A Partial Genetic Map of *Stylosanthes* Developed Using Inter-Specific F₂ Cross

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Summary Low polymorphism in *Stylosanthes* emphasizes use of inter-specific crosses for the development of mapping population for genetic linkage map. Using 78 F₂ individuals of the cross of *S. scabra* cv. Seca and *S. fruticosa*, 7 linkage groups were obtained when 81 polymorphic RAPD (Random Amplified Polymorphic DNA) markers were processed. Of these, 53 segregated in expected ratio of 3 : 1 for dominant marker in the F₂ generation, 11 markers in 1 : 1 while 17 deviated from the expected ratio. Thirty markers remain unlinked. Total genome length covered by markers in the present partial map is 630.4 cM (centi Morgan) with an average interval of 39.4 cM. The skeleton map developed is being used to place reasonable numbers of RAPD and STS (Sequence Tagged Sites) markers and to identify QTLs (Quantitative Trait Loci) for anthracnose disease, one of the most important disease of the genus *Stylosanthes*.

Key words Genetic linkage map, Inter-specific cross, Polymorphism, QTLs, RAPD, STS.

The genus *Stylosanthes* (Fabaceae) consists of approximately 40 diploid and polyploid species that are classified on the basis of morphological characteristics (Kirkbride and de Kirkbride 1985). The basic chromosome number in *Stylosanthes* is 10. Five species of this genus, namely *S. scabra*, *S. hamata*, *S. guianensis*, *S. humilis* and *S. seabrana*, have been widely used as tropical forage legumes. In early seventies this legume was introduced in India largely from Australia and South America. It is nutritive forage for animals, improve soil fertility and as pioneering colonizers reclaim degraded lands (Chakraborty *et al.* 1998, Chandra *et al.* 2006). The recent introduction of many superior lines of different species of *Stylosanthes* under an Australian Centre for International Agricultural Research (ACIAR) program and further screening and selection of germplasm yielded many suitable lines for different agroclimatic zones of the different participating countries preferably India and China (Ramesh *et al.* 2004, Chandra *et al.* 2004, Guodao *et al.* 2004). However, anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., is the most important disease of *Stylosanthes* limits their production, utilization and persistence worldwide (Chakraborty *et al.* 1998, Chakraborty 2004). Among many species, *S. scabra* is most extensively used species and few cultivars of this species have still maintained acceptable resistance to anthracnose (Edye 1987, Chakraborty *et al.* 1996). It is an allo-tetraploid ($2n=4x=40$) and appears to be highly diploidized with regular bivalent formation at meiosis, and self-pollinating species with an out-crossing rate of 1–2% (Stace 1982). *S. fruticosa* Alston synonyms *S. flavicans* Baker, *S. mucronata* Wild, *S. bojeri* Vogel and *Arachis fruticosa* Tetz. This is commonly known as African stylo and amenable to stable mixture with perennial grasses such as *Andropogon gayanus* and *Heteropogon contortus*.

Major area containing *S. humilis* was destroyed by anthracnose in 1970s and therefore this species has now of little significance in stylo-based pastures (Chakraborty *et al.* 1996). The demise of this particular species and vulnerability of many others necessitated selective breeding programs

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in Australia, Colombia and Brazil to overcome the problem of anthracnose. Kazan *et al.* (1993) have reported that inter-specific crosses may be a better vehicle for the construction of map in *Stylosanthes* since genetic variation based on RAPD analysis in agronomically important species of *Stylosanthes* indicated low level of polymorphism within each species. Additionally, the level of intra-specific variation detected on normal agarose gels of STS markers among 12 species of *Stylosanthes* have been reported to be only 3.8% (Liu *et al.* 1996). However, the greatest intra-specific variation as observed between *S. guianensis* cv Oxley and other accessions of species, but sterility in this cross generated problem for linkage analysis (Kazan *et al.* 1993). To provide durable resistance against variable pathogens the successful approach would be to incorporate more than 1 source of resistance into agronomically superior cultivars using traditional and molecular approaches. Keeping the aim to locate quantitative trait loci (QTLs) associated with anthracnose resistance a partial genetic map of *Stylosanthes* using an inter-specific cross is reported. The population in question is made using *S. scabra* cv. Seca and *S. fruticosa* as parents where inter-specific F_1 s were cytologically normal. The efficiency of mapping depends, to larger extent, on the level of DNA variation available in a given species.

Materials and methods

Mapping population and isolation of DNA

Seedlings of 2 parents namely *S. fruticosa* 41117A and *S. scabra* cv. Seca (EC440679) as a anthracnose susceptible and resistance parents respectively were raised in nursery. Along with 130 F_2 plants of the cross of *S. scabra* cv. Seca $\times$ *S. fruticosa* were raised. Seeds were properly scarified using sand paper to facilitate germination. For RAPD analysis, out of 130 F_2 78 individual F_2 plants were used in the present investigation. After 4 weeks, seedlings were transferred to big pots for their prolific growth. Seeds of all plant materials were received from Australia through NBPGR, New Delhi, India under a collaborative project. Half of the F_2 plants were raised at regional research station, Indian Grassland and Fodder Research Institute (IGFRI), Dharwad and rest half in experimental nursery at IGFRI, Jhansi, India. DNA was extracted from 4 weeks old seedlings following the method of Liu and Musial (1995). RNA was removed by incubating with final concentration of DNA in 10 μ g/ml RNase A for 30 min at 37°C. The DNA concentration was then determined by electrophoresis on 0.8% agarose gels followed by ethidium bromide staining where comparison were made to standard DNA ladders.

PCR reactions and scoring of polymorphic bands

PCR amplification for RAPD were performed in a volume of 20 μ l containing 67 mM Tris-HCL (pH 8.8 at room temperature), 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.45% (v/v) Triton X-100, 200 μ g/ml BSA, 3 mM MgCl_2 , 175 μ M of each dNTP, 7.5 pM of primer, 0.75 unit of Taq DNA polymerase (Bangalore Genei, India) and 25 ng of genomic DNA. A control PCR tube containing all components, but no template, was run with each primer to check for any contamination. DNA amplification was carried out in a MJ Research PTC-200 thermocycler (Waltham, USA) with the following reaction conditions: 40 cycles of 94°C/1 min, 37°C/1 min, 72°C/2 min and 72°C/10 min final extension. RAPD analysis was performed using 64 oligonucleotide primers obtained from Operon Technologies, Alameda (USA). Most of the primers used were known to generate clear banding patterns in *Stylosanthes* (Liu and Musial 1997). Using these primers initially DNA of few F_2 plants along with parents were carried out to pin point the polymorphic bands and then reaction was extended with 78 F_2 using primers that have shown polymorphism. Care was also taken in repeatable patterns with primers and various primers were excluded when not performed well. These primers were also observed given good reactions with the mapping populations of the cross using different species of *Stylosanthes* (Chakraborty *et al.* 1998, Thumma *et al.* 2001). Amplification products were analyzed

Fig. 1. Screening of polymorphic primers showing polymorphic bands (arrowheads). Parent one *S. scabra* cv. Seca (P1), parent 2 *S. fruticosa* 41117A (P2) and F_2 plants. Amplification with primers OPO4 (A), OPO6 (B), OPC15 (C) and OPD16 (D).

by gel electrophoresis on 1.6% agarose with $0.5\times$ TBE buffer and visualized by staining with ethidium bromide and photographed under UV radiation with Polaroid film 667. The Bangalore Genei 100 bp DNA ladder was used to estimate the molecular weight of the DNA fragments separated on gels. RAPD bands were scored using 1 to indicate presence and 0 to indicate absence of a band. Only clearly visible and thick bands present in replicated PCR were considered. In F_2 segregating population bands were scored as presence or absence depending on whether the same band is present or absent in parents. Each of 78 F_2 individual was scored for the banding phenotype. When a single primer detected more than 1 segregating markers, these markers were designated by the same primer name but distinguished by increasing Roman number (E20-1, E20-2). The decreasing number corresponded to the decreasing size of the segregating band for the primers *i.e.*, $1>2>3$.

Development of linkage map

The linkage map was constructed using MAPMAKER/EXP. 3.0 programme (Lander *et al.* 1987). Linkage relationship among the markers was established with the criteria of recombination frequency $\leq 50\%$ and the log likelihood of odd (LOD) of 3.0. The Kosambi mapping function was used to convert the recombination fractions to map distances (Kosambi 1944).

Results and discussion

In total 64 random primers were tested to amplify the DNA of both parents *i.e.* *S. scabra* cv.

Fig. 2. Ethidium bromide stained gel photographs showing segregation of RAPD bands (arrowhead) in F_2 plants with Operon primers OPN01 (top) and OPB20 (bottom). The segregating bands were scored to generate the linkage map.

Seca and *S. fruticosa* 41117A as well as a subset of F_2 plants DNA to identify the polymorphic primers and bands (Fig. 1). Of these, 13 primers (OPU17, OPV12, OPC9, OPJ13, OPP4, OPB8, OPB18, OPH12, OPAH5, OPT6, OPAH17, OPAH9 and OPAH10) did not produce any polymorphic band. Primer OPI6 produced highest 14 bands whereas 3 bands were produced by OPN1, OPC9, OPH12 and OPAH10. The maximum number of polymorphic bands scored was 4 obtained with OPAE3, OPAH3, OPE20 and OPO6 primers (Fig. 2). A partial genetic linkage map was constructed using 81 RAPD markers. Of these, 53 markers segregated according to the expected ratio of 3 : 1 for dominant marker in the F_2 generation. Eleven markers segregated in 1 : 1 ratio (bands present in 50% F_2 individuals and absent in rest 50%) and 17 markers deviated from the expected ratio. Seven linkage groups were obtained with 23 markers out of total 53 markers segregated in 3 : 1 ratio (Fig. 3). *S. scabra* has 20 chromosomes ($2n=40$). Thirty markers remain unlinked. Total genome length covered by markers in the present partial and tentative map is 630.4 cM with an average interval of 39.4 cM.

In the present study the putative map was primarily developed to visualize the linkage of polymorphic bands those generated by the primers already have been reported to be polymorphic with other inter and intra specific population of *Stylosanthes* (Chakraborty *et al.* 1998). Though the number of RAPD markers used was less, a putative map was constructed. The extent of segregation distortion with the present inter-specific population observed is significantly higher (35%) than obtained with the F_2 intra-specific population of the cross of *S. scabra* cv. Fitzroy $\times$ *S. scabra* 93116 (2%) (Thumma *et al.* 2001). High level of segregation distortion has been reported with RFLP markers in different crops (Sharma *et al.* 1994). Higher level of distortion as observed in the present study may contribute to the small population and less number of markers employed. Though in the present study the number of linkage groups were well below the expected number, higher number of linkage groups (37 and 25) than expected have been reported in *Stylosanthes* with both intra and inter-specific populations (Chakraborty *et al.* 1998, Thumma *et al.* 2001). The number of linkage groups in the present study may increase when more markers are placed. The present partial map is the second in series developed with 2 different inter-specific populations (*S. scabra* cv. Seca $\times$ *S. fruticosa*, *S. scabra* $\times$ *S. hamata*). Most of the primers used in the present investigation were of the earlier reported maps produced 7 linkage groups. Markers generated from primers like OPP12, OPAF11, OPB8, OPAH17, OPV15, OPA13, OPE11 and OPZ17 located on QTLs for anthracnose resistance also observed well segregated in the present population and found place on 7 linkage

Fig. 3. A partial genetic map based on a cross between *S. scabra* cv. Seca × *S. fruticosa*. Linkage groups are shown as vertical bars with numbers. Letter with numbers on the right side of the linkage groups represent the name of the primer and number of loci revealed by a primer. Numbers on the right side of the linkage groups indicate map distances in cM.

groups. The markers, which have made linkage groups could be better utilized in studying genetic relationship study of native *S. fruticosa* population as one of the parent used is *S. fruticosa*. This will further lead us to identify phenotypically contrast *S. fruticosa* accession could be used in developing mapping population to study the genetics of anthracnose resistance in *Stylosanthes*. Fifteen sequence-tagged-sites (STS) primers pairs (Manners *et al.* 1995, Smith *et al.* 1995, Liu *et al.* 1996, Vander Stappen *et al.* 1999) have been screened and in total 59 fragments have been amplified with the present parents would be further extended with population so that the polymorphic markers can be identified and placed to enrich the genetic map.

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