVELD	Norway	P					18	106	1	102.6	32.6	34.1
47	NGB17194	Nordgen	Ifjord 1-1-2-2	Norway	W	70°27′42″ N	27°06′30″ E		5	20	166	0	149.5	31.4	37.2
48	NGB17198	Nordgen	Karasjok 1-1-3-2	Norway	W	69°28′31″ N	25°30′23″ E		5	18	179	1	166.4	32.4	42.5
49	NGB2169	Nordgen	BODIN	Norway	CV					19	167	0	153.4	30.3	38.8
50	NGB2170	Nordgen	VÅTI7702	Norway	B					17	119	0	115.0	34.0	35.2
51	NGB2180	Nordgen	GRINDSTAD	Norway	CV					18	185	1	169.2	31.2	39.5
52	NGB2917	Nordgen	KLOMSET 01-6-54-6	Norway	W	59°28′ N	8°36′ E	130	3	19	164	0	152.3	35.1	42.5
53	NGB2918	Nordgen	HUSETER 01-9-70-1	Norway	W	59°40′ N	11°23′ E	135	2	20	164	0	150.2	32.1	39.0
54	NGB2922	Nordgen	SØRHUS 01-6-48-2	Norway	W	62°06′ N	10°40′ E	520	5	18	178	0	166.5	34.4	43.3
55	NGB2927	Nordgen	ØSTERØYA 01-9-71-1	Norway	W	59°05′ N	10°12′ E	10	2	20	182	1	164.0	34.2	42.7
56	NGB2930	Nordgen	GRAUTEKNAPP 01-6-54-7	Norway	W	59°10′ N	8°49′ E	95	2	20	194	0	174.6	34.4	44.7
57	NGB4226	Nordgen	HATLESTAD 01-7-56-3	Norway	W	61°21′ N	6°06′ E		5	17	158	0	151.6	34.9	42.2
58	NGB4227	Nordgen	HÅRKLAU 01-7-56-4	Norway	W	61°25′ N	6°15′ E		5	19	155	1	146.8	35.6	43.6
59	NGB4231	Nordgen	GJERDÅKER 01-7-58-1	Norway	W	60°40′ N	6°30′ E	100	5	19	156	1	143.6	31.3	38.4

60	NGB4508	Nordgen	ENEBO 01-6-48-5	Norway	W	61°15′ N	12°19′ E	550	4	20	161	0	149.2	33.2	38.9
61	NGB4523	Nordgen	FOSS 01-9-71-3	Norway	W	59°25′ N	11°21′ E	100	2	19	188	1	170.8	35.1	45.3
62	NGB7548	Nordgen	NAMSVATN 01-5-40-1	Norway	W	64°58′ N	13°34′ E	500	5	18	150	0	140.8	29.8	35.4
63	NGB7551	Nordgen	SOLEM 01-5-42-1	Norway	W	63°45′ N	9°45′ E	20	3	17	168	0	161.2	34.5	44.2
64	NGB722	Nordgen	KUOSSENJARKA JP0404	Sweden	W	66°41′ N	19°45′ E	260	5	19	115	0	108.8	29.5	32.7
65	NGB728	Nordgen	PJESKER PH0405	Sweden	W	65°32′ N	19°42′ E	350	4	18	182	0	171.2	33.9	43.4
66	NGB11428	Nordgen	JONATHAN	Sweden	CV					16	139	0	138.0	34.2	39.8
67	NGB11430	Nordgen	ARGUS	Sweden	CV					19	154	1	141.0	31.7	37.0
68	NGB13226	Nordgen	RAGNAR	Sweden	CV					17	111	0	107.9	32.8	34.3
69	NGB14224	Nordgen	SÖNDRARP IB0101	Sweden	W	57°36′02″ N	14°26′39″ E	268	2	20	175	1	158.4	33.6	42.2
70	NGB14236	Nordgen	LÖVHULT IB0103	Sweden	W	57°39′11″ N	14°45′23″ E	269	2	19	170	1	156.6	31.2	40.0
71	NGB731	Nordgen	RÖRMYRBERG JP0204	Sweden	W	64°40′ N	19°09′ E	350	4	20	182	2	163.2	31.9	41.1
72	NGB16958	Nordgen	LYA LJUNGHED FO0201	Sweden	W	56°24′13″ N	12°53′42″ E	175	1	18	152	0	142.3	30.7	37.7
73	NGB16975	Nordgen	NORRA KYLSÄTER FO0103	Sweden	W	58°36′04″ N	11°59′51″ E	117	2	19	176	1	161.8	33.5	42.2
74	NGB733	Nordgen	SÖDRA SUNDERBYN ME0101	Sweden	L	65°40′ N	21°52′ E	20	4	15	185	1	185.0	36.2	46.6
75	NGB16977	Nordgen	RYR, STORA BERGET FO0101	Sweden	W	58°48′16″ N	12°29′25″ E	47	2	19	179	1	165.0	33.5	43.1
76	NGB16981	Nordgen	BRÄCKETORP FO0501	Sweden	W	59°02′48″ N	12°29′26″E	166	2	16	153	0	149.8	33.8	41.7
77	NGB17061	Nordgen	STORA ROTHULT HAJ0201	Sweden	W	57°55′53″ N	15°40′33″ E	180	2	20	187	0	168.5	33.4	43.3
78	NGB1306	Nordgen	BRATTÅKER GB0101	Sweden	W	64°18′ N	19°33′ E	344	4	18	184	2	173.6	32.9	41.4
79	NGB1310	Nordgen	STORHÄGGSJÖ GB0104	Sweden	W	63°59′ N	20°00′ E	100	4	19	138	0	130.9	34.6	41.9
80	NGB1320	Nordgen	SKARPMYRBERG PR0601	Sweden	W	64°37′ N	16°18′ E	400	5	20	194	0	173.4	33.5	42.5
81	NGB1327	Nordgen	HAMMARN PR0401	Sweden	W	63°48′N	20°29′ E	10	4	19	162	0	151.4	33.6	41.2
82	NGB1330	Nordgen	ÄLGSJÖ SH0302	Sweden	W	64°13′ N	17°29′ E	390	4	20	185	0	166.0	34.7	43.3
83	NGB1331	Nordgen	VÄSTANSJÖ SH0102	Sweden	W	63°45′ N	18°59′ E	170	4	19	170	0	158.4	32.8	42.1
84	NGB1332	Nordgen	KLUBBSJÖ SH0301	Sweden	W	63°51′ N	19°07′ E	250	4	18	185	1	172.4	34.0	43.1
85	NGB1537	Nordgen	ESKELHEM TL0104	Sweden	W	57°29′ N	18°10′ E		2	18	139	1	131.7	30.7	36.1
86	NGB2530	Nordgen	RÄMNE GJ0301	Sweden	W	59°00′ N	12°04′ E	110	2	19	165	0	152.8	31.9	39.6
87	NGB4349	Nordgen	BENESTAD JK1506	Sweden	W	55°31′ N	13°54′ E	40	1	20	171	1	153.4	32.4	40.6
88	NGB4350	Nordgen	BOARP SB2106	Sweden	W	55°56′ N	13°47′ E	180	1	18	175	1	163.9	34.3	42.6
89	PI381926	GRIN		France	P					19	131	0	122.3	31.1	34.6
90	PI406317	GRIN		Russia	P					19	165	1	151.7	31.8	38.5

(Continued)

Table 1. (Continued).

Number code	Accession no.	Genebank	Name	Country	Cultivar type[1]	Latitude	Longitude	Altitude[2]	Veg. zone[3]	No. of ind.	Observed no. of markers	No. of private markers	A_A[4]	A_I[5]	PWD[6]
91	IHAR151908	IHAR		Germany	P					19	150	0	137.4	31.4	34.8
92	PI210426	GRIN		Greece	P					18	146	2	138.9	31.5	38.3
93	PI325461	GRIN		Russia	P					19	170	8	157.8	31.7	39.8
94	PI204480	GRIN		Turkey	P					19	158	3	144.3	31.6	37.6
95	14G2400116	RICP		Czech Republic	P					19	186	2	170.5	34.2	44.0
96	RCAT040682	RCAT		Hungary	W					20	157	6	143.5	30.5	39.1

[1]CV = advanced cultivar, L = traditional cultivar, landrace, B = breeding, research material, genetic stock, P = pending, unknown cultivar type, W = wild population, weedy.
[2]meters above sea level.
[3]vegetation zones, according to Moen 1999.
[4]corrected number of all markers in each accession.
[5]mean number of all alleles observed in each individual.
[6]mean number of pairwise differences (PWD) (Euclidean distances) between individuals in each accession.

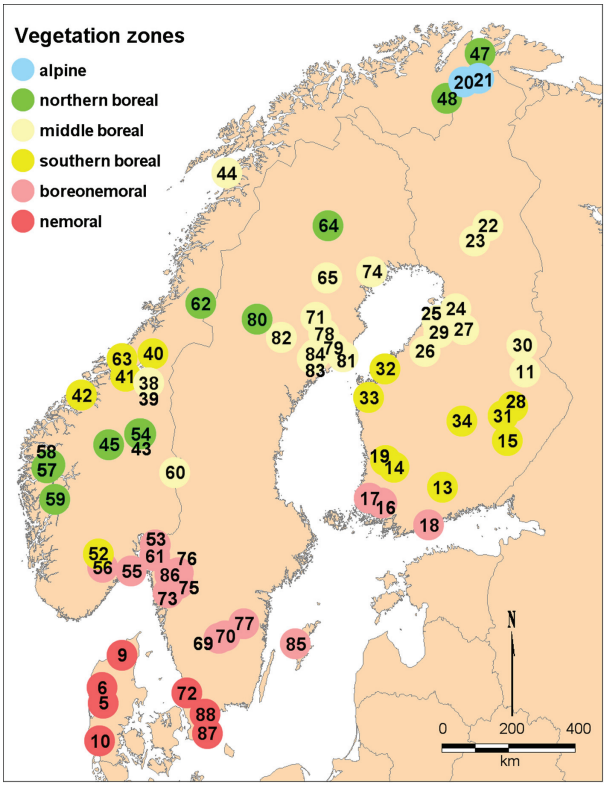

Fig. 1. Geographic location of 71 timothy accessions. Number codes are presented in Table 1.

followed by a final extension step of 7 min at 65°C. The following eight SSRs in Table 2 were amplified with the PCR program described in Cai et al. (2003). The PCR amplification reactions in 10 µl contained 0.25 U of FIREPol DNA polymerase I (Solis BioDyne OU, Tartu, Estonia), the buffer B with 2.5 mM $MgCl_2$ supplied by the enzyme manufacturer, 100 µM each dNTP, 10 ng of DNA, and 125–500 nM each primer. Suitable SSR combinations were found with the FastPCR software (Kalendar et al. 2009), and the 13 SSRs were multiplexed in five PCR (those amplified together are grouped in Table 2).

Data analyses

Allele phenotypes of the plants were visually scored using a binary code (1/0) for the presence or absence of allele peaks without knowing the doses of the alleles. When calculating genetic distances each SSR allele was thus treated as a separate marker locus. However, for the POPDIST program (Tomiuk et al. 2009), the allele phenotype was recorded locuswise i.e. the allelic content of an individual at each of the 13 SSR loci was described.

Genetic diversity of an accession was described in three different ways: 1) corrected number of all alleles (= markers) in each accession (A_A), where the observed number of alleles was corrected to a sample size n = 15

Table 2. *SSRs used in the diversity analysis of timothy accessions.*

SSR	Repeat motif	Repeat class	Fluorescent label	Exp. size (bp)[1]	Allele size range (bp)	No. of alleles	No. of alleles/ accession	Mean no. of alleles/ individual	Most common allele[2]	No. of private alleles[3]	Miss. inf. (%)	Linkage group[4]
A03A07	$(TG)_{33}$	perfect	HEX	140	94–207	50	4–17	2.10	0.64	5	2.5	unknown
C02C08	$(AAG)_{13}$	perfect	FAM	241	212–278	24	4–19	2.69	0.61	0	2.7	LG6
C01B11	$(TTC)_{16}$	perfect	TET	194	153–249	31	9–21	3.91	0.76	5	0.8	unknown
C02H01	$(TTC)_{17}$	perfect	FAM	146	95–201	53	8–26	2.93	0.44	8	2.4	unknown
D01E04	$(CAA)_8(TAA)_{10}$	compound	TET	158	95–288	56	8–23	2.82	0.67	11	1.9	LG1
B03F07	$(TC)_{14}$	perfect	HEX	130	112–172	32	2–18	1.44	0.29	2	15.1	unknown
C01E11	$(TTC)_{11}$	perfect	FAM	123	98–142	15	2–10	1.12	0.53	0	18.8	LG5
A09H08	$(TG)_{16}$	perfect	TET	255	230–267	15	4–9	3.55	0.86	5	1.1	unknown
D01G10	$(TGA)_7(CGA)_4 \ldots (TGA)_{16}$	compound and interrupted	FAM	231	208–336	74	7–24	2.51	0.44	9	2.4	LG2
B03A09	$(GA)_{19}$	perfect	TET	226	193–247	40	8–22	2.62	0.28	3	0.3	LG4
A03E06	$(TTG)_{28}$	perfect	HEX	238	166–290	49	3–22	2.46	0.91	10	0.5	unknown
D01H08	$(AAT)_{13}$	perfect	FAM	147	116–170	20	4–15	1.88	0.28	1	7.1	LG6
A10A10	$(CA)_{31}$	perfect	TET	232	161–243	40	6–22	2.68	0.68	7	3.2	LG3

[1]According to Cai et al. 2003.
[2]Occurence of the most common allele.
[3]Alleles present in only one accession.
[4]Refers to the diploid timothy map (Cai et al. 2009).

with thousand times of resampling without replacement, 2) mean number of all alleles observed in each individual (A_I) and 3) mean number of pairwise differences (PWD) (Euclidean distances) between individuals in each accession, which was counted with the program ARLEQUIN ver. 2.000 (Schneider et al. 2000). Differences in the level of diversity between different groups like countries, vegetation zones (Moen 1999), or cultivar type were analysed by ANOVA Proc GLM (SAS Enterprise Guide 4.3). Correlations were counted between diversity and latitude, longitude and altitude (Proc CORR, SAS Enterprise Guide 4.3) for those accessions where information of collection site map coordinates or elevation was available.

Genetic divergence between accessions or groups was analysed by Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using the program GenAlex 6.4 (Peakall and Smouse 2006). Significance of the results was tested by permuting the DNA marker data 999 times. A neighbor-joining (NJ, Saitou and Nei 1987) dendrogram was constructed using the program MEGA ver. 4 (Tamura et al. 2007). The genetic distances between accessions for the dendrogram were calculated with the program POPDIST (Tomiuk et al. 2009), where the estimation of genetic distances is based on grouping of allele phenotypes (distance measure of Tomiuk et al. 1998), in which case the degree of ploidy is of no importance. As far as we know, POPDIST is the only program for diversity studies that can handle codominant markers in polyploids. Principal coordinates analysis (PCA) based on Nei's genetic distances between accessions was performed using the software GenAlex 6.4 (Peakall and Smouse 2006).

Map coordinates were availabe for 71 accessions. Correlation between genetic distance (described with different distance indices: Euclidean distance from Arlequin, Nei's distance (Nei 1972) from GenAlex, and Tomiuk and Loeschke distance from Popdist) with geographic distance (km) was tested using a Mantel-test (Mantel 1967) in the software GenAlex. Mantel test was also used to compare different genetic distance indices.

RESULTS

Diversity in SSR loci

Thirteen SSRs (Table 2) were selected to assess genetic diversity in timothy accessions. Of these, five included a dinucleotide motif and eight a trinucleotide motif, of which two were compound ones. In some cases the allelic series (allele sizes fit the assumption of increments of two or three nucleotides) was perfect (C02C08, B03F07, C01E11, D01H08) but usually some alleles were missing. Generally, the allele sizes followed neatly the increment of two or three bases. However, a few extra alleles that did not fit the allele series existed in all SSRs except C01E11. In some SSRs (C02H01, D01G10, B03A09, A03E06), there seemed to be another allele series differing from the common one with one base pair (Fig. 2). The existence of this other series most probably was the outcome of an indel mutation in the SSR amplicon. As a consequence, the size of all the alleles arisen thereafter had shifted with one base pair.

The 13 selected SSRs amplified a total of 499 polymorphic alleles, the number of alleles per SSR locus varying from 15 (C01E11 and A09H08) to 74 (D01G10) (Table 2). The average repeat length of alleles in SSR loci correlated positively ($r = 0.69$) with the total number of alleles in the loci. Most of the alleles were quite rare, ca. 40% occurred in not more than 1% of all the individuals, or

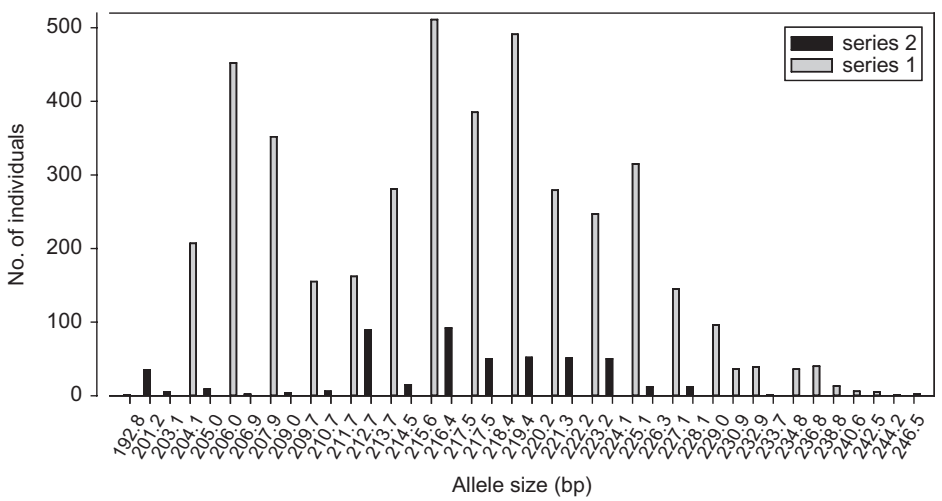

Fig. 2. An example of the two allelic series in SSR locus B03A09.

10% of the accessions. This usually caused a high occurence of the most common allele in a SSR locus (Table 2).

The mean number of alleles in an individual varied from 1.12 to 3.91 depending on the SSR locus. Very few individuals (1.2%) carried six alleles in any SSR locus. The most heterozygous SSRs were C01B11 and A09H08: 66% and 54% of the individuals in the study, respectively, contained four alleles or more in these loci. On the other hand, the alleles in the SSR loci B03F07 and C01E11 often occurred alone (in 48% and 67% of the individuals, respectively). This is perhaps spurious, due to the existence of null alleles, which is also reflected by the large number of apparent missing information in these loci (15.1% and 18.8%, respectively, Table 2).

The five SSRs with a dinucleotide repeat amplified an average of 35.4 alleles compared to 40.3 alleles amplified by the eight SSRs with a trinucleotide repeat (Table 2). The average observed number of alleles/accession was 11.7 and 13.4, and of private alleles 3.8 and 4.9 in SSRs with di- and trinucleotide repeats, respectively (results not shown). However, none of these differences were statistically significant (t-test, $p > 0.05$).

Genetic diversity within accessions

Taking all the 499 SSR alleles (= individual markers) together, the observed number in each accession ranged from 95 (NGB10785) to 203 (NGB14403) (Table 1). Most of the markers were polymorphic i.e. very few existed in all individuals of an accession. The number of private alleles i.e. alleles that did not exist in any other accession was generally low but accessions PI325461 (from Russia) and RCAT040682 (from Hungary) included eight and six private alleles, respectively. A_I ranged from 28.4 (NGB14417) to 36.2 (NGB733 and NGB757) (Table 1). Genetic diversity within accessions measured as PWD varied from 28.9 (NGB10785) to 46.8 (NGB9285) (Table 1).

Levels of diversity were found to be significantly different between countries, vegetation zones and different cultivar types (ANOVA, Table 3, 4). Finnish accessions were more diverse than Danish accessions when A_A was compared (Tukey's test, $p < 0.05$). For PWD, Denmark showed less diversity than Finland, Norway or Sweden (Tukey's test, $p < 0.05$). Danish accessions were also less diverse than Finnish, Swedish, Norwegian or exotic based on A_I. Accessions originating from southern boreal or middle boreal vegetation zone were more diverse than those from nemoral or alpine vegetation zone when A_A or PWD were compared (Tukey's test, $p < 0.05$). Vegetation zones explained 33% of the variation in diversity levels between timothy accessions (Table 3). Significant differences between vegetation zones were also observed for A_I but they were very small and explained only a minor fraction of variation between individuals (Table 3). Accessions with the cultivar type L, meaning landrace or traditional, locally cultivated accession, had a higher A_A when compared to cultivars or wild accessions. Landraces also were more diverse than cultivars based on PWD or A_I (Tukey's test, $p < 0.05$).

No correlation was observed between latitude or altitude and the diversity indices. However, a weak correlation was observed between longitude and A_A ($r = 0.29$, $p = 0.013$).

Genetic divergence between accessions and groups

AMOVA was performed in order to divide the total genetic variation into three components: variation within accessions, among accessions and among groups. According to AMOVA analysis, most of the variation (94%) in the studied timothy material was due to variation within accessions and only 5% was between accessions and 1% between countries (Table 5). No genetic divergence was observed between vegetation zones or cultivar types (AMOVA, $p > 0.05$).

No clear clustering of accessions based on countries or any other grouping was seen in either PCA (Fig. 3) or NJ dendrogram (Supplementary material Appendix A1 Fig. A1). In PCA, the first two axes explained 42.4% of the variation among the 96 accessions. Most of the accessions clustered together apart from a couple of exceptions. Genetic distance matrices counted with different ways

Table 3. *ANOVA table showing F-values, significance levels P and R^2 for comparisons of different groups for their levels of SSR diversity. Here A_I represents the number of alleles on individual level.*

		Total number of alleles (A_A)			Number of pairwise differences (PWD)			Number of alleles per individual (A_I)		
Diversity index	df	F	p	R^2	F	p	R^2	F	p	R^2
Grouping										
Accession	95							3.52	< 0.001	0.16
Country of origin	5	4.15	0.002	0.19	4.40	0.001	0.20	14.15	< 0.001	0.04
Vegetation zone	5	6.86	< 0.001	0.33	6.78	< 0.001	0.33	13.29	< 0.001	0.04
Cultivar type	2	8.58	< 0.001	0.17	4.46	0.014	0.10	6.05	0.002	0.01

Table 4. *Means, standard deviations, minimums and maximums of the three diversity indices (A_A, PWD and A_I). Groups differing significantly ($p < 0.05$) from each other in pairwise comparisons by Tukey's test are marked with different letters.*

	Number of	Corrected number of alleles per accession (A_A)					Pairwise differences between accessions (PWD)					Number of	Number of alleles per individual (A_I)				
Grouping	accessions	mean	st.dev	min	max	Tukey	mean	st.dev	min	max	Tukey	individuals	mean	st.dev	min	max	Tukey
Cultivar type																	
Cultivar	10	142.1	25.2	107.9	175.9	A	38.2	4.0	32.5	46.8	A	184	32.1	4.5	23	47	A
Landrace	17	170.0	12.6	128.0	185.0	B	42.5	2.9	35.0	46.6	B	323	33.5	4.4	19	51	B
Wild	59	152.0	18.8	86.8	174.6	A	40.2	4.0	28.9	45.3	AB	1117	32.7	4.4	17	57	AB
Country																	
Denmark	10	133.8	18.3	110.1	171.5	A	35.7	4.3	30.7	171.5	A	192	30.6	4.2	17	41	A
Finland	25	164.4	16.4	115.2	180.8	B	41.6	3.6	32.5	180.8	B	481	33.2	4.3	19	51	B
Iceland	2	143.7	45.9	111.2	176.2	AB	39.3	6.6	34.6	176.2	AB	38	32.3	4.4	23	39	AB
Norway	26	150.9	21.2	86.8	174.6	AB	40.4	3.9	28.9	174.6	B	485	33.2	4.3	20	52	B
Sweden	25	153.8	19.3	107.9	185.0	AB	40.8	3.1	32.7	185.0	B	463	33.0	4.4	19	57	B
Exotic	8	145.8	14.5	122.3	170.5	AB	38.3	3.0	34.6	170.5	AB	152	31.7	3.7	20	42	B
Vegetation zone																	
Nemoral	11	139.8	17.8	113.1	171.5	A	36.8	4.4	30.7	44.0	A	209	31.0	4.3	17	42	A
Boreonemoral	15	153.7	16.8	115.1	174.6	AB	40.5	3.6	32.5	45.3	AB	287	32.7	4.0	21	48	B
Southern boreal	14	166.5	9.3	146.6	180.8	B	42.7	1.9	39.2	46.3	B	266	33.7	3.8	20	45	BC
Middle boreal	22	166.1	11.9	130.9	185.0	B	42.6	1.8	38.9	46.6	B	412	33.7	4.8	19	57	C
Northern boreal	11	143.2	25.5	86.8	173.4	AB	38.8	4.8	28.9	43.6	AB	207	32.6	4.4	20	47	B
Alpine	2	142.5	15.7	131.4	153.6	A	37.3	3.6	34.7	39.8	A	40	32.0	3.8	23	39	ABC

Table 5. *Analysis of molecular variance in 96 timothy accessions based on SSR markers.*

Source	df	SS	MS	Variance components	% total
Among countries	5	711.41	142.28	0.25	1%
Among accessions/countries	90	6391.33	71.01	1.91	5%
Within accessions	1715	59970.62	34.97	34.97	94%
Total	1810	67073.36		37.13	100%
Stat	Value	P(rand ≥ data)			
φ_{RT}	0.007	0.001			
φ_{PR}	0.052	0.001			
φ_{PT}	0.058	0.001			

Probability, P(rand ≥ data), for PhiRT, PhiPR and PhiPT is based on 999 permutations across the full data set.
PhiRT = AC/(WA + AA + AC) = AC/TOT
PhiPR = AA/(WA + AA)
PhiPT = (AA + AC)/(WA + AA + AC) = (AA + AC)/TOT
Key: AC = est. var. among countries, AA = est. var. among accessions, WA = est. var. within accessions.

correlated well (Mantel-test) with each other: Euclidean distance (from Arlequin) with Tomiuk and Loeschke distance (POPDIST), $r = 0.83$ ($p < 0.001$), Nei's distance (GenAlex) with Tomiuk and Loeschke distance, $r = 0.87$ ($p < 0.001$), and Euclidean distance with Nei's distance $r = 0.98$ ($p < 0.001$). No significant correlation was found between genetic distance and geographic distance among accessions.

DISCUSSION

Genetic diversity within and among 96 timothy accessions mostly from Nordic countries was assessed with 13 selected SSR loci. This is the first study reporting SSR diversity in a large collection of timothy accessions. Timothy proved to be very diverse both on individual and accession level.

When levels of diversity were compared between wild accessions from different vegetation zones, southern and middle boreal vegetation zones proved to be the most variable ones. This is partly in accordance with the abundant centre model (Brown 1984), which presumes reduced neutral genetic diversity within peripheral compared to more central populations (Eckert et al. 2008). In Nordic countries timothy grows at its northern margin. At northern boreal and alpine vegetation zones, harsh winter conditions may limit survival of timothy and this may be reflected in the lower level of diversity found in these peripheral populations. However, in the more southern zones, nemoral and boreonemoral, timothy is not at the periphery of its distribution area, and it is not easily explained why a reduction in diversity was seen there. The vegetation zones with the highest diversity are also the best regions for cultivation of timothy and gene flow from cultivars may strongly affect the diversity of wild populations. In the nemoral and boreonemoral zones, other species, like *Lolium perenne*, are more commonly cultivated than timothy, which may limit gene flow from varieties to wild populations. Diversity could also be the highest in regions where timothy is best adapted to.

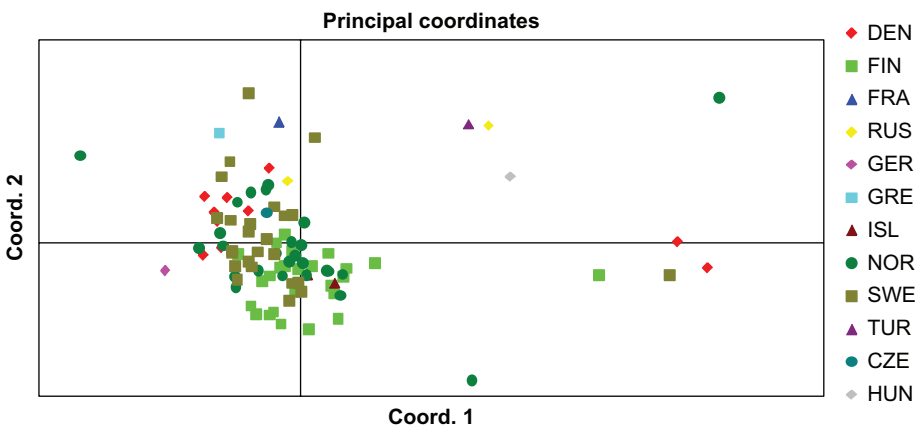

Fig. 3. Principal coordinates analysis based on Nei's genetic distances between accessions.

These could also be suitable zones for reproduction of timothy accessions from NordGen collection. Previous studies have shown that genetic diversity of plant populations may either increase or decrease with increasing altitude (Yan et al. 2009). However, in our study we did not see any correlation between the diversity indices and the altitude.

We found very little genetic divergence between accessions since most of the SSR variation (94%) was within accessions. No clear clustering of accessions was observed. Lack of geographical differentiation is partly related to the outcrossing and hexaploid nature of timothy. Timothy is a wind pollinated species and its pollen is known to travel long distances (Raynor et al. 1972). Adequate gene flow between populations or from cultivated varieties to populations could hinder divergence of populations. It has been reported that long-lived, outcrossing, late successional taxa retain most of their genetic variability within populations (Nybom 2004). In ryegrass (*L. perenne*), which is a diploid outcrossing perennial, most of the total gene diversity in isozyme loci was within populations (Balfourier 1998), and in diploid outcrossing meadow fescue (*Festuca pratensis*) varieties from Nordic countries, 79.3% of variation in AFLP markers was within populations (Fjellheim and Rognli 2005). In autotetraploid alfalfa (*Medicago sativa*), even 99.8% of genetic variation in SSR markers was within populations (Bagavathiannan et al. 2010). In timothy, significant differentiation between populations was reported on phenotypic level, although close to 90% of variation was still detected within populations for all traits scored (Casler 2001). Casler (2001) also found differentiation between varieties and wild populations of timothy for several morphological traits. However, population structure observed at phenotypic level is not always reflected at molecular level when neutral markers are used (Karhu et al. 1996). We could not detect any differentiation between cultivar types using neutral SSR markers. This could result from frequent gene flow between varieties and wild populations. Guo et al. (2003) distinguished geographical genotype groups in timothy based on neutral DNA markers; however their populations were genotyped from bulked DNA samples thus ignoring the within population variation. One possible source for low divergence between accessions could be contaminations between populations during multiplication of population samples. We have used here *ex situ* collection as a source for populations and presume that the sample collected to genebank represents the original population well. The accessions used were collected to NordGen between 1972 and 2002. However, although 34 of the 88 NordGen accessions used were multiplied once after sampling, multiplication was done in several locations and sites (20 year $\times$ location combinations). Thus it is not possible that the lack of differentiation observed was due to pollen contamination during multiplication steps. In a hexaploid plant, each locus may harbor up to six alleles which raises heterozygosity in populations. Differences between populations may rather be allele frequency differences than allele content differences; since we could not count allele frequencies here these differences could not be counted.

The SSR markers used in the study were developed by Cai et al. (2003), and to our knowledge, our study is the first one where they have been used since. Markers have to fulfill certain criteria to be useful in diversity analysis. First, they have to be highly polymorphic. In our study, the 13 selected timothy SSRs expressed immense diversity with a total of 499 alleles. Secondly, markers should be evenly distributed in the genome. Seven SSRs in our study were located on six out of seven linkage groups of the diploid timothy map (Cai et al. 2009). Thirdly, markers should be easy to interpret. In an outcrossing polyploid species individuals contain many marker bands and complex band profiles are generated, which was also seen in our study. In addition, interpretation of SSRs was challenging due to stuttering. We selected only the most easily interpreted SSRs for final analyses. Still one problem with polyploidy was that exact genotypes of plants could not be defined. In some polyploid species the allelic configurations of individuals in a given SSR locus have been determined (Esselink et al. 2004; Nybom et al. 2004), when unambiguously scorable markers with no or very few stutter bands were analysed. However, we found the evaluation of allele dosages very unreliable in the hexaploid timothy. In addition, preferential amplification of alleles in heterozygotes (Walsh et al. 1992; Weissensteiner and Lanchbury 1996; Wattier et al. 1998), and the existence of null alleles might complicate the situation. Null alleles are formed when SSR primers cannot attach due to DNA sequence differences. In our study, this was probably the reason to the appearance of no individuals with more than four alleles in two SSR loci, which might mean that one of the three timothy genomes was different from the two others in these sequence regions so that amplification of alleles did not happen. This supports the hypothesis of hexaploid timothy consisting of four doses of *bertolonii* and two doses of *rhaeticum* genome (Stewart et al. 2011). Because of the above-mentioned difficulties, each SSR allele in the current study was scored as a separate dominant marker. Therefore, we were not able to count the actual allele frequencies, which limited the discriminative power of a codominant marker system, and lead to loss of information, not in quality but in quantity. However, in the POPDIST program, it was possible to enter the allele composition of an individual (though not the exact genotype) and to gain more information. Yet, the genetic

distances counted with this program correlated well with those obtained from other programs where the alleles were scored as dominant markers, and diversity analyses (PCA, AMOVA) performed using genetic distance matrix from POPDIST could not separate different populations or countries any better (results not shown).

SSR evolution is a complex process, which is not well understood, and mechanisms and mutation models have been presented to explain the occurrence of SSRs and their allele distribution (Ellegren 2004; Oliveira et al. 2006). The mutation rates and patterns vary between repeat types, species, and also between loci (Ellegren 2004). Our study contained only 13 SSRs and therefore it is not possible to draw very extensive conclusions. However, we found higher diversity in long SSRs, which has been noticed in many organisms (Ellegren 2000; Petit et al. 2005). The shape of the allele distributions (e.g. SSR locus B03A09, Fig. 2) does not support any of the mutation models presented (Ellegren 2004). The most common allele in all SSR loci was located at the forepart of allele distribution, and if this allele is supposed to be the progenitor allele of that SSR locus then more mutations lead to longer alleles than to shorter ones. Directionality in favour of gains over losses in SSR mutation process has been found in humans and birds but there are also contradictory results (Ellegren 2004). The majority of mutations in SSR loci have been reported to represent length mutations i.e. additions or deletions of entire repeat units (Ellegren 2000). In our study single nucleotide indels were frequent as well, and they led to another allele series in 30% of the SSRs.

To conclude, the Nordic timothy material seems to be diverse enough for breeding purposes and no decline in the level of diversity was observed in varieties compared to wild timothy populations. However, no heterotic groups could be defined which could be used to enhance breeding of synthetic varieties, since most of the SSR variation observed was between individuals within accessions. Phenotypic evaluation of the timothy accessions is needed before further conclusions for variety breeding can be drawn. The plant material used in our experiment has also been phenotyped in replicated field trials and a closer comparison of molecular and phenotypic diversity is in progress. When compared to cereals, timothy varieties are closer to local and natural populations e.g. some of the varieties presently in cultivation are straight selections from local populations. Since varieties have not diverged too much from the local and natural populations, it may be relatively easy to use genebank material directly for variety development.

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Supplementary material (available online as Appendix HRD2244 at ＜www.oikosoffice.lu.se＞). Appendix A1

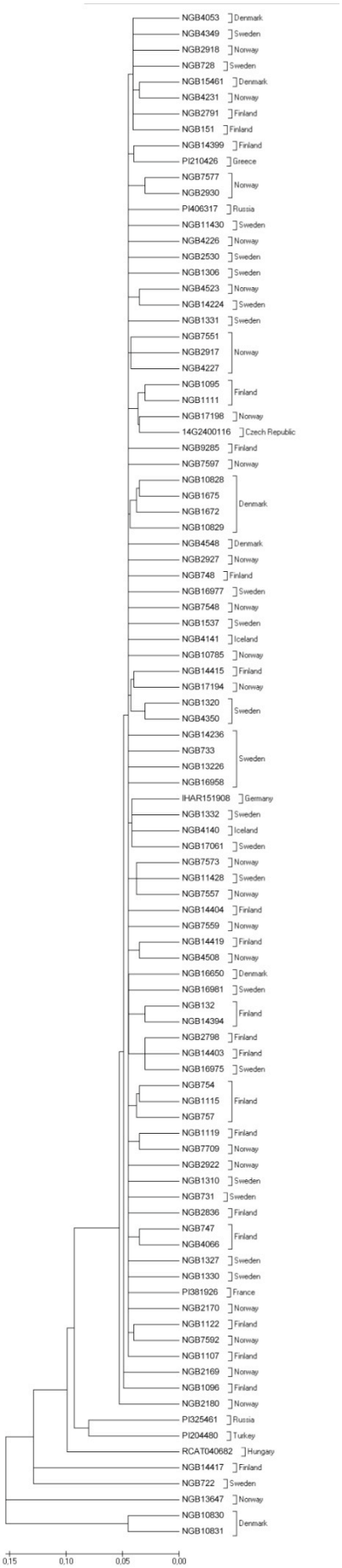
Hereditas

HRD2244

Tanhuanpää, P. and Manninen, O. 2012. High SSR diversity but little differentiation between accessions of Nordic timothy (*Phleum pratense* L.). – Hereditas 000: 000-000.

Appendix A1

Fig. A1. A NJ dendrogram of the 96 timothy accessions constructed using the program MEGA version 4. The genetic distances between accessions for the dendrogram were calculated with POPDIST.


NGB4053 Denmark
NGB4349 Sweden
NGB2918 Norway
NGB728 Sweden
NGB15461 Denmark
NGB4231 Norway
NGB2791 Finland
NGB151 Finland
NGB14399 Finland
PI210426 Greece
NGB7577 Norway
NGB2930
PI406317 Russia
NGB11430 Sweden
NGB4226 Norway
NGB2530 Sweden
NGB1306 Sweden
NGB4523 Norway
NGB14224 Sweden
NGB1331 Sweden
NGB7551 Norway
NGB2917
NGB4227
NGB1095 Finland
NGB1111
NGB17198 Norway
14G2400116 Czech Republic
NGB9285 Finland
NGB7597 Norway
NGB10828 Denmark
NGB1675
NGB1672
NGB10829
NGB4548 Denmark
NGB2927 Norway
NGB748 Finland
NGB16977 Sweden
NGB7548 Norway
NGB1537 Sweden
NGB4141 Iceland
NGB10785 Norway
NGB14415 Finland
NGB17194 Norway
NGB1320 Sweden
NGB4350
NGB14236 Sweden
NGB733
NGB13226
NGB16958
IHAR151908 Germany
NGB1332 Sweden
NGB4140 Iceland
NGB17061 Sweden
NGB7573 Norway
NGB11428 Sweden
NGB7557 Norway
NGB14404 Finland
NGB7559 Norway
NGB14419 Finland
NGB4508 Norway
NGB16650 Denmark
NGB16981 Sweden
NGB132 Finland
NGB14394
NGB2798 Finland
NGB14403 Finland
NGB16975 Sweden
NGB754 Finland
NGB1115
NGB757
NGB1119 Finland
NGB7709 Norway
NGB2922 Norway
NGB1310 Sweden
NGB731 Sweden
NGB2836 Finland
NGB747 Finland
NGB4066
NGB1327 Sweden
NGB1330 Sweden
PI381926 France
NGB2170 Norway
NGB1122 Finland
NGB7592 Norway
NGB1107 Finland
NGB2169 Norway
NGB1096 Finland
NGB2180 Norway
PI325461 Russia
PI204480 Turkey
RCAT040682 Hungary
NGB14417 Finland
NGB722 Sweden
NGB13647 Norway
NGB10830 Denmark
NGB10831
0.15
0.10
0.05
0.00