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

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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 (ne) generated by the 18 primer pairs averaged 1.3552 and ranged from 1.2069 to 1.6080; Nei’s gene diversity (He) 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
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 environmental 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. Reyn 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. viscosa* (Sawkins et al. 2001).

Of these methods, RAPD is one of the simplest, but has poor reproducibility (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. seabrans*, 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 Agropecu Aris (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. | *Stylosanthes* species     | Geographical origins | Sources  | Accession no. | *Stylosanthes* species   | Geographical origins | Sources |
|---------------|---------------------------|----------------------|----------|---------------|--------------------------|----------------------|---------|
| S001          | *S. hamata* cv. Verano    | Australia            | CATAS    | S075          | *S. guianensis* TPRC90034 | China                | CATAS   |
| S002          | *S. scabra* cv. Seca      | Australia            | CATAS    | S076          | *S. guianensis* TPRC90019 | China                | CATAS   |
| S003          | *S. guianensis* cv.CPI18750A | Australia        | CATAS    | S077          | *S. guianensis* TPRC E1   | China                | CATAS   |
| S004          | *S. guianensis* TPRC90139 | China                | CATAS    | S078          | *S. guianensis* TPRC E7   | China                | CATAS   |
| S005          | *S. guianensis* cv. Oxley | Australia            | CATAS    | S079          | *S. guianensis* TPRC E9   | China                | CATAS   |
| S006          | *S. guianensis* USF873017 | USA                  | IGAS     | S080          | *S. guianensis* TPRC R93  | China                | CATAS   |
| S007          | *S. guianensis* TPRC90144 | China                | CATAS    | S081          | *S. guianensis* GC1480    | Philippines          | IRRI     |
| S008          | *S. guianensis* TPRC90072 | China                | CATAS    | S082          | *S. guianensis* GC1576(1) | Brazil               | EMBRAPA |
| S009          | *S. guianensis* TPRC E4   | China                | CATAS    | S083          | *S. guianensis* GC1528    | Brazil               | EMBRAPA |
| S010          | *S. guianensis* cv.Graham(1) | Australia    | IGAS     | S084          | *S. guianensis* GC1463    | Brazil               | EMBRAPA |
| S011          | *S. guianensis* USF873015(1) | USA            | IGAS     | S085          | *S. guianensis* GC1576(2) | Brazil               | EMBRAPA |
| S012          | *S. guianensis* TPRC90075 | China                | CATAS    | S086          | *S. guianensis* GC1524    | Brazil               | EMBRAPA |
| S013          | *S. guianensis* cv.Endeavour(1) | Australia | CATAS    | S087          | *S. guianensis* FM07-3    | Philippines          | IRRI     |
| S014          | *S. guianensis* CIAT11364 | Colombia            | CIAT     | S088          | *S. guianensis* GC1579    | Brazil               | EMBRAPA |
| S015          | *S. guianensis* cv.Reyan No.5 | China          | CATAS    | S089          | *S. guianensis* GC348     | Brazil               | EMBRAPA |
| S016          | *S. guianensis* TPRC R292 | China                | CATAS    | S090          | *S. guianensis* FM07-2    | Philippines          | IRRI     |
| S017          | *S. guianensis* USF873016(1) | USA            | IGAS     | S091          | *S. guianensis* GC1557    | Philippines          | IRRI     |
| S018          | *S. guianensis* USF873015(2) | USA            | IGAS     | S092          | *S. guianensis* GC1517    | Brasil               | EMBRAPA |
| S019          | *S. guianensis* TPRC90069 | China                | CATAS    | S093          | *S. guianensis* GC1517    | Brasil               | EMBRAPA |
| S020          | *S. guianensis* USF873014 | USA                  | IGAS     | S094          | *S. guianensis* GC1524    | Brasil               | EMBRAPA |
| S021          | *S. guianensis* TPRC90067 | China                | CATAS    | S095          | *S. guianensis* cv.Reyan No.2 | China           | CATAS   |
| S022          | *S. guianensis* TPRC90015 | China                | CATAS    | S096          | *S. guianensis* cv.Graham(2) | Australia  | IGAS    |
| S023          | *S. guianensis* TPRC90089 | China                | CATAS    | S097          | *S. guianensis* CIAT SK    | Colombia             | CIAT     |
| S024          | *S. guianensis* TPRC R291 | China                | CATAS    | S098          | *S. guianensis* cv. Graham(3) | Australia  | IGAS    |
| S025          | *S. guianensis* CIAT1044(2) | Colombia   | CIAT     | S099          | *S. guianensis* TPRC(QI)  | China                | CATAS   |
| S026          | *S. guianensis* TPRC90105 | China                | CATAS    | S100          | *S. guianensis* TPRC(HE)  | China                | CATAS   |
| S027          | *S. guianensis* USF873016(2) | USA            | IGAS     | S101          | *S. guianensis* CIAT early blossoming | Colombia  | CIAT    |
| S028          | *S. guianensis* cv.Reyan No.10 | China         | CATAS    | S102          | *S. guianensis* ATP309    | Australia             | CATAS   |
| S029          | *S. guianensis* CIAT11371 | Colombia            | CIAT     | S103          | *S. guianensis* ATP308    | Australia             | CATAS   |
| S030          | *S. guianensis* TPRC90006 | China                | CATAS    | S104          | *S. guianensis* cv. Mineirao | Australia  | CIAT    |
| S031          | *S. guianensis* TPRC90033 | China                | CATAS    | S105          | *S. guianensis* cv.Reyan No. 18 | Colombia  | CIAT    |
| S032          | *S. guianensis* TPRC90107 | Colombia            | CATAS    | S106          | *S. guianensis* FM9405-Parcel 3 | Australia  | CATAS   |
| S033          | *S. guianensis* TPRC90005(2) | China          | CATAS    | S107          | *S. guianensis* 109       | China                | CATAS   |
| S034          | *S. guianensis* CIAT11363(2) | Colombia   | CATAS    | S108          | *S. guianensis* cv.Graham(4) | Australia  | IGAS    |
| S035          | *S. guianensis* TPRC90003 | China                | CATAS    | S109          | *S. guianensis* cv.Endeavour(2) | Australia  | CATAS   |
| S036          | *S. guianensis* CIAT1281 | Brazil              | CIAT     | S110          | *S. scabra.Seca 33260    | China                | CATAS   |
| S037          | *S. guianensis* cv.Cook   | Bolivia             | CATAS    | S111          | *S. scabra.Seca Q10042    | China                | CATAS   |
| S038          | *S. guianensis* TPRC90050 | China                | CATAS    | S112          | *S. scabra.Seca 93116    | China                | CATAS   |
| Accession no. | Stylosanthes species | Geographical origins | Sources  | Accession no. | Stylosanthes species | Geographical origins | Sources |
|-------------|----------------------|---------------------|----------|-------------|----------------------|---------------------|----------|
| S039        | S. guianensis TPRC90058 | China               | CATAS    | S13         | S. seabrana 2323     | Australia           | CATAS    |
| S040        | S. guianensis CIAT11363(1) | Colombia            | CATAS    | S14         | S. seabrana 2534     | China               | CATAS    |
| S041        | S. guianensis TPRC90093 | China               | CATAS    | S15         | S. seabrana 2539     | China               | CATAS    |
| S042        | S. guianensis TPRC90028 | China               | CATAS    | S16         | S. scabra cv. Fitzroy 40205 | Australia | CATAS    |
| S043        | S. guianensis TPRC90037(3) | China               | CATAS    | S17         | S. scabra. Seca 40292 | China               | CATAS    |
| S044        | S. guianensis TPRC R273 | China               | CATAS    | S18         | S. guianensis cv. 907 | China               | IGAS     |
| S045        | S. guianensis TPRC90085 | China               | CATAS    | S19         | S. guianensis 90005(1) | China               | CATAS    |
| S046        | S. guianensis CIAT11376 | Colombia            | CIAT     | S20         | S. guianensis 129     | China               | CATAS    |
| S047        | S. guianensis TPRC90005 | China               | CATAS    | S21         | S. guianensis 130     | China               | CATAS    |
| S048        | S. guianensis TPRC90005(4) | China               | CATAS    | S22         | S. hamata cv. Schotiekl | China               | CATAS    |
| S049        | S. guianensis TPRC90005(1) | China               | CATAS    | S23         | S. guianensis 184     | China               | CATAS    |
| S050        | S. guianensis TPRC90047 | China               | CATAS    | S24         | S. guianensis 90088   | China               | CATAS    |
| S051        | S. guianensis cv. Reyan No.7  | China               | CATAS    | S25         | S. guianensis CIAT184  | Colombia            | CATAS    |
| S052        | S. guianensis TPRC90037(2) | China               | CATAS    | S26         | S. guianensis L6       | China               | CATAS    |
| S053        | S. guianensis TPRC90095(2) | China               | CATAS    | S27         | S. guianensis 90058   | China               | CATAS    |
| S054        | S. guianensis cv. Reyan No.13 | China               | CIAT     | S28         | S. guianensis 136(1)   | China               | CATAS    |
| S055        | S. guianensis TPRC90108 | China               | CATAS    | S29         | S. guianensis 90064   | China               | CATAS    |
| S056        | S. guianensis cv. Tardio | Brazil              | CIAT     | S30         | S. guianensis 90135   | China               | CATAS    |
| S057        | S. guianensis TPRC dianbai98 | China               | CATAS    | S31         | S. guianensis 90087   | China               | CATAS    |
| S058        | S. guianensis TPRC dianbai87 | China               | CATAS    | S32         | S. guianensis cv. Graham(5) | Australia | IGAS     |
| S059        | S. guianensis TPRC90134(2) | China               | CATAS    | S33         | S. guianensis FM05-1   | Brazil              | CATAS    |
| S060        | S. guianensis FM0405 | Colombia            | CIAT     | S34         | S. guianensis 90083   | China               | CATAS    |
| S061        | S. guianensis GC1578 | Brazil              | EMBRAPA  | S35         | S. guianensis 90009-1  | China               | CATAS    |
| S062        | S. macrocephala | Philippine          | IRRI      | S36         | S. guianensis 90135   | China               | CATAS    |
| S063        | S. capitata | Philippine          | IRRI      | S37         | S. guianensis Liu-6    | China               | CATAS    |
| S064        | S. guianensis FM05-1 | Philippine          | CIAT      | S38         | S. guianensis CIAT11371 | Colombia            | CIAT     |
| S065        | S. guianensis FM05-3 | Philippine          | CIAT      | S39         | S. guianensis cv.polyploidy of Reyan No.5 | China | CATAS    |
| S066        | S. guianensis FM03-2 | Brazil              | CATAS    | S40         | S. guianensis cv.Nina  | Brazil              | EMBRAPA  |
| S067        | S. scabra.Seca CIAT50 | Australia           | CIAT      | S41         | S. guianensis cv.Temprano | Brazil  | EMBRAPA  |
| S068        | S. guianensis 58719 | China               | CATAS    | S42         | S. hippocampoides     | Brazil              | EMBRAPA  |
| S069        | S. guianensis 87830 | China               | CATAS    | S43         | S. seabrana cv.Unica  | Brazil              | EMBRAPA  |
| S070        | S. guianensis 67652 | China               | CATAS    | S44         | S. seabrana cv.Primar | Brazil              | EMBRAPA  |
| S071        | S. guianensis TPRC L8 | China               | CATAS    | S45         | S. guianensis 540     | China               | CATAS    |
| S072        | S. guianensis TPRC E3 | China               | CATAS    | S46         | S. guianensis 541     | China               | CATAS    |
| S073        | S. guianensis TPRC L1 | China               | CATAS    | S47         | S. guianensis cv.Reyan No.20 | China | CATAS    |
| S074        | S. guianensis TPRC L2 | China               | CATAS    | S48         | S. guianensis 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 resuspended three *Stylosanthes* species. Each 10 µL PCR mixture contained 50 ng genomic DNA, 0.5 µM forward primer, 0.5 µM reverse primer, and 5 µL 2× Easy Taq PCR SuperMix (TransGen biotech, Beijing, China). The mixture was overlaid with 20 µL mineral oil before thermal cycling was commenced. Amplification was carried out on a Thermal Cycler Dice™ (Bio-Rad S1000™, USA) as follows: initial denaturation at 94 ºC for 5 min, followed by five cycles of 1 min denaturation at 94 ºC, annealing at 35 ºC for 1 min and elongation at 72 ºC for 45 s. In the subsequent 30 cycles, the annealing temperature was 50 ºC for 1 min, with a final extension step at 72 ºC for 30 s, terminating with an elongation step of 7 min at 72 ºC. The amplified products were stored at 4 ºC 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× TBE buffer (0.09 mol/L Tris-H 3BO3, 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 µg/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 resuspended 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'-TGAGTCCTAAACCGGATA-3'  
5'-GACTGCGTACGAATTTTGCA-3' | 350–2000 | 10 | 1.3162 | 0.2012 | 0.3275 |
| F02–R07      | 5'-TGAGTCCTAAACCGGAGC-3'  
5'-GACTGCGTACGAATTTTGCA-3' | 180–700 | 3 | 1.6080 | 0.3207 | 0.4582 |
| F02–R10      | 5'-TGAGTCCTAAACCGGAGC-3'  
5'-GACTGCGTACGAATTTTGC-3' | 200–1600 | 7 | 1.2069 | 0.1410 | 0.2422 |
| F03–R04      | 5'-TGAGTCCTAAACCGGAAAT-3'  
5'-GACTGCGTACGAATTTTGCA-3' | 150–1600 | 9 | 1.2675 | 0.1621 | 0.2556 |
| F03–R09      | 5'-TGAGTCCTAAACCGGAAAT-3'  
5'-GACTGCGTACGAATTTTGC-3' | 250–1200 | 7 | 1.3497 | 0.2121 | 0.3317 |
| F05–R10      | 5'-TGAGTCCTAAACCGGAAAG-3'  
5'-GACTGCGTACGAATTTTGC-3' | 150–1600 | 6 | 1.4722 | 0.2695 | 0.4009 |
| F06–R04      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTTGC-3' | 250–1200 | 6 | 1.4835 | 0.2729 | 0.4039 |
| F06–R05      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTAAAC-3' | 150–2000 | 8 | 1.3232 | 0.1752 | 0.2624 |
| F06–R07      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 150–1200 | 10 | 1.2866 | 0.1855 | 0.3074 |
| F07–R09      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 200–1300 | 9 | 1.5379 | 0.3002 | 0.4459 |
| F08–R01      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 400–2000 | 12 | 1.2837 | 0.1766 | 0.3119 |
| F08–R05      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 200–1300 | 7 | 1.3260 | 0.1858 | 0.2832 |
| F08–R06      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 250–1500 | 8 | 1.3052 | 0.1975 | 0.3174 |
| F09–R02      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 150–1000 | 10 | 1.3215 | 0.1771 | 0.2722 |
| F09–R03      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 150–700 | 6 | 1.4471 | 0.2436 | 0.3616 |
| F09–R04      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 200–1300 | 7 | 1.2084 | 0.1304 | 0.2233 |
| F10–R02      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-3' | 200–900 | 6 | 1.2709 | 0.1611 | 0.2530 |
| F10–R08      | 5'-TGAGTCCTAAACCGGATG-3'  
5'-GACTGCGTACGAATTTCGA-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. hnamata*, *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 \textit{S.capitata}. The other subgroup comprised 13 accessions which contained one \textit{S. hnamata} accession (S001), four \textit{S. seabrana} accessions (S113, S114, S143, and S144), and seven \textit{S. scabra} accessions (S002, S067, S110, S111, S112, S116, and S117). Group 2 consisted of 134 accessions of \textit{S. guianensis} except for an individual \textit{S. seabrana} accession (S115). The GSC ranged from 0.62 to 0.99, and further categorized six subgroups. Group 1 comprised a single accession S056 (\textit{S. guianensis} cv. Tardio) from Brazil, belonging to the disease-resistant cultivars. Group 2 also only contained one accession S104 (\textit{S. guianensis} cv. Mineiro) from Australia and exhibited high yields. Group 3 contained ten accessions, including S095 (\textit{S. guianensis} cv. Reyan No. 2), and presented the characteristics of early blossoming and disease resistance. Group 4 included one accession, S142 (\textit{S. hippocampoides}) from Brazil. Group 5 consisted of 41 accessions, including the Chinese cultivars S105 (\textit{S. guianensis} cv. Reyan No. 18), S125 (\textit{S. guianensis} CIAT184), S118 (\textit{S. guianensis} cv. 907), S147 (\textit{S. guianensis} cv. Reyan No. 20), and S148 (\textit{S. guianensis} cv. Reyan No. 21), and the GSC ranged from 0.70 to 0.97. Most of the accessions shared relationship with \textit{S. guianensis} CIAT184. The anthracnose-resistance cultivar \textit{S. guianensis} cv. 907 was selected from the population of \textit{S. guianensis} CIAT184 by mutation breeding, \textit{S. guianensis} cv. Reyan No. 20 and \textit{S. guianensis} cv. Reyan No. 21 was selected from the population of \textit{S. guianensis} cv. Reyan No. 2 by space mutation-breeding, and \textit{S. guianensis} cv. Reyan No. 2 was selected from \textit{S. guianensis} CIAT184. Subgroup 6 included the other 80 accessions including the Chinese cultivars S015 (\textit{S. guianensis} cv. Reyan No. 5), S028 (\textit{S. guianensis} cv. Reyan No. 10), S051 (\textit{S. guianensis} cv. Reyan No. 7), and S054 (\textit{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 \textit{Stylosanthes} accessions. We found a high level of polymorphism (100%) among various accessions, which confirms that the SRAP marker technique generates highly reproducible DNA profiles for \textit{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 \textit{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 \textit{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 \textit{Stylosanthes}. Furthermore, the molecular relationships generated from this study should be useful in breeding programs for \textit{Stylosanthes}. 

![Fig. 3 Principal coordinate analysis of 148 Stylosanthes accessions based on the genetic similarity matrix generated from SRAP data](image-url)
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.

References

Budak H, Shearman RC, Gaussian RE, Dweikat I (2004) Application of sequence-related amplified polymorphism markers for characterization of turfgrass species. HortScience 39(5):955–958
Burt RL, Miller CP (1975) *Stylosanthes*—a source of pasture legumes. Trop Grassl 9(2):117–123
Costa NMS, Ferreira MB (1984) Some Brazilian species of *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 53–101
Curtis MD, Manners JM, Cameron DF (1995) Molecular evidence that diploid *Stylosanthes humilis* and diploid *Stylosanthes hamata* are progenitors of allotetraploid *Stylosanthes hamata* cv. ‘Verano’. Genome 38(2):344–348
Dongre AB, Raut MP, Bhandarkar MR, Meshram KJ (2011) Identification and genetic purity testing of cotton F1 hybrid using molecular markers. Indian J Biotechnol 10(3):301–306
Glover BJ, Gillies ACM, Abbott RJ (1994) Use of the polymerase chain reaction to investigate the delimitation of two agriculturally important species of *Stylosanthes* (Aubl.) Sw. Bot J Scoc 47(1):83–96
Huang CQ, Liu GD, Bai CJ, Wang WQ, Tang J (2014) Application of SRAP markers in the identification of *Stylosanthes guianensis* hybrids. Mol Biol Rep 41(9):5923–5929
Jiang CS, Ma XR, Zhou DM, Zhang YZ (2005) AFLP analysis of genetic variability among *Stylosanthes guianensis* accessions resistant and susceptible to the stylo anthracnose. Plant Breed 124(6):595–598
Kang SY, Lee GI, Lim KB, Lee HJ, Park IS, Chung SI, Kim IB, Kim DS, Rhee HK (2008) Genetic diversity among Korean bermudagrass (*Cynodon* spp.) ecotypes characterized by morphological, cytological and molecular approaches. Mol Cells 25(2):163–171
Kazan K, Manners JM, Cameron DF (1993) Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome 36(1):43–49
Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theor Appl Genet 103(2):455–461
Liu CJ, Musial JM (1997) *Stylosanthes* sp. aff. *S. scabra*: a putative diploid progenitor of *Stylosanthes scabra*. Pl Syst Evol 208(1):99–105
Liu GD, Phaikaw C, Stur WW (1997) Status of *Stylosanthes* development in other countries: II. *Stylosanthes* development and utilisation in China and south-east Asia. Trop Grassl 31:460–467
Liu CJ, Musial JM, Thomas BD (1999) Genetic relationships among *Stylosanthes* species revealed by RFLP and STS analyses. Theor Appl Genet 99(7):1179–1186
Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA. 76(10):5269–5273
Santos MO, Karia CT, Resende RM, Chiari L, Jungmann L, Zucchi MI, Souza AP (2009a) Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. Conserv Genet Resour 1:43–46
Santos MO, Sassaki RP, Chiari L, Resende RMS, Souza AP (2009b) Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel. Mol Ecol Resour 9(1):192–194
Santos MO, Sassaki RP, Ferreira THS, Resende RMS, Chiari L, Karia CT, Faleiro FG, Jungmann L, Zucchi MI, Souza AP (2009c) Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume. Conserv Genet Resour 1:481–485
Santos-Garcia MO, Karia CT, Resende RMS, Chiari L, Vieira ML, Zucchi MI, Souza AP (2012) Identification of *Stylosanthes guianensis* varieties using molecular genetic analysis. AoB Plants 2012:pls001
Sawkins MC, Mass BL, Pengelly BC, Newbury HJ, Ford-Lloyd BV, Maxted N, Smith R (2001) Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. Mol Ecol 10(8):1947–1958
Stace HM, Cameron DF (1984) Cytogenetics and the evolution of *Stylosanthes*. In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 49–72
Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17(16):6463–6471
Vander Stappen J, Volckaert G (1999) Molecular characterization and classification of *Stylosanthes mexicana*, *S. macrocarpa*, *S. seabrana* and *S. fruticosa* by DNA sequence analysis of two chloroplast regions. DNA Seq 10(3):199–202
Vander Stappen J, Weltjens I, Volckaert G (1999) Microsatellite markers in *Stylosanthes guianensis*. Mol Ecol 8(3):514–517
Vander Stappen J, Weltjens I, Gama Lopez S, Volckaert G (2000) Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variability of *S. humilis* accessions from South American origin. Euphytica 113(2):145–154
Vander Stappen J, Marant S, Volckaert G (2003) Molecular characterization and phylogenetic utility of the rDNA external transcribed spacer region in *Stylosanthes* (Fabaceae). Theor Appl Genet 107(2):291–298
Vieira MC, Aguiar-perecin MLR, Martins PS (1993) A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw. Leguminosae. Cytologia 58(3):305–311

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21):4407–4414

Williams RJ, Reid R, Schhultze-Kraft R, Costa NM, Thomas BD (1984) Natural distribution of *Stylosanthes* S. In: Edye LA, tace HM (eds) The biology and agronomy of *Stylosanthes*. Academic Press, Sydney, pp 73–101

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18(22):6531–6535