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ISSR Markers Reveal Genetic Polymorphism in Two Morphological Variants of *Hyptis suaveolens* Invasive to India

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ABSTRACT

A white-flowering variant of the invasive *Hyptis suaveolens* (Lamiaceae) was discovered in India and is reported in this study. Its genetic diversity was measured using 15 ISSR markers. PCR-amplified products were visualized using agarose gel electrophoresis. Traditional morphological traits such as leaf morphology, length of calyx bristles, colour of the petals, among others, were useful in unveiling infraspecific variation in *H. suaveolens.* Polymorphism was 35% between the white-flowering and typical blue forms. Maximum polymorphism was detected by primer Hy6 in 9 alleles while only one allelic variation was found for each of primers Hy10, Hy13, Hy14 and Hy15. ISSR molecular markers are useful by disclosing genetic differences underscoring morphological variations in *H. suaveolens*. Minor differences, however prevents a taxonomic status to be assigned to the white-flowering variant.

Keywords: Genetic polymorphism, India, ISSR markers, PCR, white-flowered *Hyptis suaveolens*

INTRODUCTION

The genus *Hyptis* Jacq. (Lamiaceae) includes 300 species, most of which are native to the tropical America (Harley 1988). One of them, *Hyptis suaveolens* (L.) Poit. (bamburral, pignut, stinking roger or wild spikenard) is now naturalized in most parts of India, having been introduced earlier than 1885 (Hooker 1885). It is used as mosquito repellant (Pal and Jain 1998) and for conjunctivitis (Sikarwar 1996). It invaded even protected habitats like parks and preserves, including wildlife sancturies (Murthy *et al*. 2007; Sharma *et al*. 2009). Now, it is not only a ruderal weed alongside national roadways and railway tracks but also common at the foothills of open forests, forest clearings and wastelands, particularly arid and rocky substrates, and invasive in riparian systems throughout peninsular India. It is a potent invader, capable of heavy infestations displacing the native flora (Rao *et al*. 1998; Murthy *et al*. 2007; Sharma *et al.* 2009).

A high reproductive ability, dispersal rates and allelopathic potential (Rao *et al.* 1998; Raizada 2006) make *H. suaveolens* a strong competitor and successful weed (Zetkiewicz *et al.* 1994). A matrix model was presented by Schwarzkopf *et al.* (2009) for the population dynamics of *H. suaveolens*, which is a serious threat to the natural flora and causes a loss of biodiversity in different tropical habitats (Murthy *et al.* 2007).

During a field exploration for exotic flora of India (Reddy *et al*. 2008), we rediscovered a population of *H. suaveolens* in Karimnagar, with white flowers, and which was earlier recorded by Naqvi (2001). Further investigations revealed consistent differences between the typical (blue-flowering) and rare white-flowering forms (**Table 1**). However, the white-flowering variant is restricted to hedges and roadsides in the company of grasses like *Apluda mutica*, *Chrysopogon orientalis*, *Heteropogon contortus*, *Pennisetum hohenackeri* and *Setaria verticillata.* According to Harley (pers. comm. 2009), these white-flowering forms are frequently found in Brazilian populations, where this species is extremely abundant and almost certainly much

Table 1 Morphological differences between the blue- and white-flowering *Hyptis suaveolens* populations in India.

Character	Blue-flowering (typical form)	White-flowering (variant)
Petiole length (cm) (5th node)	4 cm	3.5 cm
Leaf		
Size	Relatively bigger, 5 × 4.5 cm	Relatively smaller, 4.5 × 4 cm
Base	Cordate with 2 proximal lobes	Truncate, without such lobes
Margin	Crenate, dentate	Dentate
Venation	Basal, alternate	Opposite
Vistiture	Sparse	Dense
Calyx		
Teeth	< 2 mm	> 3 mm
Vistiture	Veins of lobes glabrous	Rusty tomentose
Nutlet (length)	< 5 mm	> 6 mm

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Research Note

Fig. 1 *Hyptis suaveolens*. (**A**) Typical form; (**B**) White-flowering variant.

more variable than the populations in India, where the genetic base is probably much more restricted, originating in a relatively small number of founder introductions. Conversely, there is a need to know the extent of genetic variation and judge the taxonomic status of this white-flowering variant.

Since inter simple sequence repeats (ISSRs) are a type of molecular marker that involves amplification of DNA using polymerase chain reaction (PCR) by a single primer composed of repeated sequences anchored at the 3′ or 5′ end by 2-4 arbitrary nucleotides (Zietkiewicz *et al.* 1994), and since ISSR markers are highly reproducible, polymorphic, easy to handle, and informative (Teixeira da Silva *et al.* 2005), they were used to compare the genomic polymorphism between blue and white-flowering weedy *H. suaveolens*.

MATERIALS AND METHODS

Genomic DNA was isolated from freshly harvested young leaves of typical *H. suaveolens* (**Fig. 1A**) and the white-flowering (**Fig. 1B**) form according to Doyle and Doyle (1990). The quality of DNA was estimated by staining the agarose (Sigma-Aldrich, St. Louis, MI, USA) gel (8 g/l) with ethidium bromide and the quantity with a Nano Drop Spectrophotometer (Nano Drop ND1000, Wilmington, DE, USA). DNA was diluted to a uniform concentration of about 10 ng/µl. ISSR-PCR was conducted in a reaction volume of 15 µl containing 30 ng template DNA, 0.2 µmol/L primer, 200 µmol/L of each dNTP, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, and 1 U of *Taq* Polymerase. PCR amplification conditions were: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. PCR was performed in a 96-well plate thermal cycler (Eppendorf, Germany). The amplified products were mixed with loading dye (0.4 g/ml sucrose and 2.5 mg/ml bromophenol blue), resolved on 8 g/l agarose gel in a 0.5× Tris borate EDTA (TBE) buffer at room temperature and at constant voltage (100 V), and detected by staining with ethidium bromide (0.5 mg/ml). The gels were documented under ultraviolet light using a Bio-Rad (USA) gel documentation unit. Each amplified product was scored as either 1 or 0 corresponding to the presence or absence of a band. The frequency of microsatellite polymorphism was calculated based on the presence or absence of common bands. The polymorphism information content (PIC) value was calculated as PIC = $\sum (1\text{-}P_i^2)$ / n, where *n* is the number of band positions analyzed and *Pi* is the frequency of the i^{th} pattern. The ability of the primers to distinguish the two variants was assessed by calculating their resolving power (Rp) as Rp = $\sum I_b$, where I_b is band informativeness, $I_b = 1–[2 \times (0.5 – pi)$ and *pi* is the proportion of variants containing band *I* (Prevost and Wilkinson 1999). The genetic associations among the two variants were evaluated manually by counting the total number of bands/alleles (similar and dissimilar) observed with 15 ISSR (Hy1–Hy15) primers tested (**Table 1**). PCR amplification was performed in triplicate and the results obtained produced identical electrophoretic patterns.

RESULTS AND DISCUSSION

There is a need to restore non-crop areas invaded by exotic *H. suaveolens* in India for the preservation of wild diversity on one hand and to provide grazing ground for both wild and domesticated herbivores on the other. Therefore, the present study is relevant by providing knowledge on the variations and adaptability of its invasiveness. The morphological differences between the blue- and white-flowering forms of *H. suaveolens* occurring in India are presented in **Table 1**.

There are no varieties or forms of the exotic invasive *H. suaveolens* reported from India. However, there is *H. suaveolens* var. *mollissima* Fern. Alonso described in 1993 from Colombia. In Brazil, the native habitat of *H. suaveolens*, populations are much more variable in all characters, and the white-flowering forms are not infrequent and do not appear to be linked to any set of morphological characters (Harley, pers. comm. 2009).

The 15 primers yielded a total of 123 alleles which were considered for PIC analysis (**Table 2**). The number of bands per primer ranged from 12 (Hy12) to 7 (Hy2, 8, 9, 13 and 15) and the average number of bands per primer was 8.2. A maximum number of nine polymorphic alleles were obtained with primer Hy6 (**Table 2**) and only one allelic variation was obtained with primers Hy10 and Hy13-15. However, primers Hy2, 3 and 11 did not reveal any polymorphism between the two flowering forms (**Table 2**). ISSR-PCR amplification profiles of white and blue flowers using

Table 2 Polymorphic information content (PIC) *Hyptis suaveolens*.

ISSR marker No.	Primer sequence	Total No. alleles	Polymorphic alleles	PIC
Hy1	(AG)8C	9	4	4.43
Hy2	(AG)8G	7	0	3.42
Hy3	(GA)8T	9	0	4.41
Hy4	(GA)8C	9	8	4.46
Hy5	(TC)8C	8	4	3.95
Hy6	(TC)8G	10	9	4.96
Hy7	(AG)8YT	6	2	2.95
Hy8	(AG)8TC	7	3	3.45
Hy9	(GA) 8YT	7	3	3.45
Hy10	(GA)8YG	8	1	3.92
Hy11	(TC)8RT	8	0	3.91
Hy12	(TC)8RG	12	6	5.92
Hy13	(GATA)4	7	1	3.43
Hy14	GCC(GA)7	9	1	4.41
Hy15	ACTGCT (AG)7	7	1	3.43

Y = T or C; R = A or G.

Table 3 Percentage polymorphism in *Hyptis suaveolens*.

Alleles	Present	Percentage (%)
Common to both two forms	80	65.04
Unique to white-flowered variant	14	11.38
Unique to typical form	29	23.58
Total number	123	100.00

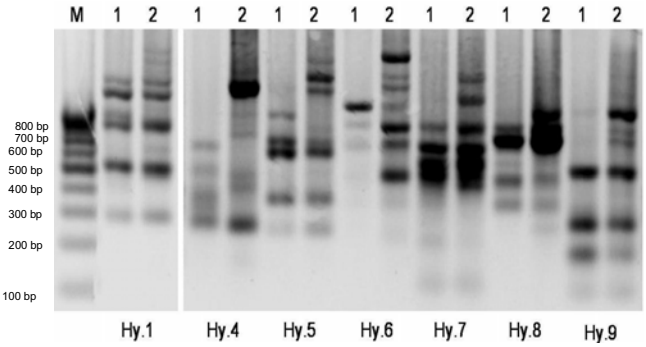

Fig. 2 ISSR-PCR amplification profiles of *Hyptis suaveolens* white and blue using primers Hy.1, Hy.4, Hy.5, Hy.6, Hy.7, and Hy.9, respectively. M: 100-bp marker; 1: White; 2: Blue.

primers Hy.1, Hy.4, Hy.5, Hy.6, Hy.7, and Hy.9, respectively is shown in **Fig. 2**. Furthermore, of the 15 primers, Hy12 showed a maximum PIC of 5.92 while Hy7 showed a minimum PIC of 2.95 (**Table 2**). Of the total of 123 alleles scored (**Table 3**), 80 (65.04%) were monomorphic and 43 (34.96%) were polymorphic for both forms. Out of 43 polymorphic alleles, 29 alleles (23.6%) were exclusive to the blue-flowering (typical) form and 14 alleles (11.4%) were unique to the white-flowering form.

From the morphological and genomic polymorphism data in this study, white-flowering *H. suaveolens* has a distinct morphological and genetic basis from the blue form. However, some limitations exist in our interpretations: (i) the degree of differences cannot be weighed; (ii) the field observations and genetic material sampled are limited since the white-flowering form is not found everywhere; (iii) the formal recognition of the white-flowering variant with a taxonomic status as a *forma* or *variety*, outside the native range of occurrence of the species, has perhaps little relevance.

Intra-population variation in seed germination (Wulff 1973) and seed size (large and small) and germination (Afolayan 1993) in *H. suaveolens* was demonstrated to be of strategic advantage to burial depth and seedling performance (Mandal *et al*. 2008) The establishment of a strategic advantage of white-flowers (perhaps to nocturnal moth pollination) of *H. suaveolens* might need to be proved. The present observations can only be used to note genetic diversity within the species.

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