Polymorphism analysis in identification of genetic variation and relationships among *Stylosanthes* species

Chunqiong Huang¹ · Guodao Liu¹ · Changjun Bai¹

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Abstract A total of 148 accessions representing six important species of the genus *Stylosanthes*, including *S. guianensis*, *S. hamata*, *S. scabra*, *S. seabrana*, *S. macrocephala*, and *S. capitata*, were used to evaluate genetic variation and relationships using sequence-related amplified polymorphism markers. The results showed that the 18 selected primer pairs generated 138 distinct fragments. The fragment sizes ranged from 150 to 2000 bp. Genetic similarity coefficients among the 148 accessions ranged from 0.51 to 0.99, with an average of 0.79. The effective allele number (n_e) generated by the 18 primer pairs averaged 1.3552 and ranged from 1.2069 to 1.6080; Nei's gene diversity (H_e) ranged from 0.1304 to 0.3207, with an average of 0.2070; and Shannon's information index (I) averaged 0.3213 and ranged from 0.2233 to 0.4582. The unweighted pair-group method with arithmetic averages at the 0.69 similarity level separated the 148 accessions into two distinct groups. One group belonged to *S. guianensis*, and the other group belonged to the non-*S. guianensis* type. This study verified that *Stylosanthes* have rich genetic variation, which is an excellent basis for *Stylosanthes* breeding for new cultivars. This study demonstrates that the SRAP technique is a reliable tool for differentiating *Stylosanthes* accessions and for discerning genetic relationship among them.

Keywords Polymorphism analysis · Genetic variation · Genetic relationships · *Stylosanthes* species · Sequence-related amplified polymorphism (SRAP)

Introduction

The genus *Stylosanthes* contains approximately 48 species and is naturally distributed in the tropical, subtropical, and temperate regions of the Americas, Africa, and Southeast Asia (Costa and Ferreira 1984). The genus has two foci of diversity, the more important of which is located in central Brazil. This includes 45% of all *Stylosanthes* species and exhibits the greatest degree of phenotypic variation and endemism. Mexico and the Caribbean Islands are also major centers of *Stylosanthes* diversity (Stace and Cameron 1984). The plant plays a significant role in providing nutritious forage for animals, improving soil fertility, and restoring degraded land. Four species of this genus, namely, *S. scabra*, *S. hamata*, *S. guianensis*, and *S. humilis*, have been widely used as tropical forage legumes. Each species has rich variations in morphology, physiology, and genetics. *S. guianensis* is the most widespread *Stylosanthes* species and exhibits remarkable phenotypic variability (Williams et al. 1984; Vieira et al. 1993). This species is one of the most important tropical forage legumes currently known and is native to South and Central America and Africa, where it is widely distributed. It is used for grazing cattle, for making leaf meal for livestock, for improving soil fertility in fruit-tree and rubber plantations and for cover crops in Australia, South America, and South China (Burt and Miller 1975).

Because of their adaptation to acidic and infertile soils in semiarid environments, *Stylosanthes* have been introduced to many countries, including Australia, India, the

✉ Chunqiong Huang
huangchunqiong44@163.com

✉ Guodao Liu
liuguodao2008@163.com

¹ Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences/Key Laboratory of Crop Gene Resources and Germplasm Enhancement in Southern China, Ministry of Agriculture, Danzhou 571737, Hainan, China

Philippines, Thailand, and China, to improve animal production and restore depleted soil nitrogen (Liu et al. 1997). Introduction of *Stylosanthes* from Australia, Africa, and South America to China began in the late 1960s and has continued to the present. *Stylosanthes* is principally well-adapted to the environment conditions of Guangdong and Hainan province of China, and 12 *Stylosanthes* cultivars have been developed through selective- and mutation-breeding by Chinese scientist. These include *S. guianensis* cv. Reyan No. 2 in 1991, *S. hamata* (L.) Taub. cv. Verano in 1991, *S. guianensis* Sw. cv. 907 in 1998, *S. guianensis* Sw. cv. Graham in 1998, *S. guianensis* cv. Reyan No. 5 in 1999, *S. guianensis* cv. Reyan No. 10 in 2000, *S. guianensis* cv. Reyan No. 7 in 2001, *S. scabra* Vog. cv. Seca in 2001, *S. guianensis* cv. Reyan No. 13 in 2003, *S. guianensis* (Aubl.) Sw. cv. Reyin No. 18 in 2007, and *S. guianensis* cv. Reyan No. 20 in 2009, and *S. guianensis* cv. Reyan No. 21 in 2011 (Huang et al. 2014).

Different DNA markers have been used to investigate the genetic diversity and relatedness of members of the *Stylosanthes* genus. Techniques included the use of random amplified polymorphic DNA (RAPD), which has been used to assess genetic variation in the five taxonomic groups of the *S. guianensis* (Kazan et al. 1993) and between *S. scabra* and *S. fruticosa* (Glover et al. 1994). Restriction fragment length polymorphism (RFLP) analysis has been used to investigate the genetic relationships between six unclassified taxa and 24 known species of the genus *Stylosanthes* (Liu et al. 1999) and to identify putative diploid progenitors of allotetraploid *S. hamata* (Curtis et al. 1995). Sequence-tagged sites (STS) were used to identify progenitor species for *S. scabra* (Liu and Musial 1997). Investigation of ribosomal DNA internal transcribed spacers (rDNA ITS) has been used to detect variation in the *S. guianensis* species complex (Vander Stappen and Volckaert 1999) and in *Stylosanthes* species (Vander Stappen et al. 2003). Simple sequence repeats (SSR) are available for three species of *Stylosanthes*: *S. guianensis* (Vander Stappen et al. 1999; Santos et al. 2009a; Santos-Garcia et al. 2012), *S. capitata* (Santos et al. 2009b), and *S. macrocephala* (Santos et al. 2009c). Amplified fragment length polymorphism (AFLP) has been successfully employed in assessing genetic variation in Mexican and South American *S. humilis* (Vander Stappen et al. 2000), and *S. viscisa* (Sawkins et al. 2001).

Of these methods, RAPD is one of the simplest, but has poor reproducibility RAPD (Williams et al. 1990). Although the AFLP technique has good reproducibility and reveals high levels of polymorphism, its operation is very elaborate and the costs are relatively high (Vos et al. 1995). The method based on analysis of SSR requires prior knowledge of the genome sequences of the organism to

design specific polymerase chain reaction (PCR) primers for amplification (Tautz 1989). In comparison, application of sequence-related amplified polymorphism (SRAP) markers overcomes most of these limitations (Li and Quiros 2001). This technique can generate more polymorphic fragments for the assessment of genetic diversity than can SSR, inter-simple sequence repeat (ISSR), or RAPD markers (Budak et al. 2004).

Although previous research has provided preliminary data regarding genetic diversity among the *Stylosanthes* genus, studies that investigated the levels of variation within *Stylosanthes* species are limited. Considering the advantages of SRAP markers, we used this method to describe the genetic variability within a group of accessions representing the genetic diversity available in *Stylosanthes* species germplasm.

Materials and methods

Plant materials

A total of 148 *Stylosanthes* accessions comprise six species. Of these, 132 accessions belong to *S. guianensis*, seven accessions group to *S. scabra*, five accessions belong to *S. seabrams*, two accessions belong to *S. hamata*, one accession belongs to *S. capitata*, and one accessions groups to *S. macrocephala*. Sixteen accessions were from the Genetic Resource Unit of Centro Internacional de Agricultura tropical (CIAT), 16 from Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), six accessions from International Rice Research Institute (IRRI), 12 accessions from the Institute of Guangxi Animal Science (IGAS) of China, and the remainder accessions (98 accessions) from the Chinese Academy of Tropical Agricultural Science (CATAS). A list of the accessions with their codes, accession numbers, places of origin, and source is provided in Table 1.

DNA extraction

Total genomic DNA of each accession was isolated from one plant according to the modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure described by Huang et al. (2014). The quality and quantity of genomic DNA were estimated by measuring absorbance at 260 and 280 nm using a UV spectrophotometer (BioPhotometer D30, Eppendorf, Germany). The integrity of the DNA was verified by agarose gel electrophoresis (Dongre et al. 2011). DNA concentrations were adjusted to 50 ng/μL to facilitate uniformity of PCR amplification. DNA samples were stored at −20 °C until use.

Table 1 Geographical origins of the 148 *Stylosanthes* spp. accessions investigated in the present study

Accession no.	<i>Stylosanthes</i> species	Geographical origins	Sources	Accession no.	<i>Stylosanthes</i> species	Geographical origins	Sources
S001	<i>S. hamata</i> cv. Verano	Australia	CATAS	S075	<i>S. guianensis</i> TPRC90034	China	CATAS
S002	<i>S. scabra</i> cv. Seca	Australia	CATAS	S076	<i>S. guianensis</i> TPRC90119	China	CATAS
S003	<i>S. guianensis</i> cv.CPI118750A	Australia	CATAS	S077	<i>S. guianensis</i> TPRC E1	China	CATAS
S004	<i>S. guianensis</i> TPRC90139	China	CATAS	S078	<i>S. guianensis</i> TPRC E7	China	CATAS
S005	<i>S. guianensis</i> cv. Oxley	Australia	CATAS	S079	<i>S. guianensis</i> TPRC E9	China	CATAS
S006	<i>S. guianensis</i> USF873017	USA	IGAS	S080	<i>S. guianensis</i> TPRC R03	China	CATAS
S007	<i>S. guianensis</i> TPRC90144	China	CATAS	S081	<i>S. guianensis</i> GC1480	Philippine	IRRI
S008	<i>S. guianensis</i> TPRC90072	China	CATAS	S082	<i>S. guianensis</i> GC1576(1)	Brazil	EMBRAPA
S009	<i>S. guianensis</i> TPRC E4	China	CATAS	S083	<i>S. guianensis</i> GC1528	Brazil	EMBRAPA
S010	<i>S. guianensis</i> cv.Graham(1)	Australia	IGAS	S084	<i>S. guianensis</i> GC1463	Brazil	EMBRAPA
S011	<i>S. guianensis</i> USF873015(1)	USA	IGAS	S085	<i>S. guianensis</i> GC1576(2)	Brazil	EMBRAPA
S012	<i>S. guianensis</i> TPRC90075	China	CATAS	S086	<i>S. guianensis</i> GC1524	Brazil	EMBRAPA
S013	<i>S. guianensis</i> cv.Endeavour(1)	Australia	CATAS	S087	<i>S. guianensis</i> FM07-3	Philippine	IRRI
S014	<i>S. guianensis</i> CIAT11364	Colombia	CIAT	S088	<i>S. guianensis</i> GC1579	Brazil	EMBRAPA
S015	<i>S. guianensis</i> cv.Reyan No.5	China	CATAS	S089	<i>S. guianensis</i> GC348	Brazil	EMBRAPA
S016	<i>S. guianensis</i> TPRC R292	China	CATAS	S090	<i>S. guianensis</i> FM07-2	Philippine	IRRI
S017	<i>S. guianensis</i> USF873016(1)	USA	IGAS	S091	<i>S. guianensis</i> GC1557	Philippine	IRRI
S018	<i>S. guianensis</i> USF873015(2)	USA	IGAS	S092	<i>S. guianensis</i> GC1517	Brazil	EMBRAPA
S019	<i>S. guianensis</i> TPRC90069	China	CATAS	S093	<i>S. guianensis</i> GC1517	Brazil	EMBRAPA
S020	<i>S. guianensis</i> USF873014	USA	IGAS	S094	<i>S. guianensis</i> GC1524	Brazil	EMBRAPA
S021	<i>S. guianensis</i> TPRC90067	China	CATAS	S095	<i>S. guianensis</i> cv.Reyan No.2	China	CATAS
S022	<i>S. guianensis</i> TPRC90015	China	CATAS	S096	<i>S. guianensis</i> cv.Graham(2)	Australia	IGAS
S023	<i>S. guianensis</i> TPRC90089	China	CATAS	S097	<i>S. guianensis</i> CIAT SK	Colombia	CIAT
S024	<i>S. guianensis</i> TPRC R291	China	CATAS	S098	<i>S. guianensis</i> cv. Graham(3)	Australia	IGAS
S025	<i>S. guianensis</i> CIAT1044(2)	Colombia	CIAT	S099	<i>S. guianensis</i> TPRC(Q1)	China	CATAS
S026	<i>S. guianensis</i> TPRC90105	China	CATAS	S100	<i>S. guianensis</i> TPRC(HE)	China	CATAS
S027	<i>S. guianensis</i> USF873016(2)	USA	IGAS	S101	<i>S. guianensis</i> CIAT early blossoming	Colombia	CIAT
S028	<i>S. guianensis</i> cv.Reyan No.10	China	CATAS	S102	<i>S. guianensis</i> ATF3309	Australia	CATAS
S029	<i>S. guianensis</i> CIAT11371	Colombia	CIAT	S103	<i>S. guianensis</i> ATF3308	Australia	CATAS
S030	<i>S. guianensis</i> TPRC90006	China	CATAS	S104	<i>S. guianensis</i> cv. Mineirao	Australia	CIAT
S031	<i>S. guianensis</i> TPRC90033	China	CATAS	S105	<i>S. guianensis</i> cv.Reyan No. 18	Colombia	CIAT
S032	<i>S. guianensis</i> TPRC90107	Colombia	CATAS	S106	<i>S. guianensis</i> FM9405-Parcelle 3	Australia	CATAS
S033	<i>S. guianensis</i> TPRC90005(2)	China	CATAS	S107	<i>S. guianensis</i> 109	China	CATAS
S034	<i>S. guianensis</i> CIAT11363(2)	Colombia	CATAS	S108	<i>S. guianensis</i> cv.Graham(4)	Australia	IGAS
S035	<i>S. guianensis</i> TPRC90003	China	CATAS	S109	<i>S. guianensis</i> cv.Endeavour(2)	Australia	CATAS
S036	<i>S. guianensis</i> CIAT1281	Brazil	CIAT	S110	<i>S. scabra</i> .Seca 33260	China	CATAS
S037	<i>S. guianensis</i> cv.Cook	Bolivia	CATAS	S111	<i>S. scabra</i> .Seca Q10042	China	CATAS
S038	<i>S. guianensis</i> TPRC90050	China	CATAS	S112	<i>S. scabra</i> .Seca 93116	China	CATAS

Table 1 continued

Accession no.	<i>Stylosanthes</i> species	Geographical origins	Sources	Accession no.	<i>Stylosanthes</i> species	Geographical origins	Sources
S039	<i>S. guianensis</i> TPRC90058	China	CATAS	S113	<i>S. seabrana</i> 2323	Australia	CATAS
S040	<i>S. guianensis</i> CIAT11363(1)	Colombia	CATAS	S114	<i>S. seabrana</i> 2534	China	CATAS
S041	<i>S. guianensis</i> TPRC90093	China	CATAS	S115	<i>S. seabrana</i> 2539	China	CATAS
S042	<i>S. guianensis</i> TPRC90028	China	CATAS	S116	<i>S. scabra</i> cv. Fitzroy 40205	Australia	CATAS
S043	<i>S. guianensis</i> TPRC90037(3)	China	CATAS	S117	<i>S. scabra</i> .Seca 40292	China	CATAS
S044	<i>S. guianensis</i> TPRC R273	China	CATAS	S118	<i>S. guianensis</i> cv. 907	China	IGAS
S045	<i>S. guianensis</i> TPRC90085	China	CATAS	S119	<i>S. guianensis</i> 90005(1)	China	CATAS
S046	<i>S. guianensis</i> CIAT11376	Colombia	CIAT	S120	<i>S. guianensis</i> 129	China	CATAS
S047	<i>S. guianensis</i> TPRC90005	China	CATAS	S121	<i>S. guianensis</i> 130	China	CATAS
S048	<i>S. guianensis</i> TPRC90005(4)	China	CATAS	S122	<i>S. hamata</i> cv. Schotfield	China	CATAS
S049	<i>S. guianensis</i> TPRC90005(1)	China	CATAS	S123	<i>S. guianensis</i> 184	China	CATAS
S050	<i>S. guianensis</i> TPRC90047	China	CATAS	S124	<i>S. guianensis</i> 90088	China	CATAS
S051	<i>S. guianensis</i> cv.Reyan No.7	China	CATAS	S125	<i>S. guianensis</i> CIAT184	Colombia	CATAS
S052	<i>S. guianensis</i> TPRC90037(2)	China	CATAS	S126	<i>S. guianensis</i> L6	China	CATAS
S053	<i>S. guianensis</i> TPRC90095(2)	China	CATAS	S127	<i>S. guianensis</i> 90058	China	CATAS
S054	<i>S. guianensis</i> cv.Reyan No.13	China	CIAT	S128	<i>S. guianensis</i> 136(1)	China	CATAS
S055	<i>S. guianensis</i> TPRC90108	China	CATAS	S129	<i>S. guianensis</i> 90064	China	CATAS
S056	<i>S. guianensis</i> cv. Tardio	Brazil	CIAT	S130	<i>S. guianensis</i> 90135	China	CATAS
S057	<i>S. guianensis</i> TPRC dianbai98	China	CATAS	S131	<i>S. guianensis</i> 90087	China	CATAS
S058	<i>S. guianensis</i> TPRC dianbai87	China	CATAS	S132	<i>S. guianensis</i> cv.Graham(5)	Australia	IGAS
S059	<i>S. guianensis</i> TPRC90134(2)	China	CATAS	S133	<i>S. guianensis</i> FM05-1	Brazil	CATAS
S060	<i>S. guianensis</i> FM9405	Colombia	CIAT	S134	<i>S. guianensis</i> 90083	China	CATAS
S061	<i>S. guianensis</i> GC1578	Brazil	EMBRAPA	S135	<i>S. guianensis</i> 990009-1	China	CATAS
S062	<i>S. macrocephala</i>	Philippine	IRRI	S136	<i>S. guianensis</i> 90135	China	CATAS
S063	<i>S. capitata</i>	Philippine	IRRI	S137	<i>S. guianensis</i> Liu-6	China	CATAS
S064	<i>S. guianensis</i> FM05-1	Philippine	CIAT	S138	<i>S. guianensis</i> CIAT11371	Colombia	CIAT
S065	<i>S. guianensis</i> FM05-3	Philippine	CIAT	S139	<i>S. guianensis</i> cv.polyploidy of Reyan No.5	China	CATAS
S066	<i>S. guianensis</i> FM03-2	Brazil	CATAS	S140	<i>S. guianensis</i> cv.Nina	Brazil	EMBRAPA
S067	<i>S. scabra</i> .Seca CIAT250	Australia	CIAT	S141	<i>S. guianensis</i> cv.Temprano	Brazil	EMBRAPA
S068	<i>S. guianensis</i> 58719	China	CATAS	S142	<i>S. hippocampoides</i>	Brazil	EMBRAPA
S069	<i>S. guianensis</i> 87830	China	CATAS	S143	<i>S. seabrana</i> cv.Unica	Brazil	EMBRAPA
S070	<i>S. guianensis</i> 67652	China	CATAS	S144	<i>S. seabrana</i> cv.Primar	Brazil	EMBRAPA
S071	<i>S. guianensis</i> TPRC L8	China	CATAS	S145	<i>S. guianensis</i> 540	China	CATAS
S072	<i>S. guianensis</i> TPRC E3	China	CATAS	S146	<i>S. guianensis</i> 541	China	CATAS
S073	<i>S. guianensis</i> TPRC L1	China	CATAS	S147	<i>S. guianensis</i> cv.Reyan No.20	China	CATAS
S074	<i>S. guianensis</i> TPRC L2	China	CATAS	S148	<i>S. guianensis</i> cv.Reyan No.21	China	CATAS

SRAP reactions

Ninety distinct primer pairs (nine forward, ten reverse) from Yingjun Inc. (Shanghai, China) were tested for PCR analysis (Li and Quiros 2001). The 90 SRAP primer pairs were screened using three selected accessions respended three *Stylosanthes* species. Each 10 μ L PCR mixture contained 50 ng genomic DNA, 0.5 μ M forward primer, 0.5 μ M reverse primer, and 5 μ L 2 $\times$ Easy Taq PCR SuperMix (TransGen biotech, Beijing, China). The mixture was overlaid with 20 μ L mineral oil before thermal cycling was commenced. Amplification was carried out on a Thermal Cycler DiceTM (Bio-Rad S1000TM, USA) as follows: initial denaturation at 94 °C for 5 min, followed by five cycles of 1 min denaturation at 94 °C, annealing at 35 °C for 1 min and elongation at 72 °C for 45 s. In the subsequent 30 cycles, the annealing temperature was 50 °C for 1 min, with a final extension step at 72 °C for 30 s, terminating with an elongation step of 7 min at 72 °C. The amplified products were stored at 4 °C before being loaded onto a gel (Huang et al. 2014). The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel in 1.0 $\times$ TBE buffer (0.09 mol/L Tris-H₃BO₃, 0.002 mol/L EDTA, pH 8.0) at a constant voltage of 100 V for approximately 1.5 h. GoldView (TransGen biotech Beijing, China) stain (0.5 μ g/mL) was added to facilitate UV light visualization. Molecular weights were estimated using a 50 bp DNA ladder (TaKaRa Biotechnology, Dalian, China).

Data analysis

SRAP bands across the gel profiles were scored visually for their presence (1) or absence (0) at least twice for each accession. Only reproducible and unambiguous SRAP fragments were used for scoring. The data were compiled in a binary data matrix using Microsoft Excel and analyzed using the numerical taxonomy and multivariate analysis system (NTSYS) program, version 2.1 (Exeter Software, Setauket, NY, USA). Simple matching coefficients were computed using the SIMQUAL module of the NTSYS program. Cluster analysis based on GSC using the Nei and Li distance was performed according to the UPGMA in the SAHN module of the NTSYS program (Kang et al. 2008). Principal coordinate analysis (PCoA) was performed to estimate the genetic distances among the major groups using the DCENTER and EIGEN modules of the NTSYS program. Effective allele number (ne), Nei's gene diversity (He) and Shannon's information index (I) were used to compute Nei's standard genetic distance coefficients using the Popgene32 program (Nei and Li 1979).

Results

Primer pair screening

Ninety primer pairs were screened three times on three selected accessions respended three *Stylosanthes* species (S002, S003, and S114) to test the ability of primer pairs to amplify DNA fragments. The most useful primer combinations were considered to be those having the highest polymorphism rate that also generated a reasonable number of clearly detectable total fragments. Of the 90 SRAP primer pairs evaluated for their ability to amplify *Stylosanthes* DNA, 72 primer pairs were rejected, because they either yielded no amplification or no polymorphic patterns. Eighteen primer pairs from the original 90 primer pairs were selected for subsequent analysis based on the polymorphic and reproducible bands they generated. The characteristics of the 18 primer pairs are listed in Table 2.

SRAP analysis

SRAP analysis was performed employing the 18 most polymorphic-selective primer pairs for 148 accessions of *Stylosanthes*. The 18 primer pairs collectively amplified 138 reproducible fragments, ranging in size from 150 to 2000 bp, and varying in the number of amplification bands between 3 and 12 for each primer pair. All these fragments were polymorphic, showing a 100% level of polymorphism on average. The highest number (12) of amplification products was obtained using the primer pairs F08–R01 and the lowest (3) with F02–R07. The average number of fragments among the 18 primer pairs was 7.66. The effective allele number (ne) ranged from 1.2069 to 1.6080 with an average of 1.3552, Nei's gene diversity (He) ranged between 0.1304 and 0.3207 with an average of 0.2070, and Shannon's information index (I) varied between 0.2233 and 0.4582 with an average of 0.3213 (Table 2). An example of the polymorphism detected among some accessions by primer pair F1–R2, as shown in Fig. 1.

Genetic diversity analysis

The GSC values of the 148 *Stylosanthes* accessions varied between 0.51 and 0.99 with an average of 0.79. Increased genetic distance indicated diminished genotype relatedness between the genotypes. The lowest GSC (0.51) was between accessions S020 and S063, which suggests that these were the least related accessions, whereas the highest GSC was 0.99, detected between accessions S069 and S070, and accessions S073 to S074, indicating a very close relationship.

Table 2 Polymorphisms detected by SRAP primer pairs among 148 *Stylosanthes* accessions

Primer pairs	Primer pairs sequence	Band size (bp)	Total bands	Effective allele number (ne)	Nei's gene diversity (He)	Shannon's information index (I)
F01–R02	5'-TGAGTCCAAACCGGATA-3' 5'-GACTGCGTACGAATTTGC-3'	350–2000	10	1.3162	0.2012	0.3275
F02–R07	5'-TGAGTCCAAACCGGAGC-3' 5'-GACTGCGTACGAATTCGA-3'	180–700	3	1.6080	0.3207	0.4582
F02–R10	5'-TGAGTCCAAACCGGAGC-3' 5'-GACTGCGTACGAATTAGC-3'	200–1600	7	1.2069	0.1410	0.2422
F03–R04	5'-TGAGTCCAAACCGGAAT-3' 5'-GACTGCGTACGAATTTGA-3'	150–1600	9	1.2675	0.1621	0.2556
F03–R09	5'-TGAGTCCAAACCGGAAT-3' 5'-GACTGCGTACGAATTCTG-3'	250–1200	7	1.3497	0.2121	0.3317
F05–R10	5'-TGAGTCCAAACCGGAAG-3' 5'-GACTGCGTACGAATTAGC-3'	150–1600	6	1.4722	0.2695	0.4009
F06–R04	5'-TGAGTCCAAACCGGTAG-3' 5'-GACTGCGTACGAATTTGA-3'	250–1200	6	1.4835	0.2729	0.4039
F06–R05	5'-TGAGTCCAAACCGGTAG-3' 5'-GACTGCGTACGAATTAAC-3'	150–2000	8	1.3232	0.1752	0.2624
F06–R07	5'-TGAGTCCAAACCGGTAG-3' 5'-GACTGCGTACGAATTCGA-3'	150–1200	10	1.2866	0.1855	0.3074
F07–R09	5'-TGAGTCCAAACCGGTAA-3' 5'-GACTGCGTACGAATTCTG-3'	200–1300	9	1.5379	0.3002	0.4459
F08–R01	5'-TGAGTCCAAACCGGTCC-3' 5'-GACTGCGTACGAATTAAT-3'	400–2000	12	1.2837	0.1766	0.3119
F08–R05	5'-TGAGTCCAAACCGGTCC-3' 5'-GACTGCGTACGAATTAAC-3'	200–1300	7	1.3260	0.1858	0.2832
F08–R06	5'-TGAGTCCAAACCGGTCC-3' 5'-GACTGCGTACGAATTGCA-3'	250–1500	8	1.3052	0.1975	0.3174
F09–R02	5'-TGAGTCCAAACCGGTGC-3' 5'-GACTGCGTACGAATTTGC-3'	150–1000	10	1.3215	0.1771	0.2722
F09–R03	5'-TGAGTCCAAACCGGTGC-3' 5'-GACTGCGTACGAATTGAC-3'	150–700	6	1.4471	0.2436	0.3616
F09–R04	5'-TGAGTCCAAACCGGTGC-3' 5'-GACTGCGTACGAATTTGA-3'	200–1300	7	1.2084	0.1304	0.2233
F10–R02	5'-TGAGTCCAAACCGGT-3' 5'-GACTGCGTACGAATTTGC-3'	200–900	6	1.2709	0.1611	0.2530
F10–R08	5'-TGAGTCCAAACCGGT-3' 5'-GACTGCGTACGAATTCAA-3'	200–1500	7	1.3785	0.2134	0.3247
Total	–	–	138	–	–	–
Average	–	–	7.66	1.3552	0.2070	0.3213

Fig. 1 Example of an SRAP amplification profile among some accessions by primer pair F1–R2

A dendrogram was constructed to cluster the 148 accessions into two major groups at the 0.69 similarity level (Fig. 2), with most accessions from the same species tending to have high genetic similarity and clustering into the same groups or subgroups. One group belonged to *S.*

guianensis, and the other group belonged to non-*S. guianensis* type, Group 1 included 14 accessions which contained *S. hmamata*, *S. seabrana*, *S. scabra*, *S. macrocephala*, and *S. capitata*. The GSC varied from 0.60 to 0.96, and further distinguished two subgroups. One

Fig. 2 UPGMA dendrogram of 148 *Stylosanthes* accessions generated from SRAP data

accession (S063) individually separated from other accessions derived from *S. capitata*. The other subgroup comprised 13 accessions which contained one *S. hmamata* accession (S001), four *S. seabrana* accessions (S113, S114, S143, and S144), and seven *S. scabra* accessions (S002, S067, S110, S111, S112, S116, and S117). Group 2 consisted of 134 accessions of *S. guianensis* except for an individual *S. seabrana* accession (S115). The GSC ranged from 0.62 to 0.99, and further categorized six subgroups. Subgroup 1 comprised a single accession S056 (*S. guianensis* cv. Tardio) from Brazil, belonging to the disease-resistant cultivars. Subgroup 2 also only contained one accession S104 (*S. guianensis* cv. Mineirao) from Australia and exhibited high yields. Subgroup 3 contained ten accessions, including S095 (*S. guianensis* cv. Reyan No. 2), and presented the characteristics of early blossoming and disease resistance. Subgroup 4 included one accession, S142 (*S. hippocampoides*) from Brazil. Subgroup 5 consisted of 41 accessions, including the Chinese cultivars S105 (*S. guianensis* cv. Reyan No. 18), S125 (*S. guianensis* CIAT184), S118 (*S. guianensis* cv. 907), S147 (*S. guianensis* cv. Reyan No. 20), and S148 (*S. guianensis* cv. Reyan No. 21), and the GSC ranged from 0.70 to 0.97. Most of the accessions shared relationship with *S. guianensis* CIAT184. The anthracnose-resistance cultivar *S. guianensis* cv. 907 was selected from the population of *S. guianensis* CIAT184 by mutation breeding, *S. guianensis* cv. Reyan No. 20 and *S. guianensis* cv. Reyan No. 21 was selected from the population of *S. guianensis* cv. Reyan No. 2 by space mutation-breeding, and *S. guianensis* cv. Reyan No. 2 was selected from *S. guianensis* CIAT184. Subgroup 6 included the other 80 accessions including the Chinese cultivars S015 (*S. guianensis* cv. Reyan No. 5), S028 (*S. guianensis* cv. Reyan No. 10), S051 (*S. guianensis* cv. Reyan No. 7), and S054 (*S. guianensis* cv. Reyan No. 13), and the GSC values ranged from 0.72 to 0.99.

PCoA was conducted based on the genetic resemblance matrix to further understand the ecological distribution of different accessions. Figure 3 presents the distribution of the different accessions according to the three principal axes of variation using PCoA. The percentages of variance revealed by principle component 1 (PC1), principal component 2 (PC2), and principal component 3 (PC3) were 79.02, 4.12, and 1.85%, respectively, which were consistent with the results of UPGMA cluster analyses.

Discussion

Our results demonstrate that SRAP analysis effectively and efficiently provided quantitative estimates of genetic relatedness among *Stylosanthes* accessions. We found a

Fig. 3 Principal coordinate analysis of 148 *Stylosanthes* accessions based on the genetic similarity matrix generated from SRAP data

high level of polymorphism (100%) among various accessions, which confirms that the SRAP marker technique generates highly reproducible DNA profiles for *Stylosanthes* accessions. The extent of polymorphism from the SRAP analysis in the present study was higher than that from RAPD (25.6%) (Kazan et al. 1993), SSR (45.0%) (Vander Stappen and Volckaert 1999), and AFLP (95.5%) (Jiang et al. 2005). The GCG range (0.51–0.99) in this study was consistent with that found using RAPD (0.55–1.00) as reported by Kazan et al. (1993). However, these values are greater than the range of 0.30–0.90 observed with SSR (Vander Stappen and Volckaert 1999) and 0.30–0.95 for AFLP analysis by Jiang et al. (2005). The GSC values obtained in our study demonstrated that the level of genetic diversity was relatively high among *Stylosanthes* accessions. The locations of the clusters obtained from PCoA also demonstrated wide genetic variability among the clusters. These results suggest that a high level of polymorphism exists in *Stylosanthes* accessions.

In summary, the SRAP marker technique has advantages in terms of convenience, high reproducibility, and high polymorphism content and can be used as an a superior method for germplasm identification and genetic diversity studies of *Stylosanthes*. Furthermore, the molecular relationships generated from this study should be useful in breeding programs for *Stylosanthes*.

Conclusions

This study demonstrates that the SRAP technique is a reliable tool for differentiating *Stylosanthes* accessions and determining the genetic relationship among these. A high level of polymorphism among 148 accessions was found. Genetic similarity coefficients ranged from 0.51 to 0.99 indicated that the genetic basis of the accessions was large. The range of genetic similarity coefficients of the species among *S. guianensis* varied from 0.62 to 0.99 was wider than other species varied from 0.60 to 0.90. This information will be useful to determine optimal breeding strategies. Furthermore, genetic distance between parents should be considered for *Stylosanthes* breeding programs.

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Author contributions CH and GL conceived and designed the experiments; CH performed the experiment, analyzed the data, and wrote this manuscript; GL and CB were responsible for the accessions collection, and discussing and reviewing the manuscript. All authors had read, critically edited, and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest All the authors declare that they have no conflict of interest.

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