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Abstract. Molecular markers have proved their efficiency for the identification of duplicate accessions in genetic resources collections. Partners of the GENRES Carrot project decided to evaluate the use of molecular markers for the identification of carrot accession duplicates. As a model analysis, 21 presumed duplicate accessions of 'Jaune du Doubs' were selected. Only accessions that were not distinguished on a morphological basis were subjected to molecular analysis. The crucial question was to determine the threshold required to declare whether accessions were duplicates or not. We used a strategy based on the comparison between intravarietal and intervarietal genetic distances. DNA extractions were made on 4–8 bulks of five individuals per accession, and the bulks were analysed using 75 AFLP markers. An additional set of 7 bulks was extracted from one accession to provide true control replicates. With the exception of the true duplicates, all the accessions were clearly differentiated. Based on these results, a general strategy for the identification of carrot duplicates is proposed.

Introduction

Plant genetic resources collection managers have to develop strategies for characterisation, conservation and use of genetic resources. They need to evaluate the extent and distribution of genetic diversity in species of interest and to determine the proportion of duplicate accessions (redundancy) in their collections. The identification of putative duplicates is becoming a priority for genebank managers, as highlighted by the increasing number of studies on this topic. Indeed, as emphasised by Ford-Lloyd et al. (1997), the identification of redundant accessions allows curators to focus effort on characterisation, evaluation and regeneration of unique genetic material.

A few years ago, the identification of duplicate accessions was done exclusively on the documentation of the accessions and on morphological characters if the information was available. Since the advent of PCR-based techniques, molecular markers have been used successfully for the identification of redundant material. RAPD and AFLP markers have proved their efficiency for this kind of analysis in different species such as rice (Virk et al. 1995; Kumar Verma et al. 1999), wheat (Cao et al. 1998), sweet potato (Zhang et al. 2001)

and Solanum (del Rio and Bamberg 2000; McGregor et al. 2002). Virk et al. (1995) proposed two strategies where putative duplicates were first selected on the basis of the passport data. The first procedure proposed the morphological characterisation followed by molecular characterisation for accessions not distinguished on a morphological basis. In the second procedure, no morphological evaluation was done, but a preliminary molecular analysis was performed with a small number of markers followed, if necessary, by a full molecular study on samples that could not be initially discriminated. Accessions that could not be distinguished after the two analyses were declared as duplicates. Whatever the strategy employed, the crucial question is to determine the threshold required to declare that two accessions are duplicates or not. Some users rely, for this final decision, on a specific number of markers or a combination of informative primers. For example, Virk et al. (1995) within their rice germplasm collection designated accessions as duplicates if no variation was found across 100 RAPD markers. Negash et al. (2002) declared enset clones, belonging to Musaceae family, as duplicates based on the identity of 104 AFLP fragments generated by four primer pairs. However, looking for the identity of such a set of markers within allogamous plants would be unreasonable. Indeed, due to their reproductive biology, residual heterozygosity exists even in inbred lines, such as hybrid parental lines. Curators are generally not in charge of very advanced cultivars, but of open-pollinated varieties, landraces or wild populations. Therefore, the elaboration of a strategy for the identification of duplicates within allogamous species, including an adapted threshold appears to be of great importance. Carrot [*Daucus carota* ssp. *sativus* (Hoffm.) Arcangeli] is an allogamous species. Preliminary studies have shown that a residual heterozygoty exists in cultivars, and depending on the variety, intravarietal dissimilarity values are between 17 and 31% for open-pollinated varieties, around 10% for hybrids and 3–5% for stabilised parental lines (Le Clerc 2001; Le Clerc et al. 2002).

Within the context of the European GENRES (Genetic Resources) Carrot project (GENRES CT-99-105) on the management and availability of carrot genetic resources, the partners decided to evaluate molecular markers as a complementary tool to morphological markers for developing a strategy of identification of carrot duplicate accessions (Astley 2000). Taking into account the putative duplicate accessions maintained in the European genebanks, the group of 'Jaune du Doubs' accessions was selected as a model for this analysis.

Materials and methods

Plant material

'Jaune du Doubs' is an old variety with a yellow conical root approximately 18–20 cm in length, and mainly used as a fodder carrot. On the basis of passport data and information provided by curators, accessions with the same

Table 1. List of the 21 accessions analysed in the present study.

Accession name	Institute code	Accession number	Sample status	Code	Number of bulk analysed
Jaune du Doubs	FRAINH	313	5	JD	4
Jaune obtuse du Doubs	GBRHRIGRU	9053	5	JOD	4
Lopee	GBRHRIGRU	11289	5	L	5
Long red	GBRHRIGRU	10246	3	LR	5
Lobbericher	DEUGAT	K 8172/96	–	LOB	6
Gelbe Wortel	GBRHRIGRU	11146	5	GW	6
Táborská Zluta	DEUGAT	DAU 110/88	–	TZA/TZB	7
White belgian fodder carrot	GBRHRIGRU	8112	5	WB	7
Gelbe Lobbericher	GBRHRIGRU	3922	5	GL	7
Lange gele stoppel	GBRHRIGRU	11286	5	LGS	7
Fance	GBRHRIGRU	10235	3	F	7
Jaune de Lobberidr	FRAINH	312	3	JL	7
Zahedan	GBRHRIGRU	4012	5	Z	8
D. carota subsp sativus	DEUGAT	DAU211/85	–	*	
Santene Skvirskaja	DEUGAT	DAU 74/97	–	*	
Juared	GBRHRIGRU	8140	5	*	
Cape Market	GBRHRIGRU	10301	5	*	
Fiumicino	GBRHRIGRU	6042	5	*	
Lennox	GBRHRIGRU	6527	5	*	
Yellow carrot No. 12	GBRHRIGRU	10524	–	*	
Jaune obtuse du Doubs	FRAINH	77	–	*	

*Not retained for molecular analysis.
Sample status: 3 = traditional cultivar/landrace; 5 = advanced cultivar OP; – = unknown.

name, synonyms and/or yellow rooted forms were selected for the experiment. Twenty-one presumed duplicates of 'Jaune du Doubs' from the European collections were obtained for morphological and molecular analyses. For convenience, accessions were named by a code (Table 1).

Morphological characterisation

In July 2001, two replications of four linear meters per accession were sown in the field with 100 seeds/m. After visual examination of both replications and according to UPOV and IPGRI recommendations, an average score on a 1–9 scale was given to each accession for each morphological character. In late September, three foliar characters (foliage leaf dissection, width crown, anthocyanin in petiole) were scored. In November, 11 root characters such as length, diameter, shoulder shape and pigmentation were scored. All data were compiled with Excel software and registered in the European database (European Carrot/*Daucus* Inventory-ECDI- available at http://www.hri.ac.uk/gru/). As a result, accessions were declared morphologically distinguished if, according to the procedure used by the Plant Variety Rights Office, they presented at least one clearly distinctive character.

Molecular analysis

DNA extraction procedure

Root slices of previously morphologically characterised accessions were freeze-dried and ground to powder in a mortar with liquid nitrogen. Extraction followed the protocol described by Briard et al. (2000a).

To investigate the effect of bulk composition on the amplification of rare bands, some extractions and amplifications were done on bulks and compared to individual profiles. Bulks consisted of five individuals of an accession. For this purpose, root powder of five individuals was pooled in equal proportions before extraction. This was repeated and applied to five different accessions.

For each accession, extraction was realised on 4–8 bulks of five individuals to assess the genetic distance within and between accessions.

An additional set of 7 bulks was extracted for the accession 'Táborská Zluta (DAU 110/88)' in order to provide true replicates, named TZA and TZB.

AFLP and ISSR amplification

AFLP electrophoresis and silver staining procedures were carried out as described by Briard et al. (2000a). Twelve primer combinations were used for the amplification (Table 2). Polyacrylamide gels were dried overnight at room temperature. To test the AFLP reproducibility of each primer and to allow the comparison of AFLP profiles between gels, 4–5 bulks were systematically reamplified at least twice.

ISSR amplifications were realised with three primers (Table 2) chosen because they generated clear profiles with multiple polymorphic bands on

Table 2. Sequence of the three selective nucleotides for the primers Eco/Mse and sequences of the ISSR primers.

Primer	Sequence
P1	AAG/CAG
P2	AGG/CTC
P3	ACT/CAG
P4	ACT/CAA
P5	AAG/CTC
P7	ACA/CTT
P8	AAG/CAA
P9	AAG/CTT
P14	AGC/CAT
P17	AGG/CAT
P20	AGG/CTA
P21	ACT/CAC
ISSR5*	VHVCTCTCTCTCTCTCTCT
ISSR8us	GAACAAACAAACAAACA
ISSR9*	HVHTCTCTCTCTCTCTC

*V = G, A, C; H = A, T, C.

numerous carrot accessions (Le Clerc 2001). DNA amplification reactions were performed in a volume of 12.5 μl containing 2.5 μl of diluted DNA (approximately 1 ng/μl), 2 mM of each dNTP (Eurogentec), 2 mM MgCl2 (Perkin Elmer), 0.5 μM of a single primer, 0.75 unit of AmpliTaq DNA polymerase (Perkin Elmer). Amplifications were realised on a MJ research PTC-100 thermocycler using the following program: 5 min at 94 °C, 40 cycles of (30 s at 94 °C, 45 s at 50 °C, 2 min at 72 °C) with a final 7 min extension at 72 °C. Amplification products were electrophoresed on 2% agarose gels for 5 h at 100 V and detected by staining with ethidium bromide.

Gel scoring and data analysis

Only non-ambiguous and polymorphic bands were scored as present (1) or absent (0). Pair-wise genetic distances between the bulks of one accession and between accessions were calculated according to the coefficient of Sokal and Michener (1958). In addition to double presence, this index also takes into account the double absence of a marker between two individuals as recommended for intraspecific analyses (Briard et al. 2000b). For each accession, the intravarietal distance value (IAD) was obtained from the mean of the distances calculated for each pair of bulks belonging to one accession. The intervarietal distance value (IED) between two accessions was the mean of the distances calculated between all the bulks of one accession and all the bulks of the other accession. IEDm, for one accession, is the mean of its IED values with all the other accessions. A Neighbor joining dendrogram based on similarity coefficients was constructed using the Darwin 4.1 software (Perrier 1998).

To assess the reliability of cultivar identification, the assignment calculator (http://www.biology.ualberta.ca/jbrzusto/alpha/Doh.html) developed by Paetkau et al. (1995) was used. It allows assigning a bulk to the accession in which it has the greatest possibility of occurrence. The likelihood of the bulk's multilocus genotype occurring in each accession were computed. As explained on the web site, a matrix A was obtained. Each data of the matrix is a measure of how much more likely genotypes of bulks sampled in accession X are in accession X rather than in accession Y.

Results

Based on the morphological characterisation of the minimum descriptors established by the European partners, eight accessions were distinguishable and therefore, it was not necessary to make further investigations with molecular markers to declare them as original material. The other 13 accessions were subjected to the molecular analysis.

Efficiency of bulk sample to assess carrot genetic diversity and assignment calculation

For the five accessions analysed, all the major bands found in each individual profile were also amplified in the bulks of five individuals (Figure 1). Some faint bands present in one individual were sometimes absent from the bulk profile, but they were not taken into account. The amplification profiles obtained on five individual plants and on the bulks of five individuals gave exactly the same results in terms of marker analysis. Reproducibility between the two bulks of the same five individuals was absolute whereas larger bulks were not enough reliable and fail sometimes to amplify all the major bands found in each individual profile.

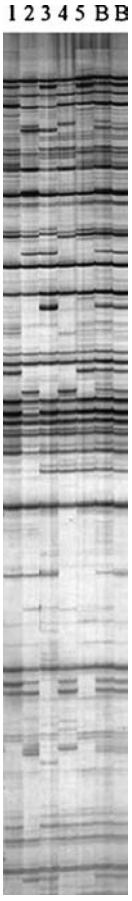

Figure 1. AFLP profiles obtained on five individuals (lanes 1–5) of the accession Gelbe Lobberricher (GL) and two bulks of the same five individuals (lanes B) after silver staining. Primer pair P20 was used for this analysis.

Estimation of the genetic distances within and between accessions

Three AFLP primer pairs (P7, P8 and P17) did not give sufficiently reliable polymorphic markers and were discarded. With nine primer pairs, 75 polymorphic markers were generated. The three ISSR primers revealed a very poor polymorphism (1 or 2 polymorphic markers per primer).

The intravarietal distance values, varied from 10 to 31%, whereas the intervarietal distance values ranged from 12 to 55% (Table 3). For each accession, distance was lower within the accession than between accessions except for TZB – the IED distance between TZA and TZB was 12%, whereas the IAD distance of TZB was 15%.

Mean genetic distances, IEDm, varied from 37% for the accessions GW and WB to 50% for the accession Z.

On the dendrogram (Figure 2), bulks of the accessions TZA and TZB, which were representing true duplicates, clustered in a single group. All the bulks of one accession clustered in one separate group except for five accessions. Accessions JOD, WB and LGS presented one bulk separated from the others, whereas accessions LOB and JL were dispersed, presenting bulks on different clusters.

The likelihood values computed for each accession with the assignment calculator were comprised between 31.01 and −0.82. (Table 4). The accessions TZ and Z presented the highest values whereas the lowest value was observed between JD and LGS (2.99). Finally, TZA bulks have no more chance to belong to TZA than to TZB and reciprocally for TZB.

Table 3. Intravarietal distances (IAD, in grey), intervarietal distances (IED) and mean intervarietal distances (IEDm) calculated according to the Sokal and Michener coefficient.

	F	GL	GW	JD	JL	JOD	L	LGS	LOB	LR	TZA	TZB	WB	Z	IEDm
F	25														43
GL	41	22													39
GW	43	33	20												37
JD	45	35	30	20											39
JL	40	36	36	40	30										40
JOD	45	37	30	30	39	25									38
L	43	44	34	31	42	33	20								40
LGS	43	35	31	28	37	31	33	23							39
LOB	39	39	39	42	34	38	39	39	31						40
LR	36	40	37	39	40	36	34	36	36	19					40
TZA	49	43	45	51	42	48	49	53	46	52	10				45
TZB	50	44	45	52	43	49	50	53	46	52	12	15			46
WB	39	33	30	30	35	32	35	33	37	33	48	49	27		37
Z	43	52	47	55	51	52	52	51	45	48	52	53	51	24	50

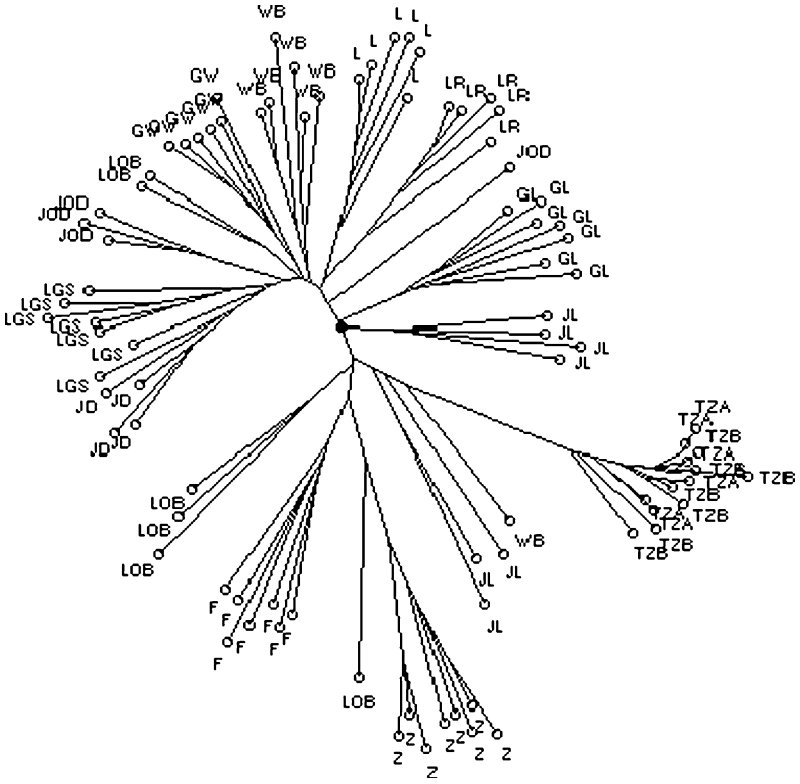

Figure 2. Dendrogram of 13 carrot accessions (described in Table 1) analysed with 75 AFLP markers and based on Neighbor Joining cluster analysis of Sokal and Michener's similarity matrix.

Table 4. Matrix A computed with the assignment calculator.

	LR	GW	WB	L	F	Z	GL	**JD**	**TZA**	**TZB**	LGS	JOD	JL	LOB
LR		12.83	7.10	10.08	10.59	21.54	15.05	13.76	27.30	31.01	10.90	10.11	8.01	12.44
GW	13.38		5.24	9.89	16.55	21.20	9.10	7.35	21.45	24.55	6.74	6.63	10.87	9.01
WB	6.96	4.08		7.85	10.29	20.37	5.96	4.11	22.16	23.46	5.49	4.41	7.21	5.11
L	10.13	9.49	8.18		15.22	23.55	16.13	7.45	24.96	26.70	7.86	7.21	10.68	12.47
F	8.96	15.03	9.95	14.38		14.61	13.44	14.26	21.99	24.14	14.14	13.24	8.41	9.91
Z	20.27	20.17	21.00	22.90	14.86		23.83	23.15	26.95	29.21	23.36	19.61	14.70	19.49
GL	14.16	8.93	6.88	16.78	13.27	24.17		9.36	19.53	22.37	9.10	10.19	10.20	7.87
JD	12.64	6.35	3.43	7.01	16.24	25.87	8.41		25.75	27.73	**2.99**	5.06	11.92	10.12
TZA	25.52	21.17	22.05	24.15	22.36	27.82	18.98	23.86		**−0.82**	26.75	20.73	17.19	15.65
TZB	29.04	23.99	23.73	26.11	25.03	29.98	21.87	26.24	**0.79**		29.93	23.18	20.33	18.08
LGS	10.89	7.17	6.74	8.81	15.81	23.49	9.76	5.02	26.11	30.34		5.28	9.85	8.83
JOD	7.88	3.82	3.17	5.41	13.84	20.03	7.78	4.07	20.32	22.95	3.06		6.20	5.72
JL	5.46	8.16	5.43	7.50	6.84	14.12	7.72	9.28	15.97	18.56	7.89	5.96		5.94
LOB	11.06	7.51	4.63	11.35	9.55	19.47	6.55	9.47	15.27	17.25	7.42	6.22	6.75	

Each value is a measure of how much more likely genotypes of bulks sampled in accession X are in accession X rather than in accession Y.

Discussion

Several molecular techniques have been used successfully to analyse carrot species variability (Grzebelus et al. 1997, 2001; Shim and Jorgensen 2000). According to our previous studies, AFLP and ISSR appear to be the most appropriate techniques for studying carrot. On average, these techniques have generated 9 and 15 polymorphic markers per primer or primer combination, respectively (Le Clerc et al. 2000, 2002; Briard et al. 2001). In the present study, the very low number of markers detected with the ISSR technique and the relatively low number of AFLP markers per combination suggested that the material was genetically closely related.

Efficiency of bulk sample to assess carrot genetic diversity

Usefulness of DNA bulks to evaluate the extent and distribution of genetic diversity in species was already tested (Dubreuil et al. 1999; Gilbert et al. 1999; van Treuren 2001). The main question, when using bulks for such analyses, concerns the representation of those bulks versus individuals and therefore, the optimal bulk size. As highlighted by Gilbert et al. (1999) and van Treuren (2001), some bands present in few individuals of large bulks might not always be amplified in the pool sample leading to a bias in the estimation of genetic similarity. However, as suggested by del Rio and Bamberg (2000), using large bulks is not inevitably a problem, on the contrary, it might be a way to minimise sampling error. Indeed, only significant differences between accessions based on differences in major bands would be taken into account (Bamberg et al. 2001). In the present analysis, we proposed to base the identification of redundant accessions on the comparison of genetic distances within and between accessions. Therefore, the use of large bulks with 20 individuals or more was not justified. Moreover, in a previous study, we tested different sizes of bulk ranging from 5 to 20 individuals (data not published). The bulks of five individuals seemed fully representative of all the individuals constituting the DNA bulks, which was not the case for the bulks with more individuals. The present analysis of the five individual bulks of five accessions confirmed the reliability of this bulk size. All the rare markers, present in only one of the constitutive individuals, were efficiently represented in the bulks.

Working on Lupin, Gilbert et al. (1999) concluded that 2–3 bulks of five genotypes should be enough to assess the genetic distances within and between accessions. For carrot, we showed in a previous study (results to be published) that the optimal number of individuals to analyse per accession depends on the level of genetic homogeneity, ranging from 5 to 35 individuals for, respectively, the identification of inbred lines to heterogeneous populations. Therefore, in the present study, investigating OP varieties (see sample status in Table 1), we decided, when possible, to analyse seven bulks of five individuals in order to reach 35 individuals per accession.

To assess genetic variability among Lupin accessions, Gilbert et al. (1999) used 2–3 pools of five genotypes by pooling DNA, i.e. DNA extractions were done on 10–15 individuals. In our case, plant material was pooled prior to DNA extraction reducing the number of extractions to seven per accession. This method limits cost: we performed a total of 80 DNA extractions and AFLP analyses instead of 400.

Estimation of genetic distances within and between accessions

In previous studies, to clearly assess genetic distances between accessions, we relied on a precise evaluation of genetic distance within accessions (Briard et al. 2001; Le Clerc et al. 2002). The more precise the analysis of each accession, the better the genetic distance estimation between them. Therefore, to determine an adapted threshold for the identification of carrot duplicates, we relied on the comparison between intravarietal and intervarietal genetic distances. We previously showed that 70 AFLP markers were sufficient to clearly identify related carrot accessions (Le Clerc et al. 2002). Therefore, when using 9 AFLP primer combinations, which resulted in 75 markers to score, we reached optimal experimental requirements.

The true duplicates TZA and TZB presented the lowest intravarietal genetic distances with 10 and 15%, respectively and therefore were the most homogeneous samples. Intervarietal genetic distance between them (12%) was slightly lower than the TZB intravarietal genetic distance and, on the dendrogram, the bulks of the two replicates clustered together in one group. With the exception of these true duplicates that could not be distinguished, most of the other accessions were clearly identified presenting intravarietal values higher than intervarietal values. On the dendrogram, those accessions were clearly discriminated without ambiguity, the bulks of one accession being grouped together. Even if the accessions WB and JOD presented one bulk separated from the others, they were clearly differentiated from the other accessions. It could be explained by the possible presence of one off-type root (a root which is not characteristic to the accession's root type) in the isolated bulk. Accessions JL and LOB, which presented the highest intravarietal genetic distances (30 and 31%, respectively), were scattered among different clusters. In this case, we could question ourselves about the opportunity to conserve such heterogeneous accessions, probably originating from a mix of different accessions.

Finally one LGS bulk is on a common branch with one JD bulk. Even if the other bulks of these two accessions are not mixed together and the IAD values are smaller than the IED values, it probably indicates some degree of genetic relationship between the two accessions. It could be interesting to analyse separately the five individuals of the two related bulks in order to determine if all the individuals are related or if it is due to only one plant coming from the other accession. Such an experiment would answer the question, is it a real relationship or pollinic pollution?

To confirm that our relatively simple procedure of comparison between IAD and IED is reliable, we used a statistical test based on assignment calculations. This assignment test was already successfully used in several population studies (Lougheed et al. 2000; Hansen et al. 2001). Kubik et al. (2001) were able to distinguish seven perennial ryegrass cultivars by correctly assigning 210 individuals to their cultivar of origin. This method assumes Hardy–Weinberg equilibrium, no linkage disequilibrium among loci, and depends on the number of loci and genotypes sampled per cultivar. In the present case, accuracy is probably not optimal due to the low number of bulks sampled per cultivar. However, it confirms the fact that no accessions were duplicates except true duplicates and that JD and LGS accessions were probably relatively close to each other.

In conclusion, even with morphologically related material, we were able to declare that the 'Jaune du Doubs' accessions were not duplicates. Therefore, it seems reasonable to think that those root types, which are morphologically but not genetically identical, are probably resulting from a phenotypical convergence created by a selection pressure on the same root characters. Moreover, with this molecular approach, we highlighted the existence of original genetic material such as the accessions TZ and Z, originating from Russia and Persia, respectively. The morphological and the molecular approaches work as complementary tools. According to these results, we are now able to propose a general strategy for the identification of carrot duplicates. After morphological characterisation, if some accessions are still considered as putative duplicates, they should be submitted to molecular analysis as follows. Seven bulks of each accession should be analysed with at least 70 AFLP markers, with accessions declared as duplicates when one of their intravarietal genetic distances is higher than their intervarietal genetic distance. It is important to notice that there is no one absolute threshold because it will depend on the data set analysed.

Despite these convincing results, one could question the validity of a molecular approach, especially when working with small collections. It could seem more reasonable for the curator to stock a few grams of each accession, even if it means keeping all the accessions, rather than starting expensive molecular analysis. This argument could be true for orthodox seeds, whereas it should be reconsidered for recalcitrant seeds, for which conservation is much more expensive. Considerations about costs of regeneration and evaluation should also be taken into account. Indeed, regeneration is expensive, especially for allogamous species, which require a high number of plants under insect-proof cages. An evaluation such as the quantification of a chemical compound by HPLC is also more expensive and tedious than a molecular analysis. Therefore, even if the identification of duplicate accessions via molecular analysis does not lead to reduction in size of germplasm collections (if it is not expensive, duplicates may be kept as safety resources), it could help curators to give priorities for the regeneration or evaluation of unique material.

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