Determining a threshold for genetic conformity in potato seedlings

Introduction

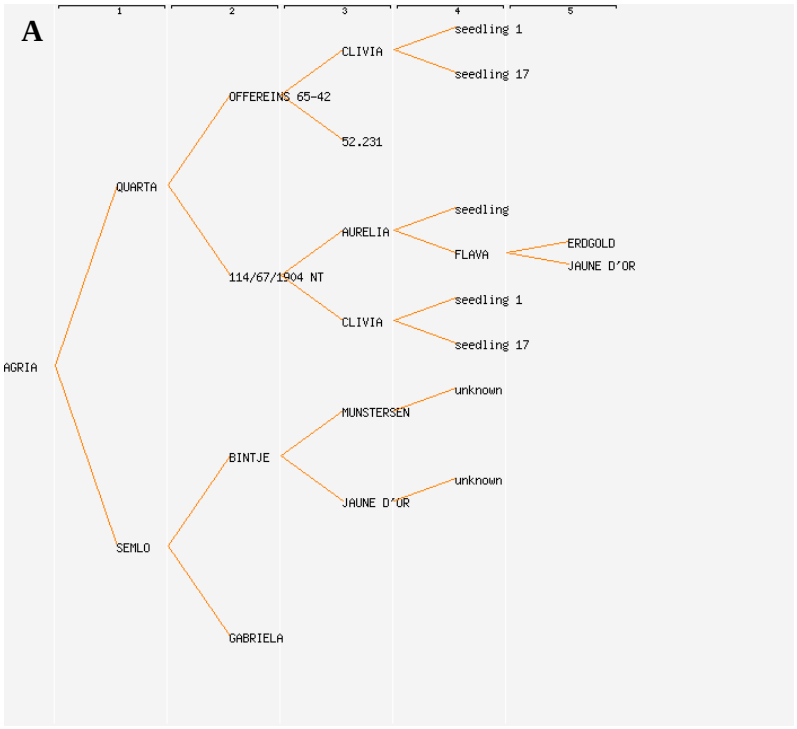
In recent years there have been numerous DNA based 'fingerprinting' studies, mainly using microsatellite markers (also known as simple sequence repeats or SSRs), which have focused on commercial potato variety identification. Between 2006 and 2008 SASA took part in a CPVO co-funded project to screen the varieties on the EU Common Catalogue using 9 microsatellite markers (Reid *et al*., 2011). In addition to these varieties SASA has built a library of over 1500 potato varieties typed with these 9 markers as well as an additional 3 markers. The results of these studies showed that commercial varieties can easily be differentiated using this technology. The exceptions to this rule are somaclonal variants such as King Edward and Red King Edward which yielded identical results and a small number of pairs of varieties which intuitively should be different but proved not to be. Most of these aberrant pairs can, however, be explained. Firstly, some varieties have been assigned different names in different countries (e.g. Asparges and Ratte). The second explanation is that at some point a mix up occurred between varieties at one location resulting in one variety subsequently been distributed with the wrong name (we know that such mix ups have occurred in the past as we also found a small number of varieties from different collections that had the same name and were proved to be totally different varieties). A third explanation is that it is possible for two *bona fide* varieties to yield identical fingerprints although the chance of this was calculated at 1 in 2.8 million during the CPVO project (based on only 9 markers). It is, however, likely that for varieties with similar lineages this number would be much lower.

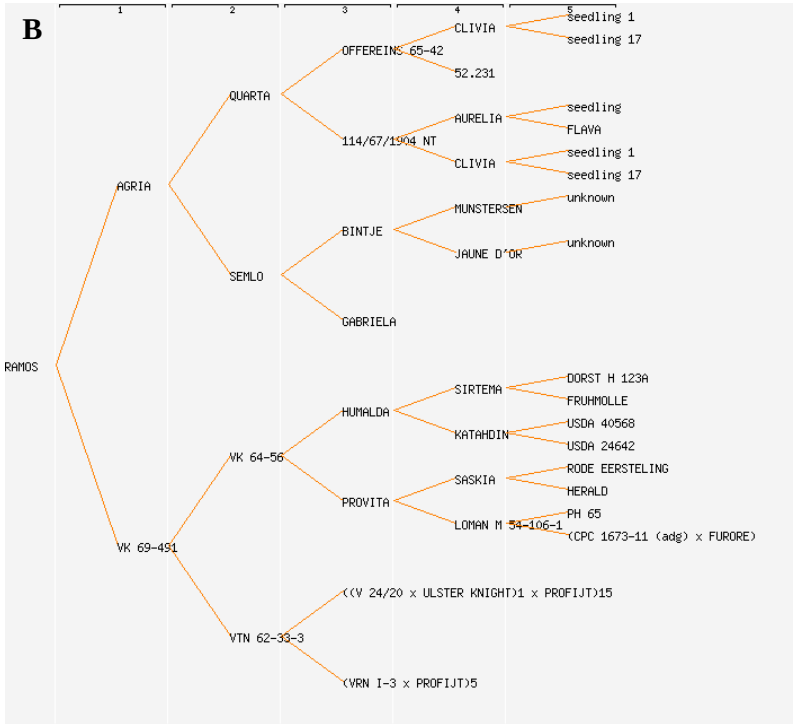
The purpose of this investigation was to determine the levels of genetic variability within seedlings (around 1000) produced by 5 crosses of 3 potato cultivars using 12 microsatellite markers and to postulate a genetic threshold for the reversal of the burden of proof for essential derivation. Numerous studies to determine the EDV threshold for other crop species have been documented, for example Maize (Heckenberger *et al*., 2005a, 2005b and 2006), barely and lettuce (van Eeuwijk and Law, 2004) and durum wheat (Noli *et al.*, 2012). Commonly the "tail principle" (van Eeuwijk and Law, 2004) is used to determine the threshold as this method does not require any *a priori* knowledge of the parents of the EDV. The tail principle involves analysis of the distribution of pairwise similarity values and the setting of a similarity value above which the disputed variety is deemed to be essentially derived from the initial variety. The International Seed Federation (ISF) have guidelines for determining the threshold for essential derivation and the reversal of the burden of proof (see http://www.worldseed.org/isf/edv.html). Below the set threshold there is no presumption of essential derivation while above it there is presumption of essential derivation and the burden of proof of non predominant derivation would fall on the breeder of the putative EDV. The threshold varies from species to species depending on the existing genetic variability within the species and the established breeding procedures. For example the threshold for lettuce is set at 0.96 Jaccard similarity using AFLPs, for oilseed rape the trigger point is a Dice value of 0.85 for both winter and spring varieties, for rye grass it is a Jaccard of 0.6, tomato is 0.78 and cotton 0.875. Maize has a two tier system at 0.82 conformity the burden of proof shifts to breeder of putative EDV while 0.9 conformity is viewed as a strong indication of predominant derivation. During the CPVO project around 900 potato varieties were assessed from the 2006 EU Common Catalogue and the mean Jaccard coefficient was calculated at around 45% with 5 pairs between 85-90% similarity and only 1 pair between 90-95% (excluding somaclonal variants and varieties suspected to have been mislabelled). However, as most of the varieties tested were either unrelated or their parentage was not known it was not possible to set a

threshold from these data hence this study.

Materials and Methods

Examination of the lineages of the 3 parent varieties (van Berloo *et al.*, 2007) show that both Fontane and Ramos have Agria as one parent, and, that the other side of the crosses also have a common background (Figure 1).





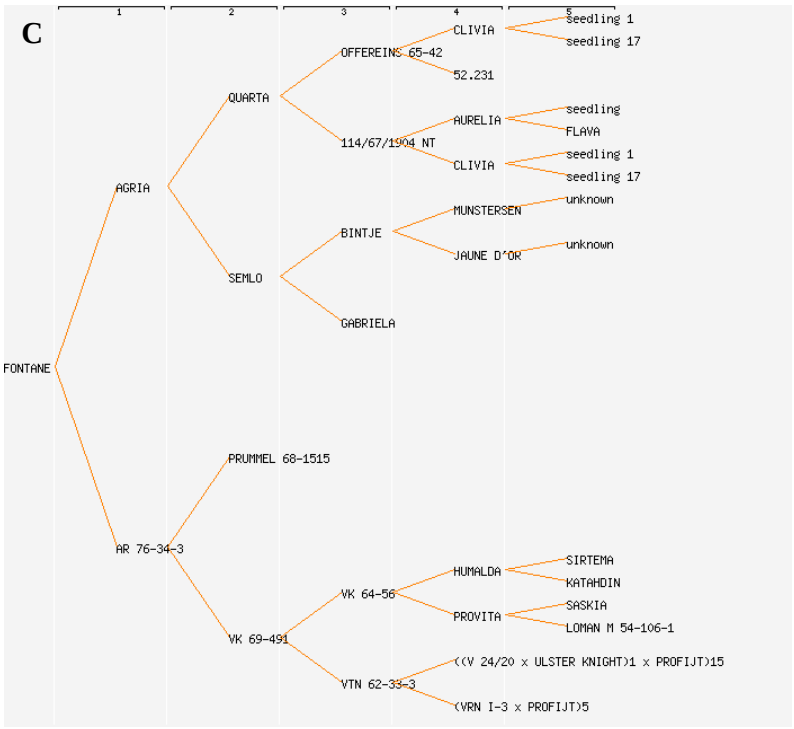


Figure 1. Pedigrees (last 5 generations) for (A) Agria, (B) Ramos and (C) Fontane. Data from Potato Pedigree Database (www.plantbreeding.wur.nl/potatopedigree).

Details of the crosses are shown in Table 1 and properties of the microsatellite markers used to analyse them in Table 2. The 12 microsatellites were amplified as per Reid *et al.* (2011) with the addition of multiplex set 4 containing the additional 3 markers. The data were stored and analysed in BioNumerics v6.1 (Applied Maths). Similarity values were calculated using the Jaccard coefficient and the results plotted using Principle Components Analysis (PCA) and Multi-Dimensional Scaling (MDS) using the default settings for analysis within BioNumerics.

Variety	**Type of cross**	**DNA Plate numbers**	**Colour code**
Ramos	Selfing	EDV#2-1 & EDV#2-2	
Fontane	Selfing	EDV#4-1 & EDV#4-2	
Agria x Ramos	Cross	EDV#6-1 & EDV#6-2	
Agria x Fontane	Cross	EDV#8-1 & EDV#8-2	
Ramos x Fontane	Cross	EDV#11-1 & EDV#11-2	
Agria	Parent from SASA db		
Fontane	Parent from SASA db		
Ramos	Parent from SASA db		

Table 1. Combinations of crosses examined in this study. The colour code shows the colours used to depict the various sets of samples in subsequent figures.

Marker name	Repeat motif	Linkage group	Number of alleles	Reference
STMS 0019	$(AT)_7$ $(GT)_{10}$ $(AT)_4$ $(GT)_5$ $(GC)_4$ $(GT)_4$	VI	10	Milbourne *et al*., 1998
STMS 1016	$(TCT)_9$	VII	16	Milbourne *et al*., 1998
STMS 1024	$(TTG)_6$	VIII	8	Milbourne *et al*., 1998
STMS 2005	$(CTGTTG)_3$	XI	6	Milbourne *et al*., 1998
STMS 2022	$(CAA)_3..(CAA)_3$	II	7	Milbourne *et al*., 1998
STMS 2028	$(TAC)_5.(TA)_3.(CAT)_3$	XII	9	Milbourne *et al*., 1998
STMS 3009	$(TC)_{13}$	VII	14	Milbourne *et al*., 1998
STMS 3012	$(CT)_4.(CT)_8$	IX	7	Milbourne *et al*., 1998
STMS 3023	$(GA)_9.(GA)_8.(GA)_4$	IV	4	Milbourne *et al*., 1998
STMS 5136	$(AGA)_5$	I	11	Ghislain *et al*., 2004
STMS 5148	$(GAA)_{17}$	V	20	Ghislain *et al*., 2004
SSR1	$(TCAC)_n$	VIII	14	Kawchuk *et al*., 1996

Table 2. Marker information showing the repeat motif of the microsatellite, linkage group, numbers of alleles and original reference.

Due to the shared pedigrees the microsatellite profiles of the 3 parent varieties could be expected to be fairly similar (Table 3). The Potato Pedigree Database also showed that an additional 8 varieties, 4 of which are in the SASA database (Arcade, Farmer, Florida and Recolta) resulted from crosses between Agria x VK 69-491 whereas Fontane is the only listed progeny of the Agria x AR 76 34-3 cross. The allelic profiles for the additional 4 varieties in the SASA database are also given in Table 3.

	Marker											
Variety	**0019**	**1016**	**1024**	**2005**	**2022**	**2028**	**3009**	**3012**	**3023**	**5136**	**5148**	**SSR1**
Agria	BF	DGLM	DEG	BDF	E	ABC	G	BCDF	AB	CDF	AIP	ADFI
Fontane	BF	DHLM	BCDE	DF	E	ABC	FG	BCDF	AB	DEFH	AJMP	ABDF
Ramos	F	DGHL	BCDG	DF	BE	ABC	G	BCF	AB	CEFH	IMOP	BDFI
Arcade	BF	DHLM	BCDG	ABD	BE	ABC	G	CD	AB	CDF	AMOP	ABDI
Farmer	BF	DGH	CEG	ABDF	BE	AB	G	BDF	AB	CDEH	IMOP	BFI
Florida	BF	DGM	CDE	ABDF	BE	BC	G	BCD	ABD	CDEH	LOP	AFI
Recolta	F	DGM	CDG	AD	E	ABC	G	CDF	ABD	EFH	IMOP	AFI

Table 3. Allelic phenotypes of 12 microsatellite markers for Agria, Fontane and Ramos and 4 additional varieties resulting from a cross between Agria and VK 69-491.

Results

All of the samples tested could be differentiated on the basis of the 12 markers with the exception of 2 progeny from the Fontane selfing (samples 319 & 320, wells G4 & H4 in the DNA plate) and 2 from the Ramos selfing (samples 99 & 102, wells C1 & F1). These pairs were examined using an additional 24 markers and could still not be differentiated. Taking into account the positioning of these pairs of plants in the DNA plates it is possible that these two pairs of DNA samples originated from the same plants.

No crosses yielded progeny identical to the parent varieties although the Ramos selfing had 3 plants that only differed by a single allele from Ramos (97.1% similarity) and 2 plants which differed by 2 alleles (94.1% similarity). The Fontane selfing yielded a single plant that

differed by two alleles from Fontane (94.5% similarity) all other plants differed by 3 or more alleles from the parent varieties (Table 4.). The closest matches to the parent varieties for the other crosses are also shown in Table 4.

Cross	Plant # with closest match to parent (% similarity)	# different alleles	Marker(s) (allelic phenotypes)
Fontane selfing	Fontane & 298 (94.5)	2	2028 (ABC & BC) 3023 (AB & A)
Ramos selfing	Ramos & 26 (97.1) Ramos & 56 (97.1) Ramos & 107 (97.1) Ramos & 4 (94.1) Ramos & 105 (94.1)	1 1 1 2 2	5136 (CEFH & EFH) 2028 (ABC & BC) 5136 (CEFH & CEF) 5136 (CEFH & CH) 2028 (ABC & BC) 3023 (AB & B)
Agria x Fontane	Agria & 689 (88.2) Agria & 739 (88.2) Fontane & 637 (83.8) Fontane & 681 (83.8)	4 4 6 6	3009 (G & FG) 3012 (BCDF & BDF) 5136 (CDF & DF) 5148 (AIP & AP) 3012 (BCDF & BCF) 5136 (CDF & DF) 5148 (AIP & AMP) 1024 (BCDE & BDE) 3012 (BCDF & BCF) 3023 (AB & A) 5136 (DEFH & CDFH) 5148 (AJMP & AJP) 1016 (GHLM & HLM) 3009 (FG & G) 3012 (BCDF & CDF) 5136 (DEFH & CDEF) SSR1 (ABDF & ABD)
Agria x Ramos	Agria & 466 (85.7) Ramos & 567 (81.1)	5 7	2005 (BDF & DF) 2022 (E & BE) 3012 (BCDF & BDF) 5136 (CDF & CDE) 1016 (DGHL & DGL) 1024 (BCDG & CDEG) 2005 (DF & BDF) 3012 (BCF & BCDF) 5148 (IMOP & IOP) SSR1 (BDFI & BFI)
Fontane x Ramos	Fontane & 900 (86.5) Ramos & 933 (83.3)	5 6	0019 (BF & F) 1024 (BCDE & BDE) 3012 (BCDF & CDF) 5148 (AJMP & IJMP) 1024 (BCDG & BCG) 2022 (BE & E) 3009 (G & FG) 5148 (IMOP & AIP)

Table 4. The closest matches to the parent varieties in the 5 crosses. Table shows the plant number and % similarity, the number of alleles that differ and the allelic phenotypes (parent variety first). Different alleles are highlighted in red.

All crosses yielded pairs of plants with greater than or equal to 90% similarity however the numbers are very different for the two classes of crosses. The Fontane selfing yielded 45 pairs while the Ramos selfing 111 pairs. In contrast the three out crosses yielded 1, 2 and 4 pairs of plants greater than or equal to 90% similarity (Table 5.).

Cross	Plant pairs with 90% similarity or greater (% similarity)	# different alleles	Marker(s) (allelic phenotypes)
Agria x Fontane	596 & 681 (91.2)	3	3009 (FG & G) 5148 (AIMP & AJMP)
	681 & 723 (90.9)	3	2005 (DF & BDF) 2028 (ABC & BC) 3012 (CDF & CD)
	634 & 662 (90.3)	3	1024 (CDE & CE) 3012 (BCDF & BCD) 5136 (CDEF & CDF)
	599 & 602 (90.0)	3	1024 (DE & CDE) 2028 (ABC & AB) 3009 (G & FG)
Agria x Ramos	545 &547 (90.3)	3	5136 (CEFH & CF) 5148 (OP & IOP)
	448 & 576 (90.3)	3	0019 (F & BF) 5136 (FH & CFH) 5148 (AJMP & AMP)
Fontane x Ramos	795 & 915 (93.1)	2	5136 (CEFH & CDEF)

Table 5. Pairs of progeny plants with 90% or greater similarity from the three out crosses and the differing allelic profiles.

Conversely there are pairs of progeny that are very different from each other. Table 6 shows the numbers of pairs with less than 40% similarity for each of the crosses and the lowest ranking pair for each cross.

Cross	# pairs <40% similarity	Plant #s most different pair (% similarity)	# different alleles/total alleles
Fontane selfing	11	228 & 380 (36.1)	22/49
Ramos selfing	8	59 &98 (35.3)	22/46
Agria x Fontane	62	640 & 767 (31.2)	28/54
Agria x Ramos	74	431 & 472 (30.8)	27/51
Fontane x Ramos	186	873 & 923 (28.6)	30/54

Table 6. The number of pairs with less than 40% similarity and the pair with the lowest ranked similarity for each of the crosses.

Cross	Most different to parent (% similarity)	# different alleles	Marker(s) (allelic phenotypes)
Fontane selfing	228 (61.1)	14	0019 (BF & F) 1016 (GHLM & GM) 1024 (BCDE & DE) 2005 (DF & D) 2028 (ABC & BC) 3012 (BCDF & CD) 5136 (DEFH & DEH) 5148 (AJMP & MP) SSR1 (ABDF & AF)
Ramos selfing	46 (64.7)	12	0019 (F & NULL) 1016 (DGHL & DH) 1024 (BCDG & BG) 2028 (ABC & BC) 3012 (BCF & BC) 3023 (AB & A) 5136 (CEFH & CEF) 5148 (IMOP & MOP) SSR1 (BDFI & BI)
	82 (64.7)	12	1016 (DGHL & DH) 1024 (BCDG & BG) 2005 (DF & D) 2022 (BE & E) 2028 (ABC & B) 5136 (CEFH & CFH) 5148 (IMOP & IOP) SSR1 (BDFI & BI)
	132 (64.7)	12	1016 (DGHL & DL) 1024 (BCDG & DG) 2005 (DF & D) 2028 (ABC & AB) 3012 (BCF & BC) 5136 (CEFH & CEH) 5148 (IMOP & MP) SSR1 (BDFI & DI)
	136 (64.7)	12	1016 (DGHL & DL) 1024 (BCDG & DG) 2005 (DF & D) 2022 (BE & E) 2028 (ABC & BC) 3012 (BCF & BC) 5136 (CEFH & CEF) 5148 (IMOP & IMP) SSR1 (BDFI & DI)

Table 7. Progeny with lowest similarity to parent varieties in selfings. The allelic phenotypes show the parental variety first and the different alleles are highlighted in red.

A small number of crosses gave unexpected results. A few plants had more than 5 microsatellite alleles for some of the markers (Table 8). Intuitively, for a tetraploid this should

not be possible although this has been observed in a small number of commercial cultivars. There are several possible explanations for this ranging from cross contamination of the sample (i.e. DNA was extracted from more than one plant), through to duplication of the microsatellite region on one or more chromosomes, and the possibility of unequal segregation of chromosomes at gamete formation.

Cross	**Marker**	**Alleles (number of plants)**
Fontane selfing	None	
Ramos selfing	None	
Agria x Fontane	5136 5148	CDEFH (1) AIJMP (2)
Agria x Ramos	1024 SSR1	BCDEG (2) ABDFI (1)
Fontane x Ramos	5136 5148	CDEFH (1) AIMOP (1)

Table 8. Information on samples yielding 5 alleles and the markers they were found in.

Two of the progeny of the Fontane selfing yielded several alleles not previously seen in commercial *Solanum tuberosum* varieties (Table 9). These plants are sufficiently different to appear to be hybrids with another *Solanum* species (other *Solanum* species were present at the time the crosses were performed). Microsatellite mutation could also be the cause of new alleles but on this scale would appear less likely given the presence of other species.

Fourteen other samples yielded alleles not seen in either parent for several of the markers (Table 9). In particular one from the Fontane selfing yielded alleles not found in Fontane for 8 of the 12 markers. Again this could have been the result of mutation but due to the number of markers exhibiting 'incorrect' alleles they are more likely to be the result of pollination by another variety.

Cross	Total # of plants	Marker	Alleles - unexpected alleles in red (# of plants)
Fontane selfing	3	0019 1016 2005 2022 2028 3009 3023 5136 5148 SSR1	A (1), I (1) C (2) B (2) BNewG (2) G (2), ADG (1) CE (2), BEF (1) BD (1) BGNew (2), ADF (1) BNew(2), GIJ (1) NewL (2), DFJ (1)
Ramos selfing	2	0019 1024 3009 3012 5136 5148	B (1) CDE (1) FG (1) BCD (1) DFH (1) IJP (1)
Agria x Fontane	1	2005	ABD (1)
Agria x Ramos	9	2022 3009 5148	BEG (5) FG (4) JOP (2)
Fontane x Ramos	1	2005 3012	BD (1) ABCF (1)

Table 9. Unexpected alleles found in progeny from the crosses. Profiles with 'New' indicate alleles not previously recorded from commercial potato varieties.

Due to the large amount of data it is not feasible to present a dendogram to show the differences between the seedlings so the data is presented as 2D PCA analysis and 3D MDS analysis. Figure 2 shows the results of the PCA analysis while Figures 3 and 4 show the MDS analysis viewed from different perspectives.

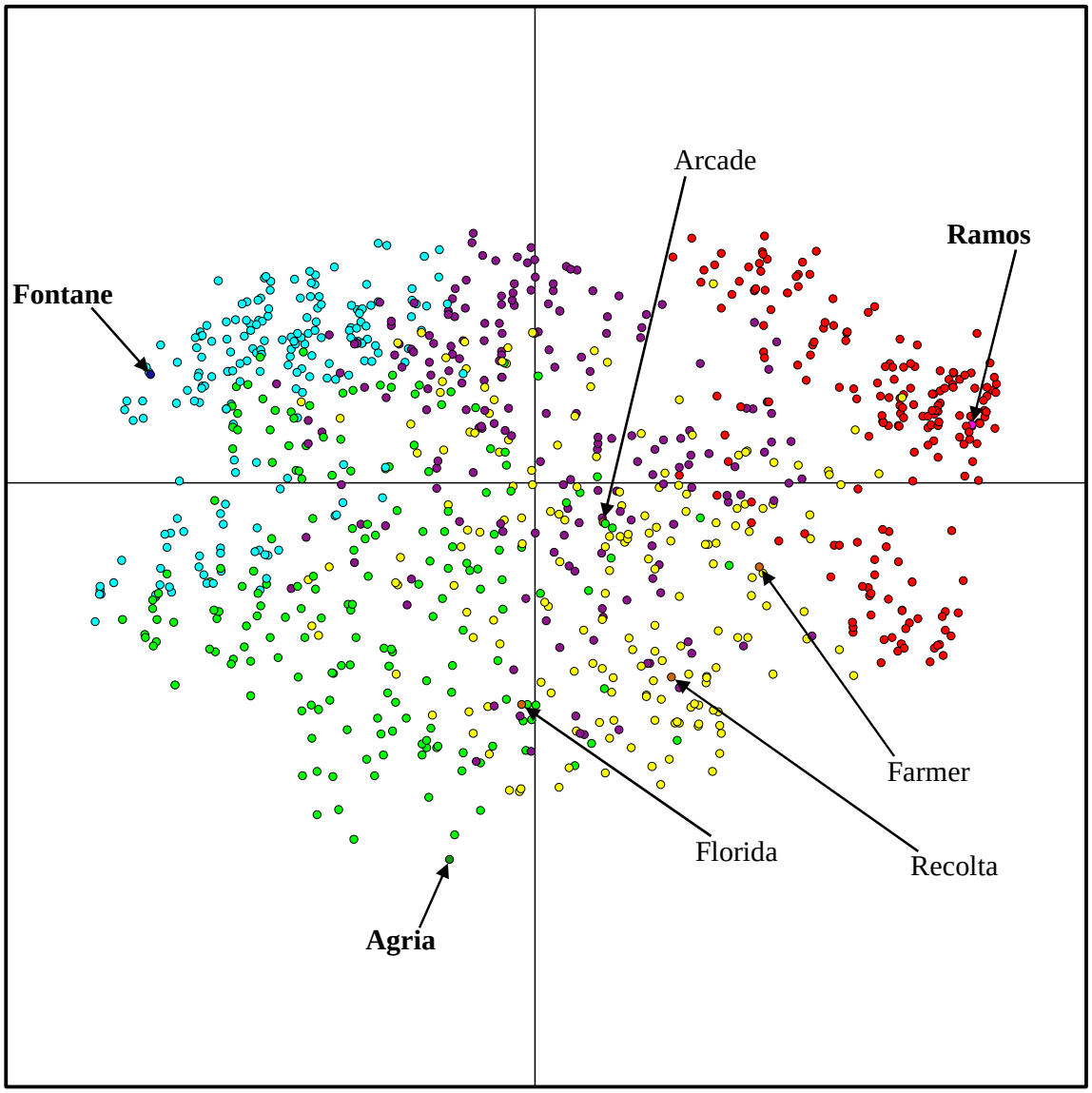


Figure 2. PCA analysis of the results from the microsatellite fingerprinting. The positions of the 3 parent varieties are arrowed along with the additional 4 varieties from the Agria x VK 69-491 cross. Note that these varieties sit between Agria and Ramos Others samples are Ramos selfing (●), Fontane selfing (●), Agria x Ramos (●), Agria x Fontane (●), Ramos x Fontane (●).

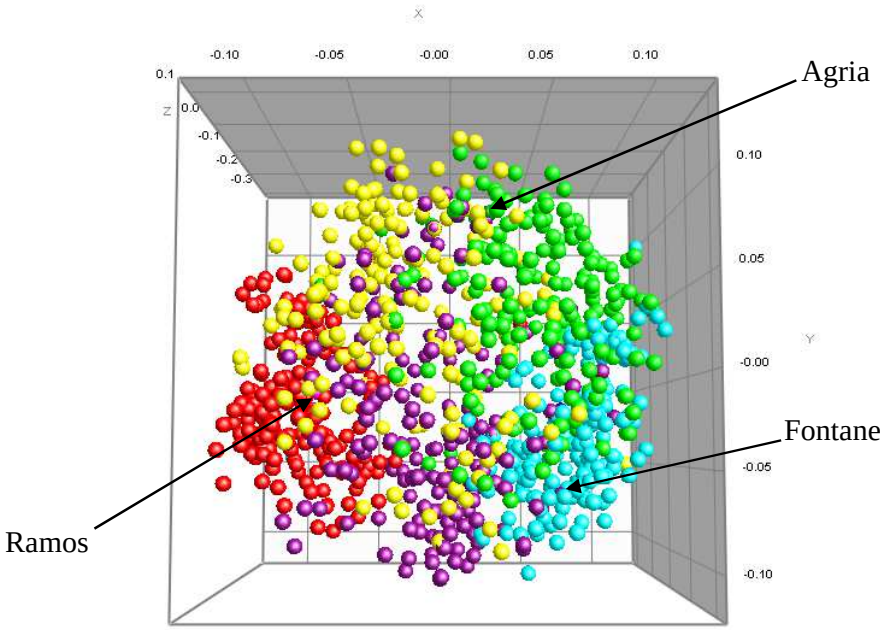


Figure 3. MDS of the microsatellite fingerprinting data viewed from front. Samples are Ramos selfing (●), Fontane selfing (●), Agria x Ramos (●), Agria x Fontane (●), Ramos x Fontane (●).The positions of the 3 parent varieties are arrowed.

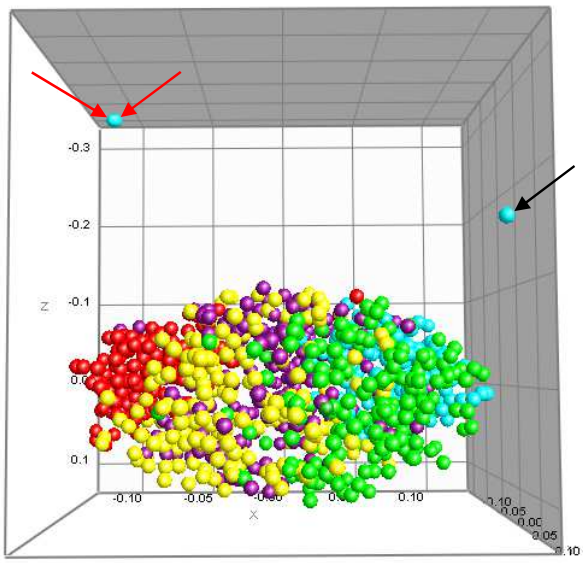


Figure 4. MDS of the microsatellite fingerprinting data viewed from above. Samples are Ramos selfing (●), Fontane selfing (●), Agria x Ramos (●), Agria x Fontane (●), Ramos x Fontane (●). The samples yielding alleles not observed in *S. tuberosum* are indicated by red arrows. A further sample from the Fontane selfing exhibiting many alleles not found in Fontane (8/12 markers) is indicated by the black arrow (the alleles in this plant are found in other commercial potato varieties).

The distribution of the pair wise comparisons of the Jaccard values calculated for approximately 900 varieties analyzed during the CPVO project showed a normal distribution with a mean similarity value of around 45% based on 9 markers (Reid *et al*., 2011). Most of the samples analyzed during the CPVO project were also screened with the additional 3 markers used in this study and all data presented for the CPVO samples in this report are based on all 12 markers (Figure 5A & 6A). Similar comparisons of the distributions of the Jaccard coefficients for the samples from the 5 crosses (excluding samples yielding alleles not found in either parent) show similarly normal distributions but with very different means (Figure 5B-F and 6B-F) and Table 10. The distributions of Jaccard similarities for the various datasets are shown in Figure 7.

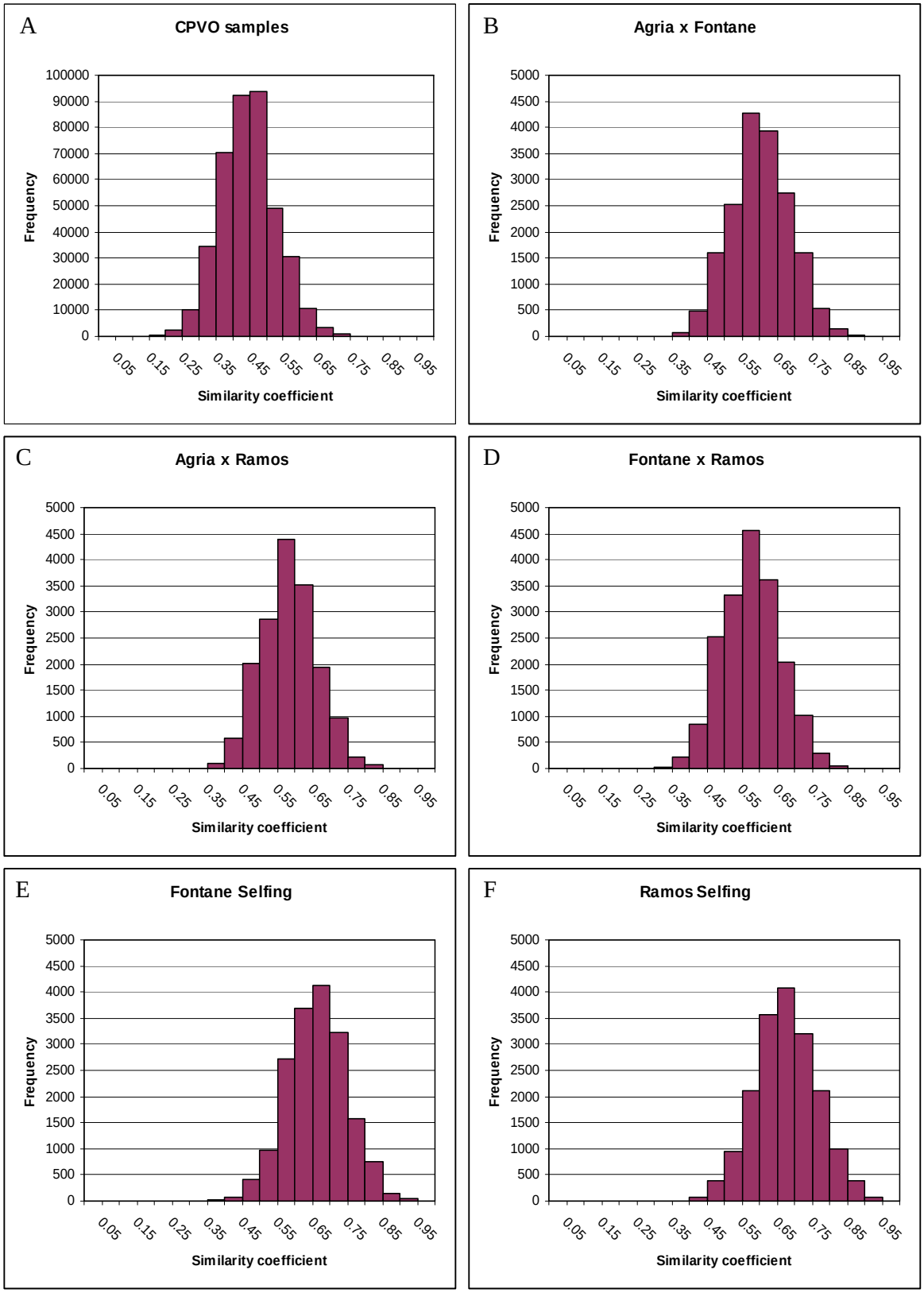


Figure 5. Frequency distribution of Jaccard coefficient pairwise comparisons. (A) From approximately 900 varieties examined during the CPVO project (based on 12 markers), (B) from Agria x Fontane cross, (C) from Agria x Ramos cross, (D) from Fontane x Ramos cross, (E) from Fontane selfing, (F) from Ramos selfing.

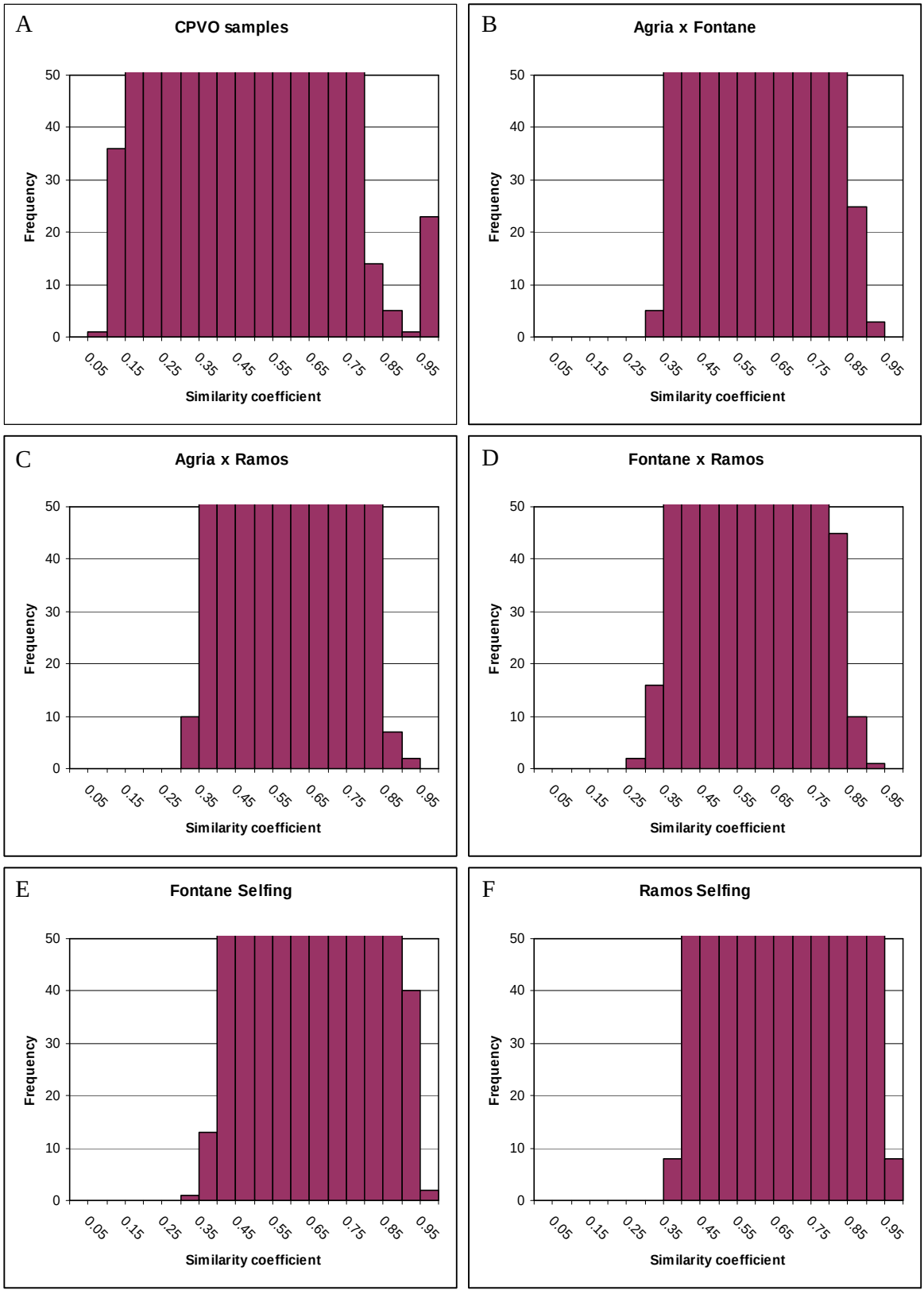


Figure 6. Frequency distribution of Jaccard coefficient pairwise comparisons with expanded frequency scale to show the tails in greater detail. (A) From approximately 900 varieties examined during the CPVO project (based on 12 markers), (B) from Agria x Fontane cross, (C) from Agria x Ramos cross, (D) from Fontane x Ramos cross, (E) from Fontane selfing, (F) from Ramos selfing.

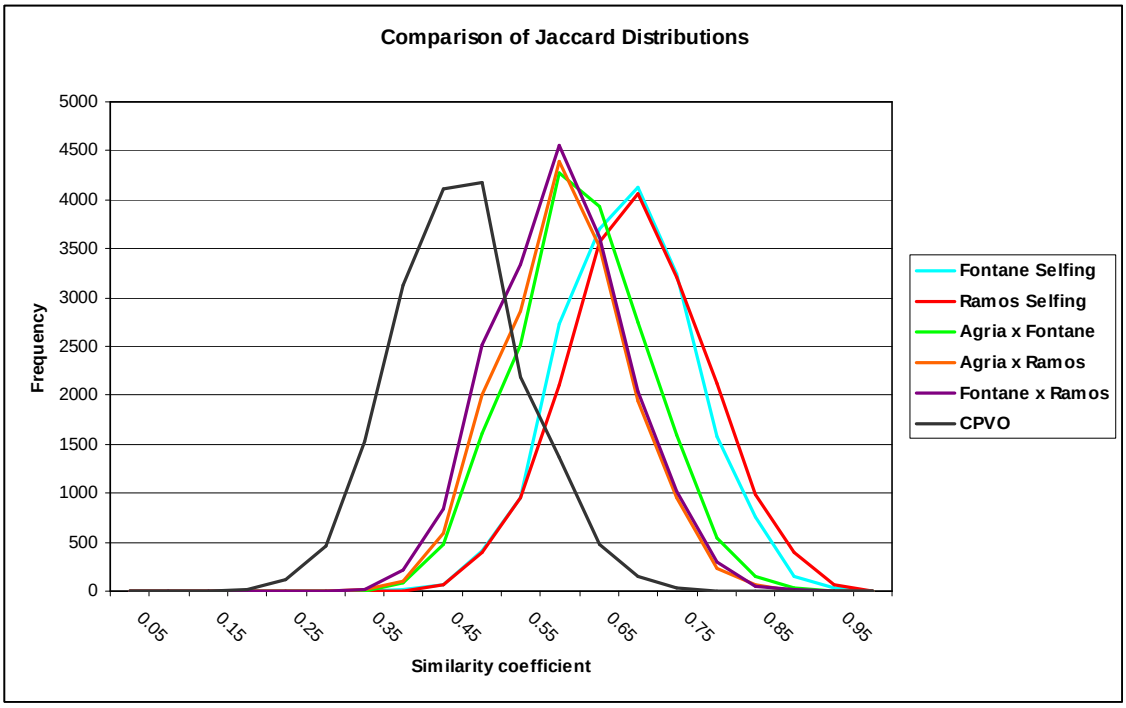


Figure 7. Frequency distributions of Jaccard coefficient pairwise comparisons for the various datasets. N.B. the CPVO data has been normalized to fit the scale of the data from this study.

Dataset	Total # of pairs	Mean % similarity	85-90% similarity (% total)	90-92% similarity (% total)	92-95% similarity (% total)	Above 95% similarity (% total)
CPVO database	399,171	44.9	5 (0.001)	0 (0)	1 (0.0002)	23 (0.006)
Agria x Fontane	17,955	60.5	25 (0.139)	3 (0.017)	0 (0)	0 (0)
Agria x Ramos	16,653	58.5	7 (0.042)	2 (0.012)	0 (0)	0 (0)
Fontane x Ramos	18,528	57.9	10 (0.054)	0 (0)	1 (0.005)	0 (0)
Fontane selfing	17,766	66.4	151 (0.850)	27 (0.152)	13 (0.073)	2 (0.011)
Ramos selfing	17,995	67.7	392 (2.178)	36 (0.200)	37 (0.206)	8 (0.045)

Table 10. The mean % similarity (Jaccard coefficient), numbers of pairs 85-90%, 90-92%, 92-95% and above 95% similarity for the various data sets. The % of pairs for each category is shown in brackets). N.B. the pairs above 95% similarity for the CPVO database (data for all 12 markers) include somaclonal variants and varieties expected to be mix ups otherwise this value would be 0.

When the crosses from this project are compared to the whole SASA database the results were even more striking (Figures 8A & 9A) with the progeny for these crosses clearly distinct from the main bulk of commercial varieties. As a comparison figures 8B & 9B show the equivalent plots with just the varieties from the CPVO project.

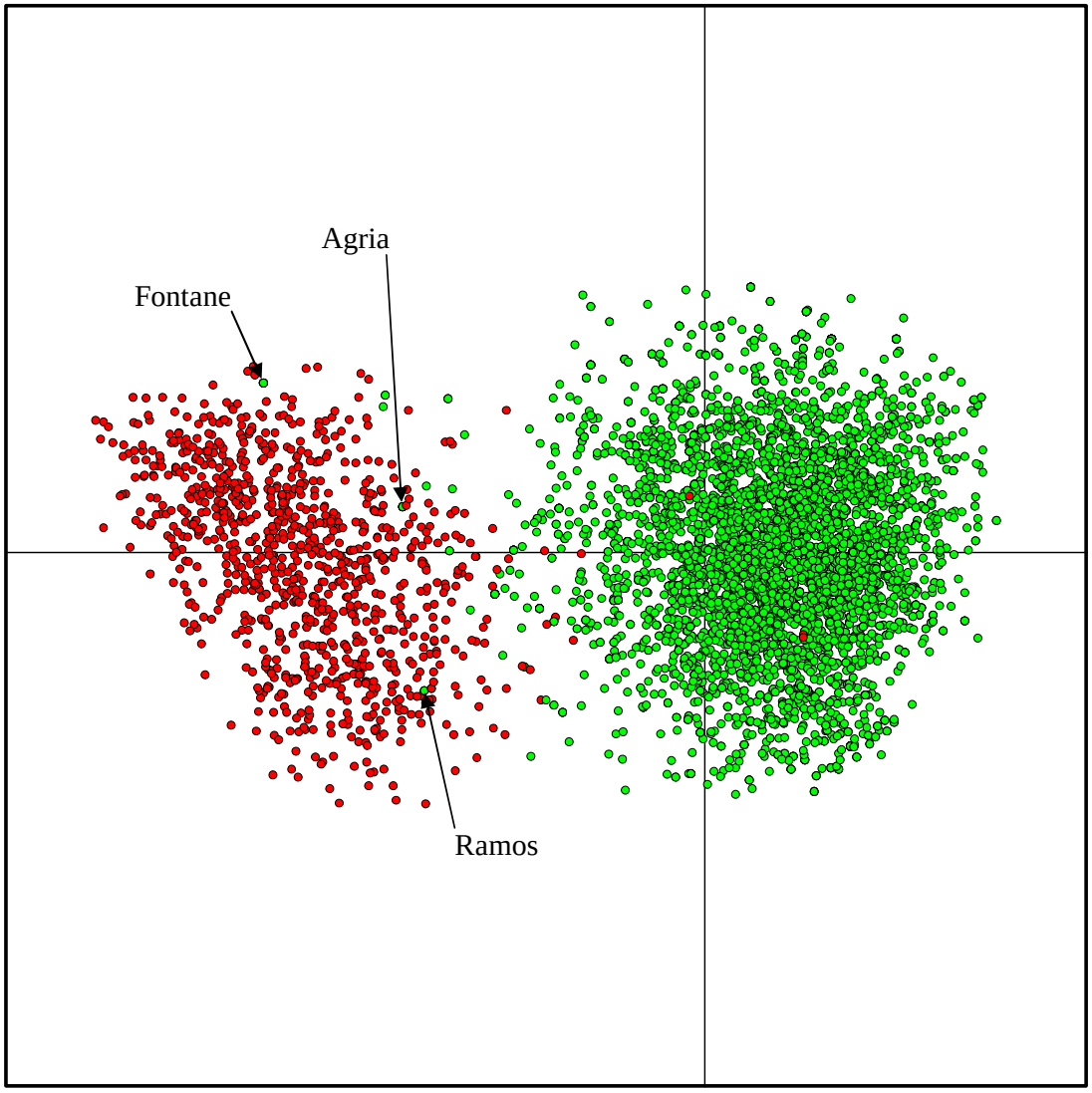


Figure 8A. PCA plot of varieties in the main SASA database (approximately 1500 varieties) (●) and progeny from the 5 crosses from this study (●).

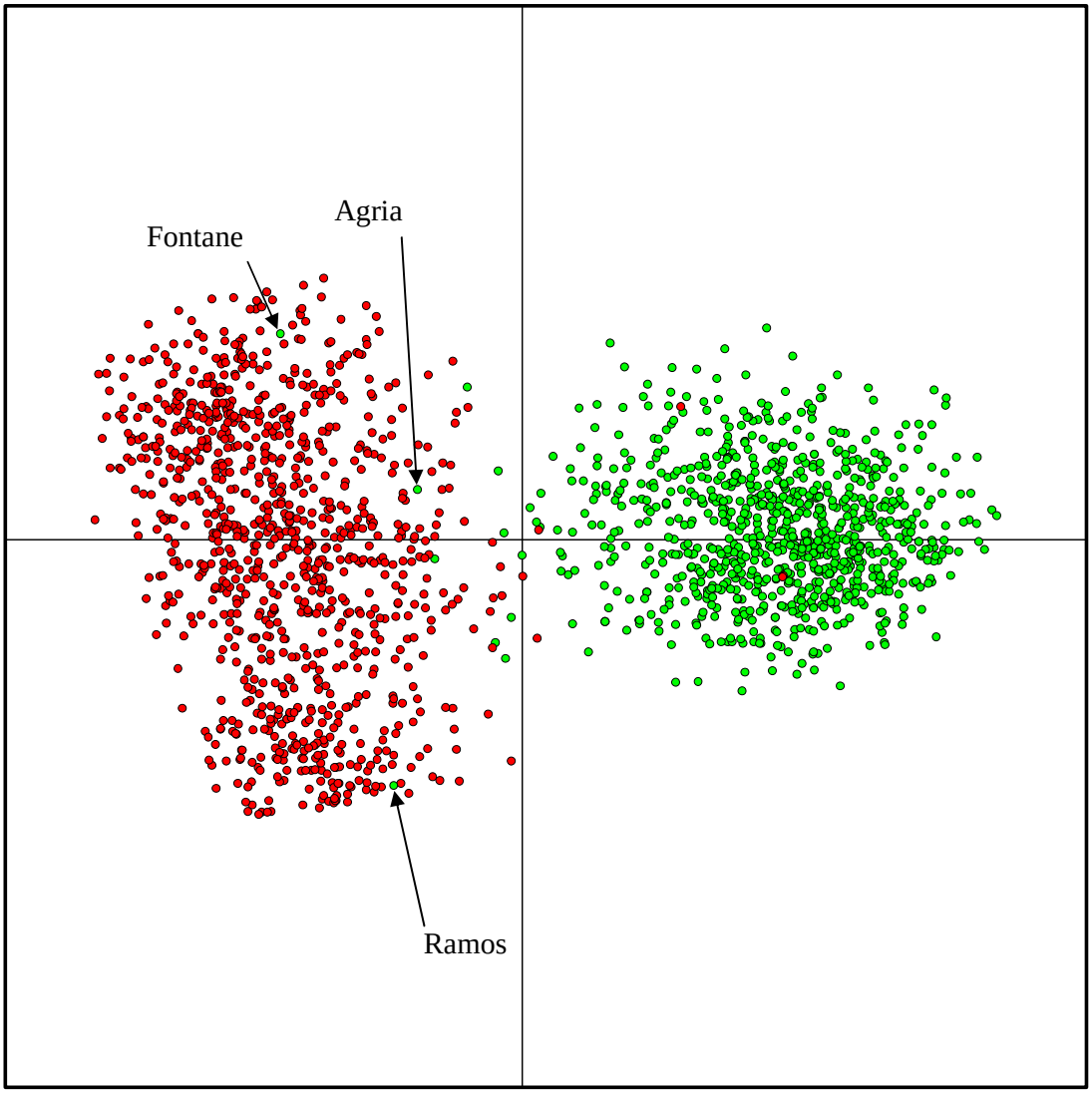


Figure 8B. PCA plot of varieties in from the CPVO project (●) and progeny from the 5 crosses from this study (●).

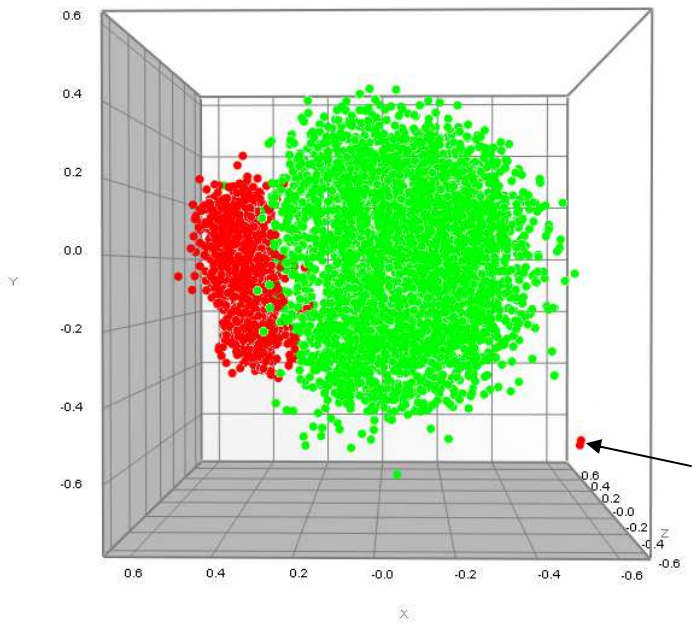


Figure 9A. MDS plot of samples in the main SASA database (approximately 6000 samples of 1500 varieties) (●) and progeny from the 5 crosses from this study (●).Samples yielding alleles not observed in *S. tuberosum tuberosum* are arrowed.

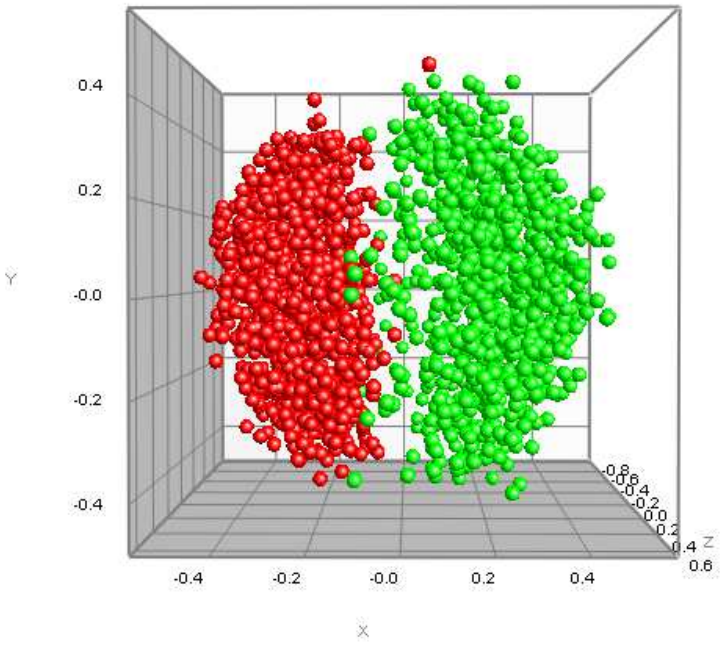


Figure 9B. MDS plot of samples from the CPVO project (●) and progeny from the 5 crosses from this study.

Discussion

As could have been expected from the lineages of the three parent varieties the progeny from the crosses show a high degree of similarity. The three ‘out crosses’ yielded a higher mean similarity (57.9, 58.5 and 60.5%) than the bulk of commercial varieties from the EUCC (44.9%), and the means for the two self crosses were even higher (66.4 and 67.7%). Adopting the tail principle for these data with a cut off set at 85% would result in 6 pairs (excluding somaclonal variants) for the EUCC varieties, 28 pairs for the Agria x Fontane cross, 9 pairs for the Aria x Ramos cross and 11 pairs for the Fontane x Ramos cross. However, adopting the same cut off for the selfings would result in 193 pairs for the Fontane selfing and 473 pairs for the Ramos selfing. Increasing the cut off to above 90% would give 3, 2 and 1 pair for the out crosses and 42 and 81 pairs respectively, setting it at above 92% 1 for the out crosses, 15 and 45 for the selfings and above 95% 2 and 8 pairs for the self crosses and none for the out crosses. It would appear on the basis of these figures that the setting of the threshold for EDV is dependant on the type of cross that has been performed.
Future directions would include examining the progeny from crosses of varieties not as closely related and the 3 in this study and to look at the F2 generation progeny of backcrosses. It would also be very interesting to see if there is any correlation between where the individual progeny plants sit within the normal distribution and their morphological and physiological characteristics.

The ESA EDV Appendix Excel file contains additional information including the similarity matrices for the 5 crosses, plate maps of the samples and a list of samples with ‘odd’ results.

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