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Detection and characterization of SNPs useful for identity control and parentage testing in major European dairy breeds

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Summary

We propose the use of single nucleotide polymorphisms (SNPs) instead of polymorphic microsatellite markers for individual identification and parentage control in cattle. To this end, we present an initial set of 37 SNP markers together with a gender-specific SNP for identity control and parentage testing in the Holstein, Fleckvieh and Braunvieh breeds. To obtain suitable SNPs, a total of 91.13 kb of random genomic DNA was screened yielding 531 SNPs. These, and 43 previously identified SNPs, were subjected to the following selection criteria: (1) the frequency of the minor allele must be larger than 0.1 in at least two of the three examined breeds, and (2) markers should not be linked closely. Allele frequencies were estimated by analysing sequencing traces of pooled DNA or by genotyping individual DNA samples. The selected SNP loci were physically mapped by radiation hybrid mapping or by fluorescence *in situ* hybridization, and tested against the neutral mutation hypothesis. The presented marker set theoretically allows probabilities of identity less than 10^{-13} for individual verification and exclusion powers exceeding 99.99% for parentage testing.

Keywords digital DNA signatures, individual identification, parentage control, single nucleotide polymorphism.

Individual identification and parentage control are essential for consumer protection and efficient management of animal populations. Today, highly polymorphic microsatellite markers are well established in cattle and used successfully for these purposes (Glowatzki-Mullis *et al.* 1995; Heyen *et al.* 1997). However, single nucleotide polymorphisms (SNPs) promise considerable advantages over microsatellite markers: (1) lower mutation rates, (2) more robust in laboratory handling and data interpretation (Krawczak 1999), (3) suitability for standardized representation of genotyping results as a digital DNA signature (Fries & Durstewitz 2001), and (4) suitability for various genotyping techniques and high potential for automation (Kruglyak 1997). One disadvantage is that any SNP has a lower information content, compared with a highly polymorphic microsatellite. But this disadvantage can be compensated for by a higher number of markers. Recently, a set of SNP markers for animal identification and paternity testing in US Beef cattle was presented by Heaton *et al.* (2002). Here we report on the development of a similar set of SNP markers for use in the major European dairy and dual-purpose breeds: Holstein, Fleckvieh and Braunvieh.

Two hundred and three bovine bacterial artificial chromosome (BAC) clones from the BAC library Bovine II, no. 754 (Buitkamp *et al.* 2000) (RZPD, Berlin, Germany) were chosen at random. The BAC ends were sequenced directly using vector-specific primers. Repetitive and coding sequences were identified by BLAST searches. To obtain polymerase chain reaction (PCR) fragments in the range of 500 to 800 bp, primers were designed with a T_m of 60 °C and a 3′-GC clamp using the Primer3 program (Rozen & Skaletsky 2000).

To identify SNPs and to estimate their respective allele frequencies, we selected unrelated bulls belonging to the *Bos taurus taurus* breeds German Holstein ($n = 35$), German Fleckvieh ($n = 33$), German Braunvieh ($n = 32$), Kerry

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($n = 1$) and Angus ($n = 1$). Additionally, one Sahiwal and one Hariana animal representing *Bos taurus indicus* were selected. Genomic DNA was extracted from frozen bull semen. To facilitate searching and evaluation of SNPs, DNA pools were prepared for German Holstein (Sbpool, $n = 33$), German Fleckvieh (Fvpool, $n = 32$) and German Braunvieh (Bvpool, $n = 28$) by adding equal amounts of DNA of the individual samples as measured by fluorometry (DyNAquant 200, Hoefer Scientific, San Francisco, CA, USA).

The PCR fragments were amplified from 50 ng genomic DNA in a total volume of 20 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 μM of each dNTP, 2 mM $MgCl_2$, 0.5 μM of each primer and 0.5 U of AmpliTaq (PE Biosystems, Foster City, CA, USA). The cycling conditions used were 3 min initial denaturation at 94 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 1 min annealing at 60 °C, and 1 min extension at 72 °C. Amplification products were sequenced using an ABI 377 sequencer (PE Biosystems). Sequence data were analysed using the Phred/Phrap/Polyphred/Consed software suite (Nickerson *et al.* 1997; Ewing & Green 1998; Ewing *et al.* 1998; Gordon *et al.* 1998).

Through this procedure, a total of 91.13 kb of genomic DNA was screened yielding 531 SNPs. From these and 43 previously identified SNPs (obtained from the laboratory of M. Georges), 58 candidates promising adequate allele frequencies were pre-selected by visual assessment of the sequencing traces. Allele frequencies at these SNP loci were determined more accurately by (1) genotyping of individual DNA samples or (2) by systematic analysis of sequencing traces from DNA pools. Individual samples were genotyped by means of an oligo ligation assay (Landegren *et al.* 1988). The PCR and cycle ligation reactions were multiplexed 5–10 times as essentially described by Baron *et al.* (1996). The 5′ ends of the allele-specific primers were modified with covalently bound pentaethyleneoxide size modifiers of various lengths. The common probes were 5′ phosphorylated and carried a fluorescent dye (6-FAM, TAMRA or HEX) on their 3′ ends. Electrophoresis and fluorescence detection were carried out on an ABI 377 DNA sequencer. Sequencing traces from the DNA pools were analysed by comparing normalized amplitude values of the two alternative bases from the pooled DNA with their normalized amplitude values from homozygous and heterozygous individuals using an algorithm described by Winter *et al.* (2002). The analysis was automated using python scripts (http://www.python.org), which are available on request.

The chromosomal locations of the 58 SNPs were determined by radiation hybrid mapping and/or by fluorescence *in situ* hybridization (FISH). Radiation hybrid mapping was performed using a 5000 rad bovine-hamster radiation hybrid panel (Womack *et al.* 1997). Breakage probabilities, θ, were converted to additive distances d (cR_{5000}) according to the formula $d = -\ln(1 - \theta)$ (Cox *et al.* 1990). The FISH analysis was performed following a standard protocol.

Based on mapping results, 37 loci with the widest physical distance from one another were selected to establish our initial SNP marker set for identity control and paternity testing (Table 1). Marker information including primers and mapping data has been deposited in UniSTS. Most of these SNPs are not known to be located closely to coding sequence (Table 2). Additionally, one SNP (*ZFXY*), which distinguishes the homologous genes *ZFX* and *ZFY* (Aasen & Medrano 1990), was used as a gender assay. For each SNP locus, the alternative genotypes were represented as a binary pair – '10', homozygous for base 1; '11', heterozygous; '01', homozygous for base 2; '00', assay failure – and concatenated as a digital DNA signature (Fries & Durstewitz 2001). Each SNP locus holds a defined position within the digital DNA signature.

To get an indication about nucleotide variation within our marker set, nucleotide diversity (π) and nucleotide polymorphism (θ) were calculated based on the sequences of three individuals each of German Holstein and German Fleckvieh. The estimates of π and θ, based on six chromosomes per breed, vary greatly (Table 2). This does not allow to draw general conclusions if the randomly chosen BAC sequences are representative of the bovine genome. However, values estimated from all obtained DNA fragments in German Holstein were $\pi = 0.00096 \pm 0.00053$ and $\theta = 0.00098 \pm 0.00058$, which is in good agreement with previous estimates (Steele & Georges 1991; Fries *et al.* 2001). To test whether the chosen SNPs might be affected by selection, Tajima's (1989) test for neutral mutation was performed (for results see Table 2). The test statistic for some SNPs deviates significantly ($P < 0.05$) from the expectation under the neutral mutation hypothesis, even when Bonferroni correction is applied. These loci may therefore be located in, as yet, unrecognized coding regions of the genome or they may be in linkage disequilibrium. Those questionable loci will be examined further and excluded in case that substantial deviation is confirmed.

Based on the allele frequency estimates shown in Table 1, we calculated a theoretical probability of identity of lower than 10^{-13} for individual identification and an exclusion power of more than 99.99% for parentage control using this marker set in the considered breeds. This shows that 37 SNPs reach the power of a typical microsatellite set commonly used for this purpose. The presented marker set contributes to a growing number of SNPs in cattle that will allow the establishment of an international standard set with sufficient exclusion powers and probabilities of identity within the whole bovine species. The usefulness of the presented SNPs within other cattle breeds will be tested in future.

We propose to extend the digital DNA signature to obtain a marker set for the entire bovine species. For identity control and paternity analysis in a given population, single nucleotide queries can be restricted to the most polymorphic positions in this population. In future, further SNP sets for detection of hereditary diseases, analysis of production traits

Table 1 The SNPs selected for identity control and parentage testing with estimates of associated allele frequencies. Probabilities of identity for identity control and exclusion probabilities for paternity control serve as power assessment parameters for the whole marker set within the examined breeds.

						Estimated frequency of allele 1		
						German Holstein	German Fleckvieh	German Braunvieh
Locus identifier	GenBank accession no.	SNP position[1]	Position in marker set[2]	Allele 1[3]	Allele 2	$n = 33$	$n = 32$	$n = 28$
ZFXY	AJ506787	243	1	T	C	NA	NA	NA
421_10	AF440368	602	2	C	G	0.48*	0.22*	0.02*
423_24	AF440366	160	3	G	A	0.42*	0.55*	0.95*
425_2	AF440371	949	4	A	G	0.41*	0.11*	0.50*
431_A2	AF440372	229	5	G	A	0.53*	0.58*	0.73*
487_67	AF440381	158	6	G	A	0.47*	0.88*	0.9*
448_67	AF440377	177	7	T	C	0.72*	0.78*	0.96*
16_2	AF440369	283	8	G	A	0.77*	0.78*	0.91*
417_16	AF440365	555	9	G	A	0.41*	0.69*	0.41*
486_67	AF440380	143	10	C	T	0.89*	0.78*	0.52*
BULGE113	AJ505155	233	11	C	T	0.69*	0.17*	0.38*
BULGE128	AJ505161	92	12	G	C	0.81*	0.18*	0.17*
BULGE101	AJ505160	87	13	C	T	0.57	0.88	0.51
BULGE105	AJ505159	192	14	A	G	0.53	0.60	0.68
004.sp6	AJ496639	356	15	G	A	0.47	0.5	0.52
007.sp6	AJ496641	415	16	A	G	0.37	0.35	0.27
013.sp6	AJ496635	280	17	T	C	0.74	0.59	0.45
018.sp6	AJ496636	379	18	C	T	0.57	NA	0.58
022.t7	AJ496762	507	19	G	A	0.38	0.34	0.60
027.sp6	AJ496763	429	20	T	C	0.66	0.58	0.31
039.t7	AJ496765	189	21	T	C	0.61	0.46	0.77
048.sp6	AJ496767	135	22	T	G	0.46	0.78	0.44
055.t7	AJ496768	143	23	G	T	0.19	0.22	0.78
060.sp6	AJ496772	522	24	C	T	0.82	0.42	0.34
064.sp6	AJ496773	339	25	C	T	0.45	0.72	0.78
070.t7	AJ496774	152	26	A	C	0.54	NA	0.83
077.t7	AJ506786	200	27	G	A	0.54	0.24	0.73
090.t7	AJ496776	622	28	G	A	0.23	0.7	0.29
107.sp6	AJ496780	231	29	C	G	0.72	0.59	0.32
118.t7	AJ496782	281	30	A	G	0.55	0.34	0.71
128.sp6	AJ496785	131	31	G	A	0.46	0.44	0.70
032.t7	AJ496786	100	32	C	T	0.68	0.88	0.59
105.sp6	AJ496789	360	33	T	C	0.34	0.44	0.44
436_C10	AF440373	389	34	C	T	0.19*	0.21*	0.37*
454_G11	AF440378	112	35	C	G	0.34*	0.05*	0.45*
058.sp6	AJ496770	107	36	A	G	0.74	0.51	0.74
116.t7	AJ496781	329	37	C	T	0.54	0.54	0.54
BULGE100	AJ505157	78	38	G	T	0.67*	0.6*	0.68*
Theoretical probability of identity of the marker set[4]						8.59E-15	8.54E-13	1.30E-13
Theoretical exclusion power of the marker set[5]						0.99998	0.99993	0.99996

[1]Numbering is according to the deposited sequence in GenBank.

[2]Indicates the order within the digital DNA signature.

[3]The two alternative bases are shown in the order of the coding in the digital DNA signature.

[4]Calculated according to Weir (1996).

[5]Calculated according to formula 3 in Jamieson and Taylor (1997).

NA, not applicable (value depends on the sex ratio of examined animals).

*Allele frequency estimates are based on individual genotypes.

n, number of animals genotyped.

Table 2 Amplicons containing highly informative SNPs with associated mapping results, nucleotide polymorphism, nucleotide diversity and Tajima's D.

							German Fleckvieh					German Holstein				
Locus identifier[1]	UniSTS accession no.	Amplification primers[2]	Closest neighbour[3]	Distance (cR_5000)	LOD score	Chromosome position[4]	Mean (θ) $\times 10^3$	Stdv (θ) $\times 10^3$	Mean (π) $\times 10^3$	Stdv (π) $\times 10^3$	Tajima's D	Mean (θ) $\times 10^3$	Stdv (θ) $\times 10^3$	Mean (π) $\times 10^3$	Stdv (π) $\times 10^3$	Tajima's D
ZFXY	BV079446	GAGTGTGGTAAAGGTTTTCGTC GATTCGCATGTGCTTTTTGA				X/Y			NA					NA		
421_10	BV079420	GCTTCTTTTAATTTCCAGGTCTCA CCATCTGAGGTGCTTCTTCG				1q43-44			NA			4.191	2.670	3.748	2.603	−0.623
423_24	BV079421	GCATAGAGCATGGGAAAGGA AGAAAAGGACCCAGGTAGGG				10q14-15			NA			1.313	1.029	1.132	0.977	−0.709
425_2	BV079443	GAAGTCAACCTGGGAACAGC GAATCATGACTGGCAGCAAA				9q17-19	0.508	0.575	0.541	0.625	0.256	0.508	0.575	1.392	1.183	6.796*
431_A2	BV079422	TGACACGTGTGACAACATGG TGGCAACTGCCATTATACTTTC				5q23-25			NA			0.779	0.882	0.949	1.043	0.851
487_67	BV079444	TGGGCCAAAGCAGAAATAAC TCATTTTGCTTATAGCATCAGATTTT				14q23-24	1.246	1.101	1.043	1.027	−0.771	0.623	0.705	0.759	0.834	0.851
448_67	BV079423	GAGGACATTTGTTTCCCTCCT CCCTAACAACATTGCTTTTCTC				2q33-36			NA			1.457	1.288	1.886	1.628	1.393
16_2[5] (*IL13*)	BV079424 (AJ132441)	GCTCAGTTCCCCTCCTCCTA GAGTCCAGGGCTGCACAGTA				7q15-21			NA			0.864	0.978	1.052	1.156	0.851
417_16	BV079425	TTGGAAGGAAGGAGGTGATG TTCACACCTGCTTTGACAGTCT				4q22-24	1.825	1.612	1.111	1.221	−1.853*	1.825	1.612	1.111	1.221	−1.853*
486_67	BV079426	GACCTCATTCTCCTACTTACTGTAAAA CATCTGTGTGGTTTGTTTACCA				3q31-32	0.899	1.018	1.095	1.204	0.850	0.894	1.011	0.680	0.896	−0.933
BULGE113	BV079447	TCCAAAAGAGTCACAGCCAAGAAC AGGCAAAAGAAGATGGCAGCAG	BMS431	9	15.0	20q11	6.041	4.401	7.356	5.466	1.180	6.041	4.401	8.276	6.012	2.006
BULGE128	BV079448	ACTGAGTCTCTTAATTGCCAC TTGAACTAGAAGAGAGCTGTATC	*GUCA A*	3	17.5	23q12-q13			NA					NA		
BULGE101[6] (*DDO*)	BV079427 (X95310)	CACTTAACTCACTGTCAGTTGC CACATCATCACTATCTTCTACCATC	*DDO*	9	13.7	9q11-q12	2.190	2.478	1.667	2.194	−0.933	2.190	2.478	2.667	2.931	0.851
BULGE105[7] (*ATP2 B4*)	BV079428 (M83364)	TCCAATCAAACCTCCCCACAAC CTTCCTGGCACTTAGAAACCACAC	*EST* 0397	14.0	21	16q12-13	1.622	1.836	1.975	2.171	0.850	1.711	1.936	2.083	2.290	0.851
004.sp6	BV079449	TTGTTCCTCCCTCTATCCTTTA AAAATGCATAAATCATTTGCAG	*XBM* 38	9	13.4	Xq26-31			NA			0.789	0.893	0.961	1.056	0.851
007.sp6	BV079450	ATGGAGGTGAGGAAAGAGAATA GTCAGCCTTGTGGAAAACTTAT	BM5004	19	11.8	20q21-23	1.810	1.599	2.204	1.935	1.032	3.775	2.750	4.023	3.073	0.355
013.sp6	BV079429	CCAAAGTTATGTTGAATCCTGA AAACTCCCAGTTTCTCACAGTT	*SLC* 34*A2*	9	14.7	6q11	7.867	5.262	8.982	6.280	0.810	6.801	4.715	5.176	4.054	−1.337
018.sp6	BV079430	ATAAGGGCAAAAATGTCCAG CTCTGCTGAAGAGACAACTAGC	*BL*41	9	14.8	3q11	0.826	0.935	0.629	0.828	−0.933	0.826	0.935	1.006	1.106	0.851
022.t7	BV079451	CTTAACTTTGAGTGGGTTGTCA CTTGACAAGTCTCCAGTTTTTG				16q13-q14	0.768	0.870	0.936	1.029	0.850	0.768	0.870	1.637	1.495	4.418*
027.sp6	BV079431	CAATTCAAGCACCTAATTTTCA TATAGCTCTGCATTCCTCTGTG	EST2187	0	21.5	1q11	1.806	1.596	1.787	1.668	−0.050	1.806	1.596	2.474	2.103	1.753
039.t7	BV079432	CACAACAAACCATGCAAAGC GGAGGGATGGACAAGACTCA	*ACADVL*	9	13.7	19q12	2.154	1.688	2.295	1.874	0.338	1.436	1.269	1.093	1.111	−1.132
048.sp6	BV079433	CTCAGATCCTCAGCTTTCTTCT AGCAAAGAATCTAAATAAGCGG	*MFGE8*	3	17.6	21q12-q14	1.537	1.358	1.520	1.420	−0.050	3.112	2.267	3.079	2.390	−0.057
055.t7	BV079452	TTTTGATGATGTTCTGGATGTT CAAGAAGTGAGAAGTGGATGAG	*PPP1 CB*	5	18.0	11q11-q12	3.808	2.774	2.899	2.396	−1.295			NA		

060.sp6	BV079434	TTTTCTATTGATGGGGCTATTT GGTTTTGTGGTTAAGAAACCTG	*EST*1042	6	16.7	18q21-q24	0.903	1.022	0.687	0.905	−0.933	0.903	1.022	1.100	1.209	0.851
064.sp6	BV079435	GTGCTCTTTTATCCTCAGTCCT CCAATTGTCAGAAGCAAAGATA	*OPCML*	19	12.4	29q11-q13	0.850	0.962	1.812	1.654	4.418*	0.850	0.962	1.036	1.138	0.851
070.t7	BV079453	TGAACAGGCTCTGATAGTATGC CATGTGCTCATTATCGAAAGTT	*GTF2IP1*	24	11.4	25q21-q23	3.893	2.836	3.407	2.721	−0.676	5.839	3.906	6.370	4.487	0.520
077.t7	BV079436	ACTTGACCATGCTTACTCCTCT ATAATCCAGGCAGAAAGAAGAC	*BE*217455	8	18.1	1q33-q34	4.027	2.934	3.065	2.534	−1.295			NA		
090.t7	BV079454	TCTCTTAGCTTTGTTCTCCTCC AATTTTAAAAGCTCTTTGGTGC	KCNMA1 (*090.sp6*)	3	20.5	28q12-q13	1.510	1.334	2.069	1.758	1.753	2.265	1.775	2.759	2.181	1.124
107.sp6	BV079437	GCTCGAAGGCTATTTGTTTATT GAAGTAAGATCACACAAGCTGC	*IDVGA* 39	6	16.7	21q24	0.749	0.847	1.595	1.456	4.417*	2.246	1.760	3.077	2.369	1.910
118.t7[8]	BV079455	GACATGACTGAGCAACTTCACT AATGTTCCCTTCACTAAGCAAT				12q12-q13[8] 22q22			n.a.			4.963	3.615	5.477	4.152	0.562
128.sp6	BV079438	GTGCTGATCAGTCCAGGGAT GCTGAGGCTTAACTAAGCAGAT	GCNT1	3	17.5	8q23-q24	1.081	1.224	1.317	1.448	0.850	1.095	1.239	0.833	1.097	−0.933
032.t7	BV079439	CTTTGGTACAGTTCTTTTGGTG CTATCACCTGTTTTTAACGCAA	*EST*0149	15	12.9	2q44-q45			NA					NA		
105.sp6	BV079440	CTGATGGATTTCAGAAGAGTTG TGGTACAGAAGGCATCACTATG	*EST*0397	21	14.0	18q11	1.007	1.139	1.226	1.348	0.850	1.007	1.139	1.226	1.348	0.851
436_C10	BV079441	TGTAATCCAGACAACTGCAAGG AAAATTACCTCCTCTTGGTGA				4q13-14	0.747	0.846	0.910	1.000	0.851	0.747	0.846	0.569	0.749	−0.933
454_G11	BV079442	TGGGAAAAATAAATAATAGAAACCAGA TTTTCCTCAAATTTATTCTTACATTG				17q24-26			NA			0.871	0.985	0.795	0.974	−0.338
058.sp6	BV079456	CTGAGTTATTGTCCATGTTGGT AGGATGCAAGAGATTCAGATTT	BMS975	3	17.5	12q21	2.346	1.839	2.143	1.821	−0.447	0.000	0.000	0.000	0.000	0.000
116.t7 (*CYP17*)	BV079457 NM_174304	CTGGATTCATATTCAATCTGGCA TATTCAGGAAGGATGAAAAGGT	*INRA*081	9	14.0	26q13-q14			NA					NA		
BULGE100[9] (*DSG1*)	BV079445 (X57784)	AAGAGAGTGGCAACAGAGTGAG GGTAAAGGGAGAAACAAGTGTG				24q21–22	2.179	2.466	2.653	2.917	0.850	2.147	2.430	1.634	2.151	−0.933

[1]Official locus symbol is provided additionally if available.

[2]Primer sequences are shown in 5′ to 3′ orientation.

[3]Closest neighbour is provided for radiation hybrid mapped amplicons.

[4]Physical position is provided if FISH results are available. Inferred physical position is provided for radiation hybrid mapped amplicons.

[5]Located in intron 2 of *IL* 13 (Buitkamp *et al.* 1999).

[6]Located in the 3′ non-coding region of *DDO* (Simonic *et al.* 1997).

[7]Located in the 3′ non-coding region of *ATP B* 4 (Brandt *et al.* 1988).

[8]Chimaeric BAC

[9]Located in the 3′ non-coding region of *DSG* 1 (Koch *et al*. 1990).

NA not applicable (no SNP detected within the three examined animals).

*Significant deviation ($P < 0.05$) from the expectation under the neutral mutation hypothesis.

or genetic diversity assessment might be added to the digital DNA signature as modules.

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