Assessment of diversity in *Harpagophytum* with RAPD and ISSR markers provides evidence of introgression

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The genus *Harpagophytum* has two species: *H. procumbens* which is an important medicinal plant in southern Africa, and *H. zeyheri.* Genetic diversity in 96 samples, obtained by germinating seeds collected from Botswana, was assessed using six inter-simple sequence repeat (ISSR) and 10 random amplified polymorphic DNA (RAPD) primers. These DNA markers yielded a total of 138 polymorphic bands. Polymorphism information content (PIC) ranged from 0.06 to 0.39 for ISSR primers, and from 0.09 to 0.43 for RAPD primers. Jaccard's similarity coefficients were highest when seedlings derived from the same fruit capsule were compared, while seedlings from different fruits on the same plant had intermediate values. The lowest values were recorded among seedlings from different plants. These results were consistent with an outcrossing breeding system in *Harpagophytum*. Analysis of molecular variance revealed significant differentiation ($P < 0.01$) between taxonomic units within *Harpagophytum*. About 39% of the variability occurred between the two species, *H. procumbens* and *H. zeyheri*. Plants with an intermediate morphology, i.e. putative hybrids (PH), showed 21% differentiation when compared with *H. procumbens* ssp. *procumbens* (PP), and 19% when compared with *H. procumbens* ssp. *transvaalense* (PT) or with *H. zeyheri* (ZZ). In addition, a deviating variant of PT was identified, here termed '*procumbens* new variety' (PN). PN showed only 9% differentiation when compared with PT, 22% when compared with PP or with PH, and 41% when compared with ZZ. Considerable differentiation between the two *Harpagophytum* species was revealed also by a cluster analysis. Introgression was, however, suggested by the intermediate position of the putative hybrid plants in a principal component analysis while inter-specific gene flow was shown by a Bayesian genetic structure analysis.

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Harpagophytum DC. ex Meisn. is a bitypic genus within the sesame family, Pedaliaceae. The two species *H. procumbens* and *H. zeyheri* are native to southern Africa, particularly Botswana, Namibia, South Africa, Angola and Zimbabwe (Ihlenfeldt 1988), where they grow in areas with low rainfall (100–200 mm year^{-1}). Although mainly adapted to the deep sands of the Kalahari desert, they can also thrive in loam soils. Typical habitats include alluvial or overgrazed plains and fossil dunes with sparse savannah vegetation. Both species are outcrossing and bee-pollinated (MCAN 2011). They are perennial herbs with several prostrate annual stems that derive from a large succulent tuberous taproot. Long-distance seed dispersal can be achieved when the large spiny fruit capsule becomes entangled in animals' fur and feet, hence their popular names 'devil's claw' and 'grapple plant'.

Two subspecies of each species are generally recognized: *H. procumbens* ssp. *procumbens* (PP), *H. procumbens* ssp. *transvaalense* (PT), *H. zeyheri* ssp. *zeyheri* (ZZ) and *H. zeyheri* ssp. *sublobatum* (ZL). PP is found in the sandy parts of Namibia and Botswana and in the northern Cape region of South Africa, PT in the northeastern Transvaal area of South Africa and just across the borders to Botswana and Zimbabwe, ZZ in Transvaal and south-eastern Botswana, and ZL in Angola and the northern parts of Namibia, Botswana, and the northeastern part of Transvaal. Although these taxa are mainly allopatric, transitional forms occur when they sometimes grow sympatrically (Ihlenfeldt 1988).

The spectacular fruit capsules play a major role in the taxonomy of the genus, while vegetative features, particularly the leaves, are less useful since they exhibit high levels of phenoplasticity (Ihlenfeldt 1988). The laterally compressed, bicarpellar and woody fruit capsule is imperfectly dehiscent along its longitudinal length. Each half of the capsule has a row of arms along the edges and each arm bears re-curved spines. The numerous seeds are stacked in two or four rows in each of two loculi, according to the species. Two rows is a diagnostic character for *H. zeyheri,* while four rows is diagnostic for *H. procumbens* (Ihlenfeldt 1988). Muzila et al. (2011)

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observed, however, that seed row number is not necessarily restricted to two or four, which may suggest introgression between the two *Harpagophytum* species.

Harpagophytum has been widely used as a medicinal plant in Africa for centuries. The indistinguishable dried tubers of both species have also been exported to the pharmaceutical industry in Europe since the 1960s, where they are known as 'Radix Harpagophyti' and used for manufacturing remedies for arthritis and other ailments (Feistel and Gaedcke 2000). In reviews on the medicinal efficacy of *Harpagophytum*, the exact origin of the tested drugs has been unknown and it has therefore not been possible to discriminate between the two species (McGregor et al. 2005; Chrubasik et al. 2007). Presently, most of the attention is, however, given to *H. procumbens* which is generally regarded as medicinally more efficient (Chrubasik et al. 2004; Mncwangi et al. 2013) although this has been contested (Stewart and Cole 2005). The rising demand for *H. procumbens* on the international market has lead to extensive collection of tubers in rural areas where this harvest constitutes a primary or even sole cash income. In Botswana, commercial export began in the 1970s. Although the Agricultural Resources Conservation Act and the Control of Goods Act carefully regulate the international trade (Stewart and Cole 2005), *H. procumbens* is presently listed as "near threatened" in Botswana.

There has been some controversy surrounding the medicinal efficacy of *Harpagophytum*-based drugs that may involve the origin of the collected tubers (Moussard et al. 1992; Baghdikian et al. 1997; Chrubasik et al. 2002; Mncwangi et al. 2013). Although the two *Harpagophytum* species are easy to distinguish in the wild, intentional adulteration with tubers of *H. zeyheri* has been reported (Mncwangi et al. 2013). There is also the possibility that unintentional adulteration occurs through inclusion of plant material resulting from introgression with *H. zeyheri*, thus decreasing the quality of some of the medicinal drugs.

Chromosome numbers have not yet been published for this genus, but our as yet unpublished counts on wild-collected flowers in Botswana suggest that *H. procumbens* is diploid, 2n = 20, while putative hybrids are mostly diploid (2n = 20, 22 and 24) and occasionally also tetraploid (Muzila et al., unpubl.). Homoploid introgression, i.e. introgression between taxa at the same ploidy level, can be very difficult to determine, and is sometimes evidenced only in the morphology of the plants or only in the nuclear DNA (Rieseberg and Brunsfeld 1998). The main objective of our research was to investigate possible introgression by performing a genetic analysis of a morphologically variable *Harpagophytum* material, including several taxa and some putatively hybridogenous plants, using random amplified polymorphic DNA (RAPD) and inter-sequence simple repeat (ISSR) markers.

MATERIAL AND METHODS

Sampling

Fruit capsules were collected from six different geographical locations in Botswana, using a systematic qualitative sampling technique; specimens that conformed to the species descriptions were collected together with specimens that deviated from the descriptions or from anything so far collected. A presumed sympatric zone of introgression in southeastern Botswana was targeted, but a few samples were also obtained from a locality where *H. procumbens* ssp. *procumbens* grows allopatrically (Fig. 1, Table 1). However, it should be noted that the *Harpagophytum* distribution stretches a little bit wider than what Ihlenfeldt and Hartmann (1970) had recorded in their original map.

Seeds from the collected fruit capsules were germinated on petri dishes at 30°C at Balsgard, SLU, Sweden. Seed germination was often unsuccessful and had to be repeated several times to produce a sufficient number of plantlets.

Morphology of taxa and putative hybrids

Harpagophytum procumbens ssp. *Procumbens*

Diagnostic features include a fruit capsule with eight long curved arms on each half of the capsule, each arm with scattered re-curved spines along the length and at the tip. The length of the longest arm is about five times the width of the fruit capsule proper. Sometimes there are only three arms in a row, but the lowest arm is then much broader and imperfectly divided. A fruit capsule usually has 25 to 30 seeds, arranged in four rows in each seed locule.

Harpagophytum procumbens ssp. *Transvaalense*

The fruit capsule usually has three arms in each row, and the length of the longest arm does not exceed twice the width of the fruit capsule proper.

A likely but undescribed variety of *Harpagophytum procumbens* – Fruit capsule is similar to that of *H. procumbens* ssp. *transvaalense* but with only three seed rows in each locule. Arm lengths for the specimens we have seen are also somewhat shorter than those of a typical *Harpagophytum procumbens* ssp. *transvaalense* capsule.

Harpagophytum zeyheri ssp. *Zeyheri*

The fruit capsule has fourshort and slightly curved arms on each half of the capsule, with recurved spines at the tips. The longest arm does not exceed the width of the fruit capsule proper. Sometimes the arms are much shorter and take the form of four rigid wings with re-curved spines. The wings sometimes bifurcate into short arms in the upper portion of the fruit. There are usually two seed rows in each locule.

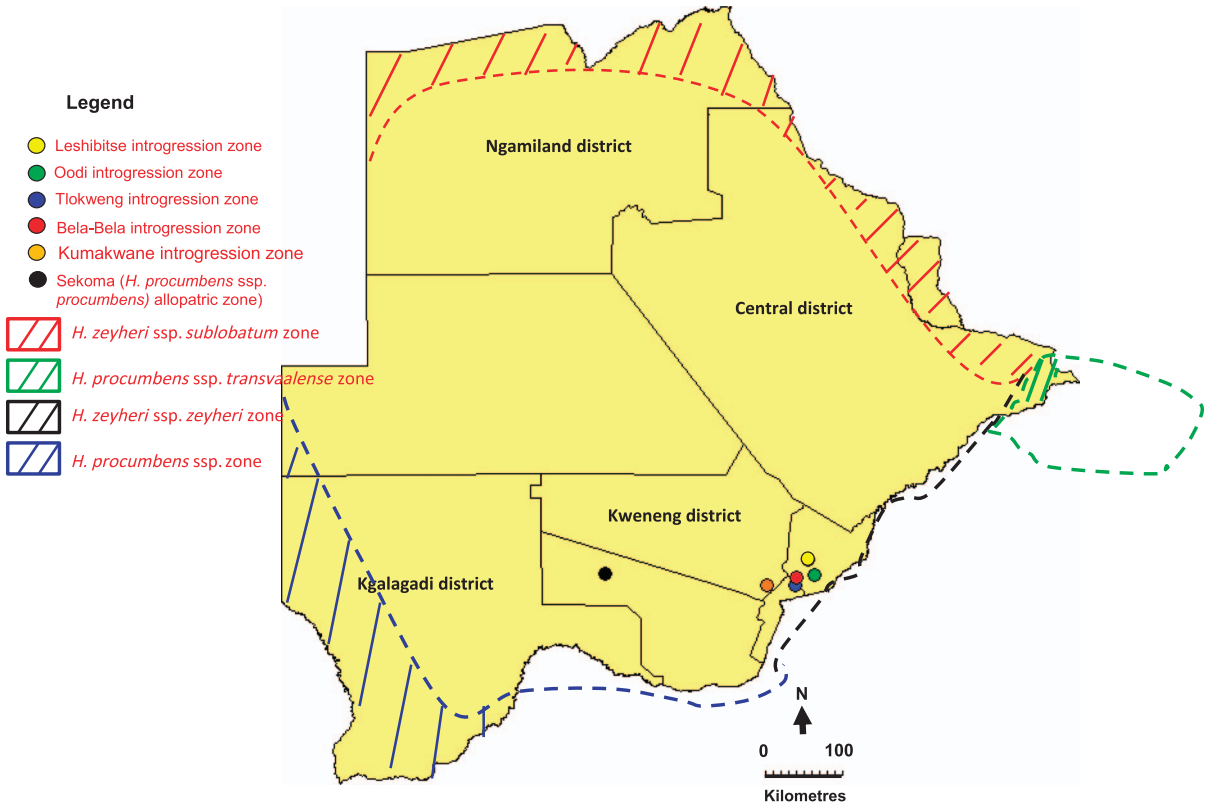

Fig. 1. Map of Botswana indicating the sampling of *Harpagophytum*.

Harpagophytum zeyheri ssp. *Sublobatum*

The fruit capsule has broad arms, with the length of the arms nearly half the width or the full width of the fruit capsule proper, but also with spines along the length of the arms and at their tips.

Putative hybrids

Four different putative hybrid plants were sampled in the field. In plant T5PH the dorsal and ventral halves of the single fruit have short arms modified into wing-like appendages with recurved spines at the ends as in *H. zeyheri*. The sinester (left) side has four arms, while the dexter (right) has only two since two of the posterior arms are fused into a single wing-like appendage. The fruit is 28–38 mm long and 11–15 mm wide, with a circumference of 105–110 mm, and has two seed rows per locule.

In plant O9PH, length of the longest arm on the single fruit is almost equal to the width of the fruit capsule proper. The sinester and dexter sides have two arms each, with the posterior-sinister arm being much wider than the rest. In addition, there are two more anteriorly placed arms that give the whole fruit capsule the shape of an inverted 'T' (cruciform) and thus deviating from the ellipsoid shape of a typical *Harpagophytum* fruit capsule. The fruit is 31–42 mm long and 18–21 mm wide, with a circumference of 102–105 mm. Each seed locule has three seed rows.

Plant K1PH has a fruit capsule shaped like those of *H. procumbens* ssp. *transvaalensis* except for having two seed rows as in *H. zeyheri*. The fruit is 55–63 mm long and 34–42 mm wide, with a circumference of 119–126 mm. This plant is probably polyploid since the fruit width of *H. procumbens* proper is only about 15–32 mm. Vegetative features also appear to be almost double the normal size of a *H. procumbens* plant.

Plant O5PH has a fruit capsule where length of the longest arm is almost equal to the fruit capsule width, thus being intermediate between *H. zeyheri* and *H. procumbens*. The fruit is 57–61 mm long and 24–25 mm wide, with a circumference of 141–153 mm, and has 3 seed rows in one locule and four seed rows in the other. Just like K1PH, this plant is probably polyploid.

DNA analysis

DNA extraction

DNA material was extracted from 96 plantlets (Table 1) using the E.Z.N.A SP DNA mini kit (Omega Bio-Tek, Norcross, GA, USA). The quality of DNA was inspected

Table 1. *Accessions of Harpagophytum subjected to DNA marker analysis.*

Location	Accession code and taxon
Bela-Bela	*H. zeyheri* ssp. *zeyheri*
Introgression zone (24°30.7S, 26°2.27E)	B1A1ZZ
Kumakwane	Putative hybrid
Introgression zone (24°37.9S, 25°41.1E)	K1A1PH
	H. procumbens ssp. *transvaalense*
	K2A1PT
	H. procumbens new variety
	K3A1PN, K3A2PN
Leshibitse	*H. procumbens* ssp. *transvaalense*
Introgression zone (24°16.9S, 26°9.41E)	L1A1PT, L2A1PT, L3A1PT
	H. procumbens new variety
	L4A1PN, L4A2PN
	H. zeyheri ssp. *zeyheri*
	L5A1ZZ
Oodi	Putative hybrid
Introgression zone (24°28.1S, 26°2.83E)	O5A1PH, O9A1PH
	H. procumbens ssp. *transvaalense*
	O1A1PT, O1B1PT, O1B2PT, O2A1PT, O2B1PT, O2C1PT, O3A1PT, O4A1PT, O4A2PT, O4A3PT, O4B1PT, O4B2PT, O4B3PT, O4B4PT, O4B5PT, O4B6PT, O4C1PT, O4C2PT, O6A1PT, O6A2PT, O6A3PT, O6A4PT, O6A5PT, O6A6PT, O6A7PT, O6A8PT, O6A9PT, O6A10PT, O6B1PT, O6C1PT, O6C2PT, O6C3PT, O6D1PT, O6E1PT, O6F1PT, O6F2PT, O6F3PT, O6F4PT, O6F5PT, O7A1PT, O7A2PT, O7B1PT, O7B2PT, O7B3PT, O7B4PT
	H. procumbens ssp. *transvaalense* new variety
	O8A1PN, O8A2PN, O8A3PN, O8A4PN, O8A5PN, O10A1PN, O11A1PN, O11B1PN
Sekoma	*H. procumbens* ssp. *procumbens*
H. procumbens ssp. *procumbens* allopatric zone (24°24.8S, 23°47.8E)	S1A1PP, S2A1PP
Tlokweng	Putative hybrid
Introgression zone (24°37.8S, 25°59.1E)	T5A1PH, T5A2PH
	H. procumbens ssp. *transvaalense*
	T3A1PT, T4A1PT, T4B1PT,T4C1PT
	H. zeyheri ssp. *zeyheri*
	T1A1ZZ, T1A2ZZ, T1A3ZZ, T1A4ZZ, T1A5ZZ, T1B1ZZ, T2A1ZZ, T2A2ZZ, T2A3ZZ, T2A4ZZ, T2A5ZZ, T2A6ZZ, T2A7ZZ, T6A1ZZ, T7A1ZZ, T7A2ZZ, T7A3ZZ, T7A4ZZ, T7A5ZZ, T7A6ZZ, T7A7ZZ

visually using a low mass ladder (Invitrogen Life Technologies, Carlsbad, CA, USA) in 2% agarose gel.

RAPD analysis

The polymerase chain reaction (PCR) protocol comprised a total volume of 25 µl, with 0.2 µl of 5 U μl^{-1} *Taq* polymerase (Amersham BioSciences, Uppsala, Sweden), 3 µl of DNA template (10 ng μl^{-1}), 1.0 µl (5 µM) of primer (Eurofins MWG Operon, Ebersberg, Germany), 16.2 µl dH_2O, 0.5 µl of 10 mM dNTPs, 1.6 µl of 25 µM $MgCl_2$ and 2.5 µl of reaction buffer. A PX2 (Thermo Hybaid, Ulm, Germany) thermocycler was used to run the PCR. The sequence had 45 cycles of 94°C for 15 s, 36°C for 45 s (with a ramp rate of 0.4°C s^{-1}), and a final extension of 72°C for 1.5 min. A total of 30 primers were tested for reproducibility and 10 (Table 2) were retained for further analyses. The PCR products were separated by electrophoresis in 1.8% agarose gel, stained in "Orange G" dye and photographed under ultraviolet illumination. Only clearly visible DNA fragments with lengths between 150 and 2176 bp were scored. Scoring for the presence or absence of DNA fragments was aided by the use of 1kb DNA ladder and the software package GeneSnap (Syngene 2008).

ISSR analysis

The PCR protocol used contained a total volume of 25 µl, with 0.2 µl of 5 U μl^{-1} *Taq* polymerase, 1 µl of DNA template (10ng μl^{-1}), 1.0 µl (5 µM) of primer, 18.3 µl dH_2O, 0.5 µl of 10 mM dNTPs, 1.5 µl of 25 µM $MgCl_2$ and 2.5 µl of reaction buffer. A PX2 thermocycler was used. The sequence had 45 cycles of 94°C for 15 s, (X)°C for 45 s (with a ramp rate of 0.4°C s^{-1}), and a final extension of 72°C for 1.5 min. The temperature (X)°C (Table 2) depended on the primer in question. Hence, preliminary pre-runs were done using automated temperature

Table 2. *Nucleotide sequence, annealing temperature, number of polymorphic (PM) and monomorphic bands (MM) and polymorphism information content (PIC) values of the RAPD and ISSR primers.*

Primer code	Marker type	Sequence (5′→3′)	Annealing temperature (°C)	PM	MM	PIC
UBC809	ISSR	$(AG)_8C$	45.1	7	1	0.389
UBC-810	ISSR	GAGAGAGAGAGAGAGAT	46.9	7	1	0.123
UBC817	ISSR	CACACACACACACACAA	48	6	2	0.057
UBC826	ISSR	$(AC)_9C$	50.6	8	2	0.151
UBC-835	ISSR	CTCTCTCTCTCTCTCTRG	49.3	8	2	0.283
UBC-890	ISSR	VHVGTGTGTGTGTGTGT	49.3	9	2	0.317
ISSR average				7.5	1.6	0.220
OPA-12	RAPD	$(GA)_6$ CC	36	7	2	0.100
OP-A07	RAPD	GAAACGGGTG	36	8	2	0.148
OPT-B11	RAPD	GTAGACCCGT	36	13	3	0.420
OPT-C05	RAPD	GATGACCGCC	36	9	2	0.067
OPT-D20	RAPD	ACCCGGTCAC	36	9	2	0.172
OPT-JO6	RAPD	TCGTTCCGCA	36	9	2	0.426
OPT-K14	RAPD	CCCGCTACAC	36	9	2	0.211
OPT-K20	RAPD	GTGTCGCGAG	36	6	1	0.266
OPT-T01	RAPD	GGGCCACTCA	36	15	3	0.087
OPT-T05	RAPD	GGGTTCCGCA	36	8	2	0.121
RAPD average				9.3	2.1	0.202
Total number of bands				138	31	

step gradient mode. Out of the 25 primers initially screened, only six (Table 2) were retained for analysis. Detection and scoring of PCR products were done as described for RAPD.

Statistical analyses

RAPD and ISSR bands were scored for 96 plants, and the polymorphic bands were used in subsequent analyses. Polymorphism information content (PIC) was determined with the formula for dominant markers, $PIC = 1 - [f^2 + (1-f)^2]$ where 'f' is the frequency of the marker in the data set (De Riek et al. 2001). A PIC value for each primer was determined as the average PIC for each polymorphic locus detected by that primer.

Pairwise genetic similarities (band sharing) were estimated using Jaccard's similarity coefficient; $S_j = n_{ab} / n_a + n_b - n_{ab}$; where n_{ab} represents the number of shared bands between lanes 'a' and 'b'; n_a represents the number of bands in lane 'a' and n_b represents the number of bands in lane 'b' (Weising et al. 2005).

The partitioning of genetic variation among and within different groups of samples (taxa and putative hybrids) was investigated with analysis of molecular variance (AMOVA), performed with GenAlEx using a matrix with Euclidean distances.

Dissimilarity between groups of samples was quantified using cluster analysis, conducted with the standard distance matrix in PAUP* ver. 4.0 (Swofford 1998), an equivalent of Euclidean distance algorithm, and the unweighted pair group method with arithmetic averages (UPGMA) mode.

When the studied samples belong to the same species or to closely related taxa, a non-hierarchical analysis may, however, yield a more appropriate representation of dissimilarity compared to a cluster analysis. Therefore, a principal component analysis (PCA) and a multi-dimensional scaling (MDS) analysis were also conducted. The PCA was performed using GenAlEx, while MDS was done with NTSYS (Rohlf 1998). Both methods used a matrix of standardized covariates as input variable.

A Bayesian analysis of genomic structure and gene flow was performed using the program Structure ver. 2.3.1 (Pritchard et al. 2000) which quantifies the number of genomes that can be derived from a population, i.e. the entire set of *Harpagophytum* samples, and how these are represented within each sample. The program was set to 50 000 iterations, with burn-in period set to 5000 runs assuming an admixture model. The a priori estimation of clusters was set to K = 1–10, while the simulation equilibrium (i.e. likelihood of K's) was around log –6000.

RESULTS

Evaluation of primers used

All bands were scored as present (1) or absent (0) in the 96 samples. This resulted in a total of 138 polymorphic and 31 monomorphic bands for the ISSR and RAPD primers taken together (Table 2). The average PIC value was 0.220

Table 3. *Genetic similarity among seedlings of Harpagophytum based on Jaccard's similarity coefficients. For accession codes, see Table 1.*

Plant type	Accession number
Seedlings originating from a common capsule	O1B1PT × O1B2PT = 0.915, O4A1PT to O4A3PT = 0.871, O4B1PT to O4B4PT = 0.850, O4C1PT × O4C2PT = 0.890, O6A1PT to O6A10PT = 0.880, O6F1PT to 06F5PT = 0.869, O7D1PT to O7D4PT = 0.856, O8A1PN to O8A5PN = 0.860, K3A1PN × K3A2PN = 0.955, L4A1PN to L4A4PN = 0.936, T1A1ZZ to T1A5ZZ = 0.927, T2A1ZZ to T2A7ZZ = 0.883, T7A1ZZ to T7A7ZZ = 0.898. **Mean = 0.893**.
Seedlings originating from a common plant but different fruit capsules	O4APT × O4BPT = 0.853, O4APT × O4CPT = 0.879, O4BPT × O4CPT = 0.876, O6APT × O6BPT = 0.770, O6APT × O6CPT = 0.835, O6APT × O6DPT = 0.790, O6APT × O6EPT = 0.820, O6APT × O6FPT = 0.874, O6BPT × O6CPT = 0. 825, O6BPT × O6DPT = 0. 772, O6BPT × O6EPT = 0.729, O6BPT × O6FPT = 0.759, O6CPT × O6DPT = 0.818, O6CPT × O6EPT = 0.819, O6CPT × O6FPT = 0.874, O6DPT × O6EPT = 0.872, O6DPT × O6FPT = 0. 803, O6EPT × O6FPT = 0.851, T1AZZ × T1BZZ = 0.922. **Mean = 0.828**.
Seedlings originating from different mother plants within a locality	O1PT × O4PT = 0.805, O1PT × O6PT = 0.817, O1PT × O7PT = 0.809, O4PT × O6PT = 0.805, O4PT × O7PT = 0.786, O6PT × O7PT = 0.805, T1ZZ × T2ZZ = 0.791, T1ZZ × T7ZZ = 0.799, T2ZZ × T7ZZ = 0.801. **Mean = 0.802**.

for the ISSR primers and 0.202 for the RAPD primers. The highest PIC values were noted for RAPD primers OPT-B11 (PIC = 0.420) and OPT-J06 (PIC = 0.423), while the lowest value was noted for ISSR primer UBC-890 (PIC = 0.06). All remaining analyses were based on a matrix with all the 138 polymorphic bands.

Similarity among seedlings

Seedlings derived from the same mother plant should be either full- or half-siblings, depending on the pollinator. In an outcrossing species, seedlings from different fruits can be expected to be more heterogeneous than seedlings from the same fruit since the former are more likely to derive from different pollination events. Jaccard's similarity coefficients among seedlings from the same fruit ranged from 0.836 to 0.955 with a mean of 0.893, while those from different fruit capsules on the same plant ranged from 0.726 to 0.922, with a mean of 0.828 (Table 3). By comparison, seedlings from different mother plants of the same taxon growing at the same locality, showed Jaccard's similarity coefficients that ranged from 0.782 to 0.817, with a mean of 0.802. These results are expected for outcrossing species.

Unfortunately, only five putatively hybridogenic seedlings were available. The similarity between two seedlings from the same capsule (T5A1PH and T5A2PH) was 0.907. By contrast, similarity among seedlings from different hybridogenous mother plants at the same locality (O5A1PH and O9A1PH) was only 0.706.

Genetic differentiation

Partitioning of genetic variation using analyses of molecular variance revealed significant differentiation ($P < 0.01$) among different sets of grouped plant material (Table 4). About 39% of the variability occurred between the two main species, *H. procumbens* and *H. zeyheri,* thus showing that they are strongly differentiated from one another. A comparison of the two recognized subspecies of *H. procumbens*, i.e. ssp. *procumbens* (PP) and ssp. *transvaalense* (PT), showed 15% infraspecific variability.

Plants with an intermediate morphology, i.e. putative hybrids (PH), showed 21% differentiation when compared with PP, and 19% when compared with either PT or with *H. zeyheri* ssp. *zeyheri* (ZZ). Obviously the molecular data place the putative hybrid at almost the same distance from the other three taxa.

In addition, a deviating morphotype, here termed '*procumbens* new variety' (PN), showed only 9%

Table 4. *Analysis of molecular variance among Harpagophytum plant groups, PN new variety of H. procumbens; PT H. procumbens ssp. transvaalense; PP H. procumbens ssp. procumbens; PH putative hybrid and ZZ H. zeyheri ssp. zeyheri. All analyses were significant at $P < 0.01$.*

Taxa compared	No. of samples compared	Molecular variance (%)
H. procumbens and *H. zeyheri*$^{\psi}$	90	39
PN and ZZ	35	41
PN and PT	66	9
PN and PP	15	22
PH and ZZ	28	19
PH and PT	58	19
PH and PP	7	21
PH and PN	18	22
PP and PT	68	15

ψ represents the comparison between *H. procumbens* and *H. zeyheri* at species level (i.e. all samples of *H. procumbens* compared to all of *H. zeyheri* regardless of the infraspecific rank).

differentiation when compared with PT, 22% when compared with PP or with PH, and 41% when compared with ZZ. This result suggests that the new variety belongs to *H. procumbens*, and is most closely affiliated with ssp. *transvaalense*. The unequal sample sizes can, however, cause some artifacts when different results are compared.

Phenetic analyses

The UPGMA tree obtained from the combined RAPD and ISSR data reflected the current circumscription of the genus *Harpagophytum* by formation of two major clusters (nodes A and B), which represent the two recognized species of the genus: *H. procumbens* and *H. zeyheri* (Fig. 2). Cluster A (node A) contains mainly *H. procumbens* ssp. *transvaalense* but also a small subcluster (node C) with the only two samples of *H. procumbens* ssp. *procumbens*. In addition, most of the seedlings of the *procumbens* new variety cluster at nodes D, I and J. Cluster B (node B), which represents *H. zeyheri*, contains the three major subclusters E, F and G together with some outliers. Moreover, all samples in subcluster E are offspring from mother plant T1, while offspring of mother plant T2 are found in subcluster F, and offspring of mother plant T7 in subcluster G. Putative hybrids were allocated to both clusters A and B, and to various subclusters.

The PCA (Fig. 3) and MDS analyses (data not shown) produced very similar results. Pronounced separation of samples was noticed, with the first (PCA1) and second (PCA2) principal components accounting for 44.3% and 15.4%, respectively, of the total variation. Analogous to the UPGMA tree, differentiation of the two *Harpagophytum* species was strongly supported by two well-separated clusters along PCA1. Furthermore, PCA1 also dispersed the putative hybrids from the edge of the *H. procumbens* cluster to the edge of the *H. zeyheri* cluster. PCA2 instead separated, although with some overlap, between *H. procumbens* ssp. *transvaalense* and the *procumbens* new variety, while *H. procumbens* ssp. *procumbens* appeared in the overlap zone.

Bayesian genome structure analysis

The Bayesian analysis of the *Harpagophytum* samples revealed a genomic structure, that is consistent with the results of the cluster analysis and the PCA. The maximum likelihood provided by the genomic structure was –ln 6000. Equilibrium for the model was reached at $K = 2$ ($\Delta K = 3.10$), roughly corresponding to the two species *H. procumbens* and *H. zeyheri* but with indication of some gene flow, especially in the samples denoted as putative hybrids (Fig. 4). For $K = 3$ ($\Delta K = 2.53$), a similar result was obtained but with two subgenomes within *H. procumbens* ssp. *transvaalense*.

DISCUSSION

Choice of method

Numerous methods for obtaining molecular-based estimates of the amount and partitioning of genetic variation have been developed in the last few decades, with increasingly sophisticated applications especially in economically important crops and in model species (Nybom et al. 2014). For low-budget projects in lesser known plant groups, multilocus methods like RAPD and ISSR are, however, likely to remain a much appreciated tool for several years to come. To our knowledge, the present study represents the first attempt to study genetic variation with nuclear DNA markers in *Harpagophytum*. Reproducible results were obtained with both RAPD and ISSR primers, yielding an average of 8.6 polymorphic bands for the 16 primer pairs employed. Similar average PIC values were found for the RAPD primers and the ISSR primers, and both methods proved highly useful. Previous studies have in general also reported quite similar PIC values when RAPD and ISSR primers have been applied to the same plant material (Weising et al. 2005; Parveen et al. 2013).

Seedling similarity reflects breeding system

Previous molecular marker-based research has shown that outcrossing species have significantly larger within-population diversity than selfing species (reviewed by Nybom 2004). Similarly, there is usually negligible marker variation within progenies derived from single mother plants in selfing species such as, e.g. *Plantago major* (Zubair et al. 2012) while the more variable progenies in outcrossing species like *Passiflora alata* and *Stylosanthes guianensis*, have allowed a precise determination of the outcrossing rate (Ferreira et al. 2010; Chiari et al. 2010). Due to the considerable problems in obtaining viable seedlings for DNA extraction in the present study, there was not sufficient material to determine the outcrossing rates. Nevertheless, the higher Jaccard's index similarities found in single-capsule offspring (mean = 0.893) compared to among offspring from different capsules on the same plant (more likely to have emanated from different pollen donors; mean = 0.828), suggest that both species are cross-pollinated thereby facilitating interspecific hybridization and backcrossing.

Differentiation among taxa of Harpagophytum

Our study clearly supported the commonly accepted classification of *Harpagophytum* into two species, with substantial interspecific differentiation according to the AMOVA, occurrence of two well-separated clusters in the UPGMA cluster analysis and the PCA, and two rather sharply delimited genomes in the Bayesian structure analysis.

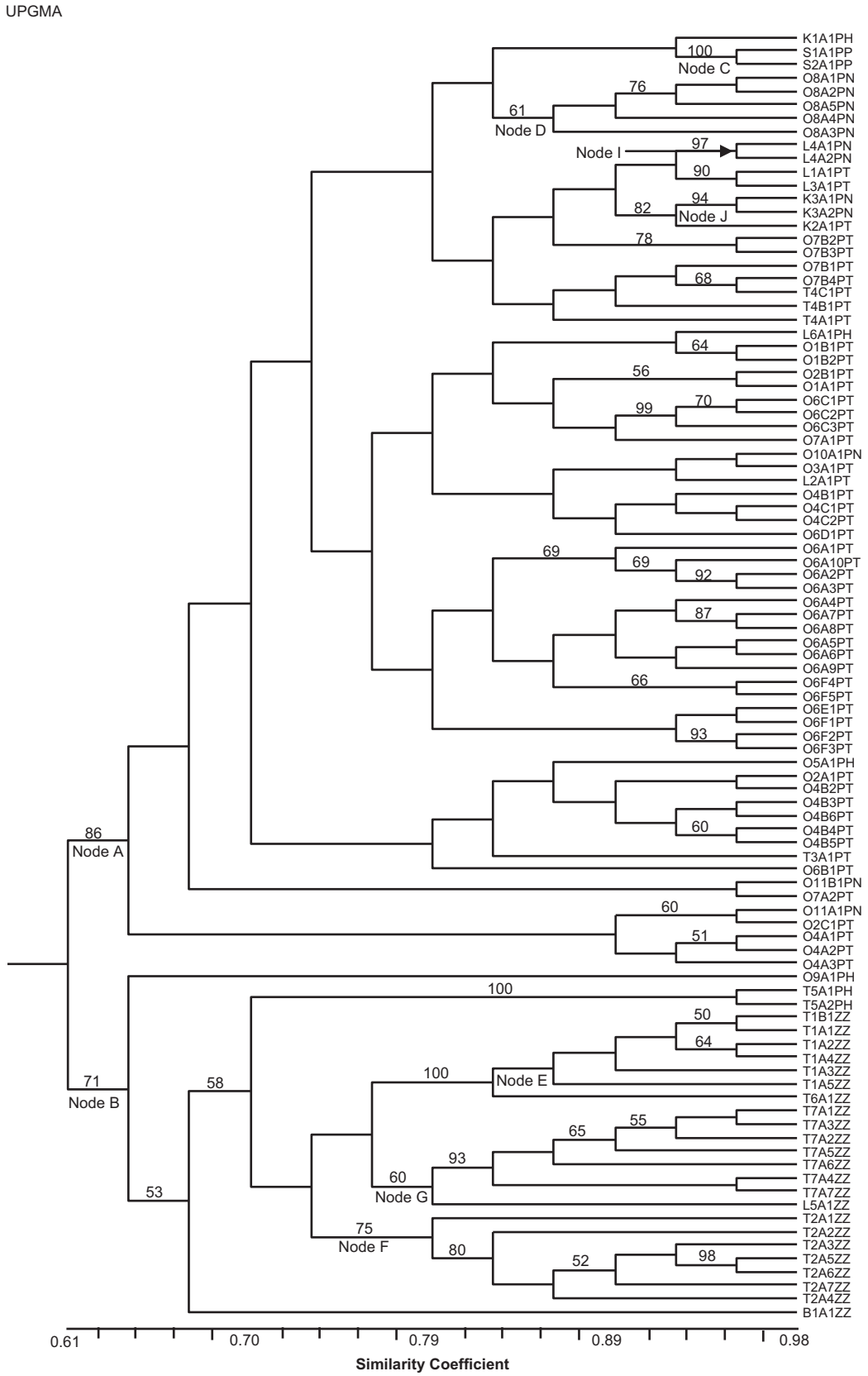

Fig. 2. Dendrogram obtained with UPGMA of ISSR and RAPD marker data in *Harpagophytum*. Labels below branches denote node identity. For accession codes see Table 1.

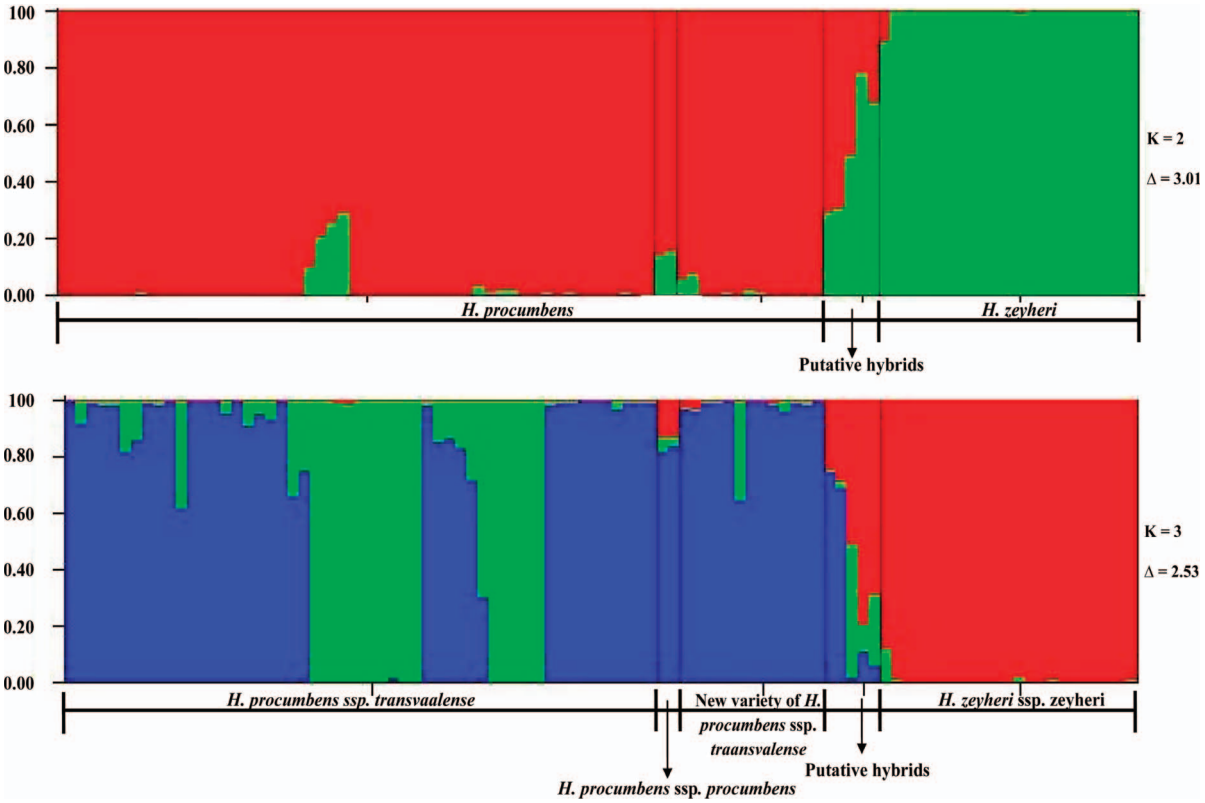

Fig. 3. ISSR and RAPD marker-based Bayesian analysis of genomic structure in *Harpagophytum.*

Harpagophytum procumbens is usually classified into two subspecies. Two seedlings obtained from a plant classified as ssp. *procumbens* and growing in an allopatric population, clustered closely together but could not be separated from ssp. *transvaalense* in the UPGMA and PCA. Quite unexpectedly, the Bayesian analysis hinted at an influence from *H. zeyheri* that was not present in ssp. *transvaalense*. However, with the limited access to ssp. *procumbens* (PP) material in our study, it was not possible to properly clarify its level of relatedness with the other taxa, and the results concerning this taxon must therefore be regarded as unproved.

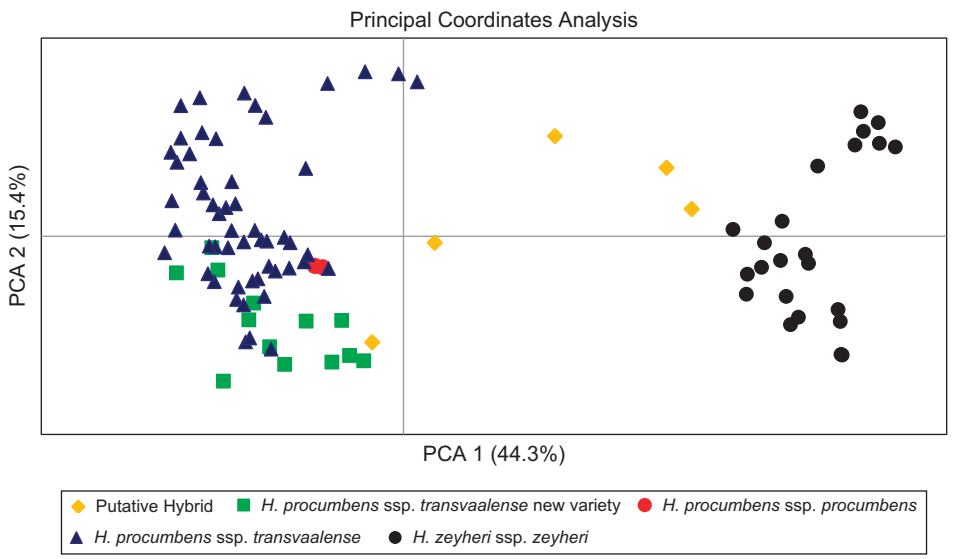

Fig. 4. Biplot of a principal component analysis using ISSR and RAPD markers in *Harpagophytum*.

Introgression and gene flow

Backcrossing in plants can lead to speciation (Grant 1971; Abbott et al. 2013; Hochkirch 2013; Soltis 2013). In this study, putatively hybridogenic plants were sampled in three locations in southeastern Botswana (Kumakwane, Oodi and Tlokweng) where both *H. procumbens* ssp. *tranvaalense* and *H. zeyheri* presently occurin spite of the more restricted distribution areas previously published by Ihlenfeldt and Hartmann (1970). One or two seedlings were analyzed from one or two plants in each of these locations. These five putative hybrid plants were equally well differentiated from the two possible parent taxa according to the AMOVA. Similarly, the PCA showed the five putative hybrid plants to bridge the gap between clusters formed by parental taxa. By contrast, these plants occur scattered within the parental clusters in the dendrogram. This is, however, typical of the topology of hybrids in hierarchical trees (Archibald et al. 2004).

The two putative hybrid plants sampled at the same locality, O5PH and O9PH, had widely different capsules, and possibly one of these plants was a polyploid. The two offspring plants, O5A1PH and O9A1PH, had relatively low Jaccard's index-based similarity (0.706) suggesting considerable genetic differentiation. Fruit capsule morphology is the major taxonomic trait in *Harpagophytum*, and capsule differences may thus be taken as an indication of hybridization as previously suggested (Muzila et al. 2011). In the latter study, fruit capsule morphometrics was carried out on material collected from four different introgressive zones. A scatter plot showed that most of the putative hybrids (although not specified as such) were allocated in between of the two species *H. procumbens* and *H. zeyheri* but with some overlapping. In the morphometrics study, the proportion of putative hybrids was about twice as high as in the present DNA-based study. Since reproductive isolation mechanisms are quite likely, especially in view of the apparent polyploidy of some hybrid plants, the need for germinating seedlings for DNA analyses may have decreased the ratio of hybridogenous plants in the present study.

A morphologically deviating material was also sampled at four locations (Tlokweng, Oodi, Leshibitse and Kumakwane) and denoted as '*procumbens* new variety' (PN). The external morphology of this material resembles that of *H. procumbens* ssp. *transvaalense*, i.e. a fruit capsule with three arms in each row, but the three seed rows in each locule are not typical of *H. procumbens*. Plants with these features were treated here as '*procumbens* new variety'. All the statistical analyses showed that PN is more similar to *H. procumbens* than to *H. zeyheri*, and there is no marker-based evidence of interspecific introgression despite the number of seed rows suggesting such an affinity. According to the UPGMA, samples of PN were intermingled among the PT whereas the PCA revealed three groups of *H. procumbens*, with PP placed in between the PN and the PT, which tended to appear in the upper left and bottom left corners, respectively, with some overlapping. Although the placement of PP is uncertain due to insufficient sampling, PN plants probably derive from backcrosses between hybrids and *H. procumbens* ssp. *transvaalense*.

This DNA study indicates the presence of introgression at Oodi, Leshibitse, Kumakwane and Tlokweng as evidenced by the presence of putative hybrids as well as *procumbens* new variety at these sites. This pattern of introgression is probably associated with the formation of patchy populations resulting from the mainly epizoochorous dispersal (attachment of fruit capsules to hooves, jaws and mouths of cattle and antelopes) of *Harpagophytum* (Ihlenfeldt 1967). The introgressive zones in our study are thus either located along an introgressive belt (Leshibitse, Oodi) which can be associated to routes followed by herders of livestock (cattle, horses, sheep and goats), or near water points (Kumakwane, Oodi, Tlokweng) where livestock could have been musting before or after drinking water. The *procumbens* new variety was also found in villages of Mmathethe (about 110 km from the introgression zones) and in Matlapaneng, Maun (about 660 km from the introgression zones), again indicating that the distribution patterns and introgression zones of *Harpagophytum* in Botswana may be closely related to livestock movements.

Introgression often results in decreased fertility. Hence, the overall lower germination rates of seeds from Botswana (5–20%) compared to those from Namibia (80%) (Ernst et al. 1988) may indicate that the introgression products have either spread further inside Botswana, or have been overrepresented in previous plant sampling expeditions. In future research, occurrence of both hybrids and the *procumbens* new variety should be investigated by sampling in South Africa and Namibia where hypothetical morphological introgressive forms have also been reported (Hachfeld 2004).

REFERENCES

Abbott et al. 2013.

Archibald, J. K., Wolfe, A. D. and Johnson, S. D. 2004. Hybridization and gene flow between a day and night-flowering species of *Zaluzianskya* (Scrophulariaceae s.s., tribe *Manuleeae*). – Am. J. Bot. 91: 1333–1344.

Baghdikian, B., Lanhers, M. C., Fleurentin, J. et al. 1997. An analytical study, antiflammatory and analgesic effects of *Harpagophytum procumbens* and *Harpagophytum zeyheri*. – Planta Med. 63: 171–176.

Chiari, L., Resende, R. M. S. and Matida, E. T. 2010. Mating system parameters in *Stylosanthes quianensis* (Aubl.) Sw. based on RAPD markers. – Afr. J. Biotechnol. 9: 5820–5822.

Chrubasik, S., Thanner, J., Künzel, O. et al. 2002. Comparison of outcome measures during treatment with the proprietary *Harpagophytum* extract Doloteffin in patients with pain in the lower back, knee or hip. – Phytomedicine 9: 181–194.

Chrubasik et al. 2004.

Chrubasik, J. E., Roufogalis, B. D. and Chrubasik, S. 2007. Evidence of effectiveness of herbal antiinflammatory drugs in the treatment of painful osteoarthritis and chronic low back pain. – Phyther. Res. 21: 675–683.

Davis, J. I. 1993. Character removal as a means of assessing stability of clades. – Cladistics 9: 201–210.

De Riek, J., Calsyn, E., Everaert, I. et al. 2001. AFLP based alternatives for the assessment of distinctiness, uniformity and stability of sugar beet varieties. – Theor. Appl. Genet. 103: 1254–1265.

Ernst, W. H. O., Tietema, T. and Veenendaal, E. M. 1988. Dormancy, germination and seedling growthof two Kalahari perennials of the genus *Harpagophytum* (Pedaliaceae). – J. Trop. Ecol. 4: 185–198.

Feistel, B. and Gaedcke, F. 2000. Analytische Identifizierung von Radix Harpagophyti procumbentis und zeyheri. – Z. Phytother. 21: 246–251.

Ferreira, T. G. T, Penha, H. A., Zucchi, M. I. et al. 2010. Outcrossing rate in sweet passion fruit based on molecular markers. – Plant Breed. 129: 727–730.

Fitzpatrick, B. M. 2009. Power and sample size for nested analysis of molecular variance. – Mol. Ecol. 18: 3961–3966.

Grant, V. 1971. Plant speciation. – Columbia Univ. Press.

Hachfeld, B. 2004. Ecology and utilisation of the medicinal plant *Harpagophytum procumbens* (Burch.) D. C. ex Meissn. (Pedaliaceae) in Southern Africa. – PhD thesis, Univ. Hamburg, Germany.

Hochkirch, A. 2013. Hybridization and the origin of species. – J. Evol. Biol. 26: 247–251.

Ihlenfeldt, H. D. 1967. Über die Abgrenzung und die naturliche Gliederung der Pedaliaceae R.Br. – Mitt. Staatsinst. Allg. Bot. Hamburg 12: 43–128.

Ihlenfeldt, H. D. 1988. Pedaliaceae. – Flora Zambesiaca 8: 86–113.

Ihlenfeldt, H. D. and Hartmann H. 1970. Die Gattung *Harpagophytum* (Burch.) DC. ex Meissn. (Monographie der afrikanischen Pedaliaceae II). – Mitt. Staatsinst. Allg. Bot. Hamburg 13: 15–69.

McGregor, G., Fiebich, B., Wartenberg, A. et al. 2005. Devil's claw (*Harpagophytum procumbens*): an anti-inflammatory herb with therapeutic potential. – Phytochem. Rev. 4: 47–53.

Mncwangi, N., Viljoen, A. M., Vermaak, I. et al. 2013. Variation of the biologically active constituent harpagoside in *Harpagophytum procumbens* and *H. zeyheri*. – Planta Med. 79: 58 DOI: 10.1055/s-0033–1336500

MCAN 2011. Indigenous natural products producer and processor organisations sub-activity EIA for Devil's Claw. Report Reference No. 5a. – Millennium Challenge Account Namibia, Windhoek, Namibia.

Moussard, C., Alber, D., Toubin, M. M. et al. 1992. A drug used in traditional medicine, *Harpagophytum procumbens*: no evidence for NSAID-like effect on the whole blood eicosanoid production in human. – Fatty Acids 46: 283–286.

Muzila, M., Setshogo, M. P. and Mpoloka S. W. 2011. Multivariate analysis of *Harpagophytum* DC. ex Meisn (Pedaliaceae) based on fruit characters. – Int. J. Conserv. Biodivers. 3: 101–109.

Nybom, H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. – Mol. Ecol. 13: 1143–1155.

Nybom H., Weising K. and Röttner B. 2014. DNA fingerprinting in botany: past, present and future. – Invest. Genet. 5:1, doi:10.1186/2041–2223-5-1

Parveen, S., Saharan, M. S., Verma, A. et al. 2013. Comparative analysis of RAPD and ISSR marker assays for detecting genetic polymorphism in *Tilletia indica*. – Europ. J. Exp. Biol. 3: 380–387.

Pritchard, J. K., Wen, X. and Falush, D. 2000. Documentation for structure software: ver. 2.3. – Google groups, Chicago, USA.

Rieseberg and Brunsfeld 1998

Rohlf, J. F. 1998. NTSYSpc numerical taxonomy and multivariate analysis system, ver. 2.0. – Applied Biostatistics Inc, New York.

Soltis, P. S. 2013. Hybridization, speciation and novelty. – J. Evol. Biol. 26: 291–293.

Swofford, D. L. 1998. PAUP, and other methods. Phylogenetic analysis using parsimony, ver. 4. –Sinauer.

Stewart, K. M. and Cole, D. 2005. The commercial harvest of devil's claw (*Harpagophytum* spp.) in southern Africa: the devil's in the details. – J. Ethnopharm. 100: 225–236.

Syngene 2008. Image analysis and processing, GeneSnap ver. 7.02. – Synoptics Group, Cambridge, UK.

Weising, K., Nybom, H., Wolf, K. et al. 2005. DNA fingerprinting in plants: principles, methods and applications. – CRC Press/ Taylor and Francis.

Zubair, M., Nybom, H., Ahnlund, M. et al. 2012. Detection of genetic and phytochemical differences between and within populations of *Plantago major* L. (plantain). – Sci. Horticult. 136: 9–16.