

Analysis based on RAPD and ISSR markers reveals closer similarities among *Citrullus* and *Cucumis* species than with *Praecitrullus fistulosus* (Stocks) Pangalo

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Abstract

A cucurbit species named *Praecitrullus fistulosus* (Stocks) Pangalo, which thrives in India, is considered to be a distant relative of watermelon. Recent experiments indicated that it has mild resistance to whiteflies (*Bemisia tabaci*). However, our attempts to cross various US plant introductions (PIs) of *P. fistulosus* with watermelon or other *Citrullus* PIs have not been successful. Thus, to determine genetic relatedness among those species, phylogenetic analysis [based on simple sequence repeat (SSR)-anchored (also termed ISSR), and randomly amplified polymorphic DNA (RAPD) markers] was conducted among PIs of *P. fistulosus*, *Citrullus lanatus* var. *lanatus* (watermelon), *C. lanatus* var. *citroides* and the wild *Citrullus colocynthis*. Phylogenetic relationships were also examined with *Cucumis melo* (melon), *Cucumis sativus* (cucumber), and wild *Cucumis* species including *C. africanus*, *C. metuliferus*, *C. anguria*, *C. meeusei*, and *C. zeyheri*. Wide genetic distance exists between *Citrullus* and *Cucumis* groups (8% genetic similarity). Phylogenetic relationships among *Citrullus* species and subspecies are closer (25–55% genetic similarity) as compared with those among most *Cucumis* species (14–68% genetic similarity). *P. fistulosus* appeared to be distant from both *Cucumis* and *Citrullus* species (genetic similarity between *P. fistulosus* and *Cucumis* or *Citrullus* groups is less than 3%). Although wide genetic differences and reproductive barriers exist among cucurbit species examined in this study, they are still considered as potential germplasm source for enhancing watermelon and melon crops using traditional breeding and biotechnology procedures.

Introduction

Citrullus Schrad. ex Eckl. et Zeyh. is a major genus of the *Cucurbitaceae*, and consists of four known diploid ($n = 11$) species: (1) *Citrullus lanatus* (Thunb.) Matsum. et Nakai that exists in tropical and subtropical climates worldwide and includes the cultivated watermelon (*C. lanatus* var. *lanatus*) and the preserving melon (*C. lanatus* var. *citroides*) (L. H. Bailey) Mansf. ex Grebo (Whitaker and Davis 1962; Whitaker and Bemis 1976; Jarret et al. 1997); (2) the perennial bitter gourd, *Citrullus*

colocynthis (L.) Schrad., which grows in sandy areas throughout northern Africa, southwestern Asia and the Mediterranean (Zamir et al. 1984; Burkill 1985; Jarret et al. 1997); (3) the perennial species *C. ecirrhosus* Cogn. (Meeuse 1962); and (4) the annual species *C. rehmii* B. DeWinter (De Winter 1990). Both *C. ecirrhosus* and *C. rehmii* are endemic to the desert regions of Namibia (Meeuse 1962). *Praecitrullus fistulosus* (Stocks) Pangalo is cultivated in India and Pakistan and is considered to be a distinct *Citrullus* (Whitaker and Davis 1962; Khoshoo and Vij 1963; Singh 1990).

It is similar in gross morphology to *Citrullus* species, but differs from them in chromosome number ($n = 12$), pollen morphology and the absence of urease in the seeds (Pangalo 1944; Whitaker and Davis 1962).

Obtaining resistance to diseases and pests is a major objective in most breeding programs of important vegetable crops. However, because of limited resistance within US plant introductions (PIs) of *C. lanatus* var. *lanatus*, limited progress has been accomplished in this respect in watermelon. Although there is great phenotypic diversity among watermelon cultivars developed in the United States, they appear to have a narrow genetic background (Levi et al. 2001a, b). Enhancing disease and pest resistance of watermelon cultivars and improving their response to environmental stress require widening their genetic background through crosses with diverse *Citrullus* accessions. Over 1600 US PIs have been collected from diverse geographical regions throughout the world and are maintained at the US Department of Agriculture, Agriculture Research Service (USDA, ARS), Plant Genetic Resources and Conservation Unit in Griffin, Georgia, USA. The US PI *Citrullus* collection contains 1400 *C. lanatus* var. *lanatus* PIs, 88 *C. lanatus* var. *citroides* PIs, 28 *C. colocynthis* PIs, and 22 *Praecitrullus fistulosus* PIs. According to the USDA, ARS, Germplasm Resources Information Network (GRIN; Online Database, National Germplasm Resources Laboratory, Beltsville, Maryland; www.ars-grin.gov), 48 PIs in this collection were reported to contain resistance to pathogens (Levi et al. 2001a) or pests (Simmons and Levi 2002a, b). In preliminary observations in the greenhouse, all *P. fistulosus* PIs appeared to have mild resistance to the B-biotype sweetpotato whitefly (*Bemisia tabaci*), which has been emerging as a major pest causing severe damage to watermelon crops in various parts of the world (Simmons and Levi 2002a). Although various cucurbit species that are resistant to diseases and pests have crossing barriers with watermelon, they are still considered potential sources of germplasm for improving this crop. "Extensive utilization of genetic resources is the ultimate objective of all undertakings in the field of germplasm resources and crop improvement" (Li et al. 1998). Accordingly, determining the phylogenetic relationships of related species is an essential part in classifying

germplasm and in identifying resistance sources that can be utilized in crop improvement using conventional breeding or biotechnology procedures (Li et al. 1998).

Praecitrullus fistulosus may be a useful source of resistance to whiteflies for the improvement of watermelons (Simmons and Levi 2002b). Based on gross morphology and cytology, Khoshoo and Vij (1963) determined that *P. fistulosus* is likely to be a distinct *Citrullus*. *P. fistulosus* was originally named as *C. vulgaris* var. *fistulosus* Duthie and Fuller. Pangalo (1944) indicated that it might be an ancestor of contemporary watermelon because of the similar morphology and suggested to name it as *P. fistulosus* (Singh 1990; Jeffrey 2001). This author also considered *P. fistulosus* as related to *Cucumis* because of similar chromosome number ($n = 12$). Navot and Zamir (1987) examined phylogeny among *Citrullus* species and showed that *P. fistulosus* is different from all *Citrullus* spp. However, there is no published information about the phylogenetic relationships of *P. fistulosus* with *Citrullus* or *Cucumis* species using DNA (ISSR and RAPD) markers. Thus, a question that has been asked is how distant is *P. fistulosus* (Stocks) Pangalo from watermelon (*C. lanatus* var. *lanatus*) as compared with other *Citrullus* and *Cucumis* species? Also, how extensive is the genetic diversity among PIs of *P. fistulosus* (collected in India) as compared with PIs of *Citrullus* and *Cucumis* species?

The primary objective of this study was to use ISSR and RAPD markers to examine the phylogenetic relationship of *P. fistulosus* (Stocks) Pangalo with watermelon (*C. lanatus* var. *lanatus*), *C. lanatus* var. *citroides*, and *C. colocynthis*. A secondary objective was to examine the phylogenetic relationships of *P. fistulosus* (Stocks) Pangalo with *Cucumis melo* L. (melon), *Cucumis sativus* L. (cucumber), *Cucumis metuliferuse*, *Cucumis africanus* L.f., *Cucumis anguria* L., *Cucumis mescusei* L. Jeffrey and *Cucumis zeyheri* Sonder.

Material and methods

Plant material

Three watermelon cultivars ('Charleston Gray', 'Black Diamond' and 'New Hampshire Midget') were provided by Syngenta seeds. Two *C. lanatus*

Table 1. Species, chromosome number (2N), and country of collection for watermelon cultivars and US PIs.

Accession	Species	2N	Country
Charleston Gray	<i>C. lanatus</i> var. <i>lanatus</i>	22	USA
New Hampshire Midget	<i>C. lanatus</i> var. <i>lanatus</i>	22	USA
Black Diamond	<i>C. lanatus</i> var. <i>lanatus</i>	22	USA
PI 169290	<i>C. lanatus</i> var. <i>lanatus</i>	22	Turkey
PI 270550	<i>C. lanatus</i> var. <i>lanatus</i>	22	Ghana
PI 299378	<i>C. lanatus</i> var. <i>citroides</i>	22	South Africa
PI 244018	<i>C. lanatus</i> var. <i>citroides</i>	22	South Africa
PI 271779	<i>C. lanatus</i> var. <i>citroides</i>	22	South Africa
PI 386024	<i>C. colocynthis</i>	22	Iran
PI 386019	<i>C. colocynthis</i>	22	Iran
PI 220778	<i>C. colocynthis</i>	22	Afghanistan
PI 381749	<i>P. fistulosus</i>	24	India
PI 174812	<i>P. fistulosus</i>	24	India
PI 381753	<i>P. fistulosus</i>	24	India
PI 271467	<i>P. fistulosus</i>	24	India
PI 217522	<i>P. fistulosus</i>	24	India
PI 271363	<i>P. fistulosus</i>	24	India
PI 381742	<i>P. fistulosus</i>	24	India
PI 381752	<i>P. fistulosus</i>	24	India
PI 381474	<i>P. fistulosus</i>	24	India
PI 381751	<i>P. fistulosus</i>	24	India
PI 381750	<i>P. fistulosus</i>	24	India
PI 381745	<i>P. fistulosus</i>	24	India
PI 381743	<i>P. fistulosus</i>	24	India
Ananas Yokneam	<i>C. melo</i>	24	Israel
SMR-58	<i>C. sativus</i>	14	USA
PI 542127	<i>C. africanus</i>	24	Botswana
PI 542135	<i>C. anguria</i>	24	Botswana
PI 376068	<i>C. meeusei</i>	48	USA
PI 532629	<i>C. zeyheri</i>	48	Zimbabwe
PI 482441	<i>C. metuliferus</i>	24	Zimbabwe
PI 482443	<i>C. metuliferus</i>	24	Zimbabwe
PI 482444	<i>C. metuliferus</i>	24	Zimbabwe
PI 482448	<i>C. metuliferus</i>	24	Zimbabwe
PI 482458	<i>C. metuliferus</i>	24	Zimbabwe
PI 482459	<i>C. metuliferus</i>	24	Zimbabwe
PI 482460	<i>C. metuliferus</i>	24	Zimbabwe
PI 505598	<i>C. metuliferus</i>	24	Zambia
PI 527568	<i>C. metuliferus</i>	24	Burundi

var. *lanatus*, three *C. lanatus* var. *lanatus*, three *C. colocynthis*, and 13 *P. fistulosus* PIs (Table 1) were obtained from the US PI *Citrullus* germplasm collection (USDA, ARS, Plant Genetic Resources and Conservation Unit at Griffin, Georgia, USA). All 15 *Cucumis* PIs (Table 1) were provided by the USDA, ARS, North Central Regional Plant Introduction Station at Ames, Iowa. Five plants of each PI were grown in the greenhouse and young leaves (2-week-old plants) were collected for DNA isolation.

Isolation of DNA

To avoid co-isolation of polysaccharides, polyphenols and other secondary compounds that damage DNA, we used an improved CTAB procedure for isolation of DNA from young leaves of watermelon or melon plants (Levi and Thomas 1999).

DNA amplification conditions and gel electrophoresis

Ten-decamer oligonucleotides were purchased from the University of British Columbia, Biotechnology Center (British Columbia, Canada) and from Operon Technologies Inc. (Alameda, California) and were used for PCR amplification as described by Levi et al. (1993) and by Rowland and Levi (1994) (Table 2). RAPD reactions were in 25- μ L reaction buffer containing 20 μ M NaCl, 50 mM Tris-HCl pH 9, 1% Triton-X-100, 0.01% gelatin, 1.6 mM MgCl₂, 200 μ M each of dATP, dCTP, dGTP and dTTP (Sigma; St. Louis, Missouri), 0.2 μ M primer, 7 units Taq DNA Polymerase supplied in storage buffer A (Promega; Madison, WI), and 25 ng template DNA. Amplification reactions were carried out for 45 cycles in a 'PTC-200 Thermocycler' (MJ Research; Watertown, Massachusetts), programmed for 60 s for DNA to denature at 92 °C, 70 s for DNA annealing at 48 °C and 120 s for DNA transcription at 72 °C. Simple sequence repeat (SSR)-anchored (also termed ISSR) primers with 15–20 decamer oligonucleotides were purchased from the University of British Columbia (primer # 800–899). The amplification conditions for ISSR primers were the same as for the RAPD primers, except for the DNA annealing temperature optimized for each primer (Table 2). Amplification products were separated by electrophoresis in 1.4% agarose gels in 0.5 \times Tris-borate buffer (Sambrook et al. 1989). The gels were stained with 0.5 μ g per mL ethidium bromide solution for 30 min and destained for 15 min in distilled water. DNA fragments were visualized under UV light and photographed using a still video system (Gel Doc 2000, Bio-Rad, Hercules, CA). The molecular weights of the amplification products were calculated using the 100-bp or 1 Kb plus DNA ladder standards (Gibco BRL/Life Technology; Rockville, Maryland).

Table 2. The nucleotide sequences of SSR-anchored (ISSR) and RAPD primers, optimal annealing temperature, and number of polymorphic markers produced by each primer.

Primer	Sequence	Annealing temperature (C)	Number of markers
808 ^a	AGAGAGAGAGAGAGAGC	59	14
809	GAGGAGAGAGAGAGAGG	59	20
810	GAGAGAGAGAGAGAGAT	53	19
812	GAGAGAGAGAGAGAGAA	53	19
813	CTCTCTCTCTCTCTT	53	19
816	CACACACACACACAT	54	8
824	TCTCTCTCTCTCTCG	54	16
825	ACACACACACACACT	54	24
826	ACACACACACACACC	62	12
827	ACACACACACACACG	62	22
829	TGTGTGTGTGTGTGC	62	8
834	AGAGAGAGAGAGAGAGCTT	59	13
835	AGAGAGAGAGAGAGAGCTC	59	14
889	AGTCGTAGTACACACACACAC	62	20
731	CCCACACCAC	49	27
B06 ^b	TGCTCTGCC	49	25
I12	AGAGGGCACA	49	21

^aPrimers from University of British Columbia.

^bPrimers from Operon, Inc.

Data analysis

A pairwise similarity matrix was generated using the Nei–Li similarity index (Nei and Li 1979) according to the equation: $\text{similarity} = 2 N_{ab} / (N_a + N_b)$, where N_{ab} is the number of PCR (ISSR + RAPD) fragments shared by two genotypes (a and b), and N_a and N_b are the total number of PCR (ISSR + RAPD) fragments analyzed in each genotype. A dendrogram was constructed based on the similarity matrix data by applying the unweighted pair-group method with arithmetic average (UPGMA) cluster analysis using the Numerical Taxonomic and Multi-Variant Analysis System for PC (NTSYS-PC version 2) (Rohlf 1993).

Results and discussion

Genetic diversity among *Citrullis* and *Cucumis* species

The ISSR and RAPD markers (Table 2 and Figure 1) revealed low genetic similarity values (8%) between *Cucumis* and *Citrullus* groups and significantly lower genetic similarity (less than 3%) with *P. fistulosus* (Stocks) Pangalo (Figure 2). The highest genetic relatedness (95% genetic similarity) exists among watermelon cultivars

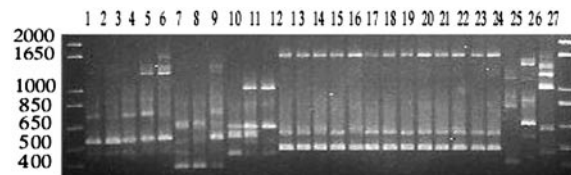


Figure 1. ISSR markers produced by primer 810 (University of British Columbia). Lanes 1–3 are watermelon cultivars Charleston Gray, New Hampshire Midget and Black Diamond, lanes 4–5 are *C. lanatus* var. *lanatus* PI 169290 and PI 270550, lanes 6–8 are *C. lanatus* var. *citroides* PI 299378, PI 244018 and PI 271779, and lanes 9–11 are *C. colocynthis* PI 386024, PI 386019, and PI 220778. Lanes 12–24 are *P. fistulosus* (Stocks) Pangalo PI 381749, PI 174812, PI 381753, PI 271467, PI 21752, PI 271363, PI 381742, PI 381752, PI 381474, PI 381751, PI 381750, PI 381745, and PI 381743. Lane 25 is melon (*C. melo*; Ananas Yokneam). Lane 26 is cucumber (*C. sativus*; SMR-58), and lane 27 is *C. metuliferus* (PI 482439). Lanes on each side are molecular size markers ‘1 Kb-plus ladder’ (GibcoBRL/LifeTechnology; Rockville, Maryland).

(Figure 2). High genetic similarities (82–87%) also exist between watermelon cultivars and PIs of *C. lanatus* var. *lanatus*. A wide genetic distance exists between PIs of *C. lanatus* var. *lanatus* and *C. lanatus* var. *citroides* (overall 55% genetic similarity), while wider genetic distance exists between *C. lanatus* and the wild species *C. colocynthis* (25% genetic similarity; Figure 2). These results are in agreement with previous studies using

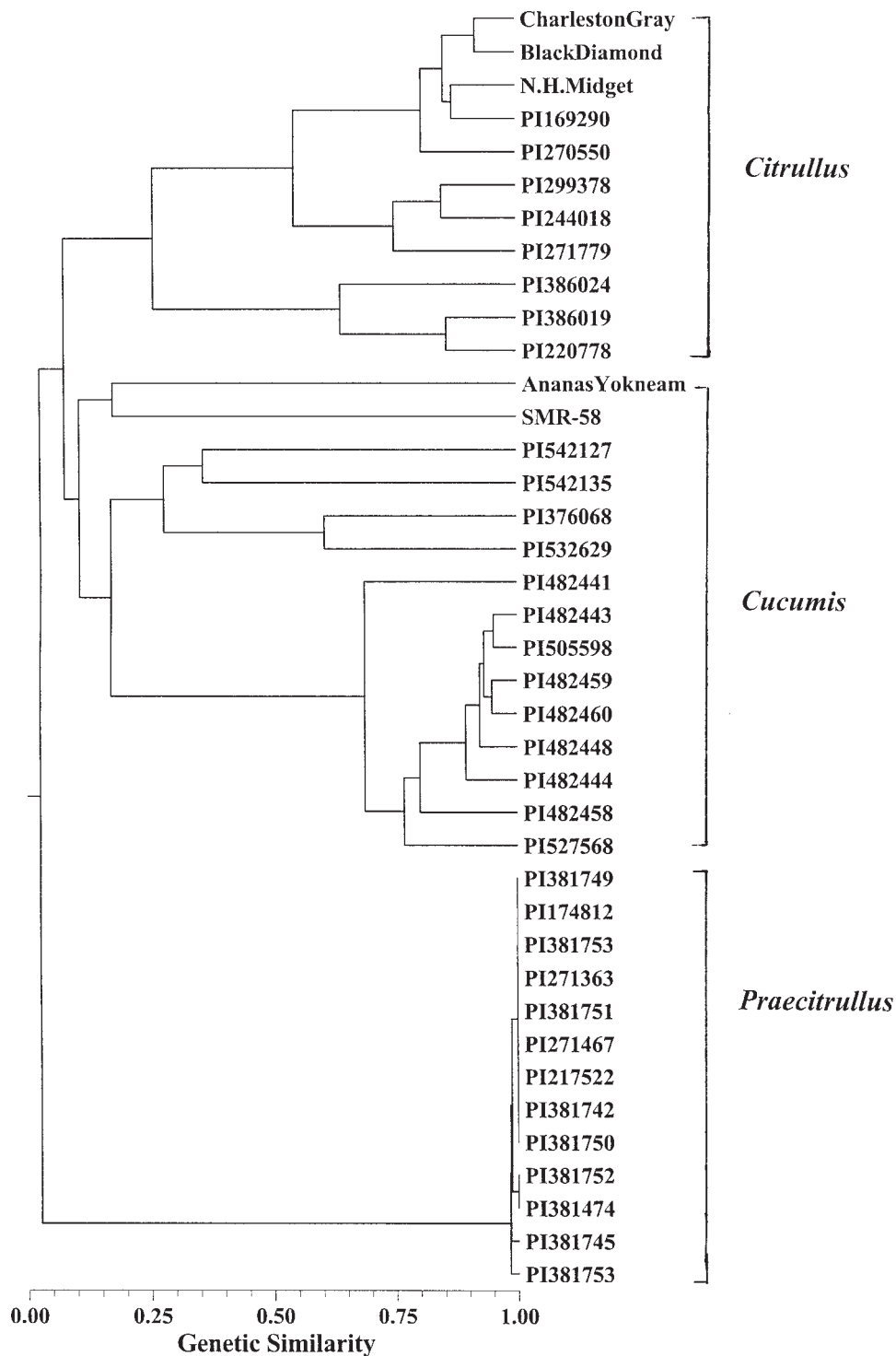


Figure 2. Dendrogram showing phylogenetic relations among PIs of *Citrullus*, *Cucumis*, and *P. fistulosus* (Stocks) Pangalo. The upper branch includes all watermelon cultivars and *Citrullus* species, the middle branch includes all *Cucumis* species, while the lower branch includes all *P. fistulosus* PIs with no genetic diversity.

isozymes (Navot and Zamir 1987) and simple sequence repeats (SSRs) (Jarret et al. 1997). Watermelon varieties and PIs of (*C. lanatus* var. *lanatus*) are readily cross-pollinated with most PIs of *C. lanatus* var. *citroides* (Whitaker and Bemis 1976). Although there is wide genetic distance between *C. lanatus* and *C. colocynthis*, there are no strong genetic barriers between these two *Citrullus* species. The F1 hybrid plants between *C. lanatus* and *C. colocynthis* may occasionally be self-sterile, but the F1 plants can be readily backcrossed or testcrossed with *C. lanatus* or *C. colocynthis* plants (Jeffrey 1975; Zamir et al. 1984; Levi et al. 2002).

Wide genetic distance exists between PIs of *Cucumis* and *Citrullus* species (overall, 8% genetic similarity; Figure 2). The genus *Cucumis* includes at least 26 known species (Kirkbride 1993). Wide genetic differences exist among representative PIs *Cucumis* species (11–61% genetic similarity) as compared with those among the *Citrullus* species (25–55% genetic similarity). There are wide genetic distances among *C. melo*, *C. sativus*, *C. anguria*, *C. africanus*, *C. meeusei*, and *C. zeyheri* (11–61% genetic similarity), while the smallest genetic distance is between the last two species (61% genetic similarity). Relatively wide genetic diversity exists within *Cucumis metuliferus* (67–97% genetic similarity among PIs), while PI 4822441 is most divergent among PIs of this group (Figure 2). *C. metuliferus*, also known as African horned cucumber, was reported as resistant to southern root-knot nematode, *Meloidogyne incognita* (Fassuliotis 1967; Wehner et al. 1991; Walters et al. 1993), powdery mildew and aphids (Clark et al. 1972), and squash mosaic virus and watermelon mosaic virus 1 (Provvidenti and Robinson 1974). However, numerous attempts to cross *C. metuliferus* with melon, *C. melo* (Fassuliotis 1977; Norton and Granberry 1980), or with cucumber, *C. sativus* (Walters and Wehner 2002) failed to produce viable seeds. Further experiments are needed in crossing *C. metuliferus* with other closely related *Cucumis* species, and in developing genetic populations that would be useful for mapping and cloning the genes that confer disease and pest resistances in that cucumis species. PIs of *C. anguria* and *C. zeyheri* were reported to be resistant to gummy stem blight [*Didymella bryoniae* (Auersw.) Rehm] (Wehner and St. Amand 1993), while PIs of *C. metuliferus* and *C. anguria* were resistant to

cucurbit yellowing stunting disorder virus (CYSDV) transmitted by whiteflies (*B. tabaci*) (Lopez-Sese and Gomez-Guillamon 2000). Genetic distances between *C. anguria* (PI 542135) and *C. africanus* (PI 542127) (35% genetic similarity) and between *C. meeusei* (PI 376068) and *C. zeyheri* (PI 532629) (61% genetic similarity) (Figure 2) are in agreement with crossability results among these species as summarized by Chen and Adelberg (2000).

Genetic relatedness among P. fistulosus and Citrullus and Cucumis species

Although *Praecitrullus fistulosus* (Stocks) Pangalo has been treated as relative of *Citrullus* spp., our data suggest that it is distinct and distant from all *Cucumis* and *Citrullus* species. The genetic similarity between *P. fistulosus* and *Cucumis* or *Citrullus* groups is overall less than 3% (Figure 2).

In agreement with the wide genetic distance (Figure 2) our pollination experiments between *C. lanatus* (PI 169290, PI 271779, PI 560901, Allsweet, Crimson Sweet, Black Diamond) or *C. colocynthis* (PI 386015, PI 386016, PI 386019, PI 386024) and *P. fistulosus* (PI 381753, PI 271467, PI 381742 and PI 381474) failed to produce viable seeds (Levi 2002; unpublished data), suggesting a complete genetic barrier between *C. lanatus* or *C. colocynthis* and *P. fistulosus*. Khoshoo and Vij (1963) also reported unsuccessful attempts to cross between *C. lanatus* and *P. fistulosus* (Singh 1990). *C. colocynthis* was recently reported to contain resistance to whiteflies (Simmons and Levi 2002b). It has the widest geographical distribution among *Citrullus*, thriving in Central and North Africa, the Middle East and in Central and South West Asia including Persia, Afghanistan, Pakistan and India (Whitaker and Davis 1962; Khoshoo and Vij 1963; Navot and Zamir 1987). Thus, a possibility of an evolutionary link between *C. colocynthis* and *P. fistulosus* has not been ruled out (Khoshoo and Vij 1963). Although *P. fistulosus* differs from all *Citrullus* species in growth habit, leaf-shape, tendrils, fruit, seed, pollen shape and size, and in basic chromosome number (Table 1), it is still treated as a *Citrullus* related type (Khoshoo and Vij 1963). Pangalo (1944) suggested that *P. fistulosus* might be a distinct genus related to *Cucumis*. This consideration is also due to the basic chromosome number (12) common to *P. fistulosus* and *Cucumis*

species (Khoshoo 1955). However, the extensive dissimilarities in DNA patterns between *P. fistulosus* and *Citrullus* or *Cucumis* (Figures 1 and 2) indicate that it is not as closely related to either genus, and should perhaps be treated as a distant cucurbit type. The status of *P. fistulosus* among cucurbit species needs further evaluation. There is little or no genetic diversity among the *P. fistulosus* PIs (Figures 1 and 2). Thus, additional *P. fistulosus* genotypes need to be collected from diverse regions in India, and evaluated for genetic diversity and for disease and pest resistances.

The present study revealed extensive differences in DNA patterns consistent with crossing barriers among wild *Cucumis* species and melon (*C. melo*) and cucumber (*C. sativus*), and between *Cucumis* and *Citrullus*. A scheme that includes traditional genetic experiments combined with molecular procedures might be considered in the overall strategy to identify and clone genes that confer disease or pest resistances in wild cucurbit species that have crossing barriers with cultivated varieties. Developing mapping populations by crossing resistant and susceptible genotypes of the same species and mapping the resistance genes is an essential step in the process of mapping and cloning resistance genes. Elucidating the resistance mechanisms is also vital prior to any attempt to introduce the genes conferring the resistance into watermelon or melon using genetic transformation procedures. In a recent study (Thies and Levi 2002) *C. lanatus* var. *citroides* PIs had higher resistance to root-knot nematode as compared with *C. lanatus* var. *citroides* PIs. However, most *Citrullus* PIs maintained at the USDA, ARS, Plant Genetic Resources and Conservation Unit (Griffin, Georgia, USA) are of *C. lanatus* var. *lanatus* (1480 PIs) while only 102 PIs are *C. lanatus* var. *citroides* (GRIN; Online Database, National Germplasm Resources Laboratory, Beltsville, Maryland, USA; www.ars-grin.gov). Thus, further expeditions and collections of wild *Citrullus* may be considered for broadening the genetic base and enhancing watermelon for disease and pest resistances.

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