# Application of molecular markers to appraise the genetic fidelity of *Ceropegia spiralis*, a threatened medicinal plant of South India

*Ceropegia spiralis* Wight (family Apocynaceae) is an endangered medicinal plant of the Western Ghats of India[1]. It is well known for its medical implications, edible tubers and ornamental flowers. The root tubers contain 'cerpegin' alkaloid which has several pharmacological activities[2,3]. Tubers also contain starch, sugars, gum, albuminoids, fats, and crude fibre and are a valuable constituent in many traditional Indian Ayurvedic drug preparations that are active against diarrhoea and dysentery[4]. Several workers demonstrated the micropropagation of *Ceropegia* species[5–7]. In *C. spiralis*, micropropagation through axillary buds, thin cell layers and *in vitro* propagation via somatic embryogenesis has been reported earlier[8–11]. The occurrence of genetic defects in the regenerants can seriously limit the broader utility of micropropagation systems[12]. Molecular markers like RAPD, ISSR, RFLP and SSRs have been suggested to be useful for evaluating genetic stability of micropropagated plants[12,13]. However, no such studies have been conducted on the genus *Ceropegia.* In this context, efforts were made to assess genetic stability of micropropagated plants of *C. spiralis* by using RAPD and ISSR markers.

Genomic DNA was isolated from fresh leaves of mother as well as 10 randomly selected micropropagated plants from the fifth subculture passage[14]. The plantlets used for molecular screening are derived from the same source plant of the fourth subculture. Quality and quantity of DNA were confirmed by electrophoresis with known quality of DNA. Each sample was diluted to 20 ng $\mu l^{-1}$ in TE buffer and stored at –20°C until further use. Sixty-four arbitrary, decamer RAPD primers (Genemed Synthesis Inc, Texas, USA) were used for screening and amplification was carried out using the method of Williams *et al.*[15]. PCR was performed in a volume of 25 µl containing 2.0 µl of template DNA (40 ng), decamer primer (2.0 µl), 10X reaction buffer (2.5 µl), 2.5 mM $MgCl_2$ (1.0 µl), 0.5 µl dNTP mix (10 mM each of dATP, dGTP, dTTP and dCTP) and 0.2 µl *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). Final volume was made using autoclaved distilled water. PCR amplification was programmed for initial DNA denaturation at 94°C for 4 min, followed by 40 cycles of 1 min denaturation at 94°C, 1 min, annealing at 35°C and 2 min extension at 72°C, with a final extension at 72°C for 10 min. The protocol for ISSR analysis was adapted from Zietkiewicz *et al.*[16].

Thirty-two ISSR primers (UBC, Canada) were screened for amplification with mother as well as micropropagated plants. A factorial experiment with varying concentrations of genomic DNA (20, 30 and 40 ng $\mu l^{-1}$), $MgCl_2$ (1.0, 2.0 and 3.0 mM) and *Taq* DNA polymerase (0.5, 0.75 and 1U) was performed to optimize PCR conditions. The amplification was programmed as follows; initial denaturation at 94°C for 6 min (one cycle), denaturation at 94°C for 1 min, annealing at 48°C, 50°C and 52°C (depending on the primer used) for 1 min and extension at 72°C for 2 min (38 cycles) and final extension at 72°C for 12 min (one cycle). PCR reactions were carried out in a thermocycler (BIOER, China).

The existing micropropagation system has produced a large number of plantlets with high survival rate during acclimatization (Figure 1)[11]. No visible morphological differences were revealed among the regenerated plantlets (Figure 1 *d*). Of the 64 RAPD primers screened, 10 primers were found to generate highly reproducible patterns with multiple discrete bands and were thus used for further analysis. Ten RAPD primers yielded 54 bands with an average of 5.4 bands per primer (Table 1). Maximum number of bands (9) was produced by primers 952 (Figure 2 *a*) and 985, whereas only 3 amplification products were detected in primers 930, 963 and 973 (Table 1).

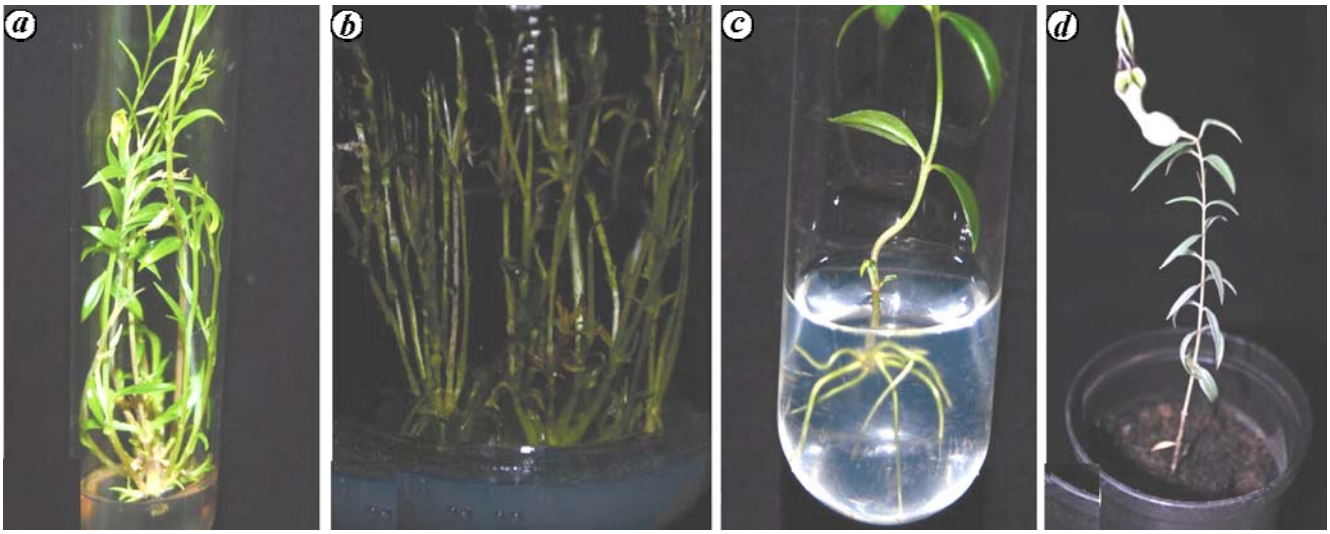


**Figure 1.** Micropropagation of *Ceropegia spiralis*. ***a***, ***b***, Shoot multiplication (MS + BAP 2.0 mg/l + TDZ 0.5 mg/l). ***c***, *In vitro* rooting (1/2 MS + IBA 1.0 mg/l), ***d***, Well-established micropropagated plant.

**Table 1.** Primers, their sequences, number and size of the amplified fragments generated by RAPD primers in *Ceropegia spiralis*

| Primer code | Primer sequence (5′–3′) | Number of bands/primer | Total number of bands | | Size range (bp) |
|---|---|---|---|---|---|
| | | | Monomorphic | Polymorphic | |
| 930 | CTGCTGTGTA | 3 | 33 | – | 350–1500 |
| 952 | CTCCAGTCAT | 9 | 99 | 3 | 300–1950 |
| 963 | ACGTGTCGAT | 3 | 33 | – | 340–550 |
| 971 | AGGCTGACTT | 6 | 66 | – | 380–900 |
| 973 | AGATCCCCTT | 3 | 33 | – | 770–1050 |
| 985 | GCTCGTCGTT | 9 | 99 | – | 300–1900 |
| 987 | TGTCGAGGTT | 4 | 44 | – | 240–900 |
| 989 | GCGTCTGGTT | 5 | 55 | – | 220–900 |
| 994 | CGCACTCAAA | 5 | 55 | – | 520–1500 |
| 995 | GTGTCGGAAA | 7 | 77 | – | 250–1500 |
| | Total | 54 | 594 | 3 | |

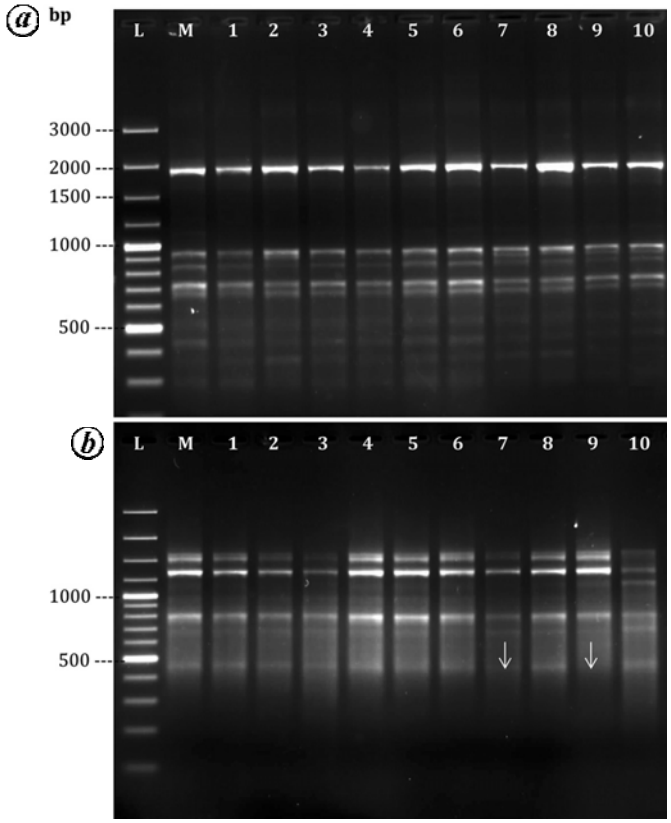


**Figure 2.** DNA fingerprints of *C. spiralis*. ***a***, RAPD primer (985); ***b***, ISSR primer (UBC-801), Lane L, 100 bp ladder; lane M, Mother plant; lanes 1–10, *In vitro* raised clones and white arrow, missing band.

A total of 597 bands was generated by 10 primers with 10 micropropagated plants and mother plant (Table 1). Out of 597 bands, 594 were monomorphic and only 3 bands showed polymorphism (primer 952). Polymorphic loci were observed in single micropropagated plant (clone 10). Similarly, RAPD markers have been shown to detect genetic variation among micropropagated plants of *Camellia sinensis*[12] and *Dictyospermum ovalifolium*[17]. However, RAPD analysis failed to detect the genetic variation in micropropagated plants of *Gerbera jamesonii*[13].

ISSR analysis was applied to investigate possible genomic changes in the clones that failed to give variation in RAPD analysis. Of the 32 ISSR primers screened, 10 primers having reproducible banding pattern were selected for further analysis. The genomic DNA at 30 ng $\mu l^{-1}$ was found to be optimum for PCR amplification. Among the three concentrations of *Taq* DNA polymerase, 1U yielded sharp and consistent bands, whereas the other concentrations produced either faint bands or smears. Sharpness of bands was improved by increasing $MgCl_2$ concentration to 2.0 mM. Information regarding the selected ISSR primers, total number of amplified fragments scored, number of bands for each primer and size range of the amplified bands is summarized in Table 2. The highest number of scorable bands (6) was obtained by primers UBC-801, UBC-810 and UBC-815, whereas the primer UBC-807 generated only two bands (Table 2). A total of 508 amplified products was generated from 10 ISSR primers with 10 micropropagated plants and mother plant. Among these, 502 bands were monomorphic and the remaining 6 bands were polymorphic. Primer UBC-801 showed two polymorphic loci in two clones (Figure 2 *b*). Polymorphic loci were observed in two different micropropagated plants (clones 9 and 10). In clone 10, polymorphism has already been detected in RAPD profile. ISSR fingerprinting has been previously found to be useful for detecting somaclonal variation among micropropagated plants of *D. ovalifolium*[17] and *G. jamesonii*[13].

A total of 1105 amplified products were generated (both RAPD and ISSR analysis), out of which 1096 bands (99.19%) were monomorphic across all the plantlets and only 9 bands (0.81%) showed polymorphism. RAPD and ISSR

**Table 2.** Primers, their sequence motifs, annealing temperatures, and number and size of the amplified fragments generated by ISSR primers in *C. spiralis*

| Primers | 5′–3′ motif/sequence | Annealing temperature (°C) | Number of bands/primer | Total number of bands | | Amplification range (bp) |
|---|---|---|---|---|---|---|
| | | | | Polymorphic | Monomorphic | |
| UBC-801 | (AT)8T | 52 | 6 | 64 | 2 | 650–2400 |
| UBC-807 | (AG)8T | 51 | 2 | 22 | – | 530–670 |
| UBC-808 | (AG)8C | 48 | 5 | 55 | 1 | 470–1600 |
| UBC-809 | (AG)8G | 50 | 5 | 54 | 1 | 680–1200 |
| UBC-810 | (GA)8T | 52 | 6 | 66 | 1 | 300–1180 |
| UBC-811 | (GA)8C | 49 | 5 | 55 | – | 600–1750 |
| UBC-813 | (CT)8T | 52 | 3 | 33 | – | 700–1200 |
| UBC-814 | (CT)8A | 52 | 5 | 54 | 1 | 600–1500 |
| UBC-815 | (CT)8G | 50 | 6 | 66 | – | 550–2100 |
| UBC-818 | (CA)8G | 48 | 3 | 33 | – | 520–1550 |
| | Total | | 46 | 502 | 6 | |

markers amplify different regions of the genome, and allow better chance for the identification of genetic variations in the clones[13]. Of the two PCR-based assays, ISSR marker was more effective in detecting polymorphism among the micropropagated plants of *C. spiralis*. Similar observations were made in the ISSR fingerprints of micropropagated plants of *C. sinensis*[12] and *G. jamesonii*[13].

In this study, the genetic stability of the *in vitro* raised clones of *C. spiralis* was confirmed using DNA-based markers. RAPD and ISSR markers proved to be effective and rapid tools for assessing the genetic stability in *C. spiralis*. Genetic stability of the micropropagated plants is the first illustration of the utility of molecular markers in the detection of genetic variation in *C. spiralis* and genus *Ceropegia* as well. These results suggest that the axillary shoot bud proliferation can be used as an efficient micropropagation tool for mass propagation of *C. spiralis*.


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