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Genetic diversity among *Ocimum* species based on ISSR, RAPD and SRAP markers

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Abstract

Genetic diversity among 37 basil accessions representing four species (*Ocimum basilicum*, *O. americanum*, *O. gratissimum* and *O. tenuiflorum*) was evaluated individually using different marker systems. The applied marker systems potentially targeted different regions of the genome and included 36 inter-simple sequence repeat (ISSR), 20 random amplified polymorphic DNA (RAPD), and ten sequence-related amplified polymorphism (SRAP) markers. Among these three marker systems, SRAP showed the highest mean value of polymorphic information content (PIC, 0.29) and resolving power (Rp, 30.19) which were much higher than those of RAPD (0.23, 5.13) and ISSR (0.19, 1.39). Basil accessions were clustered into four groups using ISSR or RAPD, but were clustered into three groups using data from SRAP or the combined data set, indicating that the genetic diversity in different target regions of the tested basil genomes was not the same. All showed very good fit of cophenetic matrices between similarity matrix and dendrogram and the results were highly correlated between marker types. In addition, the SRAP dendrogram was correlated most with the combined data set indicating that SRAP markers could be a better tool for genetic diversity analysis in basil than RAPD and ISSR markers.

Keywords: Basil, ISSR, RAPD, SRAP, Genetic diversity.
Abbreviations: ISSR_inter-simple sequence repeat; RAPD_random amplified polymorphic DNA; SRAP_sequence-related amplified polymorphism; PIC_polymorphic information content; Rp_resolving power.

Introduction

The *Ocimum* genus of family Lamiaceae, collectively named basil, is an important herb and spice crop worldwide. Basil has been used in daily life for a long time (Simon et al., 1990) and widely applied in folk medicine (Vieira and Simon 2000) and insect repellent (Umerie et al., 1998). It has also been reported that basil could provide anti-bacterial activity (Lachowicz et al., 1998; Carović-Stanko et al., 2010b) or antioxidant activity (Berić et al., 2008). The essential oil of basil consists of different compounds; hence not only the yield (Zheljazkov et al., 2008) but also the quality (Carović-Stanko et al., 2011b; De Masi et al., 2006) of essential oil varies among species. In order to improve the further breeding or collection of basil, understanding its genetic diversity is important. Morphological inspection is a direct and simple way to study basil genetic diversity. For example, morphological markers have been applied in routine screening of 46 basil accessions (Carović-Stanko et al., 2011a). However, such characters are easily affected by environmental factors which make species identification in basil more complex than expected (De Masi et al., 2006; Labra et al., 2004). DNA markers have also been demonstrated to serve as efficient tools in identifying basil species due to their characteristics which include abundance, highly polymorphism and freedom from environmental influence (De Masi et al., 2006; Labra et al., 2004; Singh et al., 2004). Various types of DNA markers were applied in basil genetic diversity studies including AFLP (amplified fragment length polymorphism) (Carović-Stanko et al., 2011b; Labra et al., 2004), random amplified polymorphic DNA (RAPD) (De Masi et al., 2006; Harisaranraj et al., 2008; Singh et al., 2004) and inter-simple sequence repeat (ISSR) (Aghaei et al., 2012) markers. Recently, sequence-related amplified polymorphism (SRAP) markers have been demonstrated to be reliable, effective and cheap in several studies including Brassica (Li and Quiros 2001), *Cucurbita pepo* (Ferrio et al., 2003), tree peony (Han et al., 2008), elephant grass (Xie et al., 2009), *Celosia argentea* (Feng et al., 2009), watermelon (Ulturk et al., 2011) and olive varieties (Isik et al., 2011), however, SRAP has not been applied in basil. Therefore, this study employed not only ISSR, RAPD but also SRAP markers. The objective of this study was to evaluate the genetic diversity among basil accessions with different marker systems including RAPD, ISSR, SRAP and the combined data set. In addition, marker systems were compared and the best marker system for genetic diversity analysis in basil was determined.

Results

Performance of different marker systems

In this study, 37 accessions belonging to four different basil species, *Ocimum basilicum*, *O. americanum*, *O. gratissimum* and *O. tenuiflorum* (Table 1) were surveyed with three marker systems including ISSR (Table 2), RAPD (Table3) and SRAP (Table 4). For ISSR analysis, 223 loci were amplified by 36 ISSR markers. Of these, 217 loci were polymorphic with an

Table 1. Basil accessions used in this study.

No.	Species	Cultivar	Source[a]
1	*Ocimum basilicum*	Asian basil	Taiwan
2	*Ocimum basilicum*	Hung Ku Chiu Tseng Ta basil	Taiwan
3	*Ocimum basilicum*	Bush Green basil	Canada
4	*Ocimum basilicum*	Greek Bush basil	Canada
5	*Ocimum basil icum*	Spicy Globe basil	Canada
6	*Ocimum basilicum*	Green Globe basil	Canada
7	*Ocimum basilicum*	Green Gate basil	Canada
8	*Ocimum basilicum*	Dark Opal basil	Canada
9	*Ocimum basilicum*	Purple Delight basil	Canada
10	*Ocimum basilicum*	Ararat basil	Canada
11	*Ocimum basilicum*	Mammoth lettuce leaf	Canada
12	*Ocimum basilicum*	Marseillais basil	Canada
13	*Ocimum basilicum*	Medinette basil	Canada
14	*Ocimum basilicum*	Napoletano lettuce leaf basil	Canada
15	*Ocimum basilicum*	Sweet basil	Canada
16	*Ocimum basilicum*	Sweet Salad basil	Canada
17	*Ocimum basilicum*	Cinnamon basil	Canada
18	*Ocimum basilicum*	Anise basil	Canada
19	*Ocimum basilicum*	Magical Michael basil	Canada
20	*Ocimum basilicum*	Nufar F1 Genovese basil	Canada
21	*Ocimum basilicum*	Genovese basil	Canada
22	*Ocimum basilicum*	Special Select FT ^{TM} Genovese basil	Canada
23	*Ocimum basilicum*	Compatto FT Genovese basil	Canada
24	*Ocimum basilicum*	Gecofure basil	Canada
25	*Ocimum basilicum*	Envigor Type Genovese basil	Canada
26	*Ocimum basilicum*	Thai basil	Canada
27	*Ocimum basilicum*	Queenette Thai basil	Canada
28	*Ocimum basilicum*	Siam Queen Thai basil	Canada
29	*Ocimum basilicum*	Mrs. Burns Lemon basil	Canada
30	*Ocimum basilicum*	Sweet Dani Lemon basil	Canada
31	*Ocimum basilicum*	Spice basil	Canada
32	*Ocimum basilicum*	Blue Spice basil	Canada
33	*Ocimum americanum*	Lemon basil	Canada
34	*Ocimum americanum*	Lime basil	Canada
35	*Ocimum gratissimum*	East Indian basil	Canada
36	*Ocimum tenuiflorum*	Sacred basil	Canada
37	*Ocimum tenuiflorum*	Purple Sacred basil	Canada

[a]The regions of basil accessions were obtained.

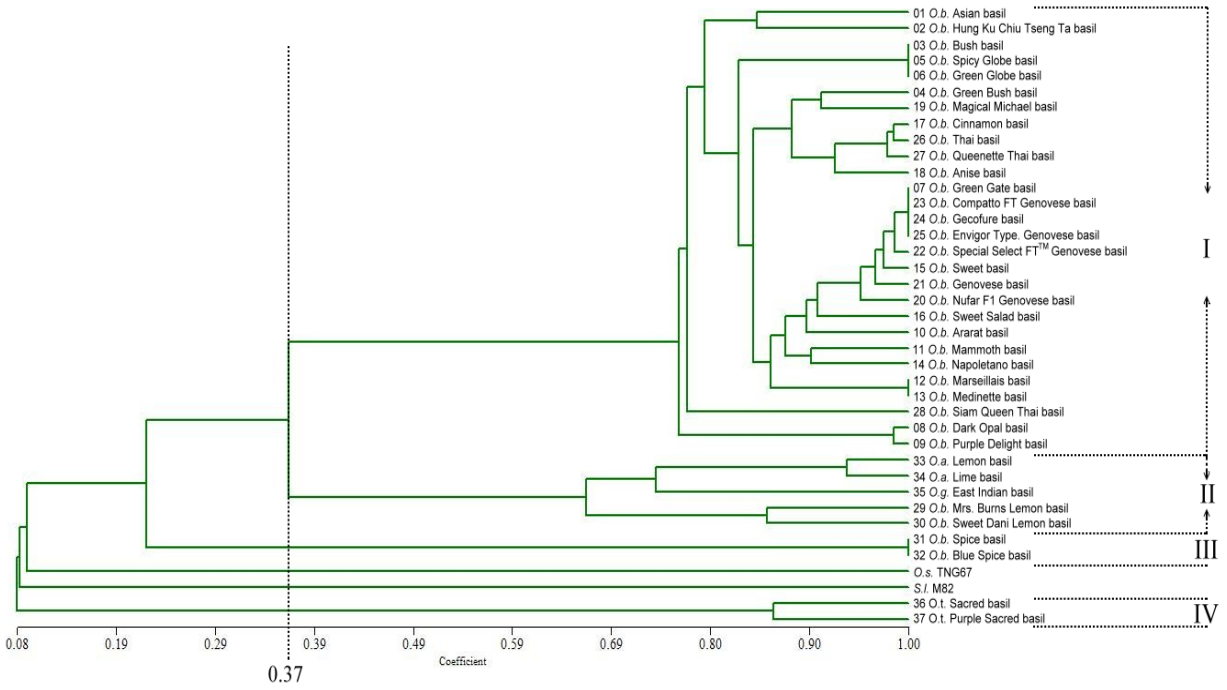

Fig 1. Jaccard's similarity dendrograms generated by UPGMA based on ISSR marker system which clustered into four groups.

average polymorphic ratio (P%) of 97% as shown in Table 2. The number of total loci (NTL) detected with ISSR markers ranged from 2 (UCB827) to 10 (UCB809, UCB817, UCB881) with an average of 6.19 loci per marker. The number of polymorphic loci (NPL) ranged from 2 (UCB827) to 10 (UCB809) with an average of 6.03 polymorphic loci per marker. Polymorphic information content (PIC) and resolving power (Rp) values for individual ISSR markers were estimated. UBC811 showed the highest PIC (0.29) and UBC850 and UBC855 gave the lowest (0.10) with an average PIC of 0.19. In contrast, UBC812 showed the highest Rp (2.65) while UBC821 and UBC861 (0.43) gave the lowest with an average Rp of 1.39 (Table 2). As shown in Table 3, a total of 329 loci (NTL) were amplified by 20 RAPD markers among which 311 loci were polymorphic with an average polymorphic ratio of 95%. The NTL ranged from 6 (OPA02) to 28 (OPA19) with an average of 16.45 loci per marker. NPL of RAPD marker ranged from 2 (OPA02, OPA03) to 28 (OPA19) with an average of 15.55 polymorphic loci per marker. The highest PIC was observed for OPA02 (0.36) and the lowest for OPA03 (0.10) with an average PIC of 0.23. In addition, OPA07 showed the highest Rp (9.30) and OPA03 the lowest (1.03) with an average Rp of 5.13 (Table 3). A total of 741 loci (NTL) were amplified by ten primer combinations of SRAP markers, among which 678 loci were polymorphic with an average polymorphic ratio of 93% as shown in Table 4. NTL ranged from 55 (me2x em5) to 101 (me2x em4) with an average of 73.4 loci. NPL ranged from 38 (me2x em5) to 94 (me2x em4) with an average of 68.5 loci per marker. The combination me1 x em6 showed the highest PIC (0.32) and the combination me2 x em5 showed the lowest (0.26) with an average PIC of 0.29. Combination me2 x em4 showed the highest Rp (42.81) and me2 x em5 had the lowest (16.49) with an average Rp of 30.19 (Table 4).

Genetic diversity analysis

Based on ISSR clustering, the Jaccard's similarity coefficients ranged from 0.065 to 1 with an average of 0.596 (data not shown). The highest value of similarity was 1 among several accessions including Spice basil and Blue Spice basil; Bush basil, Spicy Globe basil and Green Globe basil; and Green Gate basil, Compatto FT Genovese basil, Gecofure basil and Envigor Type Genovese basil. The lowest similarity (0.065) was observed between Purple Sacred basil and Siam Queen

Thai basil. A dendrogram was constructed using UPGMA clustering algorithm based on ISSR data and 37 basil accessions were clustered into four groups at a similarity coefficient of 0.37 (Fig. 1). The cophenetic correlation was estimated and showed a very good fit (r = 1.0, Table 5). Group I included 28 accessions belonging to *O. basilicum*. Group II contained five accessions representing three different species (*O. americanum*, *O. gratissimum* and *O. basilicum*). Group III contained two accessions of species *O. basilicum* including Spice basil and Blue Spice basil which could not be distinguished from each other. Group IV contained two accessions of *O. tenuiflorum* including Sacred basil and Purple Sacred basil which were distantly-related to the rest of the basil accessions in this study. Based on RAPD clustering, the Jaccard's similarity coefficients ranged from 0.21 (between Purple Sacred basil and Lemon basil) to 0.99 (between Compatto FT Genovese basil and Gecofure basil) with an average of 0.581 (data not shown). The UPGMA clustering algorithm based on RAPD data grouped 37 accessions into four groups at a similarity coefficient of 0.4 (Fig. 2). The cophenetic correlation was calculated and indicated a very good fit (r = 0.99, Table 5). A total of 28 basil accessions belonging to *O. basilicum* were found in Group I which was consistent with the ISSR data except for slightly differ in branch positions. Compatto FT Genovese basil and Gecofure basil in Group I could not be distinguished from each other based on RAPD data. Group II, Group III and Group IV of RAPD clustering (Fig. 2) were the same as those of the ISSR clustering results (Fig. 1) except for some minor changes in branch positions. Based on SRAP clustering, the Jaccard's similarity coefficients ranged from 0.28 (between Sacred basil and East Indian basil) to 0.95 (between Spice basil and Blue Spice basil) with an average 0.566 (data not shown). The dendrogram contained three groups at a similarity coefficient of 0.41 (Fig. 3) and the cophenetic correlation showed a good fit (r = 0.99, Table 5). Except for Sweet Salad basil, 27 accessions were found in Group I identical to the results of the ISSR and RAPD dendrogram analysis. Group II included six accessions which belonged to three species (*O. basilicum*, *O. americanum*, *O. gratissimum*). Group III (Fig. 3) combined Groups III and IV of the ISSR dendrogram (Fig. 1). Group III contained four accessions in which Spice basil and Blue Spice basil from species *O. basilicum* could not be distinguished from each other and as were Sacred basil and Pruple Sacred basil from species *O. tenuiflorum*. Fig. 4 shows the dendrogram based on combined data set (ISSR, RAPD and SRAP) with the three groups clustered at a similarity coefficient of 0.4. Thus, the combined data gave results similar to those of SRAP alone (Fig. 3). The cophenetic correlation was r = 0.99 which suggested a very good fit (Table 5). Group I consisted of 27 accessions of *O. basilicum* (Fig. 4) with slight difference in branch positions as compared to the SRAP dendrogram (Fig. 3). Group II and III contained accessions similar to those in SRAP clustering. Spice basil and Blue Spice basil from species *O. basilicum* in Group III could not be distinguished from each other which was similar to that of of SRAP dendrogram, contrastingly, Sacred basil (*O. basilicum*) and Pruple Sacred basil (*O. basilicum*) (Fig. 3) could be distinguished from each other using the combined data set. This indicated that the combined data set provided higher genetic resolution than that of SRAP clustering.

Correlation among different marker systems

The correlations of similarity matrices generated by individual marker systems were estimated as were the correlations of cophenetic matrices produced from individual dendrograms (Table 5). Among individual marker types, the correlations of similarity matrices between ISSR and RAPD (0.97) were higher than those between RAPD and SRAP (0.93) and between ISSR and SRAP (0.89). In addition, the correlations of cophenetic matrices between ISSR and RAPD (0.98) were higher than those between RAPD and SRAP (0.93) and between ISSR and SRAP (0.90). When their relationships were compared with the similarity matrices of the combined data set, SRAP showed the highest correlation (0.99) whereas ISSR were the lowest (0.93) (Table 5). Based on the cophenetic matrix comparison, RAPD and SRAP showed the highest correlation with the cophenetic matrix of the combined data set at 0.98 each while ISSR gave the lowest at 0.96.

Discussion

Performance of different marker systems

In this study, 37 basil accessions were surveyed with three different marker systems: ISSR, RAPD and SRAP for genetic

Table 2. Results of ISSR analysis and ISSR primer information.

ISSR	Primer (5'-3')	NTL[a]	NPL[b]	P%[c]	PIC[d]	Rp[e]
UBC801	$(AT)_8T$	8	8	100	0.22	2.32
UBC807	$(AG)_8T$	9	9	100	0.19	2.16
UBC808	$(AG)_8C$	9	9	100	0.16	1.73
UBC809	$(AG)_9G$	10	10	100	0.14	1.84
UBC810	$(GA)_8T$	8	8	100	0.17	1.56
UBC811	$(GA)_8C$	5	5	100	0.29	1.89
UBC812	$(GA)_8A$	9	9	100	0.21	2.65
UBC817	$(CA)_8A$	10	9	90	0.13	1.51
UBC821	$(GT)_8T$	3	3	100	0.13	0.43
UBC822	$(TC)_8A$	4	4	100	0.26	1.41
UBC823	$(TC)_8C$	8	8	100	0.23	2.38
UBC824	$(TC)_8G$	8	8	100	0.15	1.35
UBC825	$(AC)_8T$	3	3	100	0.26	0.97
UBC826	$(AC)_8C$	3	3	100	0.28	1.14
UBC827	$(AC)_8G$	2	2	100	0.20	0.49
UBC828	$(TG)_8A$	4	4	100	0.21	1.03
UBC829	$(TG)_8C$	4	3	75	0.15	0.76
UBC834	$(AG)_8YT$	7	7	100	0.19	1.51
UBC836	$(AG)_8YA$	4	4	100	0.17	0.76
UBC840	$(GA)_8YT$	5	5	100	0.21	1.24
UBC841	$(GA)_8YC$	7	7	100	0.17	1.4
UBC842	$(GA)_8YG$	8	8	100	0.19	1.78
UBC844	$(CT)_8RC$	7	7	100	0.20	1.73
UBC846	$(CA)_8RT$	6	6	100	0.21	1.62
UBC847	$(CA)_8RC$	7	7	100	0.18	1.73
UBC848	$(CA)_8RG$	6	6	100	0.14	1.14
UBC850	$(GT)_8YC$	5	5	100	0.10	0.54
UBC855	$(AC)_8YT$	6	5	83	0.10	0.65
UBC856	$(AC)_8YA$	3	3	100	0.17	0.59
UBC857	$(AC)_8YG$	9	9	100	0.18	1.95
UBC858	$(TG)_8RT$	6	6	100	0.23	1.89
UBC860	$(TG)_8RA$	5	5	100	0.14	0.76
UBC861	$(ACC)_6$	3	2	67	0.12	0.43
UBC864	$(ATG)_6$	5	5	100	0.15	0.81
UBC881	$GGGT(GGGGT)_2G$	10	8	80	0.14	1.57
UBC886	$VDV(CT)_7$	7	7	100	0.25	2.38
Total		223	217	3495	6.88	50.1
Average		6.19	6.03	97	0.19	1.39

a: Number of total loci (NTL)
b: Number of polymorphic loci (NPL)
c: Polymorphic ratio (P %)
d: Polymorphic information content (PIC)
e: Resolving power (Rp)

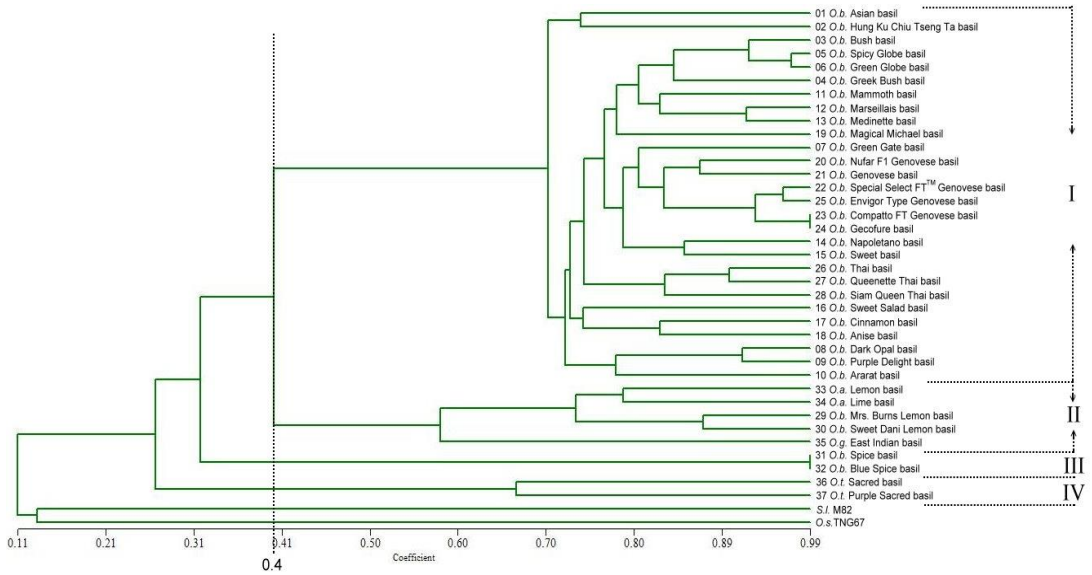

Fig 2. Jaccard's similarity dendrograms generated by UPGMA based on RAPD marker system which clustered into four groups.

diversity analysis. ISSR showed the highest polymorphic ratio (97%) followed by RAPD (95%) and SRAP (93%). The results were consistent with genetic diversity analysis of *Lentinula edodes* (Fu et al., 2010). In addition, high polymorphism of ISSR was also reported in many previous studies, for examples, in *Primula apennia* Widmer at species level (Crema et al., 2009), *Phyllanthus* species (Rout and Aparajita, 2010) and *Jatropha curcas* L. accessions (Grativol et al., 2011). Next, SRAP showed the highest average PIC and Rp at 0.29 and 30.19 compared to ISSR with values of 0.19 and 1.39 and RAPD with values of 0.23 and 5.13 in this study. This was consistent with previous reports in buffalo grass (Budak et al., 2004) and in *Cucurbita pepo* (Ferriol et al., 2003). In addition, SRAP markers have been shown a very efficient tool for genetic analysis in populations with low genetic variability such as watermelon and Turkish Olive varieties (Isik et al., 2011). Previous studies have also suggested that SRAP markers have performance than other tested molecular marker systems (Ferriol et al., 2003; Ruiz et al., 2005).

Genetic diversity analysis of basil accessions

In this study, similar average Jaccard's similarity coefficients were detected by different marker systems (0.596 by ISSR, 0.581 by RAPD and 0.566 by SRAP). However, the widest range of similarity was found in ISSR analysis (0.065 to 1) suggesting that higher genetic variations existed in the target genome regions than those targeted by RAPD (0.21 to 0.99) and SRAP (0.28 to 0.95) markers. Furthermore, four groups were obtained using ISSR or RAPD, while only three groups were obtained using SRAP or the combined data set based on clustering analysis by individual marker systems. This was consistent with the higher correlation of the ISSR and RAPD similarity matrices (0.97) and their cophenetic matrices (0.98) as compared to those between ISSR and SRAP (0.89 and 0.90) and between RAPD and SRAP (0.93 and 0.93). In previous studies, ISSR also showed more similar results in clustering of the dendrogram to RAPD than those to SRAP (Budak et al., 2004; Liu et al., 2008). This suggests that the regions targeted by SRAP are different from those by ISSR and RAPD markers. Different genome regions targeted by different DNA marker systems have been proposed in previous studies, for example, ISSR and RAPD presumably target noncoding regions whereas ISSR specifically targets regions between microsatellites (Parsons et al., 1997; William et al., 1990). On the other hand, SRAP has been reported to amplify mainly open reading frame (ORF) regions (Li and Quiros 2001). Since different DNA marker systems target different regions, mixed multiple marker systems should provide more complete genome information which is in agreement with the results obtained using the combined data set in this study and with several previous studies including genetic diversity analysis in rice (Davierwala et al., 2000), radish (Liu et al., 2008), mushroom (Fu et al., 2010), buffalo grasses (Budak et al., 2004), Jerusalem artichoke (Wangsomnuk et al., 2011), and Turkish melons (Yildiz et al., 2011). Almost all accessions belonging to *O. basilicum* were arranged in Group I, except four accessions, Mrs. Burns Lemon basil, Sweet Dani Lemon basil, Spice basil and Blue Spice basil. Interestingly, the former two, Mrs. Burns Lemon basil and Sweet Dani Lemon basil, were clustered in Group II and the latter two, Spice basil and Blue Spice basil, were clustered together in Group III in all dendrograms indicating these four accessions to be distantly related to the other *O. basilicum* accessions analyzed. Clustering of most *O. basilicum* accessions was also found in previous studies which examined genetic relationships among basil species by AFLP markers and DNA content (Carović-Stanko et al., 2010a) and which characterized *Ocimum* species by essential oils and AFLP (Carović-Stanko et al., 2011b). Furthermore, our study also obtained similar results to the result in Carović-Stanko et al. (2011b) in which Green Globe basil, Dark Opal basil, Sweet basil, Genovese basil and Queenette Thai basil were clustered in the same group. These observations suggest that these accessions are closely related to each other. The dendrogram of SRAP showed higher similarity to that of the combined data set than to those of ISSR and RAPD. For example, the basil accessions of O. *tenuiflorum* were arranged in Group IV by ISSR and RAPD but were in Group III by SRAP and the combined data set. Furthermore, all accessions of *O. americanum* and *O. gratissimum* were clustered in Group II together with two accessions of *O. basilicum* (Mrs. Burns Lemon basil and Sweet Dani Lemon basil) by ISSR and RAPD. With SRAP and the combined data set, *O. americanum* and *O. gratissimum* were clustered with three *O. basilicum* accessions, Mrs. Burns Lemon basil, Sweet Dani Lemon basil and Sweet Salad basil. Different species of basil have been studied previously using RAPD (Harisaranraj et al., 2008; Vieira et al., 2003; Singh et al., 2004) and AFLP markers (Moghaddam et al., 2011; Carović-Stanko et al., 2010a; Carović-Stanko et al., 2011b). Basils derived from different species grouping together as Group II in this study have been reported using RAPD and AFLP markers (Carović-Stanko et al., 2010a) indicating a wide range of genetic variations among *O. basilicum* accessions. In addition, no matter what marker system was used for analysis, Lemon basil, Lime basil (*O. americanum* accessions), East Indian basil (*O. gratissimum* accessions), and Sacred basil and Purple Sacred basil (*O. tenuiflorum* accessions) were clustered outside of Group I, in which most *O. basilicum* accessions grouped together. This suggests that *O. americanum* and *O. tenuiflorum* species are distant from *O. basilicum*, which is consistent with previous RAPD and AFLP studies that showed separation of *O. americanum, O. gratissimum and O. tenuiflorum* from most of the tested *O. basilicum* accessions (Carović-Stanko et al., 2011b). A total of eleven basil accessions could not be separated using ISSR clustering (nine accessions in Group I and two in Group III). Using RAPD clustering, four accessions could not be distinguished from one another including two in Group I and two in Group III. In SRAP analysis, only two accessions, Spice basil and Blue Spice basil could not be distinguished from each other and were clustered in Group III which was similar to the result using the combined data set. This observation further supports that SRAP and the combined data set showed better performance in basil accessions identification, as discussed above. This is consistent with previous reports on radish (Liu et al., 2008), buffalo grass (Budak et al., 2004), and Jerusalem artichoke (Wangsomnuk et al., 2011). Interestingly, Spice basil and Blue Spice basil were indistinguishable using all marker systems. This suggested that more primers or other type of markers would be necessary, or alternatively, these two basils might be identical accessions with different names. The correlation of similarity matrices based on individual marker system was estimated and so was the correlation of cophenetic matrix transferred from dendrogram. High correlation values (equal or greater than 0.9) were observed between almost any two marker systems, and these values were higher than similar studies in other plants species (Budak et al., 2004; Wangsomnuk et al., 2011; Yildiz et al., 2011). The combined data has been shown offering a better view of genome deviation and clear distribution in dendrogram (Budak et al., 2004; Fu et al., 2010). Therefore, the correlations of the dendrograms between the combined data set and each of the three individual marker systems were evaluated and the highest

Table 3. Results of RAPD analysis and RAPD primer information.

RAPD	Primer (5'-3')	NTL[a]	NPL[b]	P%[c]	PIC[d]	Rp[e]
OPA01	CAGGCCCTTC	11	10	91	0.24	3.73
OPA02	TGCCGAGCTG	6	6	100	0.36	2.97
OPA03	AGTCAGCCAC	9	6	67	0.10	1.03
OPA04	AATCGGGCTG	14	12	86	0.24	4.65
OPA05	AGGGGTCTTG	15	14	93	0.24	5.03
OPA06	GGTCCCTGAC	16	16	100	0.28	6.49
OPA07	GAAACGGGTG	23	22	96	0.29	9.30
OPA08	GTGACGTAGG	21	18	86	0.23	6.86
OPA09	GGGTAACGCC	17	16	94	0.17	3.62
OPA10	GTGATCGCAG	14	22	57	0.16	2.59
OPA11	CAATCGCCGT	25	14	56	0.20	6.54
OPA12	TCGGCGATAG	15	14	93	0.17	3.24
OPA13	CAGCACCCAC	15	14	93	0.17	3.14
OPA14	TCTGTGCTGG	14	13	94	0.24	4.43
OPA15	TTCCGAACCC	17	17	100	0.25	5.57
OPA16	AGCCAGCGAA	19	19	100	0.31	8.65
OPA17	GACCGCTTGT	19	19	100	0.25	6.65
OPA18	AGGTGACCGT	17	17	100	0.29	7.08
OPA19	CAAACGTCGG	28	28	100	0.22	8.32
OPA20	GTTGCGATCC	14	14	100	0.16	2.70
Total		329	311	1905	4.57	102.59
Average		16.45	15.55	95	0.23	5.13

a: Number of total loci (NTL)
b: Number of polymorphic loci (NPL)
c: Polymorphic ratio (P %)
d: Polymorphic information content (PIC)
e: Resolving power (Rp)

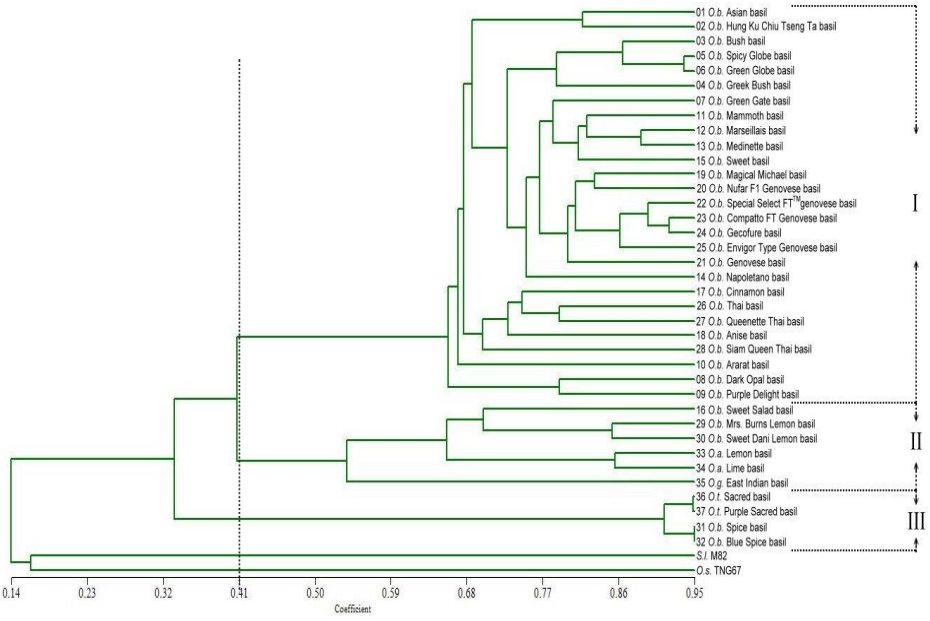

Fig 3. Jaccard's similarity dendrograms generated by UPGMA based on SRAP marker system which clustered into three groups.

correlation was detected between SRAP and the combined data set (0.99 between similarity matrices, 0.98 between cophenetic matrices). This suggests that SRAP could harbor discriminating power almost as good as the combined data set in genetic diversity evaluation among basil species.

Materials and methods

Plant materials and genomic DNA extraction

A total of 37 basil accessions representing four different species (*Ocimum basilicum, O. americanum, O. gratissimum* and *O. tenuiflorum*) including two accessions, Asian basil and Hung Ku Chiu Tseng Ta basil, were collected from KNOWN-YOU SEED company in Taiwan and the rest 35 accessions were collected from RICHTER HERBS company in Canada (http://www.richters.com/Web_store/web_store.cgi?show=list&prodclass=F002&cart_id=9734214.22070). All 37 accessions are maintained at Tainan District Agricultural Research and Extension Station (Table 1). Genomic DNA from leaves of 10 plants of each basil accession was extracted and bulked for further maker analysis (Fulton et al., 1995).

Table 4. Results of SRAP analysis and SRAP primer information.

SRAP	Primer (5'-3')		Primer (5'-3')	NTL[a]	NPL[b]	P%[c]	PIC[d]	Rp[e]
me1	TGAGTCCAAACCGGATA	em2	GACTGCGTACGAATTTGC	94	88	94	0.29	38.38
		em3	GACTGCGTACGAATTGAC	59	59	100	0.29	25.3
		em4	GACTGCGTACGAATTTGA	72	66	92	0.28	29.03
		em5	GACTGCGTACGAATTAAC	68	63	93	0.28	26.32
		em6	GACTGCGTACGAATTGCA	56	53	95	0.32	25.78
me2	TGAGTCCAAACCGGAGC	em1	GACTGCGTACGAATTAAT	90	83	92	0.31	40.59
		em2	GACTGCGTACGAATTTGC	61	56	92	0.28	24.27
		em3	GACTGCGTACGAATTGAC	85	78	92	0.28	32.92
		em4	GACTGCGTACGAATTTGA	101	94	93	0.29	42.81
		em5	GACTGCGTACGAATTAAC	55	38	69	0.26	16.49
Total				741	678	912	2.88	301.89
Average				73.4	68.5	93	0.29	30.19

a: Number of total loci (NTL)
b: Number of polymorphic loci (NPL)
c: Polymorphic ratio (P %)
d: Polymorphic information content (PIC)
e: Resolving power (Rp)

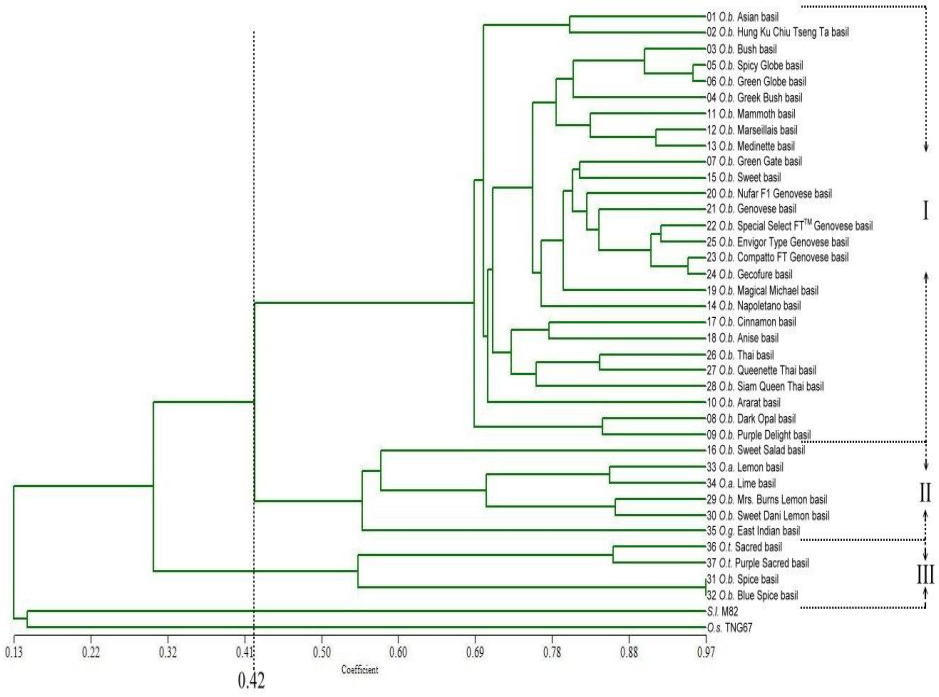

Fig 4. Jaccard's similarity dendrograms generated by UPGMA based on combined data set (ISSR, RAPD and SRAP) which clustered into three groups.

ISSR analysis

Originally, a total of 100 ISSR markers (The University of British Columbia #9, UBC #9) were screened on two accessions, *Ocimum basilicum* and *O. gratissimum*. Thirty-six ISSR markers (Table 2) which produced clear and repeatable bands were then selected for further analysis of all 37 accessions. The 12.5 µl PCR reactions contained: 20 ng DNA, 10X buffer (20 mM pH8.4 Tris-HCl, 50 mM KCl), 1 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM dNTP, and 0.8 µM primer. The PCR amplification protocol consisted of one cycle of 5 min at 94˚C, followed by 42 cycles of 30 sec at 94˚C, 1 min at 55˚C, 1 min at 72˚C and 5 min for final extension at 72˚C.

RAPD analysis

Twenty RAPD markers (Operon Technologies, Alemada, USA, Table 3) were employed for analysis. The 25 µl PCR reactions contained 40 ng DNA, 10X PCR reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 1 U *Taq* DNA polymerase, 0.1 mM dNTP, 0.4 µM primer and 1.2 mM $MgCl_2$. The amplifications were programmed as 1 cycle of 5 min at 94˚C, then 45 cycles of 1 min at 94˚C; 2 min at 36˚C; 2 min at 72˚C, followed by a final extension for 7 min at 72˚C.

SRAP analysis

Ten primer combinations of SRAP markers were performed and followed the method reported by Li and Quirons (2001) (Table 4). The 25 µl PCR reactions contained: 40 ng DNA, 10X PCR reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 1 U *Taq* DNA polymerase, 0.2 mM dNTP, 0.3 µM forward primers, 0.3 µM reverse primers and 1 mM $MgCl_2$. PCR reactions were performed as follows: initial step of 5 min at 94˚C, then 5 cycles of 1 min at 94˚C, 1 min at 35˚C, 1 min at

Table 5. Correlation coefficients among individual marker systems used and the combined data set.

Correlation	ISSR	RAPD	SRAP	ISSR + RAPD + SRAP
ISSR	1.00	0.97	0.89	0.93
RAPD	0.98	0.99	0.93	0.96
SRAP	0.90	0.93	0.99	0.99
ISSR + RAPD + SRAP	0.96	0.98	0.98	0.99

Above diagonal values represent correlation coefficients between similarity matrices and below diagonal values represent correlation coefficients between cophenetic matrices. Values on the diagonal represent cophenetic correlation for markers. Mantel's test with 1000 permutations.

72˚C followed by 35 cycles comprised 1 min at 94˚C, 1 min at 50˚C, 2 min at 72˚C, and a final extension for 10 min at 72˚C then cooling down to 15˚C. The PCR products amplified by ISSR and RAPD markers were separated in 2.0% agarose gel in 0.5X TBE buffer (44.6 mM Tris, 44.6 mM Boric acid, 1.2 mM EDTA) for 90 min at 100V, stained with 0.5 µg/ml ethidium bromide (EtBr) for 15 min, and photographed under UV (supplementary Fig. 1 and 2). PCR products amplified by SRAP markers were visualized after electrophoresis in 4% acrylamide gels in 0.5X TBE running for 2 hours at 1600 V, then silver stained as described in Bassam et al. (1991) (supplementary Fig. 3).

Data analysis

Reproducible bands from individual marker systems were scored as present "1" and absent "0" which were compiled as a binary matrix. Number of total loci (NTL) and number of polymorphism loci (NPL) were calculated for each primer. Polymorphic ratio (P%) was calculated based on NPL/NTL. For dominant markers such as ISSR, RAPD and SRAP, each locus contains the maximum of two alleles (presence and absence) instead of multiple allleles (>2) where different sizes of bands amplified by individual marker are assumed to be alleles of the same locus. The polymorphism information content (PIC) of individual locus was calculated as $2\ fi(1 - fi)$, where *fi* indicates the frequency of bands presence for locus *i* (Roldán-Ruiz et al., 2000). Resolving power (Rp) for individual marker system was calculated based on $Rp=\Sigma Ib$, where Ib (informativeness) takes the value of $1-[2 \times |0.5-p|]$, and p is the ratio of present bands among 37 tested accessions (Prevost and Wilkinson 1999). Pairwise genetic similarity of individual marker system (ISSR, RAPD, SRAP or the combined data sets) was estimated using Jaccard's coefficient (Jarccard 1908). A dendrogram was constructed by cluster analysis using the Unweighted Pair Group Method Arithmetic (UPGMA) method. To understand the relationships between the dendrograms and similarity matrices, cophenetic matrices were estimated and pairwise comparison of the matrices based on different marker systems was performed by Mantel test with 1000 permutations using software NTSYS-pc ver. 2.10e (Rohlf 2000).

Conclusions

In this study, three marker systems, RAPD, ISSR and SRAP, were applied for genetic diversity analysis of 37 basil accessions. This study has demonstrated that ISSR, RAPD, SRAP individually and in combination are efficient tools to study genetic diversity in basil accessions. Results obtained by SRAP markers analysis were more similar to those of the combined data set which were better tools for clustering basil accessions than ISSR and RAPD. This study provided valuable information for potential applications of these three marker systems in molecular breeding of basil.

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