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RAPD and ISSR analysis of some economically important species, varieties, and cultivars of the genus *Allium* (Alliaceae)

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Abstract: RAPD and ISSR markers have been used to reveal the genetic diversity and phylogenetic analysis of some economically important species of *Allium*. Two plants related to *Allium* were also included for better understanding of the phylogeny. ISSR showed more polymorphism within *A. cepa* and *A. sativum*, while RAPD showed more polymorphism within *A. porrum*. Overall, RAPD revealed more intraspecific diversity than ISSR, while ISSR showed more interspecific diversity than RAPD. This showed the usefulness of using both markers for the study of *Allium*. UPGMA-based dendrograms showed a close relationship between *Allium sativum* and *Allium porrum*. *A. porrum* showed less genetic variability than *A. cepa* and *A. sativum*. Several unique bands were identified with RAPD and ISSR, which can be converted into cultivar-specific markers. Principal coordinated analysis, however, showed some minor differences with UPGMA-based dendrograms. This study showed that rapid and cost-effective markers like RAPD and ISSR coupled with appropriate statistical tools can be successfully applied to study phylogenetic relationships at the interspecific and intraspecific level in *Allium*. These markers proved to be suitable for both phylogenetic studies among different species as well as for characterisation of different cultivars of *Allium*.

Key words: Alliaceae, AMOVA, discriminating power, molecular characterisation, polymorphism

1. Introduction

Allium L. is one of the most economically important genera of plants. Onion (*Allium cepa* L.), garlic (*A. sativum* L.), and leek (*A. porrum* L.) are among the major crops of the world. Besides these, there are some minor *Allium* crops like *A. tuberosum* Rottl. and *A. stracheyi* Baker. Many *Allium* species are used as foods, spices, and herbal remedies in widespread areas of the world (Krest et al., 2000). These crops possess a wide range of bioactive compounds, especially volatile sulphur compounds (Krest et al., 2000), which help to reduce blood lipids and cholesterol as well as platelet activity, which ultimately lowers the risk of cardiovascular diseases (Sainani et al., 1976; Kendler 1987; Augusti, 1990).

Allium is a very large genus with about 750 species (Stearn, 1992). Classification of such a large genus proved to be difficult (Hanelt, 1990). Many ambiguities remain in the phylogeny of *Allium* (Fritsch & Friesen, 2002). Additionally, there is great morphological diversity at the intraspecific level in *Allium cepa*, *A. sativum*, and *A. porrum*. These variations should be measured at the molecular level for their proper characterisation, which ultimately will be beneficial for future breeding programmes.

DNA-based molecular markers have been used previously in studies of genetic diversity and phylogenetic analysis in plants (Savolainen & Chase, 2003; Nybom, 2004). Among the different molecular markers, RAPD has been widely used for the assessment of genomic variation and genetic diversity studies in plants like *Lathyrus* L. (Chtourou-Ghorbel et al., 2002), *Digitalis* L. (Nebauer et al., 2000), *Buchloë dactyloides* (Nutt.) Engelm. (Huff et al., 1993), *Phaseolus lunatus* L. (Fofana et al., 1997), *Lens* Mill. (Ferguson et al., 1998), *Azadirachta indica* A.Juss. (Deshwall et al., 2005), *Rhus* L. (Prakash & Staden, 2007), and many others. It has also been used for solving taxonomic problems in the study of sectional, species, and subspecies classification in the genus *Carthamus* L. (Vilatersana et al., 2005), for species-level phylogeny reconstruction in *Leucaena* Benth. (Bailey et al., 2004), for elucidating relationships between subspecies of *Plantago major* L. (Wolff & Morgan-Richards, 1998), for phylogenetic analysis of different species in the section

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Oxytona Bernh. of the genus *Papaver* L. (Parmaksız & Özcan, 2011), and for assessing the relationships among some species of *Cephalaria* Schrad. ex Roem. et Schult (Göktürk et al., 2012).

Inter-simple sequence repeat (ISSR) is a PCR-based method developed by Zietkiewicz et al. (1994). ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species which include fingermillet (Salimath et al., 1995), *Vigna* Savi (Ajibade et al., 2000), sweet potato (Huang & Sun, 2000), and *Plantago major* L. (Wolff & Morgan-Richards, 1998). It has also been used for resolving problems relating to the phylogeny of Asian cultivated rice *Oryza sativa* L. (Joshi et al., 2000), wheat (Nagaoka & Ogihara, 1997), and *Diplotaxis* DC. species (Martin & Sanchez-Yelamo, 2000). Both RAPD and ISSR techniques have been used in several studies, including characterisation of Chilean *Nothofagus* Blume species (Mattioni et al., 2002), studies of genetic variability and cultivar identification in *Olea europaea* L. (Martins-Lopes et al., 2007), evaluation of genomic relationships among some populations of *Podophyllum hexandrum* Royle (Alam et al., 2009), and so on.

Considering the importance of RAPD and ISSR markers for the study of genetic diversity in various genera and species of plants, very little work has been done in different species varieties and cultivars of *Allium* with these markers (Wilkie et al., 1993; Friesen et al., 1997; Hao et al., 2002; Son et al., 2012). Other widely used markers like AFLP (Volk et al., 2004; García-Lampasona et al., 2012) and SSR (Fischer and Bachmann, 1998; Song et al., 2004; Khar et al., 2011; Lee et al., 2011; Cunha et al., 2012) have been used in the study of *Allium*. Initially, we also tried some SSR primers with our investigated species. These included some available SSR primer sequences developed from *Allium cepa* and *A. sativum*. But they gave poor amplification in *A. stracheyi* as well as in *Agapanthus africanus* L. and *Nothoscordum fragrans* Kunth. (2 species of the family Alliaceae), i.e. their cross-species transferability was low for these species. Lee et al. (2011) reported low cross-species transferability of SSR markers across different sections within the genus *Allium*. Additionally, RAPD and ISSR are cheaper and easier to use than AFLP (Hodkinson et al., 2002; Kumar et al., 2012). Therefore, we selected RAPD and ISSR markers. The objectives of our study were to study the genetic diversity within *Allium* species and to clarify the phylogenetic relationships among them with the help of RAPD and ISSR techniques, as well as to compare the applicability of these 2 markers in *Allium*.

2. Materials and methods

2.1. Materials

A total of 21 taxa from 5 species of *Allium* were investigated in this study. These included 3 varieties including 8 cultivars of *Allium cepa* L., 5 cultivars of *Allium sativum* L., 4 cultivars of *Allium porrum* L., and 2 other species of *Allium* (*Allium tuberosum* Rottl. and *Allium stracheyi* Baker). For a better understanding of the phylogenetic relationships among the *Allium* species, 2 species related to *Allium* from the family Alliaceae, viz. *Agapanthus africanus* L. and *Nothoscordum fragrans* Kunth., were included. Names of the *Allium* species, varieties, and cultivars, along with their morphological features, are given in Table 1.

2.2. DNA extraction and PCR amplification

DNA was extracted from fresh or frozen leaves, following the CTAB method of Doyle and Doyle (1987) with minor modifications. Briefly, 1 g of leaf sample was ground with liquid nitrogen and the leaf powder was then immediately transferred to a 50-mL tube containing 10 mL of 2% CTAB buffer, mixed properly, and incubated for 1 h at 65 °C with occasional gentle swirling. After incubation, the mixture was centrifuged for 10 min at 4000 rpm (2000 × *g*) at room temperature, and the supernatant was taken. It was treated with RNase for 30 min at 37 °C. The same volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the solution, mixed thoroughly, centrifuged at 4000 rpm at room temperature, and the supernatant was removed carefully. A doubled amount of isopropanol was added, incubated overnight at –20 °C, and centrifuged at 10,000 rpm at room temperature. The supernatant was discarded and the pellet was washed with 70% ethanol and again centrifuged at room temperature for 10 min at 10,000 rpm, and then dried using a vacuum desiccator. The pellet was resuspended with 50 µL of double-distilled water, allowed to dissolve, and stored at –20 °C for further use.

Primarily, 50 decamer primers obtained from Operon Technologies Inc. (Alameda, California, USA) were tested for RAPD amplification. Amplification reactions were performed based on the standard protocol of Williams et al. (1990) with some modifications. The optimum reaction mix for a 25 µL of reaction mixture contained 2.5 µL of 10X PCR buffer, 0.1 mM dNTP mix, 200 nM primer, 1U Taq Polymerase, 50 ng template DNA, and 3 mM $MgCl_2$. Amplification conditions were an initial denaturation at 94 °C for 5 min, 45 cycles at 94 °C for 1 min, 37 °C for 30 s, 72 °C for 1 min, and 5 min at 72 °C. Amplifications were performed in a PerkinElmer GeneAmp 2400 PCR system. The amplification products were separated by agarose gel electrophoresis in 1.4% gel along with molecular weight markers (mix of *Hind*III digested Lambda DNA and *Hae*III digested ϕX174 DNA). The gel was visualised under UV light following ethidium bromide staining.

ISSR analysis was performed using primers (Set No. 9) obtained from the University of British Columbia. These primers include di-, tri-, and tetranucleotide repeat motifs. PCR reactions were performed following the protocol of Zietkiewicz et al. (1994). PCR amplifications were

Table 1. List of the investigated taxa of the present study along with their morphological features.

Taxa	Cultivar	Morphological features
Allium cepa var. *cepa*	Punjab selection	Bulb 5–6 cm wide, globular, skin red.
	Pusa white round	Bulb 4–6 cm wide, flattish round, skin white.
	Agrifound light red	Bulb 4–6 cm, globular, skin light red.
	Sukh sagar	Long bulb, 4–7 cm wide, tapering towards the neck, skin dark red.
	Pusa red	Bulb 4–6 cm, flat to globular, skin bronze red.
	Puna red	Bulb 3–5 cm wide, round, skin dark red.
	Patna red	Bulb 4–6 cm, globular, skin pinkish red.
	Spring onion	Non-bulbous, leaf sheathes form a long, white, stemlike structure at the base, clustered.
Allium cepa var. *aggregatum*	-	Small cluster of 3–4 bulbs, bulbs 1–1.5 cm wide, skin red.
A. cepa var. *viviparum*	-	Bulb tunicate, small, 2–3 cm wide, pale red.
Allium sativum	Single clove	Single small bulb; 3–3.5 cm in diameter, highly pungent.
	Yamuna Safed	Bulbs compact, creamy white, bulb diameter 3.5–4.0 cm; cloves sickle shaped, 25–30 cloves per bulb, moderately pungent.
	Agrifound Parvati	Larger bulb, 5.0–6.0 cm in diameter; cloves 1.5– 1.8 cm, brownish white, 10–16 cloves per bulb, moderately pungent.
	Bote lasun small	Bulbs small, 3.0–3.5 cm in diameter; 10–13 cloves per bulb, sometimes with aerial bulblets, highly pungent.
	Bote lasun large	Bulb large, 5.0–6.0 cm in diameter, with aerial bulblets on a long, hard stalk developed from the centre of the bulb, highly pungent.
Allium porrum	Armor	Pseudostem diameter 2.3–2.7 cm; pseudostem length 13–15 cm; leaf diameter 2.8–3.1 cm.
	Alto	Pseudostem diameter 2.8–3.1 cm; pseudostem length 20–21 cm; leaf diameter 2.7–3.1 cm.
	Selecta	Pseudostem diameter 1.8–2.2 cm; pseudostem length 17–19 cm; leaf diameter 2.1–2.2 cm.
	Maridor	Pseudostem diameter 2.6–2.8 cm; pseudostem length 10–12 cm; leaf diameter 2.2–2.4 cm.
Allium tuberosum	-	Perennial rhizomatous herb; with tubers, leaves flat, 5 cm wide, typically bend outwards at the tip, flowers white.
Allium stracheyi	-	Perennial slender herb; leaves 3–12; narrowly linear; flowers light yellow.

performed in a volume of 25 µL containing 2.5 µL of 10X PCR buffer (Bangalore Genei, Bangalore, India), 2 mM $MgCl_2$, and 50 ng template DNA. Amplification reactions were carried out in a PerkinElmer Thermal Cycler 2400. Amplification conditions were as follows: an initial denaturation at 94 °C for 5 min followed by 40 amplification cycles each at 94 °C for 1 min, 45 s at annealing temperature, at 72 °C for 1 min, followed by a final extension for 5 min at 72 °C. Annealing temperature was calculated according to the Wallace rule (Thein & Wallace, 1986). The amplification products were separated by agarose gel electrophoresis in 1.4% gel along with molecular weight markers (mix of *Hind*III digested Lambda DNA and *Hae*III digested ϕX174 DNA). The gel was visualised under UV light following ethidium bromide staining.

2.3. Data analysis

To assess the genetic diversity within *Allium*, banding patterns were studied. Each taxon was represented by a specific banding pattern. To compare the efficiency (i.e. efficiency in distinguishing one taxon from another) of each assay unit (i.e. primer), the following statistical calculations were performed according to Tessier et al. (1999): a) number of banding patterns (T_p); b) frequency of each banding pattern (p_i); c) confusion probability (C_j); d) discriminating power (D_j); e) limit of D_j as N tends towards infinity (D_L); and f) effective number of patterns per assay unit (E). Band informativeness was calculated according to Prevost and Wilkinson (1999). Resolving power (R_p) of the primers was also calculated following Prakash and Staden (2007) on the basis of band informativeness.

To compare the efficiency of RAPD and ISSR, the following statistical calculations were performed according to Belaj et al. (2003): a) number of polymorphic bands (n_p); b) number of monomorphic bands (n_{np}); c) average number of polymorphic bands/assay unit (n_p/U); d) number of loci (L); e) number of loci/assay unit (n_u); f) average number of patterns/assay units (I); g) average confusion probability (C); h) average discriminating power (D); i) average limit of discriminating power (D_L); j) effective number of patterns per assay unit (P); k) expected heterozygosity of the polymorphic loci (H_{ep}); l) fraction of polymorphic loci (β); m) arithmetic mean heterozygosity (H_e); n) effective multiplex ratio (E); o) marker index (MI). The product of PCR amplification obtained with one set of primer was considered as one assay unit (U).

AMOVA was performed to analyse the genetic diversity of *Allium* using the software Arlequin ver. 3.11 (Excoffier et al., 2005) to estimate the variance components of RAPD and ISSR profiles.

For phylogenetic analysis, each amplified band was treated as a unit character regardless of its intensity and scored in terms of a binary code, based on presence (1) and absence (0) of bands. Only clear and reproducible bands were considered for scoring. For phylogenetic analysis, all the members of *Allium* along with the relative members were included. To analyse data obtained from the binary matrices, the NTSYS-pc version 2.1 statistical package (Rohlf, 2000) was used. Three datasets were used, viz. RAPD, ISSR, and combined datasets of RAPD and ISSR. The statistical method took into account the presence or absence of each band as differential features. The binary qualitative data matrices were then used to construct similarity matrices based on Jaccard similarity coefficients (Jaccard, 1908). The similarity matrices were then used to construct dendrograms using unweighted pair group method with arithmetic average (UPGMA). Bootstrapping was done using the software program 'Winboot' (Yap & Nelson, 1996). To compare RAPD- and ISSR-based dendrograms, cophenetic matrices were derived from the dendrograms using the COPH (cophenetic values) program, and the goodness-of-fit of the clustering to the 2 data matrices was calculated by comparing the original similarity matrices with the cophenetic value matrices using the Mantel matrix correspondence test (Mantel, 1967) in the MXCOMP program. Principal co-ordinate analysis (PCOORDA) was performed based on the similarity coefficients. Lastly, a combined dataset was prepared using both RAPD and ISSR data and used to calculate the combined similarity matrix, which was ultimately used to construct the phylogenetic tree and principal co-ordinate analysis. The combined phylogenetic tree was compared with RAPD- and ISSR-based trees using the Mantel matrix correspondence test.

3. Results

3.1. RAPD and ISSR amplification patterns and banding profiles

Initially, 50 RAPD and 25 ISSR primers were examined, of which 12 and 11 primers respectively were finally selected as they gave reproducible amplification patterns and scorable bands all across the investigated taxa. Details of the RAPD and ISSR profiles are given in Table 2. RAPD produced a total of 408 bands (34.00 bands per primer) ranging from 200 bp (primer OPAA 17) to 6800 bp (primer OPH 1). ISSR produced a total of 276 bands (25.09 bands per primer) ranging from 197 bp (primer UBC 811) to 2952 bp (primer UBC 857). The highest resolving power was shown by OPA 19 (14.285) among RAPD primers and UBC 810 (8.285) among ISSR primers. RAPD and ISSR profiles are represented in Figure 1a and Figure 1b, respectively.

3.2. Putative cultivar specific bands

Thirty-three and 11 unique bands were identified with RAPD and ISSR respectively, which can be converted into cultivar-specific markers following further studies based on the present observations (Table 3). All of the RAPD primers produced at least one cultivar-specific band, while only 7 ISSR primers produced such bands. However, numbers of cultivar-specific bands were not consistent with the total number of bands generated by one primer. For example, in RAPD, OPC6 and OPG2 produced 4 unique bands but produced 32 and 52 total bands, while OPA19 produced 53 total bands but 5 unique bands. In ISSR, UBC807 produced 4 unique bands with 28 total bands, but UBC810 produced only 1 unique band with 30 total bands. All of these bands were generated by different cultivars of *A. cepa* and *A. sativum*. *A. porrum* did not produce any unique bands. The highest numbers of unique bands were generated in the cultivar 'Bote lasun small' (14 for RAPD and 5 for ISSR). The sufficient number of cultivar-specific bands signifies the power of RAPD and ISSR markers in fingerprinting and diversity analysis within *Allium*.

3.3. Phylogenetic relationships

Dendrograms obtained using the UPGMA method based on RAPD, ISSR, and RAPD + ISSR data (Figure 2) clearly distinguished all the species. Two main clusters were formed in all 3 dendrograms. The first cluster was formed by the grouping of all of the *Allium* species. Within this subcluster, *A. sativum* and *A. porrum* grouped together with high bootstrap value (97.2% for RAPD, 99.3% for ISSR, and 100% for the combined dendrogram). Within the subcluster formed by *A. sativum*, 'Bote lasun small' was distinct from the other 4 cultivars. All the cultivars of *A. cepa* var. *cepa* (common onions) were grouped together, along with *A. cepa* var. *aggregatum* G.Don. Cultivar 'Spring onion' grouped with the combined subcluster of

Table 2. Description of the RAPD and ISSR fragments generated in all surveyed species of *Allium*. N_P is the number of polymorphic bands; N_T is the number of total bands; P_P is the percentage of polymorphism; R_P is the resolving power; D is the discrimination power; and D_L is the limit of D.

Marker type	Primer name	Size range of bands (bp)	N_T	N_P	P_P	R_P	D	D_L
RAPD	OPA 19	407–1030	53	53	100	14.285	0.966	0.920
	OPA 20	500–2050	31	31	100	7.238	0.966	0.920
	OPB 1	230–900	32	32	100	9.904	0.985	0.938
	OPC 5	607–909	26	26	100	6.380	0.938	0.893
	OPC 6	400–1530	32	32	100	8.952	0.957	0.911
	OPG 2	360–2300	52	52	100	13.809	0.947	0.902
	OPG 13	390–1400	17	17	100	6.380	0.933	0.888
	OPG 19	450–1630	32	32	100	13.238	0.842	0.802
	OPH 1	549–6800	31	31	100	10.952	0.919	0.875
	OPK 10	327–1780	19	19	100	6.7619	0.919	0.875
	OPN 18	259–2497	33	33	100	8.666	0.933	0.888
	OPAA 17	200–910	50	50	100	12.666	0.985	0.934
ISSR	UBC 807	415–1185	28	28	100	7.333	0.895	0.852
	UBC 808	313–892	32	32	100	6.190	0.980	0.934
	UBC 809	415–1287	19	19	100	5.047	0.895	0.852
	UBC 810	315–1375	30	30	100	8.285	0.947	0.902
	UBC 811	197–1007	43	43	100	7.714	0.966	0.920
	UBC 816	417–918	25	25	100	6.00	0.923	0.879
	UBC 825	385–883	13	13	100	4.476	0.852	0.811
	UBC 835	462–1375	23	23	100	7.523	0.933	0.888
	UBC 841	417–952	11	10	90.9	4.00	0.833	0.793
	UBC 857	408–2952	23	23	100	4.571	0.938	0.875
	UBC 861	437–1610	29	29	100	5.904	0.990	0.943

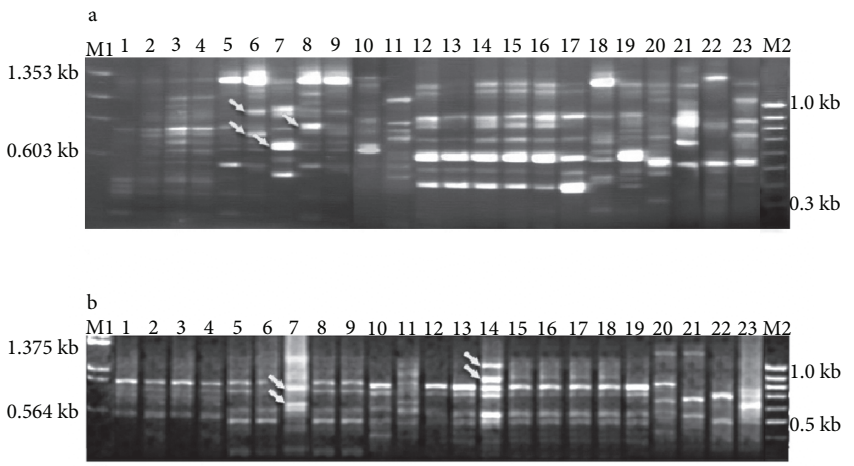

Figure 1. RAPD and ISSR patterns of the materials. **a**- RAPD profile obtained with primer OPG-2. M1: λDNA *Hind* III digest and ΦX 174 DNA *Hae* III digest marker; M2: 100 bp DNA ladder. Lane 1–4: *Allium porrum* (1: Armor, 2: Alto, 3: Selecta, 4: Maridor); 5–9: *Allium sativum* (5: Single clove, 6: Yamuna safed, 7: Bote lasun small, 8: Bote lasun large, 9: Agrifound parvati); 10: *Allium cepa* var. *viviparum*; 11: Spring onion; 12: *Allium cepa* var. *aggregatum*; 13–19: *Allium cepa* var. *cepa* (13: Pusa white round, 14: Puna red, 15: Patna red, 16: Punjab selection, 17: Agrifound light red, 18: Pusa red, 19: Sukh sagar); 20: *Allium stracheyi*; 21: *Allium tuberosum*; 22: *Agapanthus africanus*; 23: *Nothoscordum fragrans*. Arrowheads indicate unique bands. **b**- ISSR profile obtained with primer UBC-807. M1: λ DNA *Eco*RI / *Hind*III double digest marker; M2: 100 bp DNA ladder. Sequence of materials is the same as in the RAPD profile. Arrowheads indicate unique bands.

Table 3. Putative cultivar-specific markers in some *Allium* cultivars; molecular weights of the bands are indicated as suffix of the corresponding primer name.

Taxa	Cultivar	Unique bands	
		RAPD	ISSR
A. sativum	Single clove	$OPAA17_{1155}$, $OPC6_{1181}$, $OPH1_{1959}$,	----------
	Yamuna safed	$OPG2_{955}$ and $OPG2_{752}$	----------
	Bote lasun small	$OPA19_{825}$, $OPA19_{761}$, $OPA19_{711}$, $OPA20_{864}$, $OPAA17_{987}$, $OPAA17_{639}$, $OPC6_{1314,}$ $OPG2_{653}$, $OPG13_{1304}$, $OPG13_{640}$, $OPG19_{892}$, $OPH1_{910}$, $OPH1_{680}$, $OPK10_{450}$	UBC-807_{759}, UBC-807_{610}, UBC-810_{523}, UBC-811_{520}, UBC-811_{410}
	Bote lasun large	$OPG2_{820}$	----------
	Pusa white round	$OPC5_{713}$	----------
	Patna red	$OPA19_{570}$	----------
	Punjab selection	$OPA20_{655}$	UBC-857_{589}
A. cepa var. *cepa*	Agrifound light red	$OPB1_{639}$, $OPC5_{726}$	----------
	Pusa red	$OPA20_{934}$, $OPK10_{882}$, $OPK10_{482}$, $OPN18_{413}$	UBC-816_{678}, UBC-861_{680}
	Puna red	----------	UBC-807_{985}, UBC-807_{826}
	Sukh sagar	$OPA19_{562}$, $OPC5_{528}$, $OPC6_{377}$, $OPC6_{315}$	UBC-808_{559}

A. cepa var. *cepa*, and *A. cepa* var. *aggregatum*. *A. cepa* var. *viviparum* joined this combined clade. *A. stracheyi* and *A. tuberosum* was grouped together. *A. cepa*, *A. tuberosum,* and *A. stracheyi* were grouped together. The second subcluster was formed by *Agapanthus africanus and Nothoscordum fragrans*. However, there were some differences in these dendrograms. For example, in RAPD-based and combined dendrograms, cultivar 'Armor' was the most distant among the 4 cultivars of *Allium porrum* (Figure 2), while in the ISSR-based dendrogram (Figure 2), 'Maridor' was the most distant. RAPD-based and combined dendrograms could not separate *A. cepa* var. *aggregatum* from the cultivars of *A. cepa* var. *cepa*. In the RAPD-based dendrogram, *A. cepa* var. *aggregatum* shares the same clade with 2 cultivars, namely 'Pusa white round' and 'Puna red' of *A. cepa* var. *cepa*. In the combined dendrogram, however, *A. cepa* var. *aggregatum* was joined with the combined clade of 6 cultivars. On the other hand, the ISSR-based dendrogram could clearly separate *A. cepa* var. *aggregatum* from all the cultivars of *A. cepa* var. *cepa*. Additionally, 2 cultivars, namely 'Agrifound light red' and 'Pusa red', grouped together in this dendrogram. This is significant because they are geographically closer (as these are from the same state, Maharashtra) than other cultivars. In RAPD-based and combined dendrograms, 'Pusa red' maintains a separate clade that joins with the combined clade with other cultivars. However, in the ISSR-based dendrogram, cultivar 'Sukh sagar' grouped with the combined clade of 'Agrifound light red' and 'Pusa red'. In the RAPD-based and combined dendrograms, it could maintain a separate clade within *A. cepa* var. *cepa*. This is significant because this cultivar is morphologically quite different from the others.

Three-dimensional plots obtained with principal co-ordinate analysis for RAPD-, ISSR-, and RAPD + ISSR-based matrices (Figure 3) showed consistency with dendrograms with some minor deviations. The first 3 most informative PC components explained 50.09%, 41.13%, and 46.43% of the total variation, respectively, for RAPD-, ISSR-, and RAPD + ISSR-based plots. The distinctiveness of the 3 major *Allium* crops, i.e. *A. cepa*, *A. sativum*, and *A. porrum*, was highly resolved as they were distinctly separated from each other in all 3 plots. The distinctiveness of the cultivar 'Spring onion' within *A. cepa* var. *cepa* was evident in all 3 plots. However, there are some differences in the positioning of some taxa in the 3 plots. For example, within *A. sativum*, the plot of RAPD showed slightly different placement of 'Bote lasun small' (Figure 3), while the plot of ISSR showed slightly different placement of 'Single clove' (Figure 3). The combined plot, however, did not show any such distinction of cultivars (Figure 3).

3.4. AMOVA

Partitioning of the total molecular variance was analysed among species and within species. In RAPD analysis, of the total diversity, 36.23% was attributable to within-species diversity ($P < 0.0001$), whereas 63.77% was attributable to among-species diversity (Table 4). In ISSR analysis, of total diversity, 45.14% was attributable to within-species diversity ($P < 0.0001$), whereas 54.86% was attributable to among-species diversity. This showed that RAPD showed

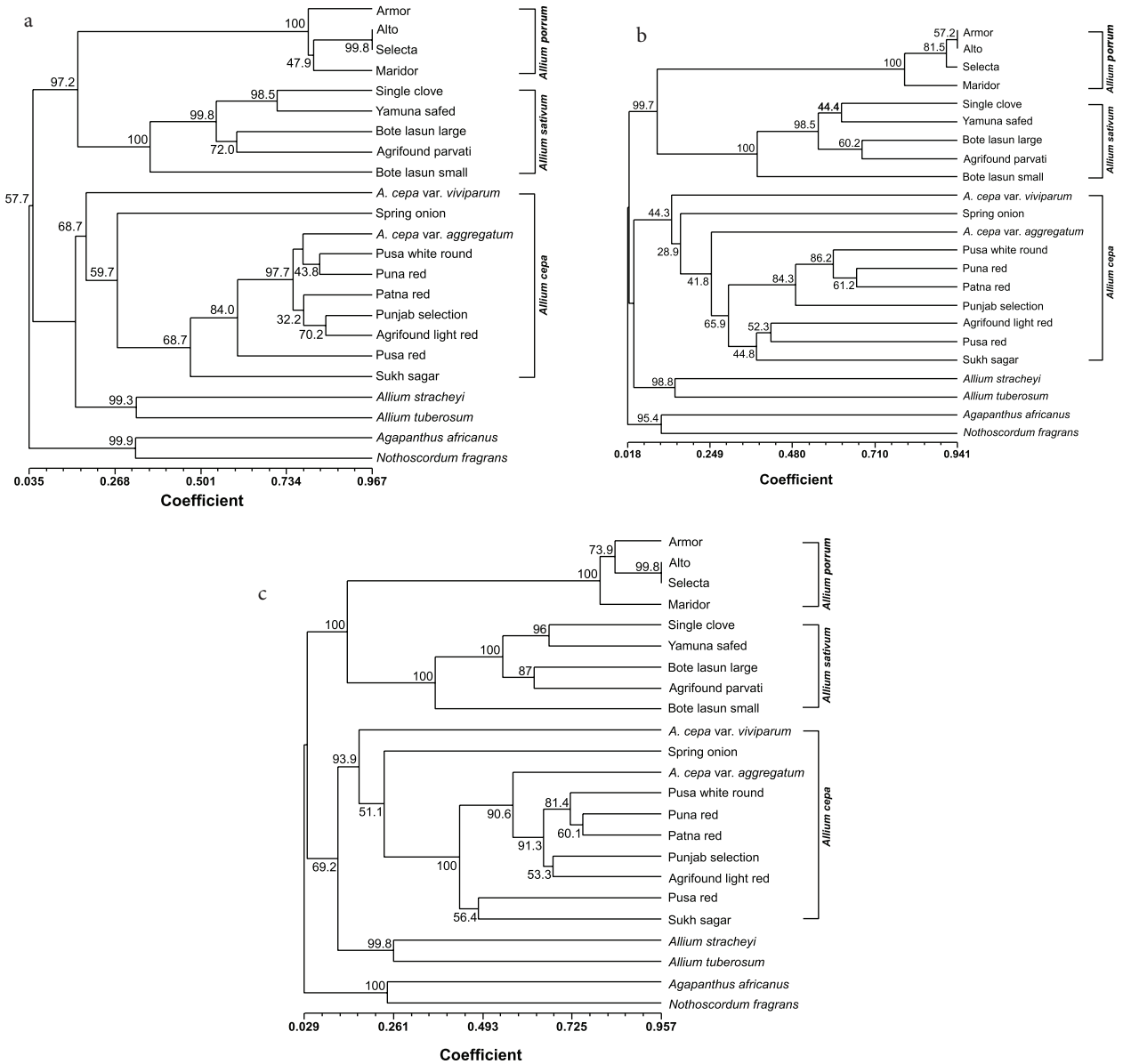

Figure 2. Phylogenetic tree of 23 taxa on the basis of Jaccard similarity coefficient. a- RAPD; b- ISSR; and c- combined dendrograms. The numbers at the fork indicate bootstrap values in percentage for the grouping of those taxa, which are to the right of the fork.

more diversity among species than ISSR, while ISSR was able to differentiate different species better than RAPD.

3.5. Comparative study of RAPD- and ISSR-based results

Three aspects of the performance of these 2 marker systems were considered: a- discriminating capacity, i.e. the efficiency of discrimination between any 2 taxa at random from the investigated taxa; b- overall efficiency of detecting polymorphisms in all the investigated taxa; c- the ability of different assays to infer genetic similarities and relationships between taxa.

A. Discriminating capacity

Both RAPD and ISSR markers proved to be highly effective in discriminating the taxa under the genus *Allium* (Table 5). The total number of polymorphic bands ranged from 365 in RAPD to 189 in ISSR. All (100%) of bands in RAPD were polymorphic at the genus level, whereas 99.47% of bands in ISSR were polymorphic. The number of polymorphic bands per assay unit was higher in RAPD (30.41) as compared to ISSR (17.18). Total number of banding patterns was also higher in RAPD (177) than ISSR (148). However, the number of banding patterns per assay unit (14.75 for RAPD and 13.45 for ISSR) was not significantly different ($t = 0.8397$; $P < 0.001$) when obtained by standard 2-sample t-test. The average

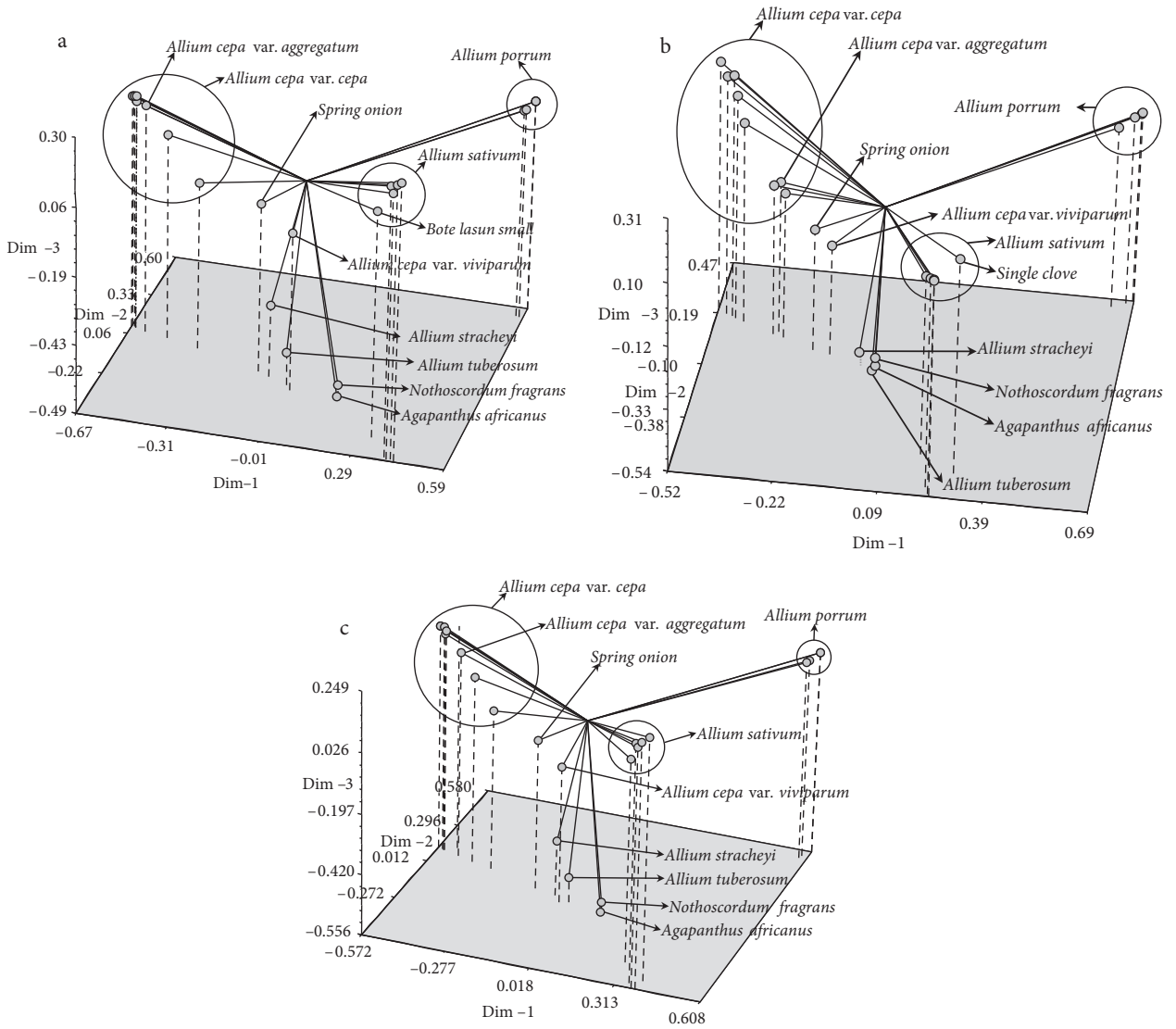

Figure 3. Three-dimensional plots obtained from principle coordinate analysis (PCOORDA) of the 23 taxa. a: RAPD, b: ISSR, and c: RAPD + ISSR.

discriminating capacity (D) was 0.941 for RAPD and 0.923 for ISSR. This parameter represents the probability that 2 randomly selected OTUs have different banding patterns. The discriminating capacities of these 2 markers were also not significantly different (t = 0.7469; P < 0.001) when obtained by standard 2-sample t-test. Average limit of discriminating power (D_L) values for both the markers were very close to the actual discriminating power (D) of both (0.896 for RAPD and 0.877 for ISSR). However, the effective number of patterns is higher in RAPD (10.56) than ISSR (9.52). The effective number of patterns indicates an ideal sample size in which all of the operational taxonomic units (OTUs) can be distinguished, given the frequencies of the patterns obtained with a marker system. This means that with any single RAPD primer almost 11 patterns can be obtained when the OTU size tends to infinity; i.e. up to 11 OTUs can be distinguished with the same primer. On the other hand, with any single ISSR primer, almost 10 OTUs can be discriminated.

B. Overall efficiency of detecting polymorphisms

Two parameters, expected heterozygosity (H_e) of each marker class and marker index (MI), were estimated to compare the performance and utility of these 2 marker systems in estimating polymorphisms in *Allium* (Table 6). Arithmetic mean heterozygosity (H_e) for ISSR (0.970) was slightly higher than for RAPD (0.939) within the genus *Allium* (Table 6). The high values of H_e indicate the extremely high polymorphism within the genus. However, intraspecific polymorphism within *A. porrum*, *A. sativum*, and *A. cepa* varied considerably, which was evident by

Table 4. The AMOVA table.

Marker type	Source of variation	d.f. (degree of freedom)	Sum of squares	Variance components	Percentage of variation	Fixation index F_{ST}
RAPD	Among species	4	574.048	34.86829	63.77	0.63767
	Within species	16	327.000	19.81250	36.23	
	Total	20	891.048	54.68079	100.00	
ISSR	Among species	4	306.026	17.50566	54.86	0.54862
	Within species	16	230.450	14.40312	45.14	
	Total	20	536.476	31.90879	100.00	

their corresponding H_e values. Within *A. porrum*, H_e value was slightly higher in RAPD (0.199) than in ISSR (0.158). On the other hand, within *A. sativum*, H_e value was slightly higher in ISSR (0.648) than in RAPD (0.640). Within *A. cepa*, H_e value was higher for ISSR (0.886) than for RAPD (0.722). The cultivars of *A. cepa* var. *cepa* showed much higher H_e value for ISSR (0.772) than for RAPD (0.468). This showed that ISSR was more polymorphic in *A. sativum* and *A. cepa* while RAPD was more polymorphic for *A. porrum*. The utility of a given marker system is a balance between the level of polymorphism detected, i.e. information content, and the extent to which an assay can identify multiply polymorphisms. A convenient estimate of marker utility may therefore be calculated by the product of information content, as measured by arithmetic mean heterozygosity (H_e), and 'effective multiplex ratio' (E). The MI value for RAPD (28.58) was higher than that of ISSR (16.76). The higher value of the 'effective multiplex ratio' in RAPD was due to a much higher number of loci per assay unit (30.416) than ISSR (17.272).

C. Genetic similarity and relationships

The correlation between RAPD- and ISSR-based similarity matrices was very high (r = 0.90435), suggesting a very high correlation between the RAPD- and ISSR-based dendrograms. It was also found that the combined dendrogram was better correlated with the RAPD-based dendrogram (r = 0.99060) than with the ISSR-based dendrogram (r = 0.95398). All 3 dendrograms showed similar groupings, though with some differences in the positioning of some of the taxa, as discussed above.

4. Discussion

4.1. PCR amplification patterns of RAPD and ISSR

RAPD and ISSR markers have been used in many studies for DNA fingerprinting and phylogenetic analysis (Divaret et al., 1999; Kumar et al., 2001; Martins-Lopes et al., 2007; Ngezahayo et al., 2007; Maslova, 2008; Mohanty et al., 2010). Nagaoka and Ogihara (1997) found that ISSR primers produce more reliable and reproducible bands than RAPD primers. In the present study, however, it was found that once the PCR conditions were well set up, high reproducibility for both RAPD and ISSR markers was

Table 5. Comparison of the discriminating capacity of RAPD and ISSR in the genus *Allium*.

Index type	Marker system	
	RAPD	ISSR
Number of assay units (U)	12	11
Number of polymorphic bands (n_p)	365	189
Number of monomorphic bands (n_{np})	0	1
Average number of polymorphic bands/assay unit (n_p/U)	30.41	17.18
Number of loci (L)	365	190
Number of loci/assay unit (n_u)	30.416	17.272
Number of banding patterns (T_p)	177	148
Average number of patterns/assay units (I)	14.75	13.45
Average confusion probability (C)	0.058	0.076
Average discriminating power (D)	0.941	0.923
Average limit of discriminating power (D_L)	0.896	0.877
Effective number of patterns/assay units (P)	10.56	9.52

Table 6. Comparison of the efficiency to detect polymorphism of RAPD and ISSR in the genus *Allium*.

Index type	Marker system	
	RAPD	ISSR
Expected heterozygosity of the polymorphic loci (H_{ep})	0.939	0.974
Fraction of polymorphic loci (ß)	1.00	0.996
Arithmetic mean heterozygosity (H_e)	0.939	0.970
Effective multiplex ratio (E)	30.41	17.20
Marker index (MI)	28.58	16.76

observed. Similar results were found by Mattioni et al. (2002) with Chilean *Nothofagus* species. In spite of the fact that *Allium* possesses a very large genome (Wilkie et al., 1993), our study showed that this technique is as readily applicable to species with large genomes like *Allium* as to species with smaller genomes like tomato (Klein-Lankhorst et al., 1991; Martin et al., 1991) and *Arabidopsis thaliana* (L.) Heynh. (Reiter et al., 1992), showing that the complexity of RAPD profiles is independent of the genome size.

While RAPD markers cover the whole genome for amplification, ISSR amplifies the region between 2 microsatellites. Hence, the polymorphisms reflect the diversity of these regions of the genome. The primers used in the present investigation were anchored at their 3' end to ensure that the annealing of the primer occurred only at the 3' end of the microsatellite motif, thus eliminating the possibility of internal priming and smear formation. The anchor also allowed only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR products expected from the priming of the dinucleotide inter-repeat regions (Zietkiewicz et al., 1994). The finding of the present study that the most polymorphic and reproducible pictures have been obtained with poly (AG) or poly (GA) microsatellites, irrespective of the anchors at the 3' end, suggests that these are the most frequent simple sequence repeats in *Allium* genome. However, poly (GT) or poly (CT) microsatellites have not given good profiles. This might be due to the fact that the distribution of these repeats in the *Allium* genome was beyond the range of amplification by *Taq* DNA polymerase. However, this is not likely the situation for poly (AT) repeats, as these are thought to be the most abundant motifs in plant species (Morgante & Olivieri 1993; Depeiges et al., 1995). However, in the present investigation, poly (AT) or poly (TA) repeats gave improper amplifications. Similar results were found in rice (Blair et al., 1999), chestnut (Casasoli et al., 2001), grapevine (Moreno et al., 1998), wheat (Nagaoka & Ogihara, 1997), and *Nothofagus* (Mattioni et al., 2002). A possible explanation is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity, and will form dimers during PCR amplification (Blair et al., 1999).

4.2. Phylogenetic relationships in *Allium*

Ajibade et al. (2000) and Galvan et al. (2003) concluded that ISSR would be a better tool than RAPD for phylogenetic studies. The present study, however, has demonstrated that both RAPD and ISSR technique along with proper statistical tools could be successfully applied to assess the genetic diversity and phylogenetic analysis in *Allium*. Although RAPD and ISSR markers showed considerable differences in detecting polymorphism and discriminating capacity, they showed similar topology in dendrograms generated on the basis of similarity matrices. A highly significant correlation between these 2 dendrograms suggested that both of these markers were equally efficient for assessing phylogenetic relationships among the investigated taxa. The combined dendrogram also showed significant correlation with RAPD- and ISSR-based similarity matrices.

Grouping of *A. sativum* and *A. porrum* in all 3 dendrograms with high bootstrap values (97.2% for RAPD, 99.7% for ISSR, and 100% for the combined dendrogram) signifies a very close relationship between these 2 species. This was also supported by several common bands that were present in all cultivars of both species. All these facts support the intraspecific classification of Hanelt et al. (1992), who placed both species into the section Allium under the genus *Allium*.

The cultivars of *A. sativum* showed high morphological diversity. In all 3 dendrograms, 'Bote lasun small' formed a separate clade from other cultivars in all 3 dendrograms with 100% bootstrap values. This cultivar also has a unique bulb morphology, having aerial bulblets just above the cloves on the central axis. This cultivar also produced several unique bands. However, in ISSR-based PCOORDA, the cultivar 'Single clove' showed slightly different placement. This cultivar also has a different morphology, having only one clove and being more pungent. This showed that PCOORDA should be used along with the dendrogram in phylogeny for better understanding of relationships within taxa.

Although *A. porrum* is a seed-propagated crop, the cultivars of this species showed much less genetic diversity than *A. sativum* and *A. cepa*. This proves the narrow genetic make-up of this species. However, further study with more varieties and accessions of this species should be performed for better understanding of the intraspecific genetic diversity of this species.

ISSR revealed better intraspecific genetic diversity than RAPD in grouping *A. cepa*. RAPD could not separate *A. cepa* var. *aggregatum* (shallot) from *A. cepa* var. *cepa*. Wilkie et al. (1993) also got similar results with RAPD analysis where shallot grouped with other onion cultivars. The ISSR-based dendrogram, however, clearly separated *A. cepa* var. *aggregatum* from *A. cepa* var. *cepa* (although with a low bootstrap value of 65.9%). This also shows that these 2 varieties are not very diverse genetically. ISSR also revealed closer relationships within the cultivars of *A. cepa* var. *cepa* than RAPD. 'Agrifound light red' and 'Pusa red' are 2 morphologically similar cultivars. In the RAPD-based dendrogram, they were grouped in separate subclusters. ISSR, on the other hand, grouped them together. 'Spring onion' maintained a separate clade within the subcluster formed by *A. cepa* in all 3 dendrograms, showing its distinctness from other *A. cepa* var. *cepa* cultivars. Morphologically, 'Spring onion' is distinct from other onions; it is non-bulbous throughout its life cycle and also less pungent than onion. *A. cepa* var. *viviparum* maintained a separate clade in all 3 dendrograms although it shares the same subcluster with other *A. cepa* varieties. Havey (1991) suggested that one of the parents of *A. cepa* var. *viviparum* is *A. cepa* as evidenced by the restriction sites in the 45s nuclear rDNA. The present RAPD- and ISSR-based clustering of *A. cepa* var. *viviparum* and other *A. cepa* varieties support these findings. In all 3 dendrograms, *A. tuberosum* and *A. stracheyi* formed a clade with very high bootstrap values (98.9% for RAPD, 98.8% for ISSR, and 99.8% for the combined dendrogram), and this clade grouped with *A. cepa*. According to Hanelt et al. (1992), *A. cepa* and *A. tuberosum* belong to the same subgenus, Rhizirideum, under the genus *Allium*. The present investigation supports this view. However, no sectional or subgeneric positions have been found for *A. stracheyi*. High confidence level for the grouping of this species with *A. tuberosum* indicates the closeness of this species with *A. tuberosum*. Further analysis of this species using other datasets may help to find the proper position of *A. stracheyi* within *Allium*. The grouping of *Nothoscordum fragrans* and *Agapanthus africanus* supports the view of Dahlgren et al. (1985) that these 2 species belong to the family Alliaceae.

4.3. Comparison of RAPD and ISSR techniques

Wilkie et al. (1993) reported that at the interspecific level in *Allium* a considerable amount of polymorphism was revealed with RAPD, although polymorphism at the intraspecific level was low in *A. cepa*. In this study, both RAPD and ISSR primers revealed 77.23% and 91.66% polymorphism respectively within the cultivars of common onion (*A. cepa* var. *cepa*). This result suggests that although modern onion cultivars are intensely selected by breeders, ISSR rather than RAPD revealed higher genetic diversity within them. The lower level of polymorphism revealed with RAPD seems to be associated with the low variability of the whole genome of *A. cepa* var. *cepa*. The low variability of the whole genome in *Allium* was also supported by Bennett et al. (2000). These facts were reflected in the RAPD analysis, which targets the whole genome. Repetitive sequences, on the other hand, have been shown to be clustered together and are associated with other specific repetitive sequences (Lu et al., 1996). Delsney et al. (1988) postulated that as repetitive sequences do not undergo the same evolutionary processes as the rest of the genome, their rate of evolution is faster than single copy sequences. As ISSR covers the repetitive regions of the genome, it could reveal higher genetic diversity within *A. cepa* var. *cepa*.

ISSR also revealed a higher level of polymorphism (75.67%) than RAPD (61.68%) within *A. sativum*. Morphologically, cultivars of *A. sativum* were different from each other. Roy (1978) differentiated some *A. sativum* populations on the basis of Giemsa banding pattern of chromosomes. Talukder and Sen (1999) showed that the different cultivars of *A. sativum* vary considerably with respect to C value. They also opined that heavy heterochromatin content plays a very important role in producing genetic diversity in *A. sativum*. As heterochromatic regions are mainly composed of repetitive DNA, garlic cultivars might show higher polymorphism with ISSR than with RAPD. Polymorphism within *A. porrum* was comparable with both RAPD and ISSR (28.78% and 25.71%, respectively). Both of these values were very low compared to those of *A. cepa* and *A. sativum*. This might be due to the fact that only commercial varieties were included in the present study. Modern breeding processes may have narrowed the genetic make-up of this species.

The mean F_{ST} values obtained with AMOVA analysis were 0.63767 for RAPD and 0.54862 for ISSR. The difference between these 2 values was significant at $P < 0.05$ level (simple pairwise t-test). According to Wright (1978), F_{ST} exceeding 0.15 would indicate high genetic variability. Thus, the present study showed that RAPD revealed higher genetic variability than ISSR in *Allium*. This may be due to the fact that a higher number of bands per primer were produced by RAPD (30.41) than by ISSR (17.27). While the F_{ST} value of ISSR was lower than that of

RAPD, the 'expected heterozygosity' (H_e) value was slightly higher (although not significantly different) in ISSR than in RAPD. Expected heterozygosity is a good measure of detecting polymorphism (Powell et al., 1996). However, as RAPD markers generated more bands, its discriminating capacity was also significantly higher than that of ISSR. The higher value of 'effective number of patterns per assay units' (P) for RAPD compared to ISSR gave evidence of the higher discriminating capacity of the former. The marker index (MI) is a product of 'expected heterozygosity' and 'effective multiplex ratio' (E). As E depends on the number of loci per assay unit, which was much higher in RAPD than in ISSR, marker index was also higher in RAPD than in ISSR.

In conclusion, the present study demonstrated that rapid and cost-effective markers like RAPD and ISSR coupled with proper statistical tools could be successfully applied to studying phylogenetic relationships at the interspecific and intraspecific level in *Allium*. The relationship between *Allium* and related plant species also supports the traditional taxonomic treatments of these species. Further analysis together with data from other methods can be used to make a more accurate reconstruction of the genus *Allium*. Additionally, the large number of unique bands obtained in the present study signifies the power of RAPD and ISSR markers in fingerprinting and diversity analysis, especially at the lower taxonomic level. However, owing to the large number of accessions available for each of these species and varieties of *Allium*, it is important to screen the specificity of these bands over a large sample size before affirming their diagnostic capacity.

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References

Ajibade SR, Weeden NF & Chite SM (2000). Inter-simple sequence repeat analysis of genetic relationships in the genus *Vigna*. *Euphytica* 111: 47–55.

Alam MA, Naik K & Mishra GP (2009). Congruence of RAPD and ISSR markers for evaluation of genomic relationship among 28 populations of *Podophyllum hexandrum* Royle from Himachal Pradesh, India. *Turkish Journal of Botany* 33: 1–12.

Augusti KT (1990). Therapeutic and medicinal values of onions and garlic. In: Rabinowitch HD & Brewster JL (eds.) *Onions and Allied Crops,* vol. 3, pp. 94–108. Boca Raton: CRC Press.

Bailey CD, Hughes CE & Harris SA (2004). Using RAPDs to identify DNA sequence loci for species level phylogeny reconstruction: an example from Leucaena (Fabaceae). *Systematic Botany* 29: 4–14.

Belaj A, Satovic Z, Cipriani G, Baldoni L, Testolin R, Rallo L & Trujillo I (2003). Comparative study of the discriminating capacity of RAPD, AFLP, and SSR markers and of their effectiveness in establishing genetic relationships in olive. *Theoretical and Applied Genetics* 107: 736–744.

Bennett MD, Johnston S, Hodnett GL & Price HJ (2000). *Allium cepa* L. cultivars from four continents compared by flow cytometry show nuclear DNA constancy. *Annals of Botany* 85: 351–357.

Blair MW, Panaud O & McCouch SR (1999). Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* 98: 780–792.

Casasoli M, Mattioni C, Cherubini M & Villani F (2001). Genetic linkage map of European chestnut (*Castanea sativa* Mill.) based on RAPD, ISSR, and isozyme markers. *Theoretical and Applied Genetics* 102: 1190–1199.

Chtourou-Ghorbel N, Lauga B, Brahim NB, Combes D & Marrakchi M (2002). Genetic variation analysis in the genus *Lathyrus* using RAPD markers. *Genetic Resources and Crop Evolution* 49: 363–370.

Cunha CP, Hoogerheide ESS, Zucchi MI, Monteiro M & Pinheiro JB (2012). New microsatellite markers for garlic, *Allium Sativum* (Alliaceae). *American Journal of Botany* 99: e17–e19.

Dahlgren RMT, Clifford HT & Yeo PF (1985). *The Families of the Monocotyledons: Structure, Evolution and Taxonomy.* Berlin: Springer.

Delsney M, Grellet F, Tremousaygue D, Raynal M & Panabières F (1988). Structure, évolution, et expression de l'ADN nucléaire. *Bulletin de la Société Botanique de France* 135: 23–38.

Depeiges A, Goubely C, Lenoir A, Cocherel S, Picard G, Raynal M, Grellet F & Delseny M (1995). Identification of the most represented motifs in *Arabidopsis thaliana* microsatellite loci. *Theoretical and Applied Genetics* 91: 160–168.

Deshwall RPS, Singh R, Malik K & Randhawa GJ (2005). Assessment of genetic diversity and genetic relationships among 29 populations of *Azadirachta indica* A. Juss. using RAPD markers. *Genetic Resources and Crop Evolution* 52: 285–292.

Divaret I, Margale E & Thomas G (1999). RAPD markers on seed bulks efficiently assess the genetic diversity of a *Brassica oleracea* L. collection. *Theoretical and Applied Genetics* 98: 1029–1035.

Doyle JJ & Doyle JL (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissues. *Phytochemical Bulletin* 19: 11–15.

Excoffier L, Laval G & Schneider S (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.

Ferguson ME, Ford-Lloyd BV, Robertson LD, Maxted N & Newbury HJ (1998). Mapping the geographical distribution of genetic variation in the genus *Lens* for the enhanced conservation of plant genetic diversity. *Molecular Ecology* 7: 1743–1755.

Fischer D & Bachmann K (1998). Microsatellite enrichment in organisms with large genomes (*Allium cepa* L.). *BioTechniques* 24: 796–800.

Fofana B, Vekemans X, du Jardin P & Baudoin JP (1997). Genetic diversity in lima bean (*Phaseolus lunatus* L.) as revealed by RAPD markers. *Euphytica* 95: 157–165.

Friesen N, Fritsch R & Bachmann K (1997). Hybrid origin of some ornamentals of *Allium* subgenus *Melanocrommyum* verified with GISH and RAPD. *Theoretical and Applied Genetics* 95: 1229–1238.

Fritsch RM & Friesen N (2002). Evolution, domestication, and taxonomy. In: Rabinovitch HD, Currah L (eds.) *Allium Crop Science: Recent Advances*, pp. 5–27. Wallingford, UK: CAB International.

Galvan MZ, Bornet B, Balatti PA & Branchard M (2003). Inter-simple sequence repeat (ISSR) marker as a tool for the assessment of both genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris* L.). *Euphytica* 132: 297–301.

García-Lampasona S, Asprelli P & Burba JL (2012). Genetic analysis of a garlic (*Allium sativum* L.) germplasm collection from Argentina. *Scientia Horticulturae* 138: 183–189.

Göktürk RS, Sümbül H, Çelebi A & Açık L (2012). Two new species of *Cephalaria* (Caprifoliaceae) from Turkey. *Turkish Journal of Botany* 36: 311–321.

Hanelt P (1990). Taxonomy, evolution, and history. In: Rabinowitch HD, Brewster JL (eds.) *Onions and Allied Crops, Vol 1. Botany, Physiology, and Genetics*, pp. 1–26. Boca Raton, Florida, USA: CRC Press Inc.

Hanelt P, Schultze-Motel J, Fritsch R, Kruse J, Maass HI, Ohle H & Pistrick K (1992). Infrageneric grouping of *Allium* – the Gatersleben approach. In: Hanelt P, Hammer K, Knüpffer H (eds.) *The Genus Allium: Taxonomic Problems and Genetic Resources*, pp. 107–123. Gatersleben, Germany: Institut für Pflanzengenetik und Kulturpflanzenforschung.

Hao G, Lee D-H, Lee JS & Lee NS (2002). A study of taxonomical relationships among species of Korean *Allium* sect. *Sacculiferum* (Alliaceae) and related species using inter-simple sequence repeat (ISSR) markers. *Botanical Bulletin of Academia Sinica* 43: 63–68.

Havey MJ (1991). Molecular characterization of the interspecific origin of viviparous onion. *Journal of Heredity* 82: 501–503.

Hodkinson TR, Chase MW & Renvoize SA (2002). Characterization of a genetic resource collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. *Annals of Botany* 89: 627–636.

Huang J & Sun SM (2000). Genetic diversity and relationships of sweet potato and its wild relatives in *Ipomoea* series *Batatas* (Convolvulaceae) as revealed by inter-simple sequence repeat (ISSR) and restriction analysis of chloroplast DNA. *Theoretical and Applied Genetics* 100: 1050–1060.

Huff DR, Peakall R & Smouse PE (1993). RAPD variation within and among natural populations of outcrossing buffalo grass [*Buchloë dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics* 86: 927–934.

Jaccard P (1908). Nouvelles recherches sur la distribution florale. *Bulletin de la Société vaudoise des sciences naturelles* 44: 223–270.

Joshi SP, Gupta VS, Aggarwal RK, Ranjekar PK & Brar DS (2000). Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theoretical and Applied Genetics* 100: 1311–1320.

Kendler BS (1987). Garlic (*Allium sativum*) and onion (*Allium cepa*): a review of their relationship to cardiovascular disease. *Preventive Medicine* 16: 670–685.

Khar A, Lawande KE & Negi KS (2011). Microsatellite marker based analysis of genetic diversity in short day tropical Indian onion and cross amplification in related *Allium* spp. *Genetic Resources and Crop Evolution* 58: 741–752.

Klein-Lankhorst RM, Vermunt A, Weide R, Liharska T & Zabel P (1991). Isolation of molecular markers for tomato (*Lycopersicon esculentum*) using random amplified polymorphic DNA (RAPD). *Theoretical and Applied Genetics* 83: 108–114.

Krest I, Glodek J & Keusgen M (2000). Cysteine sulfoxides and alliinase activity of some *Allium* species. *Journal of Agricultural and Food Chemistry* 48: 3753–3760.

Kumar LS, Sawant AS, Gupta VS & Ranjekar PK (2001). Comparative analysis of genetic diversity among Indian populations of *Scirpophaga incertulas* by ISSR-PCR and RAPD-PCR. *Biochemical Genetics* 39: 297–309.

Kumar V, Srivastava N, Singh A, Vyas MK, Gupta S, Katudia K, Vaidya K, Chaudhary S, Ghosh A & Chikara SK (2012). Genetic diversity and identification of variety-specific AFLP markers in fenugreek (*Trigonella foenum-graecum*). *African Journal of Biotechnology* 11: 4323–4329.

Lee G-A, Kwon S-J, Park Y-J, Lee M-C, Kim H-H, Lee J-S, Lee S-Y, Gwag J-G, Kim C-K & Ma K-H (2011). Cross-amplification of SSR markers developed from *Allium sativum* to other *Allium* species. *Scientia Horticulturae* 128: 401–407.

Mantel N (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.

Martin GB, Williams JGK & Tanksley SD (1991). Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. *Proceedings of the National Academy of Sciences of the United States of America* 88: 2336–2340.

Martin JP & Sanchez-Yelamo MD (2000). Genetic relationships among species of the genus *Diplotaxis* (Brassicaceae) using inter-simple sequence repeat markers. *Theoretical and Applied Genetics* 101: 1234–1241.

Martins-Lopes P, Lima-Brito J, Gomes S, Meirinhos J, Santos L & Guedes-Pinto H (2007). RAPD and ISSR molecular markers in *Olea europaea* L.: genetic variability and molecular cultivar identification. *Genetic Resources and Crop Evolution* 54: 117–128.

Maslova EV (2008). Differentiation of two hemp nettle species (*Galeopsis bifida* Boenn. and *G. tetrahit* L.) inferred from morphological characters and DNA markers. *Russian Journal of Genetics* 44: 312–319.

Mattioni C, Casasoli M, Gonzalez M & Ipinza R (2002). Comparison of ISSR and RAPD markers to characterize three Chilean *Nothofagus* species. *Theoretical and Applied Genetics* 104: 1064–1070.

Mohanty S, Das AB, Ghosh N, Panda BB & Smith DW (2010). Genetic diversity of 28 wild species of fodder legume *Cassia* using RAPD, ISSR, and SSR markers: a novel breeding strategy. *Journal of Biotech Research* 2: 44–55.

Moreno S, Martin JP & Oritz JM (1998). Inter-simple sequence repeat PCR for characterization of closely related grapevine germplasm. *Euphytica* 101: 117–125.

Morgante M & Olivieri AM (1993). PCR-amplified microsatellites as markers in plant genetics. *Plant Journal* 3: 175–182.

Nagaoka T & Ogihara Y (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theoretical and Applied Genetics* 94: 597–602.

Nebauer SG, del Castillo-Agudo L & Segura J (2000). An assessment of genetic relationships within the genus *Digitalis* based on PCR-generated RAPD markers. *Theoretical and Applied Genetics* 100: 1209–1216.

Ngezahayo F, Dong Y & Liu B (2007). Somaclonal variation at the nucleotide sequence level in rice (*Oryza sativa* L.) as revealed by RAPD and ISSR markers, and by pairwise sequence analysis. *Journal of Applied Genetics* 48: 329–336.

Nybom H (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology* 13: 1143–1155.

Parmaksız İ & Özcan S (2011). Morphological, chemical, and molecular analyses of Turkish *Papaver* accessions (Sect. *Oxytona*). *Turkish Journal of Botany* 35: 1–16.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S & Rafalsky A (1996). The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* 2: 225–238.

Prakash S & Staden JV (2007). Assessment of genetic relationships between *Rhus* L. species using RAPD markers. *Genetic Resources and Crop Evolution* 54: 7–11.

Prevost A & Wilkinson MJ (1999). A new system for comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theoretical and Applied Genetics* 98: 107–112.

Reiter RS, Williams JGK, Feldmann KA, Rafalski JA, Tingey SV & Scolnik PA (1992). Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and RAPDs. *Proceedings of the National Academy of Sciences of the United States of America* 89: 1477–1481.

Rohlf FJ (2000). NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System. Ver. 2.1. Setauket, New York, USA: Exeter Publishing Ltd.

Roy SC (1978). Polymorphism in Giemsa banding patterns in *Allium sativum*. *Cytologia* 43: 97–100.

Sainani GS, Deasi DB & More KN (1976). Onion, garlic, and atherosclerosis. *Lancet* 2: 575–576.

Salimath SS, de Oliveira AC, Godwin ID & Bennetzen JL (1995). Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. *Genome* 38: 757–763.

Savolainen V & Chase MW (2003). A decade of progress in plant molecular phylogenetics. *Trends in Genetics* 19: 717–724.

Son J-H, Park K-C, Lee S-I, Kim J-H & Kim N-S (2012). Species relationships among *Allium* species by ISSR analysis. *Horticulture, Environment, and Biotechnology* 53: 256–262.

Song Y-S, Suwabe K, Wako T, Ohara T, Nunome T & Kojima A (2004). Development of microsatellite markers in bunching onion (*Allium fistulosum* L.). *Breeding Science* 54: 361–365.

Stearn WT (1992). How many species of *Allium* are known? *Kew Magazine* 9: 180–182.

Talukder K & Sen S (1999). In situ cytophotometric estimation of nuclear DNA in different cultivars of *Allium* species. *Cytobios* 99: 57–65.

Tessier C, David J, This P, Boursiquot JM & Charrier A (1999). Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. *Theoretical and Applied Genetics* 98: 171–177.

Thein SI & Wallace RR (1986). The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Davies KE (ed.) *Human Genetic Diseases: a Practical Approach,* pp. 33–50. Oxford: IRL.

Vilatersana R, Garnatje T, Susanna A & Garcia-Jacas N (2005). Taxonomic problems in *Carthamus* (Asteraceae): RAPD markers and sectional classification. *Botanical Journal of the Linnean Society* 147: 375–383.

Volk GM, Henk AD, Richards CM (2004). Genetic diversity among U.S. garlic clones as detected using AFLP methods. *Journal of the American Society for Horticultural Science* 129: 559–569.

Wilkie SE, Isaac PG & Slater RJ (1993). Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. *Theoretical and Applied Genetics* 86: 497–504.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531–6535.

Wolff K & Morgan-Richards M (1998). PCR markers distinguish *Plantago major* subspecies. *Theoretical and Applied Genetics* 96: 282–286.

Wright S (1978). *Evolution and the Genetics of Populations, Vol. 4: Variability Within and Among Natural Populations.* Chicago: University of Chicago Press.

Yap IV & Nelson RJ (1996). Winboot: a program to perform bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. Manila: International Rice Research Institute.

Zietkiewicz E, Rafalski A & Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176–183.