Theor Appl Genet (2005) 110: 1290–1300
DOI 10.1007/s00122-005-1966-z

ORIGINAL PAPER

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Genetic diversity among barley cultivars assessed by sequence-specific amplification polymorphism

Received: 17 November 2004 / Accepted: 14 February 2005 / Published online: 1 April 2005

Abstract We analyzed the genetic structure and relationships among barley cultivars (*Hordeum vulgare* L.) with sequence-specific amplification polymorphisms (S-SAPs). Polymorphisms were identified in 824 individual barley plants representing 103 cultivars (eight plants per cultivar) widely grown in Canada and the United States, using PCR primers designed from the long terminal repeat of the barley retrotransposon BARE-1 and a subset of four selective *Mse*I primers. From the 404 bands scored, 150 were polymorphic either within or between cultivars. Genetic structure assessed with analysis of molecular variance attributed the largest component of variation to the within groups of cultivars (69–86%). Within-cultivar genetic variation was estimated as average gene diversity over loci and ranged from 0 (completely homogenous) to 0.076 (most heterogeneous cultivar). Only 17 out of 103 cultivars (16%) were judged to be homogenous by this criterion. Relationships among cultivars were analyzed by cluster analysis using unweighted pair-groups using arithmetic averages and found groups similar to those determined by agriculturally significant phenotypic traits such as spike morphology (two-rowed or six-rowed), cultivar type (malting or feed), seed characteristic (hull-less or hulled), and growth habit (winter or spring), with minor overlaps. Discriminant analysis of groups determined by these phenotypic traits fully supported the different groups with minor overlaps between the malting/feed. S-SAP markers generated from retrotransposons such as BARE-1 are invaluable tools for the study of genetic diversity in organisms with a narrow genetic base such as barley. In this study, S-SAP analysis revealed significant amounts of cryptic variation in closely related cultivars including somaclonal variation, which could not be inferred by the pedigree analysis.

Communicated by H.C. Becker

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Introduction

Assessment of the extent and nature of genetic variation in crop species has important implications in breeding, plant improvement, and conservation of plant genetic resources. In barley breeding programs, emphasis was placed on developing cultivars for specific end use, i.e., malting and brewing; thus, over time the genetic base of barley has become narrower compared to that of other crops (Martin et al. 1991).

The aim of breeding new barley cultivars is to produce new allele combinations. Heritable variation is created by controlled crosses in breeding programs between adapted high-yielding cultivars and breeding lines. Cultivar breeding is a directional selection and mainly based on elite germplasm, although specific germplasm may be incorporated by introgression from wild relatives and landraces in backcrossing programs (Nevo 1992). Selection for desirable traits is made in the field or greenhouse. These traits may include lodging resistance, disease resistance, yield, straw strength, and protein content of the grain. In addition, malting properties including extract yield, viscosity of grain, and malt may be selected. Selection is applied at the F_2 generation for highly heritable traits and at F_3 or F_4 for most traits in a pedigree-type breeding program. The early generations are heterozygous, which makes selection difficult, but an acceptable level of homozygosity is achieved following the sixth or seventh generation of selfing. The raw material in any breeding program is the amount of genetic diversity available in the gene pool that can be subsequently used for selection.

Several different types of DNA marker systems are available for measurements of genetic diversity in plants including, amplified restriction fragment length polymorphism (AFLP) (Vos et al. 1995; Barrett et al. 1998; Qi and Lindhout 1997; Reamon-Buttner and Jung 2000; Soleimani et al. 2002), random amplified polymorphic DNA (Williams et al. 1990; Thormann and Osborn 1993; Tinker and Mather 1993), simple sequence repeat (Tautz and Renz 1984; Akkaya et al. 1992), and retrotransposon-based marker systems including sequence-specific amplification polymorphism (S-SAP) (Waugh et al. 1997), inter-retrotransposon amplified polymorphism, and retrotransposon–microsatellite amplified polymorphism (Kalendar et al. 1999). Each of these marker systems differs in many respects, such as the distribution of target sequences throughout the genome, number of detected bands per assay (multiplex ratio), level of detected polymorphism, dominance/co-dominance nature (differentiating heterozygous from homozygous genotypes), ease of automation, assay reproducibility, and the cost associated with the marker system.

Retrotransposon-based marker systems rely on two principles. First, integration of a retrotransposon in a genomic location is fixed and behaves in a Mendelian fashion, and second, multiple integrations of the element in various genomic locations provide substrates of known DNA sequence for various PCR-based screening assays.

In barley, S-SAP markers (Waugh et al. 1997; Gribbon et al. 1999) were developed from BARE-1 element. BARE-1 is a member of the Ty1-*copia* group of retroelements (Manninen and Schulman 1993). An average of 14×10^3 retrotransposons belonging to the BARE-1 family were found in the genomes of *Hordeum* species (Vicient et al. 1999). Members of this family were found to be transcriptionally and translationally active, encoding a polyprotein and processing signals (Suoniemi et al. 1996, 1999; Jaaskelainen et al. 1999). Active transposition of this element, coupled with its wide distribution throughout the barley genome, makes it a good candidate as potential source of molecular markers for the study of the structure and evolution of barley genome.

S-SAP markers have been used for linkage and genetic diversity analysis in wheat (Queen et al. 2004), grapevine (Labra et al. 2004), pea (Ellis et al. 1998), oats (Yu and Wise 2000), maize (Casa et al. 2000), and *Medicago* (Porceddu et al. 2002). The ubiquitous presence of retrotransposons (Flavell et al. 1992; Hirochika et al. 1992; Voytas et al. 1992; Suoniemi et al. 1998; Noma et al. 1999) and their wide genomic distribution makes S-SAPs valuable tools for the study of genetic diversity in plants (Waugh et al. 1997; Labra et al. 2004).

The aims of this study were to assess genetic diversity levels within and between barley cultivars and to infer the utility of the S-SAP method for investigation of genetic diversity among a large group of barley cultivars with various agronomic characteristics. The information obtained could be used for future breeding purposes and to further improve barley cultivars.

Materials and methods

Plant materials and S-SAP reactions

Breeders' seed from 103 barley cultivars (Table 1) was obtained from the Canadian Grain Commission (Winnipeg, Man., Canada) and the Canadian Food Inspection Agency (Ottawa, Ont., Canada).

S-SAP procedure is described extensively by Waugh et al. (1997). Briefly, barley seeds were germinated on moist Petri dishes at room temperature for 1 week. Genomic DNA from 1-week-old seedlings was extracted using a DNeasy 96 plant kit (Qiagen). Half microgram genomic DNA from each plant were incubated with 5 U *Mse*I and 2.5 U *Pst*I restriction endonucleases at 37°C for 2 h. *Pst*I is a methylation-sensitive enzyme that does not contain any restriction site within the BARE-1 retrotransposon (GenBank accession no. Z17327). Both enzymes were used in excess to ensure complete digestion of the genomic DNA. *Pst*I linkers (*Pst*I-1 linker: 5′-CTCGTAGACTGCGTACATGCA-3′, *Pst*I-2 linker: 5′-TGTACGCAGTCTAC-3′) and *Mse*I linkers (*Mse*I-1: 5′-GACGATGAGTCCTGAG-3′, *Mse*I-2: 5′-TACTCAGGACTCAT-3′) were ligated to the ends of restricted fragments using T4 DNA ligase (Invitrogen). The ligation reactions were carried out overnight at room temperature. The products of the ligation reaction were diluted tenfold in a buffer of 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. S-SAP-PCR amplifications were carried out in two steps: a preamplification PCR step with *Pst*I + C and *Mse*I + C primers (where the symbol "+C" indicates the presence of the selective nucleotide C at the 3′ end of the primers) was carried out to reduce the number of amplified restricted fragments and to ensure that only *Pst*I/*Mse*I fragments were amplified. Lack of a *Pst*I restriction site within the BARE-1 element as described earlier implies that *Pst*I/*Mse*I fragments contain both host and the element DNA. The product of preamplification-PCR was subsequently used as template for selective amplification with an *Mse*I primer containing three selective bases at the 3′ end in combination with a [^{33}P]-labeled long terminal repeat (LTR)-derived primer. The PCR products were mixed with an equal volume of denaturing dye (98% de-ionized formamide, 0.025% bromo-phenol-blue, 0.025% xylene cyanol) and denatured at 94°C for 3 min. Amplification products were resolved in a 5% denaturing polyacrylamide gel and electrophoresed at 80 W (constant power) for 2–3 h. The gels were dried and exposed to Kodak X-Omat film for 3–5 days at −80°C. DNA fingerprints were evaluated by visual inspection of autoradiographs.

Table 1 Material used in sequence-specific amplification polymorphism (S-SAP) analysis of Canadian barley cultivars. *R* Row number, *G* growth habit (*s* spring, *w* winter), *S* seed characteristic (*h* hull-less, *d* hulled) *U* use (*f* feed, *m* malting), *NH* number of haplotypes, *NP* number of polymorphisms within cultivar, *NPD* number of pairwise differences $\hat{H}$ gene diversity over loci, *SD* standard deviation of $\hat{H}$

	Cultivar name	R	G	S	U	Parentage[a]	NH	NP	NPD	$\hat{H}$	SD of $\hat{H}$	Released
1	Ac Albright	6	s	d	f	Otra/6/(Weibull1514–64) Morgenrot/5/ Tammi/4/Maja/3/Opal//Hanna/Svanhals	3	2	0.5000	0.0030	±0.0038	1992
2	Ac Alma	6	s	d	f	Chapais/Leger	4	4	1.8210	0.0131	±0.0096	1996
3	Ac Bacon	6	s	h	f	Tupper/Johnston//Conquest/3/Abee/4/ Ellice/Bedford	7	14	6.4640	0.0598	±0.0361	1998
4	Ac Bountiful	2	s	d	m	Wpg843–234/Manley//AC Oxbow/Manley	2	1	0.5714	0.0041	±0.0041	1999
5	Ac Burman	6	s	d	f	Leger/Bruce/2/2*Leger	2	1	0.2500	0.0018	±0.0025	1991
6	Ac Hamilton	6	s	d	f	(Trent/Vanier)/(York//CI 10853/Parkland)/Perth	8	8	3.7140	0.0269	±0.0172	1994
7	Ac Harper	6	s	d	f	49–125/BT364/6* Galt//BT201/6* Galt/3/BT364	2	1	0.2500	0.0018	±0.0025	1996
8	AC Hawkeye	6	s	h	f	GO1-1/Tupper/3/Virden//Conquest/Post	2	1	0.7514	0.0045	±0.0046	1996
9	Ac Klinck	6	s	d	f	Cadette/Chapais	4	4	1.5000	0.0122	±0.0093	2000
10	Ac Lacombe	6	s	d	f	Klondike//Galt/Unitan	8	21	9.2850	0.0750	±0.0442	1991
11	Ac Legend	6	s	d	f	Chapais/CIMMYT-6	7	7	3.5000	0.0284	±0.0184	1998
12	Ac Malone	6	s	d	f	Callus culture of Leger	4	4	1.7850	0.0095	±0.0077	1999
13	Ac Maple	6	s	d	f	Chapais/CIMMYT-6	4	7	1.9280	0.0156	±0.0113	2000
14	AC Metcalf	2	s	d	m	Oxbow/Manley	7	10	4.6420	0.0329	±0.0205	1997
15	Ac Nadia	6	s	d	f	Leger/QB 173.26	7	9	4.0357	0.0328	±0.0208	1993
16	AC Oxbow	2	s	d	m	TR223 TR222 WPG8020 WPG823	1	0	0.0000	0.0000	±0.0000	1991
17	Ac Rosser	6	s	d	f	Galt/Johnston/3/Steptoe//BT 351/Heartland	6	12	4.3214	0.0311	±0.0195	1997
18	Ac Stacey	6	s	d	f	Otal/Melvin	2	3	1.2850	0.0093	±0.0073	1989
19	Ac Westech	6	s	d	f	82RCBB.13/Etienne	5	10	4.2850	0.0308	±0.0194	1998
20	ACCA	6	s	d	f	QB730.2/UL0072/Leger	4	6	2.3570	0.0170	±0.0118	1996
21	Argyle	6	s	d	m	Herta/UM 570//conquest/3/Bonanza	3	3	1.0350	0.0074	±0.0062	1981
22	B1202	2	s	d	m	RPB 70-268/2B75-1223//Klages	2	1	0.5357	0.0038	±0.0039	2000
23	B1602	6	s	d	m	Bumper/6B78-628//Morex/6B78-628	4	2	0.8571	0.0060	±0.0054	1991
24	Banner	6	s	d	f	OB907-33/TBC891-6	8	13	5.8210	0.0412	±0.0251	2000
25	Bedford	6	s	d	f	Keystone/4/Vantage/Jet//Vantmore/3/2* Husky/5/Cree	6	18	8.0714	0.0572	±0.0339	1979
26	Bella	6	s	d	f	Meldugres/Carlsberg 63199	5	14	5.7850	0.0410	±0.0250	1992
27	Belluga	6	s	d	f	TBB 773-6 Mingo OB 339-1	3	3	0.7500	0.0056	±0.0052	1995
28	Blankeney	6	s	d	f	OB907-33/GB8901	3	4	1.9280	0.0146	±0.0105	2000
29	Blitz	6	s	d	f	TBC51-89/AB99-13	1	0	0.0000	0.0000	±0.0000	2000
30	Bonanza	6	s	d	m	Vantage/Jet//Vantmore/3/2* Parkland/ 4/Conquest	4	4	1.9640	0.0148	±0.0106	1970
31	Brier	6	s	d	f	Leduc///Galt/York//Dickson/Galt	2	1	0.4285	0.0032	±0.0036	1989
32	Bronco	6	s	d	f	Vanier/Laurier//Perth/3/Leger	3	11	2.9285	0.0271	±0.0180	1993
33	Brooke	6	s	d	f	Sandrine/TBC51-89	1	0	0.0000	0.0000	±0.0000	2000
34	Brucefield	6	s	d	f	Maskot/Chapais	3	4	1.9285	0.0178	±0.0128	1997
35	BT954	6	s	d	m	NA	6	14	4.8920	0.0347	±0.0215	2001
36	Cadette	6	s	d	f	QB 139.7/(Min660102/Bonanza)	8	19	6.7140	0.0621	±0.0373	1986
37	CDC Battleford	6	s	d	m	M67/Bt411	4	11	5.1428	0.0369	±0.0228	2001
38	CDC Copeland	2	s	d	m	WM861-5/TR118	2	1	0.5357	0.0039	±0.0040	1999
39	CDC Earl	6	s	d	f	Duke/Heartland	7	19	8.2850	0.0767	±0.0453	1993
40	CDC Kendall	2	s	d	m	Manley SM85221	4	2	1.1428	0.0083	±0.0068	1995
41	CDC Silky	6	s	h	f	Duke/(Nordic/BT 413)(M718/B4r6705-15-1)	1	0	0.0000	0.0000	±0.0000	1994
42	CDC Sisler	6	s	d	m	M34/Argyle	2	1	0.4285	0.0032	±0.0036	1996
43	CDC Springside	6	s	d	m	M76/SM93067	1	0	0.0000	0.0000	±0.0000	2001
44	CDC Stratus	2	s	d	m	Manley/ID 810279	1	0	0.0000	0.0000	±0.0000	1994
45	CDC Thompson	2	s	d	m	Nairn/Manley	5	4	1.2850	0.0094	±0.0074	1994
46	CDC Tisdale	6	s	d	m	BT409/Foster	3	2	0.6785	0.0051	±0.0049	2001
47	CDC Yorkton	6	s	d	m	M67/BT411	3	2	0.7857	0.0059	±0.0054	1999
48	Chapais	6	s	d	f	(QB 58.14/Beacon)/BT 904	2	1	0.2500	0.0019	±0.0026	1988
49	Codac	6	s	d	f	Diamond/Duke	7	6	2.7142	0.0204	±0.0137	1993
50	Duel	6	s	d	m	Morex//6B75-1374/M31	2	1	0.2500	0.0019	±0.0026	1992
51	Duke	6	s	d	f	(BrYG3-3/Fedak)//(M65-220/Bonanza)/UA1851	2	1	0.4285	0.0032	±0.0036	1986
52	Etienne	6	s	d	f	Perth/(64-76/(OB595/Algerian//Parkland5))	2	2	1.0714	0.0075	±0.0063	1988
53	Excel	6	s	d	m	Cree/Bonanza//Manker/3/2* Robust	8	13	5.2850	0.0375	±0.0230	1994
54	Falcon	6	s	h	f	11012.2/Tern//Tulelake	5	4	1.6780	0.0132	±0.0098	1992
55	Foster	6	s	d	m	Robust/6/Glenn/4/Nordic//Dickson/Trophy/3 /Azure/5/Glenn/Karl	3	2	0.8214	0.0058	±0.0052	2000
56	Gamine	6	s	d	f	Maskot/Chapais	1	0	0.0000	0.0000	±0.0000	1996
57	Grant	6	s	d	f	P885-4/P854-35	1	0	0.0000	0.0000	±0.0000	1996
58	Harrington	2	s	d	m	KlagesX(Gazelle/Betzes//Centennial)	2	1	0.5714	0.0042	±0.0042	1981
59	Jackson	6	s	d	f	Fjola/Husky (H64-35 BT607)/Pomo	1	0	0.0000	0.0000	±0.0000	1985

Table 1 (Contd.)

	Cultivar name	R	G	S	U	Parentage[a]	NH	NP	NPD	$\hat{H}$	SD of $\hat{H}$	Released
60	Jaeger	6	s	h	f	Nopal'S "- Ager [(F10.14/Mona-EmirxBco. Mr-Gvs) Api-CM67xOre]	3	2	0.6785	0.0053	±0.0051	1999
61	Johnston	6	s	d	f	Klondike/5/Nord/3/Vantage/Jet// Vantmore/4/Bonanza	2	1	0.5357	0.0040	±0.0042	1980
62	Jolly	6	s	d	f	Laurier/(BR.M. 45-680 X (Montcalm X Byng)	2	1	0.5357	0.0040	±0.0042	1987
63	Kasota	6	s	d	f	Celaya//Mezquita/Godiva/3/Trompillo	2	1	0.4285	0.0032	±0.0036	1995
64	Klondike	6	s	d	f	Galt/NDB133 Vantage/Jet//Vantmore/3/2* Parkland/4/Dickson	2	2	0.5000	0.0038	±0.0040	1976
65	Labelle	6	s	d	f	Loyola/Laurier	3	3	0.9285	0.0070	±0.0060	1991
66	Lacey	6	s	d	m	M44/Excel//M46/M44/3/M44/Excel//Stander	8	14	6.3570	0.0457	±0.0276	2001
67	Leduc	6	s	d	f	Steptoe/Klondike	2	1	0.5357	0.0043	±0.0044	1982
68	Legacy	6	s	d	m	6B86-3517/Excel	5	4	1.6785	0.0119	±0.0088	2001
69	Leger	6	s	d	f	Trent/Vanier	2	1	0.5714	0.0045	±0.0046	1982
70	Lucky	6	s	d	f	NA	2	2	0.5000	0.0040	±0.0042	2000
71	Mahigan	6	s	d	f	Celaya//Mesquita/Godiva/3/Trompillo	1	0	0.0000	0.0000	±0.0000	1998
72	Manley	2	s	d	m	Norbert//Hecter/Kleges	5	5	2.3928	0.0169	±0.0117	1991
73	Maskot	6	s	d	f	QB 167.21/OB193.11	2	1	0.2500	0.0020	±0.0028	1989
74	McDiarmid	6	w	d	f	Tapir/Wysor	3	2	0.5000	0.0040	±0.0042	1996
75	McGregor	6	w	d	f	Tapir/Wysor	1	0	0.0000	0.0000	±0.0000	1995
76	Merit	2	s	d	m	TR490/2B80-350	2	1	0.2500	0.0018	±0.0025	1999
77	Mistral	6	s	d	f	NA	3	4	2.2500	0.0159	±0.0111	2000
78	Myriam	6	s	d	f	NA	5	4	1.6428	0.0117	±0.0087	1994
79	Nellygan	6	s	d	f	CQ-CM/Apan//RM508/3/DL69/Hiproly	4	8	3.1428	0.0222	±0.0146	1999
80	Niska	6	s	d	f	BrY63-4/Galt	1	0	0.0000	0.0000	±0.0000	1999
81	Noble	6	s	d	f	Dickson/3/CIho 4738//Traill/UM 570	2	1	0.4285	0.0030	±0.0034	1987
82	Nord	6	s	d	f	Olli/Byng	1	0	0.0000	0.0000	±0.0000	1956
83	OAC Baxter	6	s	d	f	Chapais (male sterile)//OAC Kippen/Leger	3	2	0.6785	0.0048	±0.0046	2000
84	OAC Elmira	6	w	d	f	WB74-69//Wb74-69/WB55-1	6	7	1.9280	0.0153	±0.0110	1987
85	OAC Kippen	6	s	d	f	(York//CI 10853/Parkland)/Perth	2	1	0.2500	0.0018	±0.0024	1987
86	Otal	6	s	d	f	Otra/breeding line of Weibullsholm/3/ Hanna/Svanhals//Opal/4/Tammi/5/Morgenrot	2	2	1.0714	0.0075	±0.0063	1982
87	Peregerine	6	s	h	f	H12-4816/R181//M69.77-SHI. R.KCI.NO.87/CEL-5106	1	0	0.0000	0.0000	±0.0000	1999
88	Robust	6	s	d	m	Morex/Manker	2	1	0.2500	0.0018	±0.0024	1994
89	Sabina	6	s	d	f	QB 167.21/OB193.11	2	1	0.5714	0.0040	±0.0041	1989
90	Samson	6	s	d	f	Olli/M64-69//R72-181	3	6	2.6428	0.0186	±0.0126	1985
91	Sandrine	6	s	d	f	Mingo OB3391-1 QB203-4	2	11	2.7500	0.0200	±0.0135	1995
92	Sophie	6	s	d	f	BRM 45-680 X (Montcalm/Byng)/BRM 45680/QB 113.1	7	20	7.8920	0.0576	±0.0341	1980
93	Stander	6	s	d	m	Excel//Robust/Bumper	2	2	0.5000	0.0037	±0.0039	1999
94	Stetsen	6	s	d	f	Westbred 501/Gustoe	2	1	0.2500	0.0018	±0.0025	1995
95	Stien	2	s	d	m	Norbert//Hecter/Kleges	5	6	2.5350	0.0179	±0.0122	1987
96	Tankard	6	s	d	m	Argyle/Minnesota M-34	3	2	0.6780	0.0050	±0.0047	1992
97	TB891-6	6	s	d	f	(Vanier/Keystone//BT 421(S6525/Galt)) (Vanier/Laurier//Perth)	1	0	0.0000	0.0000	±0.0000	1992
98	Trochu	6	s	d	f	Noble//DL69/DL70///Mari-Coho/Nackta//TR219	1	0	0.0000	0.0000	±0.0000	2000
99	Tukwa	6	s	d	f	I74161/Hiproly	3	6	1.6785	0.0122	±0.0090	1993
100	Virden	6	s	d	f	WA6415-66//Bonanza/NDB136/3/ UM67-739R//Bonanza/Dickson	3	3	1.4285	0.0104	±0.0080	1987
101	Vivar	6	s	d	f	Leduc//DL69/DL70/3/Noble/4/CM67-U.Saak1800//Promesa/CM67//DL70	1	0	0.0000	0.0000	±0.0000	2000
102	Viviane	6	s	d	f	NA	3	5	2.4640	0.0178	±0.0122	1999
103	Westford	6	s	d	f	NA	3	3	1.5000	0.0108	±0.0083	2000

[a]/ Primary cross, // secondary cross, *number preceding* number of backcrosses

BARE-1 primers

LTR-derived PCR primers used in this study were designed from the LTR region of the BARE-1 retrotransposon. Sequences were retrieved from GenBank at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Initially, a large set of primers was designed covering most of the BARE-1 LTR sequence. Published LTR-derived primers (Waugh et al. 1997; Kalendar et al. 2000) were also synthesized and used in a prescreening S-SAP assay on barley cultivars in this study. Only two LTR-derived primers, R1 and R4, designed in this study, were retained for further analysis. All other primers especially those that were designed from the more 3′ regions of the LTR sequences produced suboptimal amplification products. R1 and R4 (Table 2) were combined with various *Mse*I selective primers (Tables 2, 3) for final S-SAP analysis.

Table 2 Sequences of PCR primers used in this study

Primer	Sequence 5′to 3′
R1	GTTATGTAGTGGGCGAGCGAG
R4	GCATACATGTGTCCTTCGTTGCC
M2	GATGAGTCCTGAGTAACAC
M3	GATGAGTCCTGAGTAACAG
M4	GATGAGTCCTGAGTAACAT
M7	GATGAGTCCTGAGTAACTG

Table 3 S-SAP primer pair combinations, number of amplified bands, and percentage polymorphism detected among barley cultivars

Primer pairs	No. amplified bands	No. polymorphic bands	Percent polymorphism
R1M4	106	54	50.9
R4M3	102	25	24.5
R1M7	111	36	32.4
R1M2	85	35	41.2
Total	404	150	37.1

Data analysis

DNA fingerprints were scored as binary variables, 1 for the presence and 0 for the absence of bands. The total number of bands that were scored included those that were clearly polymorphic, and those that were clearly monomorphic. Bands whose presence could not be clearly discerned were ignored.

Clustering

The binary data were used to generate a genetic similarity matrix with SIMQUAL routine using the DICE formula (Dice 1945), which is equivalent to Nei and Li (1979) genetic similarity coefficient from the NTSYS-pc statistical package (Rohlf 2000). Clustering of genotypes was performed using SAHN, also in NTSYS-pc, based on the genetic similarity matrix with the unweighted pair-group using arithmetic average (UPGMA) method.

Gene diversity estimates

The mean gene diversity ($\hat{H}$) and gene diversity over loci were calculated from the binary data based on Nei's (1987) formula using Arlequin program (Schneider et al. 1997). $\hat{H}$ is defined as the probability that two randomly chosen haplotypes are different in the sample.

Analysis of molecular variance

Variance components for barley populations and the relationships among haplotypes determined by a minimum spanning tree were calculated using analysis of molecular variance (AMOVA) with the Arlequin program (Schneider et al. 1997).

Regression

A linear regression analysis was carried out to determine the effects of selfing/inbreeding on the level of within cultivar variation. Inbreeding levels were determined, where possible, from variety description. These levels refer to the level of inbreeding in breeders' seed source. Seed samples used in this study are assumed to have derived from unselected bulks derived from breeders' seeds. They are therefore more highly inbred, but residual heterozygosity would be manifested as heterogeneity of haplotypes within the seed source.

Discriminant analyses

Group determination, i.e., classes, was preset according to the following sets of agronomic traits (or use): two-row/six-row, malting/feed, winter/spring, hulled/hull-less. The analyses were carried out separately for each trait set. Binary variables need not disqualify the various discriminant analyses (Rao 1952; Cochrane and Hopkins 1961; Gilbert 1968; Krzanoswski 1975). Canonical discriminant analysis (DA) (Kshirsagar 1972), an ordination method, was used to assess if there was justification to support each of the agronomic (or use) trait classifications. Canonical DA (in this case, using S-SAP variables) assumes prior knowledge of the grouping of the individuals and does not use the variables (in these cases the class variables) by which those individuals were grouped for the analysis. Classificatory DA was performed to obtain the appropriate discriminant functions to be used to identify an unknown sample based on its S-SAP profile, using a reduced set of band patterns. The approach taken was a conventional (Anderson 1984; Kshirsagar 1972) or parametric method, i.e., using the linear classification function. Mahalanobis distances based on the pooled covariance matrix were used, in which variables with zero variance, i.e., invariant, were deleted. Based on the pooled covariance matrix squared distances, between-class means were computed. The test of the distances between these class means, i.e., the Mahalanobis distances, was ignored, because the variables that were generated were binary and therefore do not comply with the assumption of normality. Therefore, the analyses were used as a guide only without relying on the various test statistics. To find the nature of the group differences, the within-canonical structure was examined, and the variables most highly associated with each of the first and only axis were summarized. Plots of the first and only canonical variable were made for visual examination to determine the degree of overlap between the two classes, separately for each set of agronomic traits. Cross validation (Lachenbruch and Mickey 1968) was also available as part of the SAS program and used to assess how the discriminant function classifies the observation that it excludes from the main data matrix. All computations were done using SAS (SAS Institute 2002).

Results

DNA fingerprints of 824 barley plants were obtained using two specific primers derived from the LTR region of BARE-1 element in combination with four selective *Mse*I primers (Table 2). Two LTR-derived primers, R1 and R4, were selected for the final S-SAP analysis based on the result of a prescreening S-SAP assay to select the most optimal primer combinations. The R1 primer sequence extends from nucleotide position 70 on the antisense DNA strand of BARE-1 LTR, and the R4 primer sequence extends from nucleotide position 384 of the sense strand of the BARE-1 LTR sequence. Generally, primers that were designed from the more 3′ regions of the LTR sequences yielded suboptimal amplification products and were therefore eliminated from the final analysis. An example of S-SAP profile for three barley cultivars, eight plants per cultivar, is shown in Fig. 1.

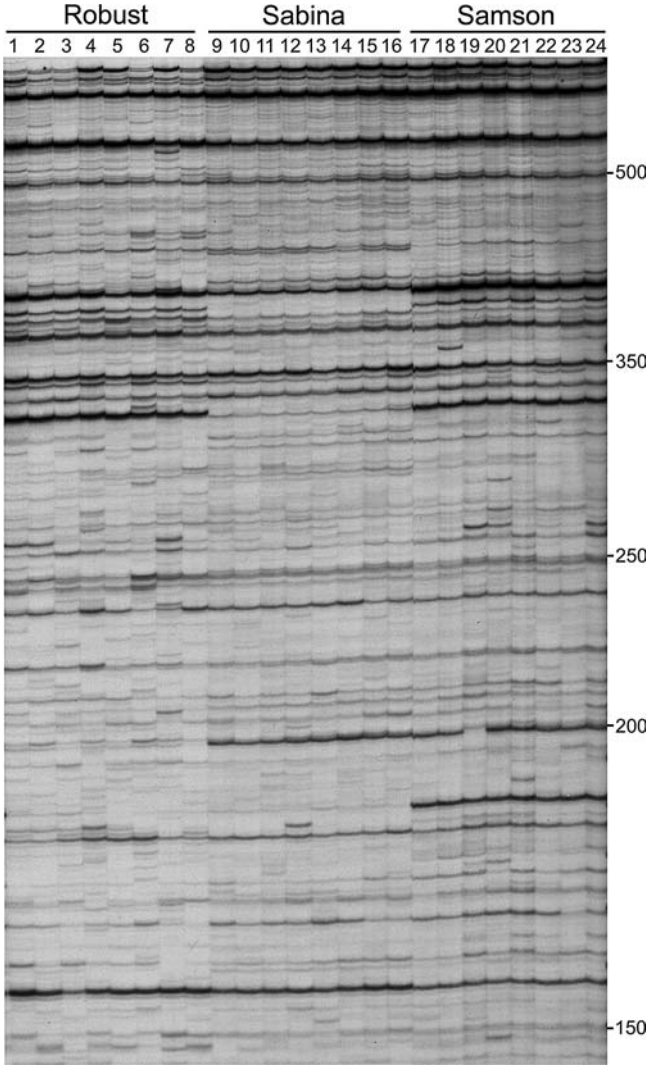

Fig. 1 Portion of sequence-specific amplification polymorphism (S-SAP) profile of three barley cultivars with R4/M3 primer pair combination. For each cultivar, 'Robust', 'Sabina', and 'Samson', DNA was isolated from eight individual plants, and S-SAP profiles were generated as described in "Materials and methods." *Numbers on the right* indicate DNA fragment size in base pairs

Genetic diversity/similarity estimates

Four primer pair combinations generated 404 amplified bands, of which 150 were polymorphic either within or between cultivars (Table 3). The highest and the lowest multiplex ratios were observed for the R1M7 and R1M4 primer pairs ("R" stands for the LTR-derived primer and "M" for *Mse*I-selective primer), 111 and 85, respectively. However, there was no correlation between high multiplex ratio and the level of polymorphism resulting from each primer pair. Indeed, the R1M7 fingerprint had the lowest percentage of polymorphism despite having the highest number of amplified bands.

Clustering and DA

UPGMA clustering produced distinct groups for two-rowed, winter, and hull-less cultivars (not shown), but there was some overlap between malting and non-malting six-rowed spring cultivars. This trend was more obvious with DA (Fig. 2). Furthermore, DA resulted in a clear separation of the phenotypes winter versus spring, two-rowed versus six-rowed, and hulled versus hull-less. However, a small amount of overlap between six-rowed malting and six-rowed feed cultivars was seen (Fig. 2). The grouping of barley cultivars obtained by DA was largely supported by the minimum spanning tree obtained from Arlequin analysis (not shown). Both DA and cluster analysis showed spring six-rowed cultivars to be the most diverse group in this study.

AMOVA analysis: partitioning of genetic variance

AMOVA (Table 4) was performed separately for four different groups as indicated in Table 1. The 824 barley plants used in this study were grouped into 441 haplotypes after removing genotypes with common DNA profiles. A haplotype is defined as a genotype with a defined set of alleles. Haplotype sharing was observed exclusively in intra-cultivar hierarchy, (i.e., common genotypes belonging to the same cultivar). However, no inter-cultivar haplotype sharing was observed. Therefore, all individual haplotypes clustered within their own cultivar. In all cases (Table 4), AMOVA resulted in highly significant ($P < 0.001$) genetic variance within and between groups. The variance among cultivars in the same group accounted for the largest percentage of the total variance (69–86%). In the analysis of six-rowed cultivars based on the malting versus non-malting grouping, the smallest variance component (4%) was obtained between groups indicating the absence of a

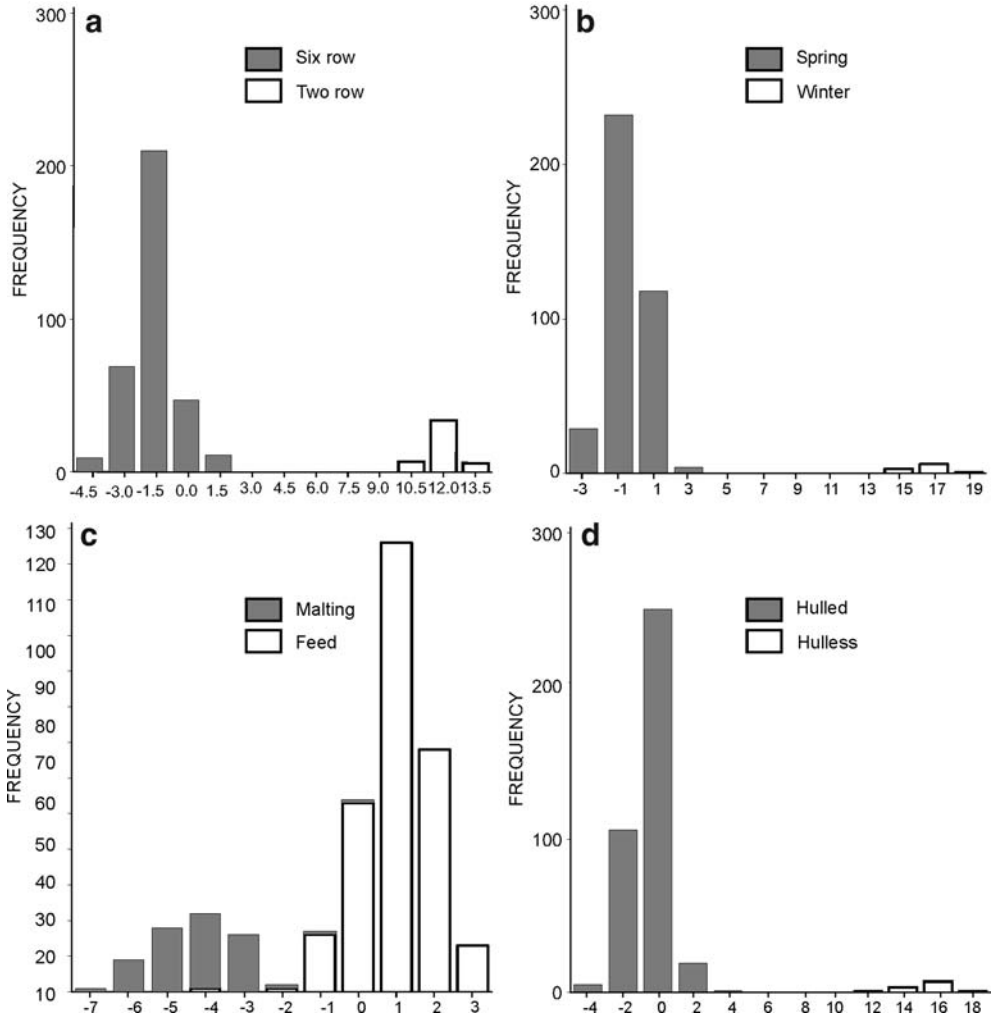

Fig. 2 Discriminant analysis (DA) of barley cultivars based on preset morphological criteria. Plot of canonical variables on axis 1: **a** two-rowed vs six-rowed group, **b** spring vs winter group, **c** feed vs malting group, and **d** hulled vs hull-less group. Groups were completely delineated by DA analysis except in **c**, where there was a minor overlap between malting and feed cultivars

Table 4 Summaries of analysis of molecular variance analyses among various agronomic groups of barley cultivars

Level	*df*	Sum of squares	Variance	Percent variation
Two-rowed/six-rowed				
Among groups	1	299.25	3.61	22.6
Among cultivars	89	3,167.43	11.12	69.57
Within cultivars	193	241.21	1.24	7.82
Winter/spring				
Among groups	1	116.59	3.98	23.22
Among cultivars	78	2,914.13	11.87	69.27
Within cultivars	165	212.53	1.28	7.52
Hull/hull-less				
Among groups	1	94.96	2.52	16.04
Among cultivars	78	2,935.76	11.93	75.79
Within cultivars	165	212.53	1.28	8.18
Feed/malting				
Among groups	1	97.68	0.57	4.13
Among cultivars	78	2,933.04	11.96	86.55
Within cultivars	165	212.53	1.28	9.32

major genetic structure on this level. Within-cultivar component of variation ranged from 7.5% to 9.3%.

Genetic heterogeneity within cultivars

Genetic heterogeneity was detected in 84% of all barley cultivars in this study (Table 1). The number of haplotypes within each cultivar ranged from one (no genetic variation within cultivar) for 17 homogenous cultivars to eight (maximum heterogeneity within cultivar in which each plant within the cultivar could be uniquely distinguished) for six completely heterogeneous cultivars (Table 1). Within-cultivar average gene diversity over loci was estimated to be $0.0134 \pm (-0.009)$. When within-cultivar genetic variation, measured as the mean number of pairwise differences between genotypes of the same cultivar, was plotted against the level of inbreeding, i.e., number of generations of selfing, a downward trend was observed (Fig. 3), indicating an inverse relationship between increased number of generations of selfing and the

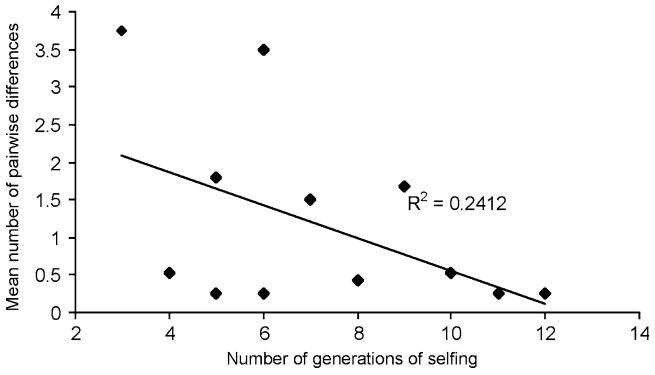

Fig. 3 Plot of number of generations of selfing vs genetic diversity within cultivars measured as the mean number of pairwise differences among individual plants in the cultivar. A subset of 13 cultivars, ranging from F_3 to F_{12} generations of selfing, was used

level of genetic heterogeneity within cultivars. Six-rowed spring type (comprising malting and non-malting cultivars) constituted the most diverse group in this study. Average genetic similarity within this overall group was estimated to be 0.61 (not shown), with no significant difference between mean genetic similarity between feed and malting cultivars. The two-rowed group formed a separate subgroup with an average genetic similarity of 0.85 (not shown), the highest value of mean genetic similarity for a group of cultivars in this study. Six-rowed winter and six-rowed spring hull-less cultivars also formed two unique subgroups.

Discussion

In this study, a substantial level of intra- and inter-cultivar genetic diversity was detected by S-SAP analysis. The ubiquitous nature of retrotransposons in plant genomes (Flavell et al. 1992; Kumar and Bennetzen 1999; Suoniemi et al. 1999), and their broad genomic distribution makes retrotransposon-based markers ideal tools for a wide range of applications in plants including measurements of biodiversity, genome evolution, linkage analysis, and mapping. Once a retrotransposon is stably integrated into a genomic location, it behaves in a Mendelian fashion. Therefore, integration sites shared between two plants are likely to have been present in their last common ancestor. S-SAP-based polymorphism may result from transpositional activity of retroelements and/or a restriction site polymorphism, as is the case for AFLP and some restriction fragment length polymorphisms.

In general, most plant retrotransposons studied to date have not demonstrated mobility within the recent past. In contrast, the BARE-1 element of barley is actively transposing (Jaaskalainen et al. 1999; Suoniemi et al. 1999) and furthermore, both biotic and abiotic stresses cause retroelement-induced mutations in wild barley (Kalendar et al. 2000). Liu and Wendel (2000) used Southern transfers to analyze introgressed rice lines and found a significant increase in the transpositional activity of the rice retrotransposon Tos17. Tissue culture was also shown to activate retrotransposition in rice (Hirochika et al. 1996). Most retrotransposon have been found in the non-coding region of the plants, often within a locus harboring other repetitive elements in a nested fashion (SanMiguel et al. 1996; Shirasu et al. 2000). However, many insertions have also been found in and around normal plant genes (White et al. 1994) including *bm3* mutation in maize (Vignols et al. 1995).

Measurements of genetic diversity, cluster analysis, and DA

The objective of breeding new crops cultivars is to create new allele combinations, followed by selection of desirable phenotypes during selfing generations. In an inbreeding crop such as barley, the level of heterozygosity for unlinked loci is reduced by approximately half, following each generation of selfing. The trend continues until a pure line is obtained in which almost complete homozygosity over all loci is achieved. Selection pressure is also directed towards achieving a homogenous and stable population of plants exhibiting minimum within-cultivar variation.

Biotypes are variant plants that belong to the same cultivar and possess different DNA profiles. In this study, 84% of all cultivars had biotypes. Although the aim of a breeding program is to produce a homogenous population of plants for a given cultivar, biotypes have been detected in many crop cultivars studied to date (Olufowote et al. 1997; Soleimani et al. 2002; 2003; Perry 2004). The possibility of contamination of seed samples as the cause of this finding was ruled out, because all the biotypes of the same cultivar fell within the same cultivar, and because the genetic distances among biotypes within cultivars were substantially lower compared to the distances between cultivars. Therefore, despite selection pressure aimed at cultivar homogeneity, a substantial level of genetic (molecular) diversity still exists within each cultivar. Regression analysis between the level of inbreeding and within-cultivar biodiversity level showed that inbreeding/selfing had a negative effect on the level of genetic diversity (Fig. 3). The regression analysis was limited to a selected group of cultivars from Table 1 for which a complete cultivar description was available. The level of selfing in this subgroup ranged from F_3 to F_{12} generation. Other factors in a breeding program that may also affect the extent of genetic heterogeneity within a cultivar, such as the choice of parents and the numbers of backcrosses among others, were not factored in. As described earlier, selection in inbred crops such as barley results in a heterogeneous population of homozygous plants. Although the exact sources of this genetic variation are difficult to predict, a multitude of factors may be responsible for maintaining within-cultivar variation. These may include breeding practices, spontaneous mutations, and induction of

transposition system. Retrotransposon insertions in or near genes can play an important role in species evolution (Shapiro 1999), and many retrotransposon insertions have been found in association with normal plant genes (White et al. 1994). Studies have also shown that environmental stress can induce transposition as described earlier. The effects of transposition on genetic variation are more likely to be profound in plants with an active transposon system such as barley. In order to partially address the source of genetic variation within cultivars, we analyzed families with these lines (parent1-parent2-progeny). Four cultivars were found in this category: 'AC Alma' ('Chapais' × 'Leger'), 'AC Klinck' ('Cadette' × 'Chapais'), 'Gamine' ('Maskot' × 'Chapais'), and 'AC Metcalfe' ('AC Oxbow' × 'Manley'). Comparison of gene diversity (from Table 1) between parents and progeny indicates that there are two likely sources for the observed genetic variation within each cultivar. Some of the variation may be residual, which is carried over from parents to progeny as in 'AC Klinck' and 'AC Metcalfe', in which gene diversity in the progeny is not significantly different from that of the parents. In other cases such as 'AC Alma', the level of genetic variation in the progeny was significantly higher than those of the parents, indicating that retrotransposition may have played a major role in these observations. We also analyzed band-sharing data between parents and progeny of the above-mentioned cultivars (not shown) and found that between 2% and 5% of bands that were present in progenies could not be assigned to either parent.

This study demonstrated the usefulness of S-SAP markers for the study of cultivated barley. S-SAPs were found informative in revealing genetic variations both at the intra- and inter-cultivar levels. These markers could be used for future breeding purposes and assessing cultivar purity testing in addition to their use for intellectual property right protection (such as Plant Breeders' Rights in Canada). Recent S-SAP-based mapping studies in wheat (Queen et al. 2004) and barley (Waugh et al. 1997) have shown that these markers had wide genome distribution along all chromosomes in these organisms.

Studies using pedigree data have shown that the gene pool of the malting barley cultivars is narrow (Martin et al. 1991; Horsely et al. 1995). The reduction in the genetic base of cultivated barley is thought to be the result of using local germplasm with reduced genetic base. However, these studies were based on the estimation of the coefficient of parentage and genetic measurements involving phenotypic traits such as malting characteristics. Estimates of genetic diversity based on the coefficient of parentage may be misleading due to various assumptions made regarding the relatedness of ancestors with unknown genealogies, linkage, and unequal contribution of parents to progenies (Barrett et al. 1998; Soleimani et al. 2002).

In this study, many cultivar pairs with close pedigrees were found to be substantially diverse from each other genetically. Example of such cultivar pairs included, 'CDC Yorkton' and 'CDC Battleford', and 'AC Malone' and 'Leger'. Indeed, 'AC Malone', which was developed via somaclonal variation from callus culture of 'Leger', had only 0.73 (73%) genetic similarity to 'Leger', based on the analysis of 404 S-SAP markers. In other words, 27% of S-SAP markers were polymorphic between 'Leger' and 'AC Malone'. This is a very substantial amount of genetic diversity between two cultivars in which one is derived from the other by means of tissue culture.

In conclusion, an objective assessment of the extent of genetic diversity in genome depends on the ability to detect the most prevalent and widely distributed types of genomic mutations and rearrangements. The S-SAP markers reported here were not mapped, and therefore, no conclusions can be made regarding their genomic locations and their possible linkage to agronomically important traits in barley. However, the results of the discriminant analysis (Fig. 2) indicate that at least some of these markers may potentially be linked to agriculturally significant traits such as malting/feed. In this study, the emphasis was placed on BARE-1-related polymorphisms. There are various other Ty1-*copia* retroelements in barley genome with their unique history of transposition and genome distribution (Shirasu et al. 2000). Simultaneous analysis of a diverse group of retroelements with S-SAP in a multi-retrotransposon approach (Leigh et al. 2003) may reveal additional polymorphisms. Whether the diversity in DNA sequence measured here could be translated into phenotypic diversity is beyond the scope of this study. However, there is a growing body of evidence implicating the role of retrotransposition in altering the phenotype. Insertion of retrotransposon in and around genes (Miyao et al. 2003; Kidwell and Lisch 1997) could alter their expression pattern, resulting in a change in phenotype. Whether these mutants survive in the subsequent generations will in turn depend on the fitness of individuals bearing the mutations. Breeding may be viewed in part as a man-made directional selection in which individual plants are subjected to various biotic and abiotic stresses. In this scheme, there are various macro- and micro-evolutionary processes that could potentially affect the genetic makeup of the resulting population. In the presence of an active transposition system, a high degree of genome plasticity is expected to result during the breeding process and associated selection pressure. Therefore, retrotransposon-based marker systems such as S-SAPs can be a useful tool for crop genetic studies, as they target retrotransposon insertion sites in genome and can be used as powerful tools for crop improvement.

Acknowledgements The authors would like to thank Ajit Singh Sahota from the Canadian Food Inspection Agency (CFIA) and Jean Mellish from the Canadian Grain Commission (CGC) for providing plant material. We would also like to thank Dr. Thin-Meiw (Alek) Choo of Agriculture and Agri-Food Canada for providing cultivar description of barley material. We are indebted to him for his advice and discussions. This study was partially funded by the Automated Quality Testing (AQT). This paper benefited from comments by Dr. Steve Molnar and Dr. Nick

Tinker of Agriculture and Agri-Food Canada, and we thank them for their valuable comments and advice on an earlier draft of the manuscript.

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