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B. Arnholdt-Schmitt

RAPD analysis: a method to investigate aspects of the reproductive biology of *Hypericum perforatum* L.

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Abstract Recent interest in breeding strategies for *Hypericum perforatum* L. requires a better understanding of the floral biology of this medicinal plant. The aim of the present study was to check, whether RAPD fingerprinting may be a useful tool for research on the mode of reproduction of this species. Progenies from three defined single plants of two accessions, as well as progenies from a random sample of seeds of a wild population, of *H. perforatum* were characterized by RAPD analyses using six primers. The results obtained by DNA fingerprints indicate the predominance of an identical mode of reproduction for this species, obviously due to apomixis. Nevertheless, non-identical reproduction was evident as a minor effect in *H. perforatum*, as could be demonstrated by significant deviations in the RAPD fingerprints of progenies from one single plant. It is concluded that RAPD fingerprint analysis is a suitable technique to discover identity or non-identity *in H. perforatum* populations. Therefore, RAPDs may be used in addition to cytological studies to confirm the mode of reproduction by apomixis versus self-pollination, haploid parthenogenesis or cross-fertilization.

Key words Apomixis · Breeding · Cross-pollination · *Hypericum perforatum* · RAPD fingerprints

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B. Arnholdt-Schmitt
Institut für Pflanzenernährung, Justus-Liebig-Universität Giessen, Südanlage 6, D-35390 Giessen, Germany
e-mail: Birgit.Arnholdt-Schmitt@ernaehrung.uni-giessen.de

Present address:
Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Caixa Postal 1065, 60001-970 Fortaleza, Ceará, Brasil
e-mail: birgit@ufc.br

Introduction

During recent years, interest on *Hypericum perforatum* L. (St. John's Wort) as a natural antidepressant in phytotherapy is rapidly growing. Traditionally, this medicinal plant is collected from wild growing populations. To meet the increase in demand, nowadays, an increasing proportion of *H. perforatum* plants is produced by cultivation. Effective breeding strategies are required, therefore, to produce cultivars of St. John's Wort, which give a homogeneous quality of extracts and exhibit favorable agronomic characteristics to ensure stable quality and yield production.

However, up to now, the basic question for breeding purposes of whether and to what extent cross-pollination occurs naturally in *H. perforatum* is still unresolved. *H. perforatum* is tetraploid (2n=4*x*=32) and is assumed to be an allopolyploid (Mártonfi et al. 1996). In early investigations, Noack (1939) reported that apomixis is by far the most frequent event (about 97%) in the reproductive biology of this species. Halusková and Cellárová (1997) performed RFLP analysis of *H. perforatum* to characterize somaclones and their progenies. The use of rDNA probes gave hints for the occurrence of sexual recombination in *H. perforatum* plants. In comparison to RFLP studies RAPD analysis is easier to handle and can be performed with different primers that cover not only one but several sites of a genome. Therefore, large regions of selected genomes of various origins can be checked very rapidly for differences. The aim of the present study was to investigate whether RAPD fingerprint analysis is a proper technique to elucidate aspects of the mode of reproduction by indicating identity or non-identity in studies with individuals of *H. perforatum* L.

Materials and methods

Plant material

H. perforatum L. seeds of three accessions (A,B,C) were from the botanical garden in Marburg. Whereas seeds of accession A were collected from various plants and subsequently propagated togeth-

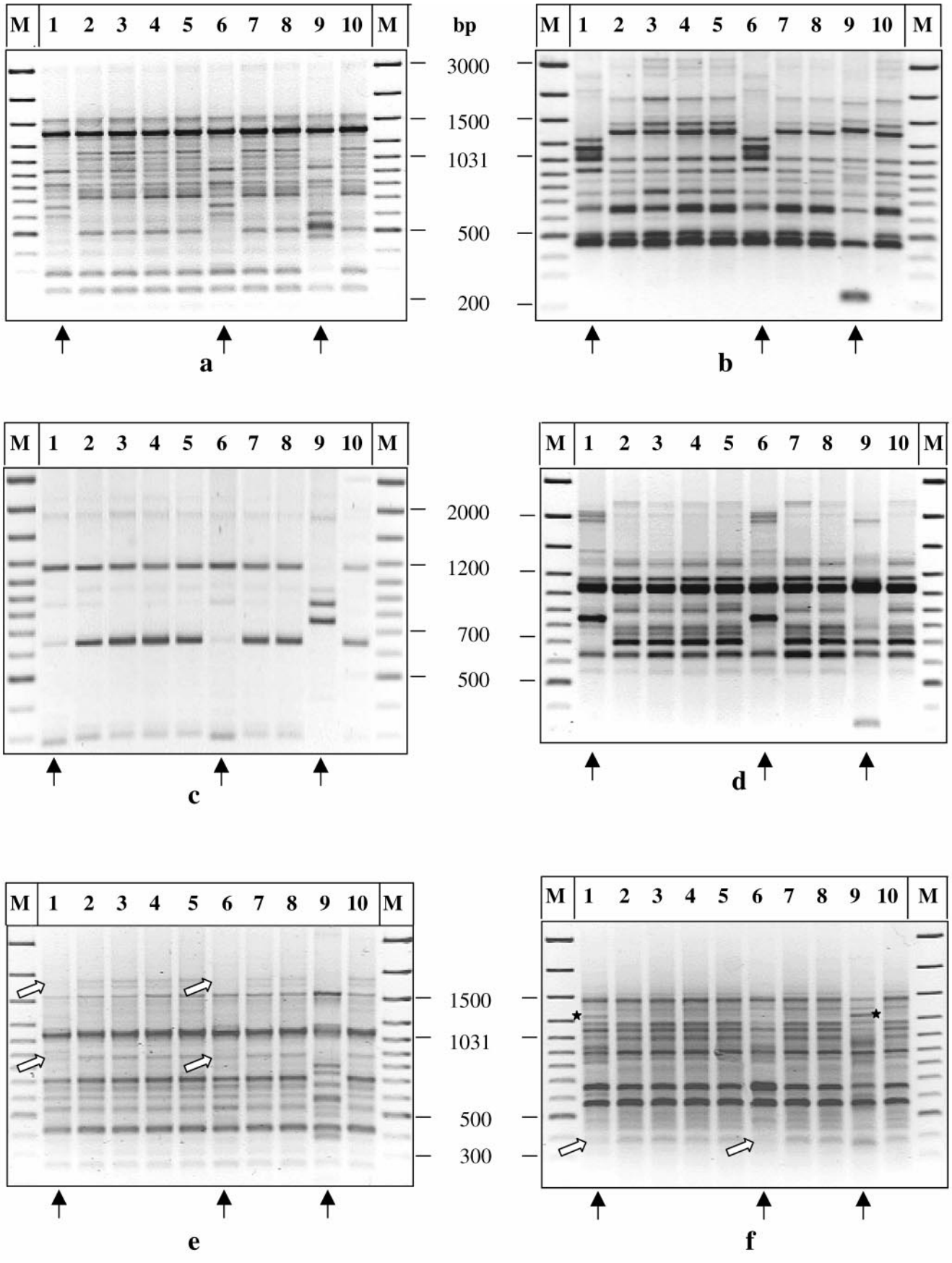

Fig. 1a–f RAPD fingerprints of ten progenies of accession A obtained with six primers. **a** primer 025, **b** primer 026, **c** primer 020, **d** primer 029, **e** primer 006, **f** primer 001

er during two generation cycles, seeds of accessions B and C originated from a single plant each and were respectively isolated for propagation. From accession B, progenies of two single plants (B/1 and B/2) were available. For analysis, seedlings were grown under sterile conditions. Leaves of ten plants were harvested separately after 6–7 weeks of vegetative growth under sterile conditions. At harvest, the leaves were washed twice in sterile aqua dest, shock frozen with liquid nitrogen and stored at –80°C until DNA isolation.

DNA isolation

The DNA was isolated by using the DNeasy Plant Mini Kit from Qiagen (Germany). DNA quality and quantity was checked in a 1% agarose gel in comparison to a concentration range of lambda high-molecular-weight DNA (Sigma). Quantitation was done by densitometric measurements with RFLPscan (Scanalytics, Inc.) software.

RAPD analysis

Ten nanograms of high-molecular-weight DNA was used for 25-µl PCR assays. Random primers were from Operon Technologies (020, 025, 026 and 029), obtained from Roth (Germany), as well as from Pharmacia (001 and 006). The assays were performed with Rapid analysis beads (Amersham Pharmacia) that contain buffer components, nucleotides and polymerases in a Ready-to-Use kit. PCR conditions were as follows: 5 min at 95°C; 45 cycles of: 1 min at 95°C, 1 min at 36°C, 2 min at 72°C; and as a last step 5 min at 72°C. Amplifications were carried out using a Techne thermocycler (model Progene).

Six random primers were selected for the RAPD analyses of progeny plants. In a pre-screen with 44 primers, the chosen primers indicated quantitative or qualitative polymorphism between two randomly chosen plants of accession B: Primer 001: 5´-GGTGCGGGAA-3´; Primer 006: 5´-CCCGTCAGCA-3´; Primer 020: 5´-TCTCCCTCAG-3´; Primer 025: 5´-TGAGCGGACA-3´; Primer 026: 5´-ACCTGAACGG-3´; Primer 029: 5´-CTCTG-GAGAC-3´.

PCR fragments were separated in 1.5% agarose and stained in 1.5 µg/ml of ethidium bromide solution. Documentation and evaluation were performed with a videodensitometer and RFLPscan software (Scanalytics, Inc.). Reproducibility of the RAPD methodology used for the present investigations was checked very carefully before application for repeated extractions, the use of various thermocyclers and the effect of different personnel working with this method. Differences in RAPD fingerprints were only accepted if they could be confirmed in two replicates.

Results

Variation between progenies of accession A

In Fig. 1a–f the RAPD analyses of ten progeny plants from accession A are given for all six primers employed. The results demonstrate that three genotypes can be identified among the ten investigated single-plant genomes independently of the primer applied. The predominant genotype for this accession is represented by plants 2, 3, 4, 5, 7, 8 and 10. A second genotype is shown by plant 9, whereas a third genotype corresponds to plant 1 as well as to plant 6. All primers applied were able to discriminate the same genomes as being different from each other. But the clarity of the differences between the fingerprints was dependent on the specific primer. While primers 025, 026 and 029 (Fig. 1a, b and d) produced strongly different fingerprints for the three genotypes observed, primer 020 (Fig. 1c) characterises the genomes of plants 1 and 6 only by quantitative polymorphism. Primers 006 and 001 indicated clear differences between the predominant genome and the genome of plant 9, but only slight, although unequivocal, differences were obtained with these primers for plants 1 and 6 in comparison to the predominant genome (see arrows in Fig. 1e and f). Primer 001, additionally, revealed a slight difference between the fingerprints of plants 1 and 6 by a distinct fragment of 1240 bp (see asterisk). This fragment may be due to cross-pollination (see below). A fragment of comparable size is part of the fingerprint of plant 9 (see asterisk).

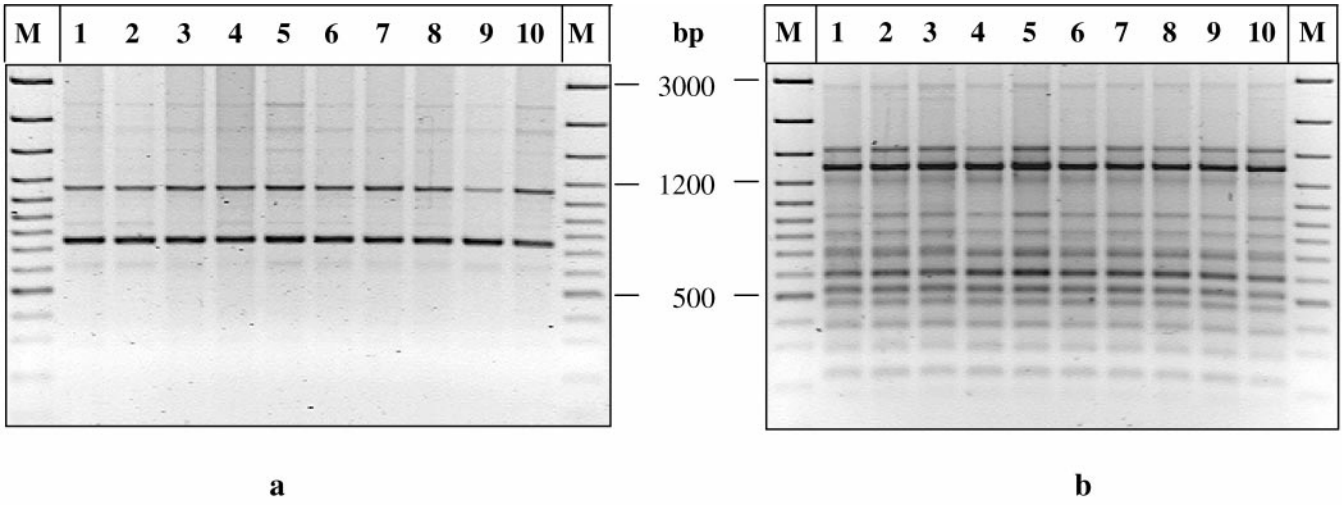

Fig. 2 RAPD fingerprints of ten progenies of accession C obtained with primers **a** 020 and **b** 025

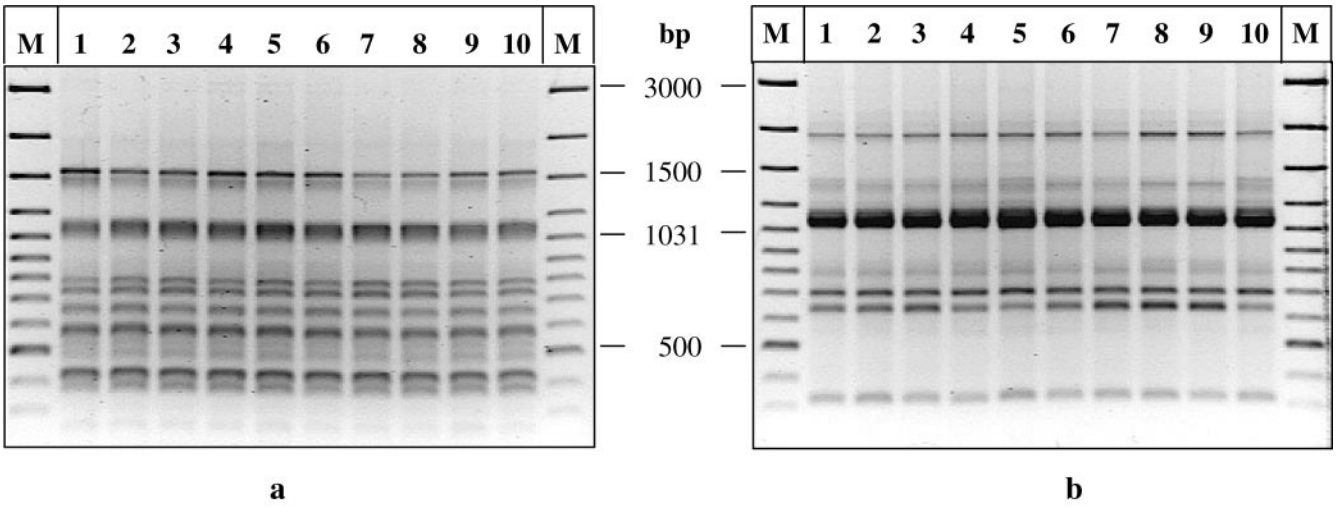

Fig. 3 RAPD fingerprints of ten progenies from plant B/2 of accession B obtained with primers **a** 001 and **b** 029

Variation between progenies that originate from single plants

By comparing the RAPD fingerprints of ten progenies that originated from a single plant of accession C no variation was observed with any of the six selected primers. The results for two primers are given as an example in Fig. 2a,b. Also the ten progenies of accession B from plant B/2 did not show any significant differences in the fingerprints produced (two examples are given in Fig. 3a,b). Nevertheless, progenies from another plant of the same B accession, named B/1, display significant variation. In Fig. 4, it can be seen from the fingerprints produced by primer 029, for example, that the seedlings from B/1 originate from the same accession as the progenies of plant B/2 (see Fig. 4b and Fig. 3b for comparison). However, in comparison to the typical fingerprint of the predominant genotype of the progenies from plant B/1 and plant B/2, progenies 1 and 7 from plant B/1 show significant deviations in their fingerprints (Fig. 4a–c). The polymorphism shown by each respective primer is due to a single band (see arrows). That was also true for fingerprints produced by the other three primers (data not shown).

Discussion

Breeding of *H. perforatum* and a greater demand for homogeneity in plant production requires a good knowledge of the mode of reproduction of this species. Since the significance of cross-pollination is still an open question for the species the present investigations were done to check whether the RAPD fingerprint technique may contribute to elucidating its mode of reproductive biology without the need for morphological and physiological studies and independent of the developmental stage of the plants. This is the first report on RAPD analysis of the medicinal plant *H. perforatum* L.

The investigations on ten progenies of a collection of a wild-grown population of *H. perforatum* plants and ten progenies, respectively, from three single plants of two accessions, indicate that the predominant mode of reproduction by this species is identical propagation. This is shown primarily by the large number of identical fingerprints of the progenies from single plants (80–100% of the progenies display identity, see Figs. 2–4) and of the progenies from a random sample of different plants of accession A (seven plants are identical and two additional plants are nearly identical as is shown with five of the six primers, see Fig. 1).

Further, the fingerprints of three genotypes that are found among the progenies from plants of a wild population of accession A indicate very different genomes. This is evident by the fact that all of the six primers used could discriminate the three genotypes and that, additionally, about 67% of the primers show differences between the genomes not only at one but at several sites. This situation is characteristic for wild populations when self-pollination or vegetative propagation is predominant, leading to a mixture of highly distinctive groups of individuals. In the case of predominant cross-pollination a more uniform population would have been expected for progenies of wild-grown plants.

Nevertheless, RAPD analysis on progenies of plant B/1 from accession B indicates that cross-fertilization may occur naturally in *H. perforatum* as a minor event. Although it was obvious that no sexual recombination was involved in the production of the analysed seeds from a plant of accession C, as well as from plant B/2 of accession B, 20% of the progenies of B/1 indicate non-identical reproduction by the presence of additional bands for their RAPD fingerprints in comparison to the fingerprint that predominantly characterizes the progenies of accession B (see Figs. 3 and 4). RAPD analysis has also proved to be efficient in revealing, or excluding, somaclonal variations normally due to minor genomic variations, like mutation or rearrangement (Brown et al.

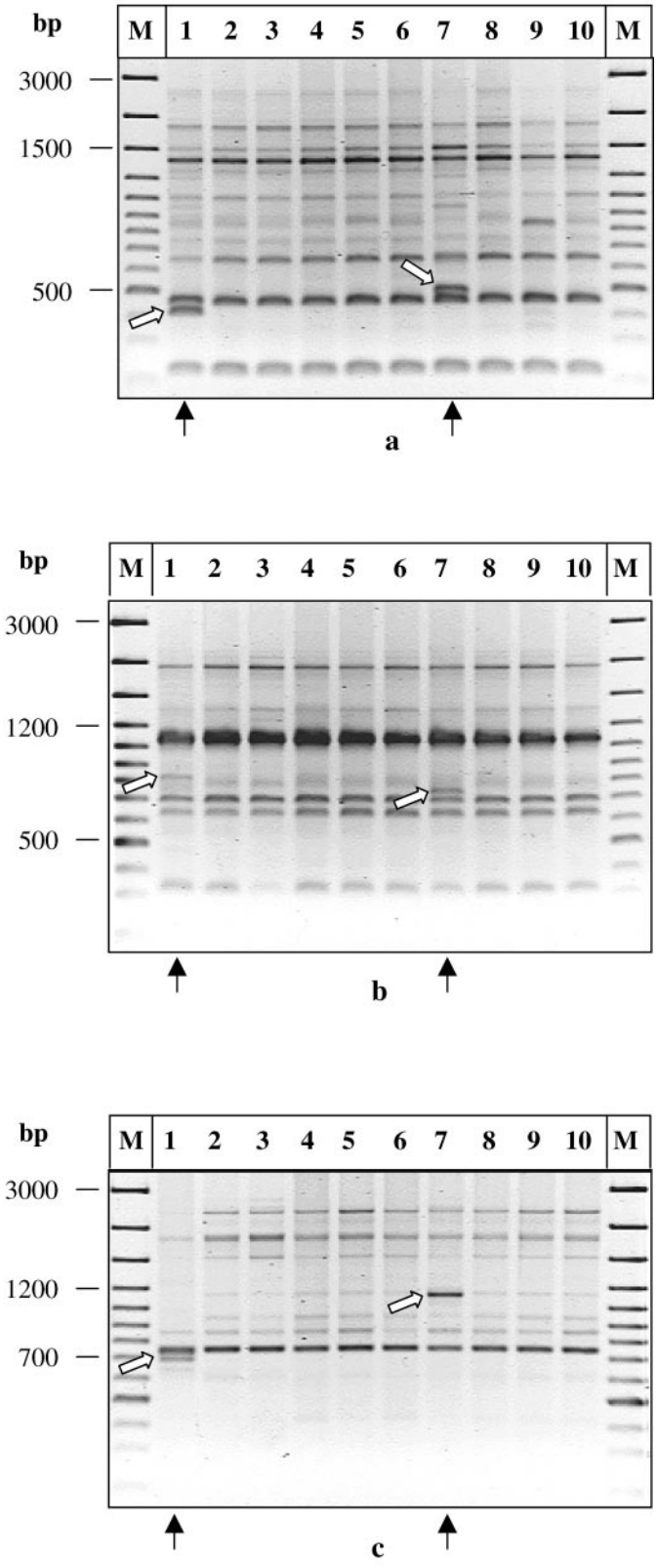

Fig. 4 RAPD fingerprints of ten progenies from plant B/1 of accession B obtained with primers **a** 026, **b** 029 and **c** 020

1993; Piccioni et al. 1997; Shoyama et al. 1997). However, for the present study it is not assumed that the observed deviations from the predominant genome could be due to mutation or rearrangement, since changes occurred in 10% of the tested progenies from the two plants of accession B and could be found at several sites in the genomes of progenies 1 and 7 by the use of various primers.

In conclusion, RAPD analysis proved to be a valuable technique to discriminate between identical and non-identical individuals of *H. perforatum* and therefore may be helpful in studies to elucidate the mode of reproduction of this species. On the basis of the results presented, crossing experiments with selected individuals of *H. perforatum* that differ with respect to RAPD fingerprints can now be performed in order to calculate the percentage of cross-pollination. It has to be considered, however, that genetic variations, like genomic mutations, can be missed by RAPD analysis, as was shown by Fourré et al. (1997). Additionally, haploid parthenogenesis may occur in *H. perforatum* resulting in aberrant RAPD fingerprints due to the dominance of the marker. Also, homogeneous progenies may theoretically originate from sexually homozygous plants arising after restitution nucleus formation at meiosis-II with parthogenesis following in the mother plant. Since these aspects seem to be relevant, analyses on the cytology of *H. perforatum* reproduction should be a valuable tool to complement RAPD analysis.

The established DNA fingerprint technique may now be employed to reconsider the question of whether identical genotypes are, in fact, caused by apomixis, as Noack (1939) reported in early investigations, or whether self-pollination may be of importance in *H. perforatum*, as was recently discussed in Germany on the occasion of testing cultivars for protection. By following up several generations after crosses with selected individuals, it should be possible to very easily reveal whether the hybrid genome of the F_1 generation will be maintained by clonal propagation or whether inbreeding takes place.

It is recommended, in general, to take comparable tissues of the same developmental stage for RAPD analyses, since different organs, tissues and the age of the plants show physiologically dependent variations in the repeated DNA fraction which, in our experience, may interfere with the identification of RAPD fingerprints (Arnholdt-Schmitt 1995; Arnholdt-Schmitt et al. 1998, 1999).

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