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ORIGINAL PAPER

Development of a rapid identification method for potato cytoplasm and its use for evaluating Japanese collections

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Abstract The cytoplasm of potatoes, characterized by the presence of T-type chloroplast DNA and β-type mitochondrial DNA, is sensitive to nuclear chromosomal genes that contribute to various types of male sterility. Past breeding efforts with various potato varieties have resulted in several different cytoplasms other than T/β. Varieties with *Solanum stoloniferum*-derived cytoplasm (W/γ) show complete male sterility, while those with *S. demissum*-derived cytoplasm (W/α) produce abundant, but non-functional pollen. Thus, identification of cytoplasmic types is important for designing efficient mating combinations. To date, only T-type chloroplast DNA can be accurately identified by a PCR marker. Here, we report a rapid identification technique by multiplex PCR, followed by restriction digestion with *Bam*HI in one reaction tube, and propose a new nomenclature for potato cytoplasm types (T, D, P, A, M, and W). Using this new technique, our collections of 748 genotypes, including 84 Japanese named varieties, 378 breeding lines and 26 landraces, and 260 foreign varieties and breeding lines, were grouped into cytoplasm types: T (73.9 %), D (17.4 %), P (4.5 %), A (1.5 %), M (0.3 %), and W (2.4 %). The utility of this marker system for breeding is discussed.

The Hawkes (1990) classification system is tentatively adopted throughout the text.

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Introduction

In potato breeding, cytoplasmic differences have been identified in several agronomic traits by reciprocal crosses. For example, the cytoplasm of the common potato (*Solanum tuberosum* L. ssp. *tuberosum*, $2n = 4x = 48$) was associated with high percentage tuberization, high tuber yield, many tubers, poor flowering, early vine maturity, low pollen stainability, and poor pollen shedding (Sanford and Hanneman 1979, 1982; Hoopes et al. 1980; Hilali et al. 1987; Maris 1989). Potato has at least seven different cytoplasmic sterility factors ([ASF^s], [Fm^s], [In^s], [SM^s], [Sp^s], [TA^s], and [VSA^s]) that confer sterility in the presence of dominant nuclear chromosomal genes (*ASF*, *Fm*, *In*, *SM*, *Sp*, *TA,* and *VSA*) (Grun et al. 1977). Consequently, breeders often encounter sterility problems in genotypes with desirable traits.

The cytoplasmic genome of the common potato is characterized by the presence of T-type chloroplast DNA (ctDNA) (Hosaka 1986) and β-type mitochondrial DNA (mtDNA) (Lössl et al. 1999). Although cytoplasmic sterility factors typically reside on mtDNA (Hosaka et al. 1988; Lössl et al. 2000), β-type mtDNA has shown complete association with the T-type ctDNA (hereafter, T/β cytoplasm) (Lössl et al. 2000). Thus, the T/β cytoplasm is predominant in the common potato (Hosaka and Hanneman 1988; Waugh et al. 1990; Powell et al. 1993; Bryan et al. 1999; Provan et al. 1999; Lössl et al. 2000), and sterility problems are unavoidable with the T/β cytoplasm.

In addition to intrinsic sterility, specific male sterility in association with cytoplasmic genomes has been reported. Cultivars carrying Ry_{sto} (a resistance gene to *Potato virus Y*), released mainly in Germany (Ross 1986), exhibit male sterility caused by association with the characteristic mtDNA derived from *S. stoloniferum* Schlechtd. et Bché. (the W/γ cytoplasm) (Brown 1984; Ortiz et al. 1993; Lössl

et al. 2000). This specific male sterility is called "tetrad sterility" (Abdalla and Hermsen 1971) or "lobed sterility" (Grun et al. 1962) because anthers shed small quantities of pollen that mostly cluster in tetrads and have a four-lobed appearance. With the T/β and W/γ cytoplasms, sterility is always characterized by visible abnormalities such as the absence of pollen, no or poor pollen shedding, or various deformities of pollen and anthers (Abdalla and Hermsen 1971; Grun 1979). In contrast, the cytoplasm derived from *S. demissum* Lindl. is deceitful because the F_1 and backcrossed progenies carrying the *S. demissum* cytoplasm (the W/α cytoplasm) produce abundant and normal-looking pollen; however, this pollen is non-functional onto *S. tuberosum* (Dionne 1961). In potato breeding, *S. demissum* is the most frequently used wild species as a source of resistance to the most serious potato disease, late blight (*Phytophthora infestans*) (Ross 1986; Plaisted and Hoopes 1989). Consequently, the W/α cytoplasm is found in 40 % of German potato varieties (Lössl et al. 2000). Therefore, accurate identification of the cytoplasm is important in designing efficient mating combinations.

Among cultivated potatoes, five ctDNA types (W, C, T, A, and S) and five mtDNA types (α, β, γ, δ, and ε) have been distinguished by restriction fragment length polymorphism (RFLP) analysis (Hosaka 1986; Lössl et al. 1999). In addition, a set of polymerase chain reaction (PCR) primers flanking a 241-bp deletion that defines the T-type ctDNA (Hosaka et al. 1988; Kawagoe and Kikuta 1991) has been developed (Lössl et al. 2000; Hosaka 2002) and has been frequently used worldwide for various purposes (Gavrilenko et al. 2007; Spooner et al. 2007; Ames and Spooner 2008; Chimote et al. 2008). Likewise, PCR primers specific for the mtDNA types α, β, and γ were developed by Lössl et al. (2000), and have been used for cultivar assessment (Lössl et al. 2000; Chimote et al. 2008) and cytoplasmic diversity evaluation of Andean cultivated and wild potatoes (Hosaka and Sanetomo 2009). In addition to the T/β, W/α, and W/γ cytoplasms, A/ε, S/ε, and W/δ cytoplasms are found in potato cultivars (Lössl et al. 1999; Chimote et al. 2008).

A large survey of ctDNA variation in Andean cultivated potato species and the closely related wild species using high-resolution markers, such as simple sequence repeat (SSR, or microsatellite) markers, separated the W- and C-type ctDNAs into many different types. A relatively high correlation between ctDNA types and nuclear DNA differentiation was observed (Sukhotu et al. 2004). In our recent study using 476 accessions of 7 cultivated and 32 wild potato species, 6 mtDNA and 9 ctDNA markers revealed 63 different mtDNAs and 129 different ctDNAs, resulting in 164 haplotypes (Hosaka and Sanetomo 2009) (Fig. 1). The S- and A-type ctDNAs of cultivated potatoes formed relatively distinct groups. However, unlike ctDNA typing, mtDNA typing did not clearly indicate informative groups. For example, mtDNA types α, β, and γ determined using the PCR marker ALM_4/ALM_5 (Lössl et al. 2000) showed a random association with ctDNA types: the W/α cytoplasm was not specific to the *S. demissum* cytoplasm, and the W/γ was not specific to the *S. stoloniferum* cytoplasm (Hosaka and Sanetomo 2009). Alternatively, we found a *S. demissum* cytoplasm-specific DNA marker, named "Band 1," which was maternally inherited from *S. demissum*, but the intracellular origin as to whether it is a part of ctDNA or mtDNA remained unknown (Sanetomo and Hosaka 2011).

Although various cytoplasmic markers, particularly SSR markers for ctDNA (Provan et al. 1999), have been available for evaluating cytoplasmic diversity, imperceptible distinctions, such as the classification into 164 haplotypes (Hosaka and Sanetomo 2009), are not practical for breeding purposes. Furthermore, since ctDNA SSR markers are mostly derived as polymorphic mononucleotide repeats, detection of such single-base differences requires polyacrylamide or capillary gels (Powell et al. 1995; Provan et al. 2001), which are not always available and are inconvenient for large-scale screening. In this study, we developed a rapid and simple marker technique using multiplex PCR, followed by restriction digestion with *Bam*HI in the same reaction tube and ordinary agarose gel electrophoresis. Based on this marker system, we propose a new nomenclature for potato cytoplasm types T, D, P, A, M, and W and have used this system to evaluate our collections. The importance of distinguishing cytoplasmic types for potato breeding is discussed.

Materials and methods

Plant materials

To evaluate the markers, 165 accessions of 37 species were used, including Andean cultivated species and their closely related wild species, which covered all 164 different cytoplasms previously distinguished by Hosaka and Sanetomo (2009) and the *S. demissum* cytoplasm (Table 1). These accessions were obtained either from the US Potato Genebank (NRSP-6; Sturgeon Bay, Wisconsin) or the CIP gene bank (Lima, Peru). A further survey was conducted of our collections of 748 genotypes, including 84 Japanese named varieties, 378 breeding lines and 26 landraces, and 260 foreign varieties and breeding lines (Supplementary Table).

DNA extraction

To evaluate the markers, DNA was extracted from fresh leaves as described by Hosaka and Hanneman (1998) and

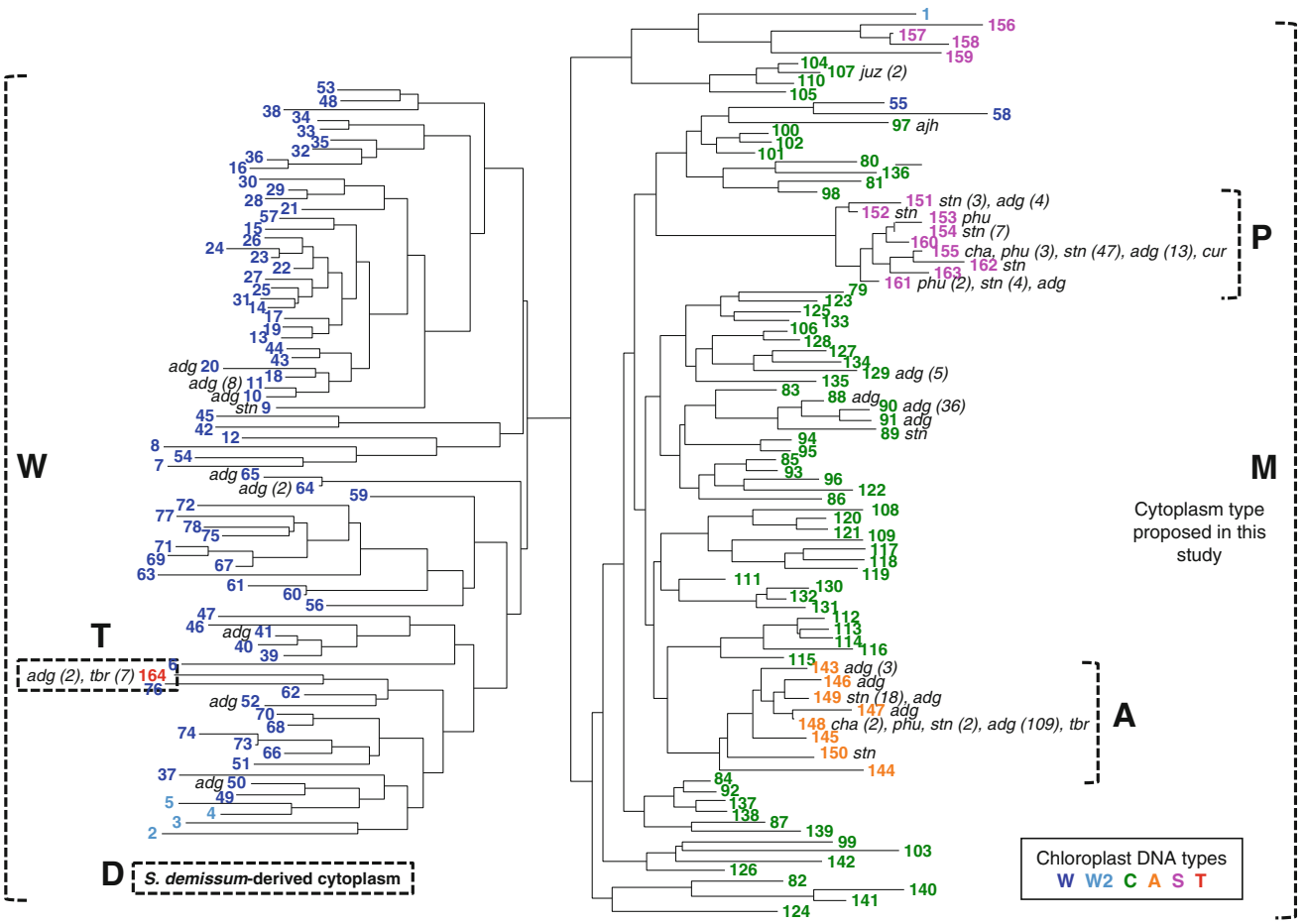

Fig. 1 Relationships among haplotypes of Andean cultivated potatoes and relatives based on chloroplast and mitochondrial DNA polymorphisms (modified from Hosaka and Sanetomo 2009). Haplotype identity numbers are colored by their chloroplast DNA types. Haplotypes found in cultivated species accessions are denoted with haplotype identity numbers and species abbreviations (when more than one accession shares the same type, the number of accessions is parenthesized): *ajh S. ajanhuiri*, *juz S. juzepczukii*, *cha S. chaucha*, *phu S. phureja*, *stn S. stenotomum*, *adg S. tuberosum* ssp. *andigena*, *tbr S. tuberosum* ssp. *tuberosum*, and *cur S. curtilobum*. In addition, the approximate correspondences of the cytoplasm types proposed in this study to previous haplotypes are shown

adjusted to a concentration of 5 ng/μl. For a wide survey of our collections, DNA was extracted by the CTAB method (Doyle and Doyle 1987) or a simpler method, called 1-min DNA extraction (Hosaka 2004), and used without adjusting the DNA concentration.

Cytoplasmic marker development

Various PCR markers have been previously developed and tested in our laboratory. Among them, five PCR markers were selected (Table 2). The T marker was designed by Hosaka (2002) to detect a 241-bp deletion in the T-type ctDNA. The S marker is the SSR marker NTCP6, developed by Provan et al. (1999). The D marker was developed previously as the *S. demissum*-derived cytoplasm-specific marker harboring Band 1 (Sanetomo and Hosaka 2011). The SAC and A markers were newly developed in this study by the following method. According to Hosaka (1986), in the *Bam*HI restriction fragment patterns of ctDNA, the S-, A-, and C-type ctDNAs showed a characteristic band of 19.5 kbp and lacked 16.3- and 3.66-kbp bands. The A-type ctDNA further possessed a shorter band of 3.44 kbp instead of 3.79 kbp. These 16.3-, 3.66-, and 3.79-kbp fragments correspond to fragment nos. 1, 11a, and 10, respectively, of the physical map of *Bam*HI digests constructed by Heinhorst et al. (1988). Referring to the complete sequence of potato ctDNA (Chung et al. 2006), the corresponding regions were estimated, and the PCR products from the estimated regions of the variant types were sequenced. We found that the S-, A-, and C-type ctDNAs lost a *Bam*HI recognition site between fragment nos. 1 and 11a because of a base change from GGATCC to AGATCC, whereas the A-type ctDNA had an extra *Bam*HI recognition site within fragment no. 10 because of a base change from GGATCG to GGATCC. Primers flanking these *Bam*HI recognition sites were designed and named as the SAC and A markers, respectively (Table 2).

Table 1 Evaluation of cytoplasms in cultivated potato species and the closely related wild species

Taxonomic series and species	Accession	Previous typing[a]		Marker banding pattern type[b]					Cytoplasm type	ALM_4/ALM_5[c]
		Haplotype	ctDNA	T	S	SAC	D	A		
Series *Pinnatisecta* (Rydb.) Hawkes										
S. pinnatisectum Dun.	PI 184764	45	W	1	1	2	0	2	W	0
	PI 275230	42	W	1	1	2	0	2	W	0
Series *Yungasensa* Corr.										
S. chacoense Bitt.	chc 525-3	72	W	1	1	2	0	2	W	0
	PI 537025	37	W	1	1	2	0	2	W	3
S. tarijense Hawkes	PI 498399	51	W	1	1	2	0	2	W	2
Series *Megistacroloba* Cárd. et Hawkes										
S. boliviense Dun.	PI 498215	5	W2	1	1	2	0	2	W	2
	PI 545964	49	W	1	1	2	0	2	W	2
S. megistacrolobum Bitt.	PI 265874	98	C	1	1	1	0	2	M	1
	PI 473356	81	C	1	1	1	0	2	M	1
	PI 473361	124	C	1	1	1	0	2	M	1
	PI 545999	80	C	1	1	1	0	2	M	1
S. raphanifolium Cárd. et Hawkes	PI 473371	99	C	1	1	1	0	2	M	1
S. sogarandinum Ochoa	PI 230510	56	W	1	1	2	0	2	W	1
Series *Conicibaccata* Bitt.										
S. chomatophilum Bitt.	PI 266387	54	W	2	2	2	0	2	–	1
	PI 365327	8	W	1	2	2	0	2	–	1
S. irosinum Ochoa	PI 568985	1	W2	1	2	2	0	2	–	1
Series *Piurana* Hawkes										
S. acroglossum Juz.	PI 498204	6	W	1	2	2	0	2	–	1
S. blanco-galdosii Ochoa	PI 442701	7	W	2	2	2	0	2	–	1
Series *Tuberosa* (Rydb.) Hawkes (Wild species)										
S. acroscopicum Ochoa	PI 365314	55	W	1	1	2	0	2	W	1
	PI 365315	103	C	1	1	1	0	2	M	2
S. brevicaule Bitt.	PI 498110	30	W	1	1	2	0	2	W	2
	PI 498111	28	W	1	1	2	0	2	W	2
	PI 498112	29	W	1	1	2	0	2	W	2
	PI 498113	24	W	1	1	2	0	2	W	2
	PI 498114	23	W	1	1	2	0	2	W	2
	PI 498115	40	W	1	1	2	0	2	W	2
	PI 498218	34	W	1	1	2	0	2	W	1
	PI 545967	38	W	1	1	2	0	2	W	1
	PI 545968	21	W	1	1	2	0	2	W	1
	PI 545970	13	W	1	1	2	0	2	W	2
S. bukasovii Juz.	PI 210042	138	C	1	1	1	0	2	M	1
	PI 210051	113	C	1	1	1	0	2	M	1
	PI 265876	114	C	1	1	1	0	2	M	1
	PI 275271	115	C	1	1	1	0	2	M	1
	PI 283074	120	C	1	1	1	0	2	M	1
	PI 310937	118	C	1	1	1	0	2	M	1
	PI 365304	93	C	1	1	1	0	2	M	1
	PI 365318	163	S	1	3	1	0	2	P	1
	PI 365321	92	C	1	1	1	0	2	M	1
	PI 365349	84	C	1	1	1	0	2	M	1

Table 1 continued

Taxonomic series and species	Accession	Previous typing[a]		Marker banding pattern type[b]					Cytoplasm type	ALM_4/ALM_5[c]
		Haplotype	ctDNA	T	S	SAC	D	A		
	PI 365350	85	C	1	1	1	0	2	M	1
	PI 365355	94	C	1	1	1	0	2	M	1
	PI 414155	134	C	1	1	1	0	2	M	1
	PI 442698	95	C	1	1	1	0	2	M	1
	PI 458379	122	C	1	1	1	0	2	M	1
	PI 473447	121	C	1	1	1	0	2	M	1
	PI 473450	116	C	1	1	1	0	2	M	1
	PI 473453	130	C	1	1	1	0	2	M	1
	PI 473491	157	S	1	1	1	0	2	M	2
	PI 473492	144	A	1	1	1	0	1	A	1
	PI 498219	100	C	1	1	1	0	2	M	1
	PI 498220	137	C	1	1	1	0	2	M	1
	PI 568932	82	C	1	1	1	0	2	M	1
	PI 568933	123	C	1	1	1	0	2	M	1
	PI 568939	139	C	1	1	1	0	2	M	1
	PI 568944	106	C	1	1	1	0	2	M	1
	PI 568949	108	C	1	1	1	0	2	M	1
	PI 568954	145	A	1	1	1	0	1	A	1
S. canasense Hawkes	PI 246533	117	C	1	1	1	0	2	M	1
	PI 283080	140	C	1	1	1	0	2	M	1
	PI 310938	119	C	1	1	1	0	2	M	1
	PI 310956	160	S	1	3	1	0	2	P	1
	PI 473346	86	C	1	1	1	0	2	M	1
	PI 473347	87	C	1	1	1	0	2	M	1
	PI 473348	141	C	1	1	1	0	2	M	1
S. candolleanum Berth.	PI 498227	131	C	1	1	1	0	2	M	1
	PI 545972	111	C	1	1	1	0	2	M	1
	PI 568969	133	C	1	1	1	0	2	M	1
S. coelestipetalum Vargas	PI 473354	127	C	1	1	1	0	2	M	1
	PI 590904	126	C	1	1	1	0	2	M	1
S. dolichocremastrum Bitt.	PI 498234	142	C	1	1	1	0	2	M	4
S. immite Dun.	PI 365330	59	W	1	1	2	0	2	W	1
	PI 498245	12	W	1	1	2	0	1	–	1
S. leptophyes Bitt.	PI 283090	32	W	1	1	2	0	2	W	2
	PI 320340	44	W	1	1	2	0	2	W	2
	PI 458378	33	W	1	1	2	0	2	W	1
	PI 473342	35	W	1	1	2	0	2	W	2
	PI 473343	16	W	1	1	2	0	2	W	2
	PI 473344	36	W	1	1	2	0	2	W	2
	PI 473445	109	C	1	1	1	0	2	M	1
	PI 473451	132	C	1	1	1	0	2	M	1
	PI 473495	46	W	1	1	2	0	2	W	2
	PI 545895	2	W2	1	1	2	0	2	W	2
	PI 545896	4	W2	1	1	2	0	2	W	2
	PI 545985	43	W	1	1	2	0	2	W	2
	PI 545986	19	W	1	1	2	0	2	W	2

Table 1 continued

Taxonomic series and species	Accession	Previous typing[a]		Marker banding pattern type[b]					Cytoplasm type	ALM_4/ALM_5[c]
		Haplotype	ctDNA	T	S	SAC	D	A		
	PI 545987	53	W	1	1	2	0	2	W	1
	PI 545988	22	W	1	1	2	0	2	W	2
	PI 545990	105	C	1	1	1	0	2	M	2
	PI 545991	14	W	1	1	2	0	2	W	2
	PI 545992	15	W	1	1	2	0	2	W	2
	PI 545993	17	W	1	1	2	0	2	W	2
	PI 545995	48	W	1	1	2	0	2	W	1
S. marinasense Vargas	PI 210040	96	C	1	1	1	0	2	M	1
	PI 310946	136	C	1	1	1	0	2	M	1
S. medians Bitt.	PI 210045	128	C	1	1	1	0	2	M	1
	PI 442703	79	C	1	1	1	0	2	M	1
	PI 473496	135	C	1	1	1	0	2	M	1
S. multidissectum Hawkes	PI 210043	102	C	1	1	1	0	2	M	1
	PI 210044	101	C	1	1	1	0	2	M	1
	PI 210052	112	C	1	1	1	0	2	M	1
	PI 210055	156	S	1	1	1	0	2	M	0
	PI 473349	83	C	1	1	1	0	2	M	1
	PI 473353	159	S	1	1	1	0	2	M	2
	PI 498304	158	S	1	1	1	0	2	M	2
S. multiinterruptum Bitt.	PI 275272	125	C	1	1	1	0	2	M	1
	PI 498267	58	W	1	1	2	0	2	W	2
S. oplocense Hawkes	PI 435079	66	W	1	1	2	0	2	W	2
	PI 442693	39	W	1	1	2	0	2	W	2
	PI 458390	47	W	1	1	2	0	2	W	2
	PI 498067	3	W2	1	1	2	0	2	W	2
	PI 545876	62	W	1	1	2	0	2	W	1
	PI 545908	74	W	1	1	2	0	2	W	2
	PI 545910	73	W	1	1	2	0	2	W	2
S. pampasense Hawkes	PI 275274	61	W	1	1	2	0	2	W	0
	PI 442697	60	W	1	1	2	0	2	W	0
S. sparsipilum (Bitt.) Juz. et Buk.	PI 498136	18	W	1	1	2	0	2	W	2
	PI 498138	31	W	1	1	2	0	2	W	2
	PI 498139	57	W	1	1	2	0	2	W	2
	PI 498140	25	W	1	1	2	0	2	W	2
	PI 498305	26	W	1	1	2	0	2	W	2
S. × *sucrense* Hawkes	PI 473506	76	W	1	1	2	0	2	W	1
S. vernei Bitt. et Wittm.	PI 458373	63	W	1	1	2	0	2	W	0
	PI 458374	67	W	1	1	2	0	2	W	0
	PI 473306	69	W	1	1	2	0	2	W	0
	PI 473311	75	W	1	1	2	0	2	W	0
	PI 500067	77	W	1	1	2	0	2	W	0
	PI 545884	27	W	1	1	2	0	2	W	2
	PI 558148	71	W	1	1	2	0	2	W	0
	PI 558151	78	W	1	1	2	0	2	W	0
(Cultivated species)										
S. ajanhuiri Juz. et Buk.	CIP 702677	97	C	1	1	1	0	2	M	1

Table 1 continued

Taxonomic series and species	Accession	Previous typing[a]		Marker banding pattern type[b]					Cytoplasm type	ALM_4/ALM_5[c]
		Haplotype	ctDNA	T	S	SAC	D	A		
S. juzepczukii Buk.	CIP 700895	107	C	1	1	1	0	2	M	3
S. phureja Juz. et Buk.	CIP 703275	153	S	1	3	1	0	2	P	1
S. stenotomum Juz. et Buk.	CIP 701165	151	S	1	3	1	0	2	P	1
	CIP 701985	152	S	1	3	1	0	2	P	1
	CIP 702583	9	W	1	3	1	0	2	P	1
	CIP 703088	89	C	1	3	1	0	2	P	1
	CIP 703710	161	S	1	3	1	0	2	P	1
	CIP 703808	162	S	1	3	1	0	2	P	1
	CIP 703933	150	A	1	1	1	0	1	A	1
	CIP 707297	154	S	1	3	1	0	2	P	1
S. tuberosum L. ssp. *andigena* Hawkes	CIP 700790	147	A	1	1	1	0	1	A	1
	CIP 703268	146	A	1	1	1	0	1	A	1
	PI 243363	143	A	1	1	1	0	1	A	1
	PI 246497	129	C	1	1	1	0	2	M	1
	PI 255508	65	W	1	1	2	0	2	W	1
	PI 265882	50	W	1	1	2	0	2	W	2
	PI 281080	148	A	1	1	1	0	1	A	1
	PI 281105	90	C	1	1	1	0	2	M	1
	PI 292089	91	C	1	1	1	0	2	M	1
	PI 365345	52	W	1	1	2	0	2	W	1
	PI 473285	11	W	1	1	2	0	2	W	2
	PI 473391	10	W	1	1	2	0	2	W	2
	PI 473393	41	W	1	1	2	0	2	W	2
	PI 498076	64	W	1	1	2	0	2	W	1
	PI 498294	88	C	1	1	1	0	2	M	1
	PI 498310	155	S	1	3	1	0	2	P	1
	PI 546017	20	W	1	1	2	0	2	W	2
	PI 546023	149	A	1	1	1	0	1	A	1
S. tuberosum L. ssp. *tuberosum*	CIP 703252	164	T	3	1	2	0	2	T	1
Series *Acaulia* Juz.										
S. acaule Bitt.	CIP 761143	110	C	1	1	1	0	2	M	3
	PI 210030	104	C	1	1	1	0	2	M	3
Series *Longipedicellata* Buk.										
S. stoloniferum Schlechtd. et Bché.	PI 186544	70	W	1	1	2	0	2	W	2
	PI 195167	68	W	1	1	2	0	2	W	2
Series *Demissa* Buk.										
S. demissum Lindl.	PI 186551	–	W	1	1	2	1	2	D	2

[a] Haplotypes and chloroplast DNA (ctDNA) types cited from Hosaka and Sanetomo (2009)

[b] See Fig. 1 for banding pattern types of each marker

[c] A set of primers ALM_4/ALM_5 is a mitochondrial DNA marker developed by Lössl et al. (2000). The banding pattern type (see Fig. 1) is given for each accession

PCR and restriction digestion

The PCR reaction was performed in volume of 5 µl consisting of 1 µl of template DNA (approximately 5 ng/µl), 2.5 µl of Ampdirect® Plus (Shimadzu Co., Japan), 0.125 U of *Taq* DNA polymerase (BIOTAQ™ HS DNA Polymerase, Bioline Ltd., UK), and 0.5 µl each of 3 µM forward and reverse primers. The reaction was performed

Table 2 Diagnostic PCR markers for identification of cytoplasm types in potato

Marker	Primer (5′–3′ sequence)	Conc. (μM)	References
T	GGAGGGGTTTTTCTTGGTTG	2	Hosaka (2002), H1 in Hosaka (2003)
	AAGTTTACTCACGGCAATCG		
S	GGTTCGAATCCTTCCGTC	2	NTCP6 (Provan et al. 1999)
	GATTCTTTCGCATCTCGATTC		
SAC	TTGGAGTTGTTGCGAATGAG	2	The present study
	GTTCCCTAGCCACGATTCTG		
D	CGGGAGGTGGTGTACTTTCT	3	Band 1-F11 and -R6 (Sanetomo and Hosaka 2011)
	ACGGCTGACTGTGTGTTTGA		
A	AACTTTTTGAACTCTATTCCTTAATTG	3	The present study
	ACGCTTCATTAGCCCATACC		

Primer concentrations in 10× primer mix are shown

using a thermal cycler (96-well GeneAmp PCR® System 9700, Applied Biosystems) with a thermal profile of one cycle of 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, and terminated with one cycle of 5 min at 72 °C. After the PCR reaction, the samples for detecting the SAC or A marker were mixed with 5 μl of digestion mix consisting of 1 μl of 10× NE Buffer 3 (New England Biolabs), 0.1 μl of 100× BSA (10 mg/ml; New England Biolabs), and 6 U of *Bam*HI (New England Biolabs). Restriction digestions were performed at 37 °C for more than 3 h using the thermal cycler.

Rapid identification method

For multiplex PCR, 0.5 μl each of 3 μM forward and reverse primers in the PCR reaction were replaced by 0.5 μl of 10× primer mix (all primers premixed at the concentrations given in Table 2), and the extension time increased to 1.5 min. After the PCR reaction, the samples were digested with *Bam*HI as described above.

mtDNA typing with ALM_4/ALM_5 primers

mtDNA types α, β, and γ were characterized by the presence of a 2.4-kbp fragment, or a 1.6-kbp fragment or the absence of a fragment, respectively, when amplified using the primer set ALM_4 (5′-AATAATCTTCCAAGCGGAGAG-3′) and ALM_5 (5′-AAGACTCGTGATTCAGGCAAT-3′) (Lössl et al. 2000). The PCR reaction was performed in volume of 5 μl consisting of 1 μl of template DNA (approximately 5 ng/μl), 2.5 μl of Ampdirect® Plus, 0.125 U of *Taq* DNA polymerase (*TaKaRa LA Taq*® HS, Takara Bio Inc., Japan), and 0.5 μl each of 3 μM ALM_4 and ALM_5. The reaction was performed with a thermal profile of one cycle of 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 57 °C, and 1.5 min at 72 °C, and terminated with one cycle of 5 min at 72 °C.

Electrophoresis and visualization

After PCR or *Bam*HI digestion, samples with volume of 10 μl were mixed with 3 μl of loading dye I (sterile water:10× dye = 17:13), or those with volume of 5 μl were mixed with 3 μl of loading dye II (sterile water:10× dye = 11:4) (10× dye = 0.25 % bromophenol blue, 0.25 % xylene cyanol, and 25 % Ficoll Type 400). Sample volumes loaded on an agarose gel were either 6.5 μl (T, S, D, ALM_4/ALM_5, and the rapid method) or 10 μl (SAC and A). The samples of A or ALM_4/ALM_5 markers were loaded on a 1.4 % agarose gel in 1× TAE buffer (40 mM Tris–acetate and 1 mM EDTA). The electrophoresed gels were stained in 1× TAE buffer, containing 2.5 μl of Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany) per 100 ml, for 30 min with gentle shaking, followed by destaining in used 1× TAE buffer for 30 min with gentle shaking. Samples of the T, S, SAC, and D markers and those of the rapid method were loaded on a 3 % agarose gel in 1× TBE buffer (89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA). The electrophoresed gels were stained and destained in 1× TBE buffer. Photographic images were captured using a UV lamp.

Results

Variability of markers

Six markers, including the mtDNA marker ALM_4/ALM_5, were tested against 165 accessions, covering 164 different cytoplasms, and the *S. demissum* cytoplasm (Table 1; Fig. 2). The phenotypes obtained with these markers were compared with the previously determined haplotypes and the ctDNA types (Table 1).

The T marker generated three banding patterns or types. Type 1 was the most prevalent (162 out of 165 accessions).

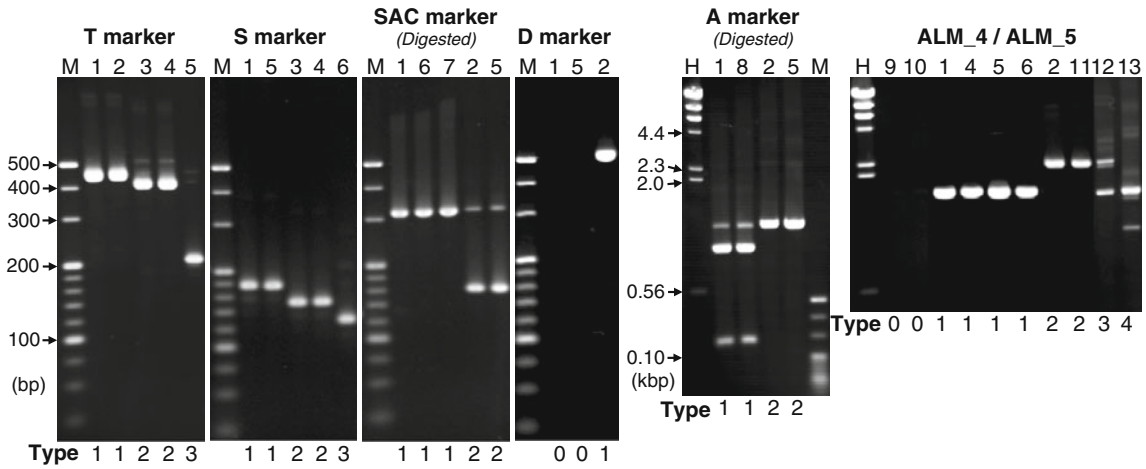

Fig. 2 Banding pattern types scored for the evaluation of cultivated potatoes and their close relatives. The T, S, SAC, and D markers were detected on a 3 % agarose gel in 1× TBE buffer, and the A and ALM_4/ALM_5 markers were detected on a 1.4 % agarose gel in 1× TAE buffer. The following samples were used: 1 *S. bukasovii* (PI 568954), 2 *S. demissum* (PI 186551), 3 *S. blanco-galdosii* (PI 442701), 4 *S. chomatophilum* (PI 266387), 5 *S. tuberosum* ssp. *tuberosum* (CIP 703252), 6 *S. stenotomum* (CIP 701165), 7 *S. stenotomum* (CIP 702583), 8 *S. immite* (PI 498245), 9 *S. pinnatisectum* (PI 275230), 10 *S. vernei* (PI 473306), 11 *S. stoloniferum* (PI 195167), 12 *S. acaule* (PI 210030), and 13 *S. dolichocremastrum* (PI 498234). M 20-bp ladder markers. H λDNA *Hin*dIII digests

Two accessions (*S. blanco-galdosii* Ochoa PI 442701 and *S. chomatophilum* Bitter PI 266387) exhibited a slightly shorter band (Type 2), which likely corresponded to the band reported by Ames et al. (2007), possessing a novel 41-bp ctDNA deletion found specifically in *S. chiquidenum* Ochoa, *S. chomatophilum*, *S. jalcae* Ochoa, and *S. blanco-galdosii*. Type 3 was found only in the T-type ctDNA carrier, characterized by a 241-bp deletion (haplotype 164).

The S marker generated three different band types (Fig. 2). Type 1 was the most prevalent (149 out of 165 accessions). Type 2 was found in all five accessions of the series *Conicibaccata* and *Piurana*. Type 3 was found in accessions of haplotypes 151–155 and 160–163, all having the S-type ctDNA (Table 1). Thus, cultivated potatoes having the S-type ctDNA were exclusively Type 3. Two additional *S. stenotomum* accessions (CIP 702583 and CIP 703088) exhibited the Type 3 band; however, the former had the W-type ctDNA (haplotype 9), and the latter had the C-type ctDNA (haplotype 89) (Table 1). The S-type ctDNA was found in the other six wild species accessions, which were separated into Type 1 (haplotypes 156–159) and Type 3 (haplotypes 160 and 163) by the S marker.

The SAC marker generated two band types: Type 1 with a single 312-bp band and Type 2 with a digested, half-sized band (Fig. 2). For an unknown reason, Type 2 always exhibited the expected digested band and a faint band at the same position as the undigested band, even after prolonged digestion time with a larger amount of the restriction enzyme (Fig. 2). This was also the case for the A marker (below) and the H3 marker in our previous study (Hosaka 2003). The SAC marker classified the accessions into two groups: one with the S-, A-, or C-type ctDNA (Type 1) and another with the other ctDNA types (Type 2) (Table 1). The only exception was *S. stenotomum* accession CIP 702583, which exhibited Type 1 by the SAC marker but had the W-type ctDNA. This accession was also an exception in the aforementioned S-marker analysis, indicating that its ctDNA type had been erroneously determined (the correct ctDNA type is probably S type).

The D marker generated a single band only in the *S. demissum* accession. A faint band of the same size was sometimes amplified in other accessions but was not reproducible in repeated experiments.

PCR products of the A marker from accessions with the A-type ctDNA were digested into two bands (Type 1), while those with the other ctDNA types were not digested (Type 2) (Fig. 2). The only exception was *S. immite* PI 498245, which had the W-type ctDNA but exhibited Type 1 (Fig. 2).

We obtained five different banding patterns with the ALM_4/ALM_5 mtDNA primers (Fig. 2). According to Lössl et al. (2000), Types 0, 1, and 2 corresponded to γ-, β-, and α-type mtDNA, respectively. The association between the ctDNA/mtDNA types and species, namely T/β and *S. tuberosum*, W/α and *S. demissum*, and W/γ and *S. stoloniferum* found among cultivars (Lössl et al. 2000), was not validated. W/α and W/β were found in various species and W/γ in *S. pinnatisectum*, *S. chacoense*, *S. pampasense*, and *S. vernei*. The two accessions of *S. stoloniferum* analyzed in this study were both W/α (Table 1).

Nomenclature of cytoplasm types

Based on the combinations of marker band types, we propose a new nomenclature system for potato cytoplasms

(Table 3). Cytoplasms of potatoes were classified by the SAC marker into cytoplasm types M and W (SAC marker Type 1 and Type 2, respectively) (Fig. 2). The P-type cytoplasm was distinguished from the M-type cytoplasm by a diagnostic S-marker Type 3 band, and the A-type cytoplasm was distinguished from the M-type cytoplasm by a diagnostic A-marker Type 1 band (Fig. 2). The T-type cytoplasm was distinguished from the W-type cytoplasm by a diagnostic T-marker Type 3 band and the D-type cytoplasm from the W-type by the presence of a specific D-marker band (Fig. 2). According to this definition, 67, 11, 8, 71, 1, and 1 out of 165 accessions were determined to be the M-, P-, A-, W-, T- and D-type cytoplasms, respectively (Table 1). Six wild species accessions could not be determined because they carried rare marker-band types. The M-type cytoplasm was found in 63 accessions with the C-type ctDNA and four wild-species accessions with the S-type ctDNA. The P-type cytoplasm was found in nine accessions with the S-type ctDNA and one each with the C- and W-type ctDNAs. The latter accession with the P-type cytoplasm and W-type ctDNA had previously been erroneously determined as described above. The A-, W-, T-, and D-type cytoplasms corresponded perfectly with the A-type ctDNA, W- or W3-type ctDNA, T-type ctDNA, and the *S. demissum* cytoplasm, respectively.

Multiplex marker system

PCR products amplified with the S and D markers were not digested by *Bam*HI (data not shown), whereas those amplified with the A and SAC markers were digested (Fig. 3). In addition, PCR products amplified with the T marker were digested (Fig. 3) because there exists a *Bam*HI recognition site within a 241-bp deletion (Hosaka et al. 1988). Primers for the T, S, SAC, D, and A markers were combined at the concentrations shown in Table 2 in one reaction tube. After the PCR reaction, *Bam*HI digestion was performed in the same tube. Marker banding patterns obtained in a 3 % agarose gel were identical to the sum of the respective *Bam*HI-digested marker-banding patterns (Fig. 3). A faint, pseudo-band generated by the D marker, if present, could be distinguished by comparing the band intensities with those of flanking marker bands (data not shown).

Evaluation of our collections

Using the multiplex marker system, our collection of 748 genotypes, including 84 Japanese named varieties, 378 breeding lines and 26 landraces, and 260 foreign varieties and breeding lines were surveyed (Supplementary Table). These genotypes were grouped into cytoplasm types: T (73.9 %), D (17.4 %), P (4.5 %), A (1.5 %), M (0.3 %), and W (2.4 %) (Table 4). Other types were not found in our collections. Based on the available pedigree records, the D-type cytoplasm was apparently derived from *S. demissum*, as described previously (Sanetomo and Hosaka 2011). The P-type cytoplasm was found in recent Japanese cultivars and breeding lines, all descended maternally from *S. phureja*. The A-type cytoplasm was found in the Japanese landraces Murasaki-imo and Nemuro-murasaki, which were previously reported as A-type ctDNA carriers (Hosaka 1993). Four Japanese breeding lines also had the A-type cytoplasm. Furthermore, the A-type cytoplasm was found in Maris Piper (from England, its distinct ctDNA reported by Powell et al. 1993), LT-7 and V-2 (from Peru), Katiusha (from Russia), and Rankoku 3 (from Korea, previously reported as a Japanese landrace in Hosaka 1993). The M-type cytoplasm was found only in the Japanese breeding line 86106-16 and the USA breeding line MN82328, their cytoplasmic origins being unknown. Genotypes with the W-type cytoplasm were further examined using the ALM_4/ALM_5 marker. Two Japanese breeding lines (Hokkai 56 and WB88055-8) were γ-type mtDNA carriers, derived maternally from *S. stoloniferum*. Four and five breeding lines had α- and β-type mtDNAs, respectively. Their cytoplasmic origins are unknown. All of the foreign genotypes with the W-type cytoplasm possessed

Table 3 Nomenclature of potato cytoplasm types

Cytoplasm type	Named after	Marker banding pattern type					Note[a]
		T	S	SAC	D	A	
M	**M**other type, or an ancestral type of Andean cultivated potatoes	1	1	1	0	2	C/ε?
P	Derived from *S. **p**hureja* into the common potato gene pool	1	3	1	0	2	S/ε
A	The most prevalent *S. tuberosum* ssp. ***a**ndigena* type	1	1	1	0	1	A/ε
W	**W**ild species	1	1	2	0	2	W/α, γ or δ
T	The most prevalent *S. tuberosum* ssp. ***t**uberosum* type	3	1	2	0	2	T/β
D	Derived from *S. **d**emissum* into the common potato gene pool	1	1	2	1	2	W/α

[a] Possible chloroplast/mitochondrial DNA type combination, deduced from Lössl et al. (1999, 2000), Hosaka and Sanetomo (2009) and Sanetomo and Hosaka (2011)

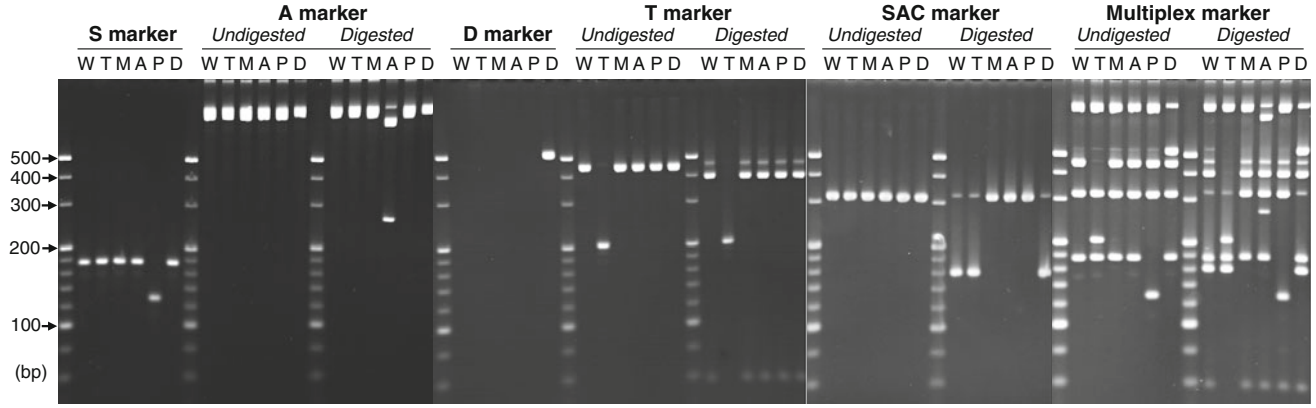

Fig. 3 Rapid identification of the W-, T-, M-, A-, P-, and D-type cytoplasms using the S, A, D, T, and SAC markers separately or as a multiplex marker, detected on a 3 % agarose gel in 1× TBE buffer. The following samples were used: W *S. brevicaule* (PI 545970), T *S. tuberosum* ssp. *tuberosum* (CIP 703252), M *S. canasense* (PI 246533), A *S. tuberosum* ssp. *andigena* (PI 281080), P *S. tuberosum* ssp. *andigena* (PI 498310), and D *S. demissum* (PI 186551). Each gel has 20-bp ladder marker in the far left lane

γ-type mtDNA: Saginaw Gold (from USA); Alwara, Verdi, and GLKS 58-1642-4 (from Germany); $(VT^n)^2$62-33-3 and GLKS 1642/4 (from The Netherlands); and Serrana Inta (from Argentina). According to the pedigrees, Saginaw Gold, Alwara, and Serrana Inta appeared to have *S. stoloniferum*-derived cytoplasm, whereas GLKS 58-1642-4, $(VT^n)^2$62-33-3, and GLKS 1642/4 appeared to have *S. vernei*-derived cytoplasm.

By observation of acetocarmine-stained pollen, Serrana Inta, Hokkai 56, and WB88055-8 showed tetrad sterility (Fig. 4b–d), which confirmed that their cytoplasms were derived from *S. stoloniferum*. Verdi, for which the pedigree was unavailable, also exhibited tetrad sterility (Fig. 4e), suggesting that the cytoplasm was derived from *S. stoloniferum*. In contrast, GLKS 1642/4 exhibited normal-appearing pollen (Fig. 4a), indicating that the cytoplasm was not derived from *S. stoloniferum*.

Discussion

Definition and verification of the new cytoplasm types

mtDNA typing by Lössl et al. (1999) was validated only within the common potato gene pool. Based on the combinations of the four ctDNA markers and D marker (intracellular origin unknown, Sanetomo and Hosaka 2011), we propose a new nomenclature for the cytoplasmic genomes of the cultivated potatoes and their closely related wild species (Table 3). The T-, D-, P-, A-, M-, and W-type cytoplasms are found in the common potato gene pool (Table 4) and shared with Andean cultivated potatoes, ancestral wild species, and the closely related wild species (Table 1).

Previously, we demonstrated that cytoplasmic differentiation was positively correlated with nuclear genomic differentiation (Sukhotu et al. 2004; Sukhotu and Hosaka 2006; Hosaka and Sanetomo 2009). Cultivated potatoes and the ancestral wild species (closely related species *S. bukasovii* Juz., *S. canasense* Hawkes, *S. candolleanum* Berth., and *S. multidissectum* Hawkes) were clearly differentiated from other wild species based on nuclear DNA RFLP analysis (Sukhotu and Hosaka 2006). The former and latter groups included the northern member and southern member species of the *S. brevicaule* complex referred to by Spooner et al. (2005) and also corresponded to the S-, A-, and C-type ctDNA groups and the W-type ctDNA group, respectively (Sukhotu and Hosaka 2006). It has been thought that potatoes were first domesticated from the former group (Spooner et al. 2005; Sukhotu and Hosaka

Table 4 Cytoplasmic diversity in the Japanese potato collections

Origin	T	D	P	A	M	W[a]	(W/α)	(W/β)	(W/γ)	Total
Japan	352 (72.1 %)	87 (17.8 %)	31 (6.4 %)	6 (1.2 %)	1 (0.2 %)	11 (2.3 %)	4	5	2	488
USA[b]	73	13	2	0	1	1			1	90
Europe	117	20	0	2	0	5			5	144
Others	11	10	1	3	0	1			1	26
Total	553 (73.9 %)	130 (17.4 %)	34 (4.5 %)	11 (1.5 %)	2 (0.3 %)	18 (2.4 %)				748

[a] Samples with W-cytoplasm type were further investigated to determine the mitochondrial DNA type using ALM_4/ALM_5 marker

[b] Including Canada

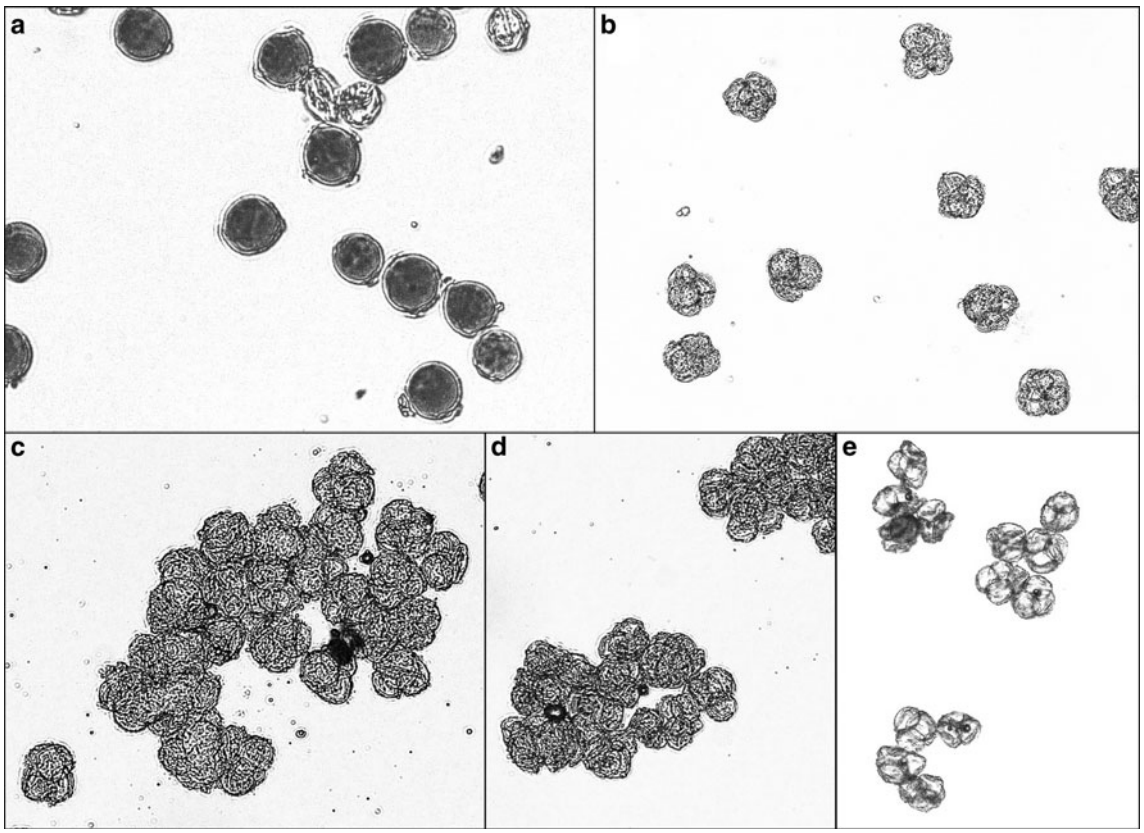

Fig. 4 Pollen stained with acetocarmine showing normal pollen in GLKS 1642/4 (**a**) and tetrad sterility in Serrana Inta (**b**), Hokkai 56 (**c**), WB88055-8 (**d**), and Verdi (**e**)

2006). Thus, the separation of the M-type cytoplasm from the W-type cytoplasm is likely associated with an evolutionary trend toward domestication.

The S-type ctDNA was found in two distinct groups (Fig. 1): one in wild species only and the other in cultivated species, particularly in the cultivated diploid species *S. stenotomum* and *S. phureja* (Hosaka and Sanetomo 2009). Of note, the P-type cytoplasm was defined only in those of the latter group. The T- and D-type cytoplasms and the P- and A-type cytoplasms are relatively distinct cytoplasmic types within the W- and M-type cytoplasms, respectively, each of which has diverse cytoplasmic variations (Sukhotu et al. 2004; Hosaka and Sanetomo 2009; Sanetomo and Hosaka 2011). Most of cultivated accessions were classified into one of these distinct cytoplasmic types (Fig. 1). The A-type cytoplasm was the most prevalent type in *S. tuberosum* ssp. *andigena*, whereas the T-type cytoplasm was the most prevalent type in the common potato. The P-type cytoplasm was introduced from *S. phureja* (Mori et al. 2011), whereas the D-type cytoplasm was introduced from *S. demissum* into the common potato gene pool (Sanetomo and Hosaka 2011).

The proposed cytoplasm types, however, are not supported as distinct clades in the ctDNA phylogeny (Spooner and Castillo 1997). Thus, these cytoplasm types may not necessarily reflect phylogenetic relationships. Nevertheless, compared with the previously distinguished 164 haplotypes, the newly defined cytoplasm types represent a rather small but informative number because they readily indicate the cytoplasmic ancestors of cultivars and the cytoplasmic identities of cultivated potatoes and the closely related wild species, as illustrated in Fig. 1.

Rapid identification method for cytoplasm types

We developed a method for distinguishing six cytoplasm types by mixing all diagnostic primers into one PCR reaction (multiplex PCR), followed by restriction digestion with *Bam*HI in the same tube followed by electrophoretic separation in an ordinary agarose gel. Previous methods for distinguishing potato cytoplasmic genomes included restriction digestion of purified ctDNA, Southern hybridization, and polyacrylamide or capillary gel electrophoresis (Hosaka 1986; Waugh et al. 1990; Bryan et al. 1999; Provan et al. 1999; Chimote et al. 2008). A PCR-based

determination method has been developed for mtDNA typing by Lössl et al. (1999). However, the PCR results with the mtDNA markers of Lössl et al. (1999) varied with different *Taq* DNA polymerases (data not shown), probably because the primers were designed for amplifying rather large DNA fragments. In our study, all primers were designed to amplify regions of less than 1.2 kbp, enabling easy PCR amplification with various *Taq* DNA polymerases (data not shown). Consequently, this new method is rapid, simple, and inexpensive, enabling analyses of hundreds of samples in a short period of time. This method can be easily adopted in laboratories that use marker-assisted selection or any type of DNA markers.

In a previous study (Hosaka and Sanetomo 2009), the S marker (=SSR marker NTCP6) generated ten polymorphic bands (127, 142, 143, 171, 172, 173, 174, 175, 176, and 177 bp) on a polyacrylamide gel. However, on a 3 % agarose gel, these polymorphic bands were detected as only three different bands (Fig. 2). Using the NTCP6 marker, Provan et al. (1999) distinguished three bands (174–176 bp) in European potato cultivars, while Martyrosyan et al. (2007) distinguished four bands (172–176 bp) in Russian potato cultivars. Chimote et al. (2008) distinguished three bands (172–174 bp) in Indian potato varieties and reported the total absence of the 127-bp band in an Indian potato gene pool. Although the ctDNA diversity information obtained from discriminating among the 171- to 177-bp bands or between the 142- and 143-bp bands with the NTCP6 marker was lost, the S marker is useful for accurately detecting the 127-bp band, which is a diagnostic band for the P-type cytoplasm (Table 3).

Cytoplasmic diversity in the common potato gene pool

Among Japanese potatoes, including cultivars, breeding lines, and landraces, 72.1 % possessed the T-type cytoplasm characterized by the T-type ctDNA (Table 4). This percentage changed little when examining only named cultivars (73.8 %) or when foreign cultivars and foreign breeding lines were included (73.9 %). These percentages of T-type cytoplasms are comparable to the 78.6 % (44/56; Powell et al. 1993) or 84.8 % (151/178; Provan et al. 1999) of European cultivars, 73.4 % (94/128) of Indian cultivars (Chimote et al. 2008), and 63.5 % (40/63) of Russian cultivars (Gavrilenko et al. 2007). These high percentages are attributed primarily to the breeding history: almost all modern cultivars are descended from a single clone, 'Rough Purple Chili' (Plaisted and Hoopes 1989; Provan et al. 1999). In contrast, German cultivars have a relatively low frequency (47 %) of T-type cytoplasm (Lössl et al. 2000). This is because *S. demissum*-derived late blight resistance and *S. stoloniferum*-derived *Potato virus Y* resistance were used extensively in German breeding programs (Ross 1986; Lössl et al. 2000).

Accounting for cytoplasm types in breeding

Among the four cross combinations in *S. tuberosum* ssp. *tuberosum* (T) and *S. tuberosum* ssp. *andigena* (A), T × A hybrids produced higher tuber yields than A × T or T × T (inter-varietal) hybrids (Maris 1989). Thus, the T-type cytoplasm is attractive for breeding high-yielding varieties (Hoopes et al. 1980; Sanford and Hanneman 1982; Maris 1989). A negative attribute of the T-type cytoplasm is the occurrence of various types of male sterility (Grun 1979), which has long bothered breeders by limiting the choice of male parents (Glendinning 1983). Moreover, the D- and *S. stoloniferum*-derived W/γ-type cytoplasms are increasing in the common potato gene pool; i.e., 17.2 and 1.2 %, respectively, in our collections (Table 4) and 40 and 10 %, respectively, in German collections (Lössl et al. 2000). This is because parental clones with the D or W/γ cytoplasm were functionally male sterile (Dionne 1961; Grun et al. 1962; Abdalla and Hermsen 1971; Brown 1984; Ortiz et al. 1993; Lössl et al. 2000), so that these were used only as female parents, resulting in cytoplasmic invasion into the common potato gene pool. Without knowing the cytoplasm types of the breeding lines, D or W/γ cytoplasm would continue to invade the gene pool, and male sterility problems would continue to worsen. The choice of male parents will be strictly limited, as warned previously by Provan et al. (1999).

Wide cytoplasmic diversity exists in the Andean cultivated potatoes (Hosaka and Hanneman 1988; Sukhotu et al. 2005). Cytoplasmic effects or the effects of nuclear–cytoplasmic interactions on agronomic traits and fertility are important issues to address. We have demonstrated that P-type cytoplasm does not contribute to male and female sterility (Mori et al. 2012). We have also noticed that some genotypes with the D-type cytoplasm can function as pollen parents. Thus, it may be possible to deliberately improve parental clones by replacing cytoplasms with those that are superior in terms of male fertility and heterotic effects between nuclear and cytoplasmic genomes. Alternatively, a fertility-restoring gene, such as the *Rt* gene, which partially circumvents male sterility caused by the nuclear and T-type cytoplasm interactions (Iwanaga et al. 1991), can be searched for among genotypes with the D- or W/γ-type cytoplasm. For these purposes, our newly developed rapid identification method and cytoplasm classification system will be helpful.

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