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ORIGINAL ARTICLE

Genetic Diversity in Various Accessions of Pineapple [*Ananas comosus* (L.) Merr.] Using ISSR and SSR Markers

Jian-sheng Wang[2] · Jun-hu He[1] · Hua-rui Chen[1] · Ye-yuan Chen[1] · Fei Qiao[1]

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Abstract Inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers were used to assess the genetic diversity of 36 pineapple accessions that were introduced from 10 countries/regions. Thirteen ISSR primers amplified 96 bands, of which 91 (93.65%) were polymorphic, whereas 20 SSR primers amplified 73 bands, of which 70 (96.50%) were polymorphic. Nei's gene diversity ($h = 0.28$), Shannon's information index ($I = 0.43$), and polymorphism information content (PIC = 0.29) generated using the SSR primers were higher than that with ISSR primers ($h = 0.23$, $I = 0.37$, PIC = 0.24), thereby suggesting that the SSR system is more efficient than the ISSR system in assessing genetic diversity in various pineapple accessions. Mean genetic similarities were 0.74, 0.61, and 0.69, as determined using ISSR, SSR, and combined ISSR/SSR, respectively. These results suggest that the genetic diversity among pineapple accessions is very high. We clustered the 36 pineapple accessions into three or five groups on the basis of the phylogenetic trees constructed based on the results of ISSR, SSR, and combined ISSR/SSR analyses using the unweighted pair-group with arithmetic averaging (UPGMA) method. The results of principal components analysis (PCA) also supported the UPGMA clustering. These results will be useful not only for the scientific conservation and management of pineapple germplasm but also for the improvement of the current pineapple breeding strategies.

Keywords Pineapple · Genetic diversity · SSR markers · ISSR markers

Jian-sheng Wang and Jun-hu He contributed equally to this work and they are co-first authors.

✉ Jun-hu He
hejunhu123@163.com

[1] Tropical Crops Genetic Resources Institute of Chinese Academy of Tropical Agricultural Science, Danzhou 571737, Hainan Province, China

[2] Pingdingshan University, Pingdingshan 467000, Henan Province, China

Introduction

One important aspect of horticulture is the cultivation of plants for food, fiber, biofuel, medicine, and other products that are used to sustain and enhance human life. Horticulture plays a key role in sedentary human civilization, whereby farming of domesticated species created food surpluses that nurtured the development of civilization (Mishra et al. 2015; Nemli et al. 2015; Ipek et al. 2016; Tsou et al. 2016).

Pineapple [*Ananas comosus* (L.) Merr.], an important commercial fruit crop, is cultivated both in the tropical and subtropical regions of the world. It was domesticated long before its first discovery (Morrison 1973) and its historical origin is South America (Smith and Downs 1979; Leal 1990). The excellent nutritional and bioactive properties of pineapple have played a significant role in its recent increase in global consumption. Moreover, its global production in 2014 was estimated to be 2.54×10^7 tons (FAOSTAT 2014). Pineapple ranks as the third major tropical fruit following citrus and banana. In addition to its edible value, pineapple has also been explored in manufacturing paper (Marques et al. 2007), proteolytic enzymes (Maurer 2001), and secondary metabolites (Manetti et al. 2009). The original shape and colorful hues of pineapple fruits are also of ornamental value (Souza et al. 2012).

Pineapple cultivars are often divided into different groups according to differences in their leaf and fruit characteristics. To date, several main cultivar groups have been documented, which include Cayenne, Spanish, Queen, Maipure, Abacaki, Mordilona, and Pernambuco (Samuels 1970; DeWald et al. 1988; Duval and d'Eeckenbrugge 1993; Noyer et al. 1997). These cultivar groups have distinct morphological traits, although there may be inconsistencies in the classification of some cultivars as the environment may influence certain external features.

The limitations of phenotype-based genetic markers have led to the development of DNA-based markers. Molecular markers are independent of environmental, pleiotropic, and epistatic effects, and thus provide new tools to support cultivar identification. Although different DNA-based marker techniques such as RFLP, RAPD, and AFLP have been applied to assess the genetic diversity and relationships among pineapple genotypes (Duval et al. 2001; Ruas et al. 2001; Kato et al. 2004; Boczkowska et al. 2012), the relationships among pineapple accessions have not been fully elucidated. Microsatellites are regions of short, tandemly repeated DNA sequences of 1–6 base pairs in length that are ubiquitous in eukaryotic genomes.

Two different marker strategies have been used based on microsatellites, namely, simple sequence repeats (SSRs) and inter simple sequence repeats (ISSRs). SSRs have advantages over various other markers because these are highly abundant and polymorphic, co-dominantly inherited, analytically simple, and readily transferable (Weber 1990) and have been widely utilized in plant genomic studies (He et al. 2003; Goulão and Oliveira. 2001; Baranski et al. 2012; Viruel et al. 2005; Zhang et al. 2012). Although a few SSR primers have been developed for pineapple, its application to the assessment of genetic diversity in this particular species has not been reported.

ISSRs (Zietkiewicz et al. 1994) are a different microsatellite-based method that circumvents the need for prior knowledge of the genome, cloning, or primer design. The abundance, multi-allelic behavior, high polymorphism, dominant inheritance, and excellent reproducibility of ISSR markers make these ideal for genetic diversity studies and marker-assisted selection in plant breeding. Despite their numerous advantages, ISSR markers have not yet been exploited in genetic diversity studies in pineapple. Moreover, no reports on the estimation of genetic diversity pineapple cultivars using multiple markers have been published, and no studies involving both dominance and co-dominance markers on the same pineapple cultivar has been simultaneously conducted.

Thus, in the present study, we employed ISSR and SSR molecular markers for the first time to assess the genetic diversity of 36 pineapple accessions from different countries. Because applying only one type of molecular marker may underestimate polymorphism and genetic diversity levels, we used a combined set of SSR and ISSR markers to cover the pineapple genome. Moreover, we assessed the levels of polymorphism that were detected by the two different methods to discuss the efficiency of ISSR and SSR markers in pineapple diversity analysis.

Materials and Methods

Plant Materials

Thirty-six pineapple accessions were analyzed in the present study (Table 1). These accessions were collected from major countries/regions that produce pineapple, which include Australia, Brazil, Costa Rica, Guangdong of China, Hainan of China, Indonesia, Japan, Mauritius, Taiwan of China, and Thailand. Sample plants were collected from the Tropical Crops Genetic Resources Institute of the Chinese Academy of Tropical Agricultural Science (CATAS, Hainan province, Danzhou, China).

DNA Extraction

Total genomic DNA was extracted from pineapple leaves using the SDS method (Dellaporta et al. 1983) with minor modifications. Briefly, approximately 0.2 g of leaf tissue was ground to a fine powder in liquid nitrogen and then transferred to a 2.5-mL microcentrifuge tube. A 800-μL volume of extraction buffer [100 mM Tris–HCl (pH 8.5), 50 mM EDTA (pH 8.0), 500 mM NaCl, 20% (w/v) SDS] was immediately added, and the mixture was incubated at 65 °C for 1.0–1.5 h, with one time shaking at intervals of 20–25 min. Then, the mixture was centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant (~600 μL) was gently collected and then transferred to a new microcentrifuge tube, to which an equal volume of chloroform: isoamyl alcohol (24:1) was added. The tubes were mixed thoroughly by gentle inversion and were centrifuged at 10,000 rpm for 10 min at 4 °C. Subsequently, the supernatant was transferred to the new microcentrifuge tube with the proper volume of isopropanol and incubated for 1 h at 4 °C. After

Table 1 Pineapple accessions used in this study

Accession number	Name	Source	Accession number	Name	Source
A1	Creampine	Taiwan Island	A19	OK-2	Japan
A2	Xuli	Taiwan Island	A20	Early ripening perfume	China Hainan
A3	Tainong 19	Taiwan Island	A21	Perola	Brazil
A4	Bogoul	Japan	A22	Spininess pineapple	Indonesia
A5	Perfume	Taiwan Island	A23	Tainong 17	Taiwan Island
A6	Sugarloaf	Costa Rica	A24	Australian pineapple	Australia
A7	MD2	Costa Rica	A25	PZS-2	China Hainan
A8	Spineless pineapple	Indonesia	A26	Victoria	Mauritius
A9	Honey bright	Japan	A27	Comte de paris	Indonesia
A10	PZS-1	China Hainan	A28	Xuwen	China Guangdong
A11	Tainong 4	Taiwan Island	A29	Golden pineapple	Taiwan Island
A12	N67-10	Japan	A30	Tainong 7	Taiwan Island
A13	Phuket	Thailand	A31	Mibao	Taiwan Island
A14	Soft touch	Japan	A32	No 8	Brazil
A15	Tainong 18	Taiwan Island	A33	Tainong 14	Taiwan Island
A16	Cacaine	China Hainan	A34	Tainong 16	Taiwan Island
A17	Red Spanish	Indonesia	A35	Gold diamond	Taiwan Island
A18	Red skin pineapple	Taiwan Island	A36	Pearl	Taiwan Island

centrifugation, the precipitated DNA was washed thrice with 70% precooled ethanol, air-dried, and then resuspended in TE [10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The DNA samples were stored at −20 °C until PCR analysis.

ISSR Analysis

Primer sequences used in the amplification of the ISSR markers (Table 2) were as reported elsewhere (Goulão and Oliveira 2001; Grativol et al. 2011; Hammami et al. 2014). These primers was synthesized by Shanghai Life Technology Co., Ltd. (China). A total of 31 ISSR primers were initially screened, of which 13 primers generating good and clear amplified bands were used in the analysis (Table 2). The PCR reactions were performed in a total volume of 20 μL containing 25 ng of genomic DNA template, 10× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U

Table 2 Primer sequences used for ISSR analysis in this study

	ISSR primer	Sequence (5′–3′)	Polymorphism/non-polymorphism
1	ISSR1	CACACACACACACACAGT	Polymorphism
2	ISSR2	GAGAGAGAGAGAGAGACG	Polymorphism
3	ISSR3	GAGAGAGAGAGAGAGA(CT)C(AG)	Polymorphism
4	ISSR4	CACACACACACACACATG	Polymorphism
5	ISSR5	CACACACACACACACAG	Polymorphism
6	ISSR6	GAGAGAGAGAGAGAGAGT	Polymorphism
7	ISSR7	AGAGAGAGAGAGAGAG(CT)T	Polymorphism
8	ISSR8	CTCTCTCTCTCTCTCTG	Non-polymorphism
9	ISSR9	GAGAGAGAGAGAGAGAC	Polymorphism
10	ISSR10	GCACACACACACACAC	Polymorphism
11	ISSR11	ATATATATATATATATG	Non-polymorphism
12	ISSR12	AGAGAGAGAGAGAGAGT	Non-polymorphism
13	ISSR13	AGAGAGAGAGAGAGAGC	Non-polymorphism
14	ISSR14	AGAGAGAGAGAGAGAGG	Non-polymorphism
15	ISSR15	GTGTGTGTGTGTGTGTA	Non-polymorphism
16	ISSR16	GTGTGTGTGTGTGTGTC	Polymorphism
17	ISSR17	GTGTGTGTGTGTGTGTT	Non-polymorphism
18	ISSR18	ACCACCACCACCACCACC	Polymorphism
19	ISSR19	AGCAGCAGCAGCAGCAGC	Non-polymorphism
20	ISSR20	AGTAGTAGTAGTAGTAGT	Non-polymorphism
21	ISSR21	ATGATGATGATGATGATG	Polymorphism
22	ISSR22	GAAGAAGAAGAAGAAGAA	Non-polymorphism
23	ISSR23	GATAGATAGATAGATA	Non-polymorphism
24	ISSR24	GACAGACAGACAGACA	Polymorphism
25	ISSR25	TGCATGCATGCATGCA	Non-polymorphism
26	ISSR26	GGATGGATGGATGGAT	Non-polymorphism
27	ISSR27	TGTGTGTGTGTGTGTGA	Non-polymorphism
28	ISSR28	ACACACACACACACACT	Non-polymorphism
29	ISSR29	ACACACACACACACACC	Non-polymorphism
30	ISSR30	TGTGTGTGTGTGTGTGAGC	Non-polymorphism
31	ISSR31	CACCACCACCACCACAT	Non-polymorphism

Taq DNA polymerase, and 0.8 mM of the ISSR primers. ISSR amplification was performed in an Eppendorf Mastercycler Gradient PCR (Eppendorf, Germany) under the following conditions: an initial pre-denaturation step at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30, 45 s annealing at 50–58 °C (depending on primer used), and extension at 72 °C for 90 s, and ending with a final extension at 72 °C for 5 min. The amplified products were separated by 1.5% agarose gel electrophoresis followed by staining using Gelred-Biotium (Fig. 1). To confirm the reproducibility of the banding patterns, the PCR experiments were repeated twice.

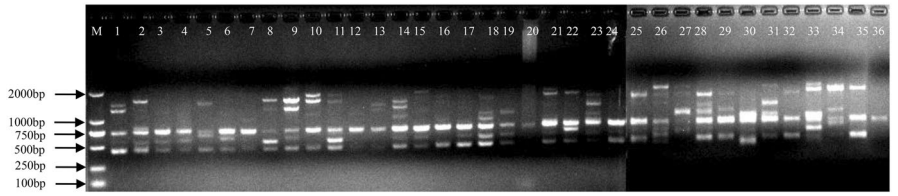

Fig. 1 Electrophoretic pattern of 36 pineapple accessions as amplified using primer ISSR16. *Lanes 1–36* represent pineapple genotypes A1–A36, as shown in Table 1. M is the standard molecular marker (1000-bp ladder)

SSR Analysis

A total of 20 out of 73 SSR primers (Table 3) were screened and then used for pineapple genetic diversity analysis. SSR amplification was conducted in a 20 μL reaction volume containing 10× reaction buffer, 1.5 mM $MgCl_2$, 0.15 mM of each dNTP, 0.3 μM of each primer, 1.2 U of *Taq* DNA polymerase, and 40 ng of genomic DNA template, respectively. The PCR conditions were as follows: 2 min and 30 s at 94 °C (initial denaturation step), followed by 35 cycles consisting of 94 °C for 45 s, annealing at a specific temperature between 52 and 56 °C (depending on primer used) for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 8 min. After mixing each reaction with 5 μL of loading buffer and denaturing at 95 °C for 5 min, the PCR products were electrophoresed on a 6% denaturing polyacrylamide gel at a constant power of 90 W for 40–50 min. The gels were fixed with acetic acid, stained with silver nitrate, and developed using anhydrous sodium carbonate. Finally, the gel was dried at room temperature, and the resulting band was analyzed (Fig. 2).

Data Scoring and Statistical Analysis

The ISSR and SSR bands in all pineapple accessions were scored as present (1) or absent (0). Only reproducible, clear, and well-resolved bands were considered, and smeared and weak bands were excluded from the analysis. To estimate the level of genetic diversity, the following parameters, including the percentage of polymorphic bands (PPB), average number of effective alleles (ne), observed heterozygosity (Ho), and expected heterozygosity (He) were calculated using the program POPGENE 1.32 (Yeh et al. 1997) for each SSR primer. Also, polymorphic information content (PIC), as an important parameter of primers, was also estimated. The PIC value for each locus was calculated using the following formula (Roldan-Ruiz et al. 2000):

$$\mathrm{PIC}_i = 2f_i(1 - f_i),$$

where PIC_i is the polymorphic information content of the locus i, f_i is the frequency of the amplified fragments (band present), and $1 - f_i$ is the frequency of non-amplified fragments (band absent). The frequency was calculated as the ratio

Table 3 Primer sequences used for SSR analysis in this study

	SSR primers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Polymorphism/non-polymorphism
1	AY098521	GTATATTACCGTGGATGCGGGAG	AGCATCAAGGGGTCCCGAGTT	Polymorphism
2	AY149881	CTTAGGGTTGAATGGTCC	ATCTGAGACCCAAGTTCG	Non-polymorphism
3	CO730886	CTCATCTCACTCCATACC	CCATTCGATATCGCCTTC	Non-polymorphism
4	CO730888	CGCATCAGCGCCAAACGC	GGAAGCGAAAGGAGATCG	Polymorphism
5	CO730928	TTCTGCTCCGCTCTTCTTC	AATTGAGGCGATCGATGC	Polymorphism
6	CO731235	ATTTCGAGCCCTTGGTCG	TTTATGGGGTCGCGTCGG	Polymorphism
7	CO731287	AGGGAAGCTTTGGAGGTGCAG	TGCAATAGCGATGATAAACCCCAG	Polymorphism
8	CO731330	GTAACACAACTACTCTACTCGGCA	CCTCCTCCAATTAAGATCCCTCAA	Non-polymorphism
9	CO731431	CACATGTCCACGTATTGG	GTAGCTCCACTCAGCCC	Non-polymorphism
10	CO731753	CTTTTGGGCTATGTTGCG	TGCTAAAGTACCCACCAG	Polymorphism
11	CO731816	CTCCTCAGCTTCGTCGCC	GACGAGATTGGCGTATCCC	Polymorphism
12	CO731871	AGTGAGAGGGAATCCTC	CTTATTCACCTCGTTGCC	Non-polymorphism
13	AJ845033	TCCACAGTGGGCGCAAAC	AAAGGACATGAGGTAGGCC	Non-polymorphism
14	AJ845035	GTATACCCCTCACCACCCAAG	GCGCAATCCATAGCGCAAGTC	Non-polymorphism
15	AJ845036	AGGTGAAGGTGGAGCTCACC	GTCGCCGTTAATCGACACGTG	Non-polymorphism
16	AJ845038	TGATCATGGCGACGACCCAG	TCATTGTCGCGGCACCATG	Non-polymorphism
17	AJ845039	TTGGAGCCGATATTATCGTCC	ACGATCTCACAATGCTCCTCG	Non-polymorphism
18	AJ845040	TCGCGTATTATTCAAACAGCC	ACTGAGGGGGTTCACGAG	Non-polymorphism
19	AJ845048	TCATCACCCCGCGCCTTTGC	TGCCAAGCCATCCTCAGACG	Non-polymorphism
20	AJ845052	CAGTGGTGATTGAAGCCATGC	TTCACACCGAGAAAGCCCGG	Non-polymorphism
21	AJ845056	TGCTGGCTCTGTGGGATG	TTAGGTTTTCAGTGGAGAGAG	Polymorphism
22	AJ845060	TGTAGGCATATGGTGGGTCTG	ATCTCTTAATCCAAGGGCCG	Polymorphism
23	AJ845062	GCCTCGAAAACACTGCTAGGC	GGTGTGTCAATTAGGCCTGAC	Non-polymorphism
24	AJ845069	TCCCCCTAATCATCGGAAGCC	GGATGATGATGGTCACCTCTG	Non-polymorphism
25	AJ845076	ACCCAGCCATTGTCGTGCCTG	AGTTTACAAGGCGCATAGG	Non-polymorphism

Table 3 continued

	SSR primers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Polymorphism/non-polymorphism
26	AJ845077	TCATTTAGGATGCTGCATGG	TCTCTCATGCGCACATGC	Non-polymorphism
27	AJ845081	ACATTCCTCAGAGTGACCAGC	CACTAATCCTTGACCCAGACC	Polymorphism
28	G134	GTTCGCAGGAGAATAGAG	CGTTGTAATGCAGTGAAC	Non-polymorphism
29	ISSR03_386_Ab	AGGAAGATCTGGACCGT	TCAATTCTGCGGGTACT	Non-polymorphism
30	ISSR03_662_Ac	GTATTTTGGGTGAGGTCG	TGACGCTACCCACTTCA	Non-polymorphism
31	ISSR13_591_Ac	ACATGGAGCTGTTCATGC	ACGTACAACACCCACCA	Non-polymorphism
32	ISSR19_571	ATGGGATGTGGCATTGT	GCACCCAAATATGCATCG	Non-polymorphism
33	SSR_ISSR06_369	AGAGAGAGCTGGCTGAATG	AGAGAACGGGCACAAACCT	Non-polymorphism
34	ISSR09_443_Ab	CACACACACACACTGTAG	GGATCACTAGCATACCATTC	Non-polymorphism
35	OPM06_1072_Ac	ACATGATCGTTGTTGAGCC	CTAGACAATTCACTCACAGG	Non-polymorphism
36	OPM10_583	TCTGGCGCACATGGTGA	TGAGCGACAATCGTGGATGA	Non-polymorphism
37	OPQ18_425	CTGGGTGATGGTACTAGA	ACAAGTATGGTGCCAAC	Non-polymorphism
38	OPR13_1036	GACAGCCTAATCTTACTTGG	CACAAGAACTAGCTCAGC	Non-polymorphism
39	OPR15_385_Ac	GACAACGAGTGACGTGG	GGACAACGAGGATTCAACA	Non-polymorphism
40	OPR15_841_Ab	TGGATACGATGGTCAGTC	CTGTTCAATCTACCAGAACG	Non-polymorphism
41	OPU16_515_Ab	TGGATCGTTGACAAATCC	GAAGCGAGTTTACTGCA	Non-polymorphism
42	OPU18_755_Ab	CTAATCGCATCCAAATGC	AGGAAGCTTTGTGTAGC	Non-polymorphism
43	OPU18_765_Ab	ATGCTTGTCCTTGAGCT	CCTACCACTGAGACCAG	Non-polymorphism
44	OPX18_1186_Ac	GTTATTGGTTTGGCCAGT	ACTAGGTGGTCACTTAGG	Non-polymorphism
45	DT337383	TTTAATCGGGTGGAGTAAGGA	GCCAATATGAACAGGGGAAA	Non-polymorphism
46	DT335877	TAGAGGTCGGGAGAACGAAA	GCGGAGGCTACTGATGCTAC	Non-polymorphism
47	DT336678	CCACAACAACGAGAGAACCA	AAAAGACACCTTGCGAGCAC	Non-polymorphism
48	CO731649	CCGTTGAGATCGGAGAAATG	ACCACACATGAGCAAAACGA	Non-polymorphism
49	DT338176	CTCCTCATCTACCGCACCTC	CCCTAGACGACGACGAAGAG	Polymorphism
50	CO731264	GGCACAATTTTTGTGGCTGT	GAGGATGGAGAAACCCATGA	Non-polymorphism

Table 3 continued

	SSR primers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Polymorphism/non-polymorphism
51	DT336954	CATCCATCCATCCATCCAAT	GTCGTTGATCATTCGCAAAA	Polymorphism
52	DT336292	AAGCGCAGGTTCGTAATTTG	TCGAAATCCACAGAACACCA	Non-polymorphism
53	DT338035	TGCTCTGTTGCTGATGAGGT	CCAGCCTCTCCTTCTCCTTC	Non-polymorphism
54	DT336561	GCAAATGAGGCCACAAACTT	GGGTGGTGTGGACTTTCTCT	Polymorphism
55	DT337038	CCCTGAAGGTGGAGATTGTG	AAAAACCAAAACCCTGGACA	Polymorphism
56	DT336932	GCATGCCAAAGGAAAGAGTT	CCCTGAACAAATCACCCAAC	Polymorphism
57	CO731629	AGAAGCGGAAGCGTGTTG	GCGGAGATCGAAGCACTC	Polymorphism
58	DT337663	CCAAATTCACCACCGAAGAC	GCAATCTCAAAGCCATCCAT	Polymorphism
59	DT337868	CGAGAGAGATTGTGCGTTTG	GGGGGAACACACTGCTAAAG	Non-polymorphism
60	DT336852	CTTTTGCTCAGAAAGCAGGTT	TGCGTGCTTGACCTCTGTTA	Polymorphism
61	DT335782	GCAACCCCAATACCCTAACC	GTACTCCGCCATTGTTGGTG	Non-polymorphism
62	DT338083	TTCTGATCAATGAGTGGACACC	TCCTGAATCCAAAGGCAAAG	Non-polymorphism
63	DT338084	TTACACATGCACGGAGTAC	CTAAGAGACAACCCAGGAAC	Non-polymorphism
64	DT338085	GCCAATAACAACCTCAAGC	TCCATACACACAGTACGTCG	Polymorphism
65	DT338086	ACCTACAAGTGGTACGTCG	GGAGCAAGGAGTTATTCAG	Non-polymorphism
66	DT338089	TAATCGGGTGGAGTAAGG	GCTCACATAGGCCAATATG	Non-polymorphism
67	DT338091	GCTGCTCTTGCTGCCAT	AAGCCATAGGACCACCAC	Polymorphism
68	DT339694	CGTGCCGCATAAATCAT	TATCTCCTCGCTCCTCTTG	Non-polymorphism
69	DT339172	CAGCCAATAACAACCTCAAG	TCCATACACACAGTACGTCG	Non-polymorphism
70	DT338171	TAGTGAGTCAGGAGGAGAATG	CAAATAAACGGAGCGGAT	Non-polymorphism
71	DT338494	GCCAATAACAACCTCAAGC	TCCATACACACAGTACGTCG	Non-polymorphism
72	DT337096	ATGGTGGTTCACTTATCAGC	AGACATTCAAAGCGGAGAG	Non-polymorphism
73	CO731867	GCGAGCCTGTTAGACTTTGT	ACGATCTCAGCTGGACCTT	Non-polymorphism

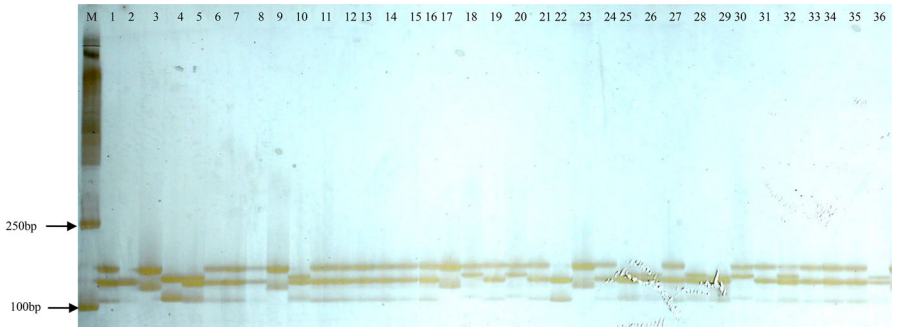

Fig. 2 Electrophoretic pattern 36 pineapple accessions as amplified using primer DT336561. *Lanes 1–36* represent pineapple genotypes A1–A36, as shown in Table 1. M is the standard molecular marker (1000-bp ladder)

between the number of amplified bands at each locus and the total number of accessions (excluding missing data). The PIC of each primer was calculated using the average PIC value from all loci of each primer.

Marker index (MI) was also calculated according to the following formula:

$$\mathrm{MI} = \mathrm{PIC} \times \beta \times \alpha,$$

where β is the proportion of polymorphic bands, and α is the number of loci per assay unit (Powell et al. 1996).

NTSYS-PC version 2.10 (Rohlf 2000) was used in cluster analysis and principal coordinate analysis for all pineapple accessions. Genetic similarity among accessions was evaluated by calculating the simple matching (SM) coefficient (Sokal and Michener 1958). Clustering of genotypes was conducted using the SAHN method based on the genetic distance matrix with the unweighted pair group method with arithmetic averaging (UPGMA). Principal Coordinate Analysis (PCA) was performed using DCENTER module and Eigen values based on the variance covariance matrix calculated from the marker data with the software package NTSYS-PC.

Results

ISSR Analysis

We screened 13 ISSR primers according to their ability to generate unambiguous and polymorphic bands. A total of 96 bands were scored for all accessions, of which 91 (93.65%) were polymorphic (Table 4). The number of amplified bands per primer ranged from 4 to 14, with an average of 7.38 bands per primer. Primer ISSR24 generated the highest number of bands (14), whereas primer ISSR18 resulted in the lowest number of bands (4). All ISSR primers, except for ISSR6,

Table 4 Total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphic bands (PPB), average of effective alleles (ne), Nei's gene diversity (h), Shannon information index (I), marker index (MI), and polymorphism information content (PIC) of various pineapple accessions using ISSR primers

Primers	TNB	NPB	PPB (%)	Ne	h	I	MI	PIC
ISSR1	10	10	100.00	1.31	0.20	0.33	2.00	0.20
ISSR2	5	5	100.00	1.17	0.14	0.26	0.65	0.13
ISSR3	8	8	100.00	1.29	0.19	0.34	1.60	0.20
ISSR4	5	5	100.00	1.55	0.34	0.51	1.65	0.33
ISSR5	5	5	100.00	1.45	0.29	0.46	1.45	0.29
ISSR6	5	4	80.00	1.19	0.14	0.24	0.72	0.18
ISSR7	8	7	87.50	1.36	0.24	0.39	1.68	0.24
ISSR9	6	6	100.00	1.47	0.30	0.47	1.80	0.30
ISSR10	8	6	75.00	1.12	0.10	0.19	0.78	0.13
ISSR16	12	12	100.00	1.34	0.23	0.37	2.76	0.23
ISSR18	4	3	75.00	1.46	0.25	0.36	0.99	0.33
ISSR21	6	6	100.00	1.61	0.36	0.54	2.16	0.36
ISSR24	14	14	100.00	1.38	0.25	0.40	3.50	0.25
Total	96	91	–	–	–	–		–
Mean	7.38	7	93.65	1.36	0.23	0.37	1.67	0.24

ISSR7, ISSR10, and ISSR18, showed the highest percentage polymorphic bands (100%). Calculation for Shannon's information index and gene diversity was conducted to further understand the genetic diversity of pineapple accessions. The mean value of Nei's gene diversity and Shannon's Information index using the ISSR primers were 0.23 and 0.37, respectively. The marker index for ISSR primers varied between 0.65 and 3.50, and the mean value was 1.67. PIC varied from 0.13 for ISSR2 and ISSR10 to 0.36 for ISSR21, and the average was 0.24.

ISSR analysis indicated that the genetic similarity coefficients (GSCs) of all pineapple accessions ranged from 0.50 to 0.89, with an average of 0.74 (data not shown). The highest GSC (0.89) was observed between the accessions "N67-10" and "Sugarloaf" and between "Early-ripening-perfume" and "Red-skin pineapple." The lowest GSC (0.50) was detected between "Victoria" and "Xuwen." Based on the corresponding genetic similarity coefficient among the tested 36 pineapple accessions, phylogenetic reconstruction was performed using the UPGMA approach (Fig. 3). The dendrogram showed that the 36 accessions could be further classified into five major clusters (clusters I–V) at a similarity coefficient of 0.72. Among the five clusters, cluster II was the largest, consisting of 30 accessions from Australia, Brazil, China, Costa Rica, Indonesia, Japan, Taiwan, and Thailand. The individual accession "Creampine" from Taiwan and "Red Spanish" from Indonesia formed clusters I and IV, respectively. Cluster III consisted of two accessions, namely, "Victoria" from Mauritius and "Mibao" from Taiwan. The two pineapple accessions "Perola" and "Xuwen" formed V. PCA roughly divided the 36 pineapple accessions into four groups, which showed minimal differences from

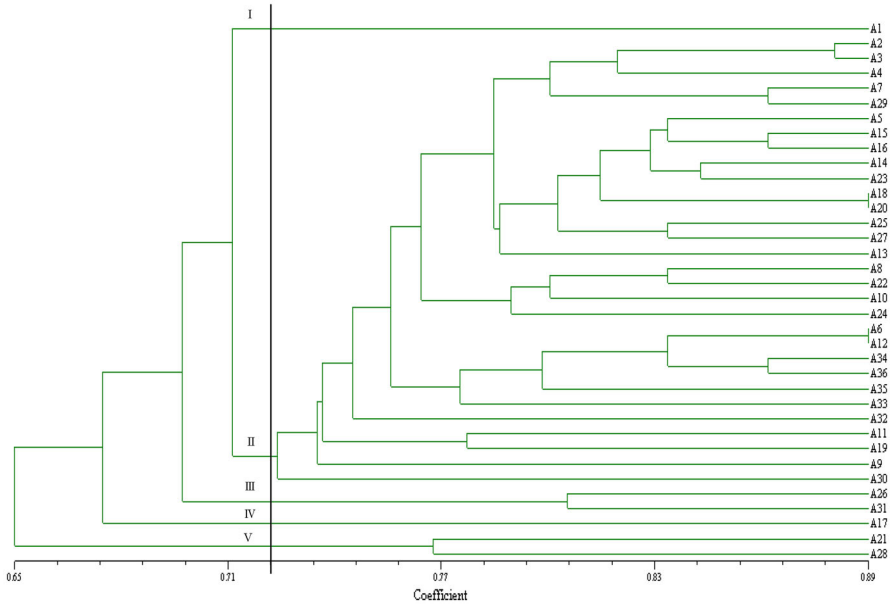

Fig. 3 Phylogenetic reconstruction of 36 pineapple accessions using UPGMA; the dendrogram was constructed from estimated simple matching genetic distances based on ISSR markers

the clustering results of UPGMA; e.g., groups II and IV produced a tight cluster. The first three components explained 23.91% of the observed total variation (Fig. 4).

SSR analysis

For SSR analysis, 20 SSR primers showing high polymorphism and strong-signal bands were selected for evaluating all pineapple accessions. These primers amplified a total of 73 bands in all pineapple accessions, of which 70 were polymorphic, thereby accounting for 96.50% of the total number of bands. The average number of bands and polymorphic bands per primer was 3.65 and 3.50, respectively. The effective alleles per primer varied between 1.16 and 1.70, with an average of 1.44. The mean value of Nei's gene diversity was 0.28, and Shannon's information index was 0.43. He ranged from 0.38 for DT338091 to 0.54 for CO731753, with an average value of 0.48. Ho varied between 0.11 for DT336954 and 0.56 for CO730928, with an average value of 0.31. Calculated PIC values were 0.13–0.49, and the highest PIC value was observed with CO730928 (Table 5).

In the present study, GSC values for pineapple accessions based on SSR analysis varied from 0.34 to 0.95, with a mean of 0.61 (data not shown). The highest GSC (0.95) was observed between "Pearl" and "PZS-1," and the lowest GSC (0.50) was detected between "Red Spanish" and "Red skin pineapple." Considering a threshold value 0.55, the 36 accessions were clustered into three major clusters (clusters I–III) (Fig. 5).

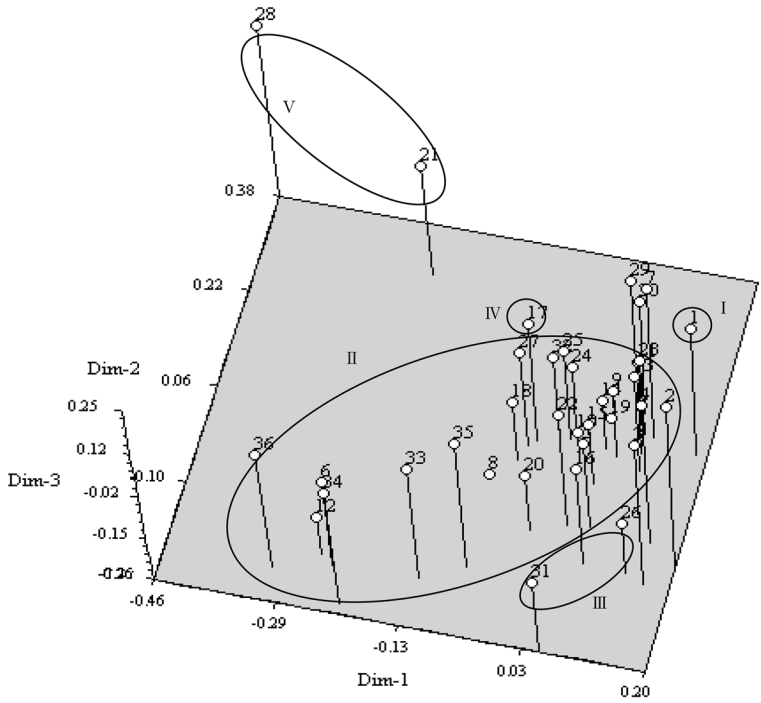

Fig. 4 Three-dimensional plot of principal coordinate analysis of 36 pineapple accessions using ISSR markers. The contribution of PC1, PC2, and PC3 was 8.67, 7.85, and 7.39%, respectively

Cluster I contained the highest number of pineapple accessions and included 13 accessions from Taiwan, five from Japan, one from Costa Rica, three from Indonesia, five from China, one from Thailand, two from Brazil, one from Australia, and one from Mauritius. Cluster II consisted of two accessions, i.e., "MD_2" and "Golden pineapple." The two pineapple accessions "Red Spanish" and "Gold diamond" showed lower similarity coefficients compared to the other accessions and formed a distinction group. Clustering of the individual accessions was confirmed with PCA. The spatial distribution of the three groups in the PCA scatter plot largely corresponded with the results of the cluster analysis (Fig. 6), and the first three components accounted for 32.83% of the total variation.

SSR-ISSR Analysis

To obtain more comprehensive genetic estimates, a combined analysis was conducted using the ISSR and SSR data together. In this analysis, GSC ranged from 0.48 to 0.86, with a mean of 0.69 (data not shown). The GSC matrix based on the combined data was used to generate a dendrogram showing genetic relationships among the accessions. Based on the dendrogram, we grouped the 36 accessions into three main clusters (Fig. 7). Cluster I was the biggest cluster that consisted of 34

Table 5 Total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphic bands (PPB), average of effective alleles (ne), Nei's gene diversity (h), Shannon information index (I), observed heterozygosity (Ho), expected heterozygosity (He), marker index (MI), and polymorphism information content (PIC) of various pineapple accessions using SSR primers

Primers	TNB	NPB	PPB (%)	ne	h	I	Ho	He	MI	PIC
CO731235	3	3	100.00	1.39	0.26	0.41	0.39	0.47	0.78	0.26
AJ845056	3	3	100.00	1.34	0.25	0.41	0.33	0.51	0.75	0.25
CO730888	6	5	83.33	1.34	0.22	0.36	0.17	0.51	1.35	0.27
CO731816	2	2	100.00	1.35	0.24	0.40	0.33	0.51	0.48	0.24
CO731753	2	2	100.00	1.58	0.32	0.48	0.39	0.54	0.64	0.32
CO730928	3	2	66.67	1.64	0.33	0.45	0.56	0.51	0.98	0.49
DT338091	6	6	100.00	1.52	0.32	0.49	0.28	0.38	1.92	0.32
DT336561	6	6	100.00	1.65	0.38	0.56	0.28	0.44	2.28	0.38
DT336954	5	5	100.00	1.60	0.35	0.53	0.11	0.51	1.75	0.35
CO731629	3	3	100.00	1.58	0.36	0.55	0.28	0.51	1.08	0.36
DT337663	4	4	100.00	1.19	0.14	0.26	0.28	0.47	0.56	0.14
DT338085	4	4	100.00	1.47	0.31	0.49	0.39	0.44	1.28	0.32
DT336932	5	5	100.00	1.16	0.13	0.23	0.44	0.51	0.80	0.16
AJ845081	3	3	100.00	1.35	0.23	0.37	0.39	0.39	0.69	0.23
DT336852	4	4	100.00	1.48	0.30	0.48	0.39	0.44	1.20	0.30
AJ845060	2	2	100.00	1.29	0.19	0.32	0.22	0.51	0.38	0.19
DT337038	2	2	100.00	1.55	0.35	0.54	0.39	0.44	0.70	0.35
AY098521	5	4	80.00	1.42	0.26	0.40	0.22	0.51	1.04	0.26
DT338176	3	3	100.00	1.70	0.39	0.57	0.22	0.51	1.17	0.39
CO731287	2	2	100.00	1.25	0.19	0.34	0.22	0.49	0.38	0.19
Total	73	70	–	–	–	–				–
Mean	3.65	3.5	96.5	1.44	0.28	0.43	0.31	0.48	1.01	0.29

accessions and was further divided into two subclusters. Cluster I-1 included two accessions from Taiwan Island, one accession from Costa Rica, one accession from Brazil, and one accession from Japan. Cluster I-2 contained 29 accessions from Japan, Costa Rica, Indonesia, China, Thailand, Brazil, Australia, and Mauritius. Clusters II and III comprised one accession each, "Xuwen" from China Guangdong and "Red Spanish" from Indonesia. The PCA results corroborated those of cluster analysis (Fig. 8), and the first three components explained 23.21% of the observed total variation.

Discussion

Assessment of the genetic variability in various pineapple accessions is important in pineapple breeding and the conservation of genetic resources; it is particularly useful in the characterization of individual accessions and cultivars. The ability to

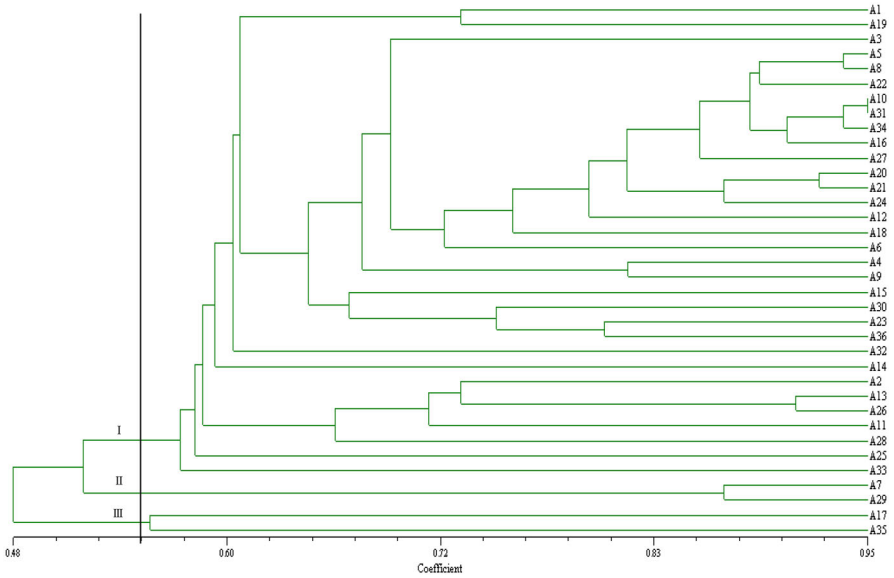

Fig. 5 Phylogenetic reconstruction of 36 pineapple accessions using UPGMA; the dendrogram was constructed from estimated simple matching genetic distances based on SSR markers

reliably distinguish pineapple accessions could be invaluable for pineapple diversity studies, and molecular markers offer an effective approach to unveil genetic diversity based on DNA polymorphisms. In the present study, we used microsatellite-based markers, SSR and ISSR, for the molecular characterization of pineapple accessions. Previous studies offer limited information on genetic variations in pineapple species (Duval et al. 2001; Ruas et al. 2001; Kato et al. 2004) primarily because of the types of molecular markers used. Duval et al. (2001) used RFLP markers to study molecular diversity in 301 pineapple accessions and found that the variation was mostly at the intraspecific level, particularly in the wild species *Ananas ananassoides* and *Ananas parguazensis*. Ruas et al. (2001) observed moderate intraspecific genetic variation between the genera *Ananas* and *Pseudananas* using RAPD markers. However, our work shows the sensitivity of a combined SSR and ISSR approach that enables the identification of discrete DNA differences for each pineapple accession.

Although SSR and ISSR have several advantages compared to other molecular markers, it seldom employed in studying the genetic diversity of pineapple accessions. The present study generated detailed information of various pineapple accessions using SSRs and ISSRs. The frequency of polymorphic SSR markers was 27.4%, which is higher than the results (13.1%) obtained by Feng et al. (2013). This discrepancy may be due to differences in the materials that were utilized in each study. The wide geographical distribution of pineapple accessions used in the present study determined that these have a high level of variation at the DNA level. The mean number of bands amplified by each SSR primer in pineapple was 3.65,

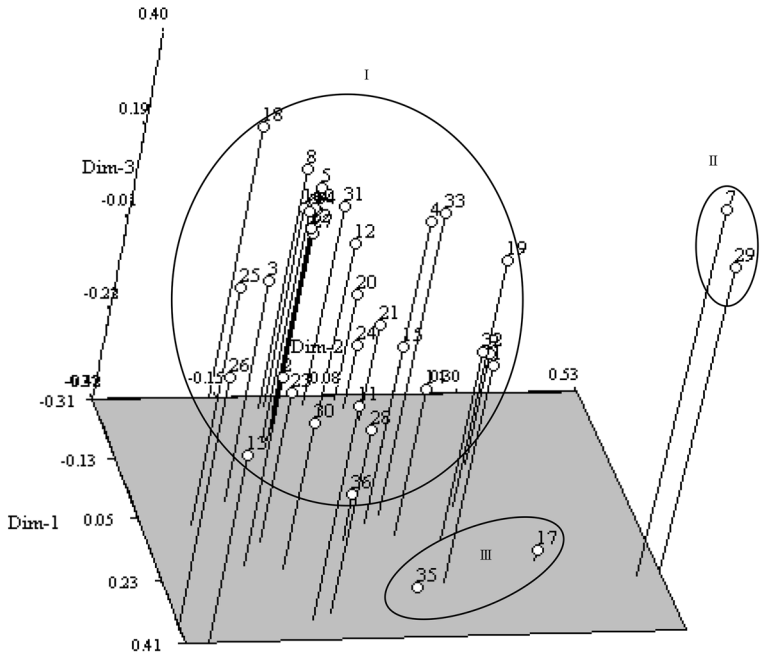

Fig. 6 Three-dimensional plot of principal coordinate analysis of 36 pineapple accessions using SSR markers. The contribution of PC1, PC2, and PC3 was 14.36, 10.27, and 8.20%, respectively

which is lower than that of apple (6.46) (Goulão and Oliveira 2001) and of moraceae (5.13) (Zhao et al. 2007). The findings may possibly be related to the distinct characteristics of SSRs in different species. Polymorphic SSR markers generally have a higher percentage (53.7%) of GA/TC repeats than non-polymorphic markers (46.9%), which may also be the condition our investigation of diversity among various pineapple accessions. A similar phenomenon was also observed using ISSR markers.

We also compared the related information of ISSRs and SSRs as genetic markers in pineapple. PPB (93.25%), ne (1.44), He (0.28), I (0.43), and PIC (0.29) at the accession level using SSR primers were higher than those using ISSRs (PPB = 92.72%, ne = 1.36, h = 0.23, I = 0.37, and PIC = 0.24). Similar results were also observed in apple (Goulão and Oliveira 2001), Poaceae (Hammami et al. 2014), potato (McGregor et al. 2000), and mulberry (Zhao et al. 2007). A possible explanation for these differences in the resolution of the two marker systems is that ISSR and SSR techniques target different parts of the genome. These differences may also be related to species specificity of each molecular marker. Our findings indicate that SSR is species-specific to pineapple but not ISSR. There was also a poor correlation between the individual ISSR and SSR systems (r = 0.15) in our study. Previous investigations have reported little correlation between marker systems such as in wheat (Bohn et al. 1999), maize (Pejic et al. 1998), fig (Hidetoshi

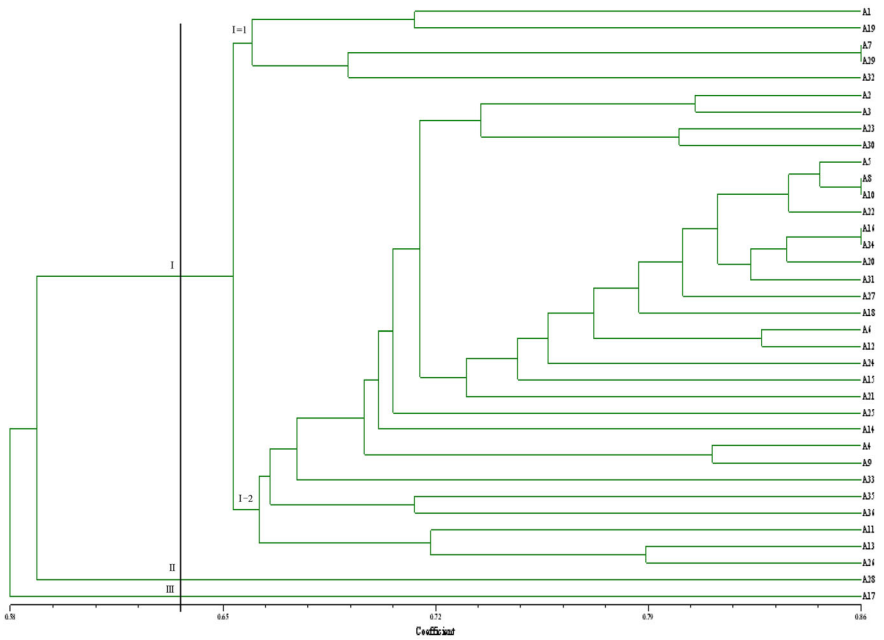

Fig. 7 Phylogenetic reconstruction of 36 pineapple accessions using UPGMA; the dendrogram was constructed from estimated simple matching genetic distances based on combined SSR+ISSR markers

et al. 2009), and ruthenia medic (Li et al. 2013). In contrast, both the individual ISSR and SSR marker systems showed a positive significant correlation with the combined marker system ISSR+SSR ($r = 0.73$ and 0.79, respectively), suggesting that the information revealed by the combined marker system is more comprehensive than the results obtained using individual markers.

Different dendrograms obtained using SSRs and ISSRs in this study also showed that the pineapple accessions could be further classified, i.e., clustered into five groups using ISSR markers and into three groups using SSR markers. Interestingly, the pineapple accessions from the same region were not always clustered together, but different original accessions were usually clustered into a group, which is indicative of the complex genetic relationship among various pineapple accessions. The genetic similarity coefficient showed that although the diversity level detected by ISSR and SSR markers differed, both markers revealed a wide genetic variability among pineapple accessions. The current high level of genetic diversity among different pineapple accessions could be attributed to the wide geographical distribution of the study materials evaluated in this study, which were collected from 10 countries/regions. A previous study has shown that widely distributed plants often have higher genetic diversity than narrowly distributed ones (Godt et al. 2004). Several studies have extensively investigated the genetic diversity within *A. comosus* (Aradhya et al. 1994; Duval et al. 2001; Ruas et al. 2001; Kato et al. 2004); however, these studies did not explore the diversity among different accessions.

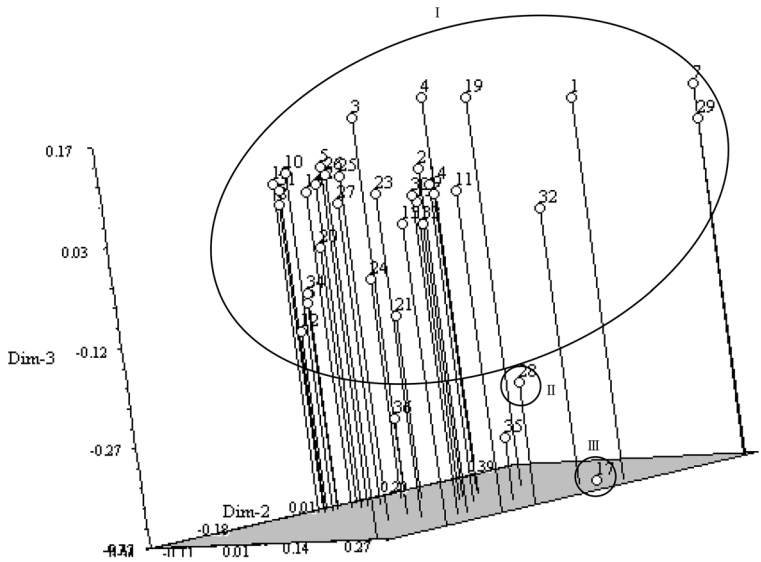

Fig. 8 Three-dimensional plot of principal coordinate analysis of 36 pineapple accessions using combined SSR+ISSR markers. The contribution of PC1, PC2, and PC3 was 9.89, 7.07, and 6.25%, respectively

Pineapple breeding programs include hybridization of different accessions for the selection of elite field variants. However, crossbreeding proved to be a difficult approach for pineapple because of its high level of genome heterozygosity and genome instability (Kato et al. 2004). Several variants used for pineapple breeding originate from vegetative reproduction and tissue culture propagation, which generate numerous phenotypic variant forms (Dewald et al. 1988; Wakasa 1977, 1979; Collins 1960; Pérez et al. 2011). However, this also resulted in a narrow genetic background in pineapple accessions, wherein distinction could solely be based on morphological characters and thus discrimination of accessions has become a challenge. Thus, an identification index based on molecular markers is very important. Our study proves that ISSR and SSR methodologies are highly effective in demonstrating pineapple genetic variation; it also revealed that Chinese cultivated pineapples have a high level of genetic diversity. Our results will be useful in the scientific conservation and management of pineapple germplasm, as well as in improving the current pineapple breeding strategies in China.

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