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# Evolution of Hypervariable Microsatellites in Apomictic Polyploid Lineages of *Ranunculus carpaticola*: Directional Bias at Dinucleotide Loci

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ABSTRACT

Microsatellites are widely used in genetic and evolutionary analyses, but their own evolution is far from simple. The mechanisms maintaining the mutational patterns of simple repeats and the typical stable allele-frequency distributions are still poorly understood. Asexual lineages may provide particularly informative models for the indirect study of microsatellite evolution, because their genomes act as complete linkage groups, with mutations being the only source of genetic variation. Here, we study the direction of accumulated dinucleotide microsatellite mutations in wild asexual lineages of hexaploid *Ranunculus carpaticola*. Whereas the overall number of contractions is not significantly different from that of expansions, the within-locus frequency of contractions, but not of expansions, significantly increases with allele length. Moreover, within-locus polymorphism is positively correlated with allele length, but this relationship is due solely to the influence of contraction mutations. Such asymmetries may explain length constraints generally observed with microsatellites and are consistent with stable, bell-shaped allele-frequency distributions. Although apomictic and allohexaploid, the *R. carpaticola* lineages show mutational patterns resembling the trends observed in a broad range of organisms, including sexuals and diploids, suggesting that, even if not of germline origin, the mutations in these apomicts may be the consequence of similar mechanisms.

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SOON after their discovery, microsatellites (short sequence repeats *sensu* Ellegren 2000a) became widely used in genetic and evolutionary analyses and are currently one of the most popular sources of genetic markers in population biology (Goldstein and Schlötterer 1999; Selkoe and Toonen 2006). A correct understanding of their evolutionary dynamics and typical stable microsatellite allele-frequency distributions (*e.g.*, Garza *et al.* 1995; Queney *et al.* 2001) is vital to fully exploiting these sequences, especially for accurate reconstructions of population processes (Ellegren 2000a; Schlötterer 2000). It has become increasingly clear that microsatellite evolution is a complex mutational process influenced mainly by DNA slippage (Levinson and Gutman 1987), mismatch repair efficiency, selection, length constraints, and also by other factors (such as repeat type, flanking sequence, and the degree of perfection of the repeats; Ellegren 2000a, 2004; Schlötterer 2000; Huang *et al.* 2002). At equilibrium, the distribution of microsatellite alleles seems to be balanced by biased mutation processes and point mutations acting toward decreasing the lengths of pure repetitive DNA (Ellegren 2000a; Sibly *et al.* 2003).

Although more and more studies have addressed the mutational behavior of microsatellites, we still lack a clear understanding, mostly because of conflicting observations and exceptions (Ellegren 2000a, 2004; Schlötterer 2000). For example, microsatellites are known to have a high rate of gain and loss of repeat units mainly due to DNA slippage. Therefore, they should mutate stepwise, with only occasional saltatory changes in repeat count (usually $<20\%$ of total mutations; *e.g.*, Sturzeneker *et al.* 2000; Udupa and Baum 2001; Brohede *et al.* 2002; Vigouroux *et al.* 2002; Beck *et al.* 2003; Wilson *et al.* 2003). A few studies, however, have reported mainly multi-step changes at microsatellite loci (*e.g.*, 63% of mutations in dinucleotide human microsatellites; Huang *et al.* 2002). Similarly, most reports indicate that the mutation rate of microsatellites increases with the length of the repeated array (*e.g.*, Wierdl *et al.* 1997; Ellegren 2000b; Hile *et al.* 2000; Brohede *et al.* 2002; Huang *et al.* 2002; Vigouroux *et al.* 2002; Dettman and Taylor 2004; Thuillet *et al.* 2004), but other studies have failed to find such a relationship (*e.g.*, Harr *et al.* 1998; Schug *et al.* 1998; Cole 2005). The most heterogeneous findings are probably from studies describing directional bias for

Sequence data from this article have been deposited with the GenBank Data Libraries under accession nos. DQ118782–DQ118860 and DQ640998–DQ641024.

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microsatellite mutations. The spectrum ranges from bias toward additions rather than deletions (*e.g.*, Primmer *et al.* 1996; Eckert *et al.* 2002; Vigouroux *et al.* 2002, 2003; Wilson *et al.* 2003) to an overall tendency toward deletions (*e.g.*, Di Rienzo *et al.* 1998; Huang *et al.* 2002; Weetman *et al.* 2002) through more neutral results showing a similar rate of expansions and contractions (*e.g.*, Hile *et al.* 2000; Brohede *et al.* 2002; Beck *et al.* 2003) to studies finding a length-dependent mutational bias (*e.g.*, Wierdl *et al.* 1997; Harr and Schlötterer 2000; Xu *et al.* 2000; Udupa and Baum 2001; Huang *et al.* 2002; Lai and Sun 2003).

To infer the mutational dynamics of microsatellites, several approaches have been applied (Ellegren 2000a, 2004; Schlötterer 2000): (1) direct observation of mutations in pedigrees (*e.g.*, Brohede *et al.* 2002; Beck *et al.* 2003) or in somatic/carcinogenic cell lines (*e.g.*, Di Rienzo *et al.* 1998; Hile *et al.* 2000; Sturzeneker *et al.* 2000; Eckert *et al.* 2002) and (2) indirect approaches such as modeling microsatellite evolution (*e.g.*, Calabrese and Durrett 2003; Lai and Sun 2003; Sibly *et al.* 2003) or analyzing historically accumulated mutations in populations (*e.g.*, Estoup *et al.* 1995; Weetman *et al.* 2002; Wilson *et al.* 2003). Despite their many advantages, pedigree studies are methodologically limited because of the rarity of naturally occurring mutations; a large amount of data must typically be accumulated to observe a few mutation events (Rose and Falush 1998; Schlötterer 2000; Brohede *et al.* 2002). An alternative is given by sexually reproducing populations that have experienced a severe bottleneck during historic times, after which microsatellite loci usually undergo a process of regenerating their genetic variability (Estoup and Cornuet 1999). If the bottleneck event is recent enough in such populations, most alleles should be derived by single mutational steps from the most frequent alleles that were probably conserved throughout the bottleneck (Estoup *et al.* 1995; Estoup and Cornuet 1999).

Particularly informative models might be provided by asexual organisms (Sunnucks *et al.* 1996; Weetman *et al.* 2002; Wilson *et al.* 2003). Lacking meiosis, recombination, and syngamy, the genomes of purely asexuals are inherited as giant linkage groups, with mutations being the only source of variation between generations (Richards 2003). In addition, such lineages could also experience severe reductions of allelic variation at multiallelic loci (such as microsatellites) when founding new populations with a single colonist, even if generally a heterozygous one (Samadi *et al.* 1999; van Dijk 2003; Haag and Ebert 2004; Paun *et al.* 2006a). For example, on the basis of the assumption of the recent introduction of the snail *Potamopyrgus antipodarum* in Britain and using an analysis based on minimum spanning networks, Weetman *et al.* (2002) showed that within the most common clonal lineage of *P. antipodarum*, microsatellites experience a strong tendency to delete repeats (>88% of total mutations). The authors suggest that, for the persistence of microsatellites in this clone, the switch toward this tendency must have occurred in relatively recent evolutionary time and may be associated with polyploidy, rather than with asexuality.

Through a methodology based on simple coalescence, we investigate the mutational dynamics of two hypervariable dinucleotide microsatellites in wild asexual lineages of the hexaploid apomictic *Ranunculus carpaticola* (apomixis is here defined as agamospermy, *i.e.*, reproduction via asexually formed seeds in higher plants; Asker and Jerling 1992). *R. carpaticola* is a species of the *R. auricomus* complex (Ranunculaceae), a Eurasiatic group of a few sexuals and hundreds of apomictic taxa (Hörandl 1998). Intensive cytoembryological and experimental research has shown that most members of the *R. auricomus* complex are aposporous and pseudogamous (*e.g.*, Häfliger 1943; Izmaiłow 1973, 1996; Nogler 1984, 1995). Apospory relates to the mitotic formation of an unreduced embryo sac (in the complete absence of meiosis) arising directly from somatic nucellar cells (Asker and Jerling 1992), which outcompetes the later-developed sexual megaspores and develops further parthenogenetically into an embryo. In apomictic *R. auricomus*, both reduced sperm nuclei fuse with two unreduced polar nuclei for endosperm formation. Thus, fertilization is still required, but only for endosperm development (pseudogamy; Koltunow and Grossniklaus 2003). The pollen is meiotically reduced and, although partly aborted, is still functional (Häfliger 1943; Izmaiłow 1996; Hörandl *et al.* 1997). Meiotic embryo sac formation and fertilization are therefore still possible in predominantly apomictic populations, but are expected to occur at low frequencies. Individual plants are perennials, living in cultivation for ∼20 years, but plants set seeds after just 2–3 years.

Apomictic reproduction for eight hexaploid populations of *R. carpaticola* from northern Slovakia has been inferred from the clonal population structure revealed by isozymes and AFLPs, with very restricted genotypic variation and high levels of heterozygosity, a uniform polyploid chromosome number ($2n = 48$), and the very high proportion (up to 80%) of sterile and malformed pollen grains (Hörandl 2002; Hörandl and Greilhuber 2002; Paun *et al.* 2006a). These apomictic populations are most probably of relatively recent (inter- or postglacial) and unique hybrid origin from divergent sexual ancestors (*i.e.*, diploid *R. carpaticola* and autotetraploid *R. cassubicifolius*; Hörandl and Greilhuber 2002; Paun *et al.* 2006a,b) and have since undergone rapid dispersal. Character incompatibility and genotype/genodive analyses of AFLP genotypes indicate a lack of recombination events in all populations except one. Therefore, most of the sites have been colonized with single genotypes, followed by their spread by asexual means (Paun *et al.* 2006a).

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**TABLE 1**

**Asexual lineages of *R. carpaticola* used in this study**

| Lineage | No.[a] | $N_{AFLP}$[b] | Population acronym | Locality | Voucher |
|---|---|---|---|---|---|
| I | 37 | 98 | TRE | Strážovské vrchy (near Trenčín), between Kubra and Kubrica, close to the bus stop at Kyselka | E. Hörandl, O. Paun, K. Mládenková, s.n., 30.04.2004 (SAV) |
| II | 22 | 101 | VRU1 | Turčianska kotlina, Vrútky-Piatrová, behind the cottage | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| III | 8 | 96 | VRU1 | Turčianska kotlina, Vrútky-Piatrová, behind the cottage | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| IV | 30 | 87 | VRU2 | Turčianska kotlina, Vrútky-Piatrová, behind the cottage | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| V | 9 | 88 | TUR | Turčianska kotlina (near Martin), Turčianska Štiavnička, Révayovský Park | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| VI | 11 | 87 | LUB | Vel'ká fatra, L'ubochňa, close to the village Dolina | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| VII | 17 | 89 | RUZ | Liptovská kotlina, Ružomberok, right side of the road, direction from Žilina | E. Hörandl, O. Paun, K. Mládenková, s.n., 01.05.2004 (SAV) |
| VIII | 18 | 93 | HRA | Liptovská kotlina, Liptovský hrádok, close to the river Váh | E. Hörandl, O. Paun, K. Mládenková, s.n., 02.05.2004 (SAV) |
| IX | 20 | 94 | IVAC | Liptovská kotlina, Ivachnová (meadow across the highway and adjacent to Carpinus forest) | E. Hörandl, *8492*, 03.05.1998 (WU); E. Hörandl, O. Paun, K. Mládenková, s.n., 02.05.2004 (SAV) |
| X | 6 | 78 | IVAC | Liptovská kotlina, Ivachnová (meadow across the highway and adjacent to Carpinus forest) | E. Hörandl, *8492*, 03.05.1998 (WU); E. Hörandl, O. Paun, K. Mládenková, s.n., 02.05.2004 (SAV) |

SAV, Herbarium of the Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia; s.n., without collection number; WU, Herbarium of the Faculty Centre for Botany, University of Vienna, Vienna.

[a] Number of individuals included in the analysis.

[b] Number of shared present AFLP fragments supporting each lineage.

Nevertheless, studying the variation at two microsatellite loci in these populations significantly changed the levels of genetic and genotypic diversity detected with AFLP and confirmed the degree of heterozygosity to be the highest possible (Paun *et al.* 2006a). The specific goals of this study are (1) to identify possible microsatellite mutational biases and constraints within the purely asexual lineages of *R. carpaticola*; (2) to compare them with microsatellite mutational behavior in other sexual and asexual organisms; (3) to trace potential switches in microsatellite evolution produced by the relatively recent hybridization, polyploidization, and the change toward apomixis in this group (Paun *et al.* 2006b); and (4) to fit a model of microsatellite mutation to our data for further use in studies on genetic diversity and the origin of apomictic taxa. Because of the high number of sampled individuals and mutations, we expect our approach to give a comprehensive image of microsatellite evolution.

## MATERIALS AND METHODS

**Apomictic lineages:** Fresh leaves of *R. carpaticola* were collected from eight sites in northern Slovakia (the polyploid populations from Paun *et al.* 2006a) and stored in silica gel. Sampling was performed randomly to ensure that genotype frequencies were not biased; for the same reason we chose plants that were at least 3 m apart. Within these populations, a total of 10 local asexual lineages (178 individuals, Table 1) were identified by AFLP fingerprinting (Paun *et al.* 2006a). Divergent genotypes within populations (23 of 201 total hexaploids analyzed) that occurred in only one to two individuals and were probably the results of infrequent sexual processes were removed from the analysis. Each of the 10 asexual lineages was supported by an identical multilocus AFLP profile in all the individuals (Table 1), with a total of 249 fragments scored in three primer combinations. Although AFLPs are dominant markers masking potential segregation of multiple copies of the same alleles, the highest possible degree of heterozygosity ($H_o = 1$) found at microsatellite loci studied in these populations (Paun *et al.* 2006a) rejects the possibility of background sex and/or segregation within these lineages. The uniform hexaploid level also contradicts the occurrence of partly sexual events, which would alter ploidy levels (meiosis on its own would result in triploids; fertilization on its own would result in nonaploids).

**Microsatellite loci:** For capturing microsatellite sequences, DNA was extracted according to the standard procedure of Doyle and Doyle (1987) and enriched for $(GAC)_6$, $(CA)_{10}$, $(AT)_{12}$, and $(AG)_{12}$ following a modified protocol from Fischer and Bachmann (1998). DNA was digested with *Rsa*I, ligated to a 5′-phosphorylated adaptor, hybridized to biotinylated microsatellite oligonucleotides, and captured on streptavidin-coated magnetic beads (Dynabeads). Captured DNA was amplified by polymerase chain reaction (PCR), using one adaptor strand as the primer, and then cloned into pGEM-T Easy Vector with JM109 competent cells (Promega, Madison, WI). Insert-containing colonies were amplified using M13 primers. PCR products with a minimum of 400 bp were

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**TABLE 2**

**Characteristics of microsatellite loci for *R. carpaticola***

| Locus | GenBank accession no. | Primers (5′–3′) | $T_a$[d] | $MgCl_2$ (mM) | Repeat motif in clone | FL[e] |
|---|---|---|---|---|---|---|
| 1207[a] | DQ118785 | F[b]: AGCCGAGACCAGGTATGAG<br>R[c]: CGTTCAGCTAGCGTTCCTAC | 52 | 2.5 | $(GT)_{19}$ | 132 |
| 1407N | DQ118795 | F: GGAG(AT)TGCAGTGGTGGAGA<br>R: CAGCAACCACCTTCTTCAAC | 55 | 1.5 | $(GA)_{30}$ | 47 |
| 3313 | DQ118820 | F: GTTCTTGCTTGCGGAGATT<br>R: AGCTGGTAAAGACACACACA | 52 | 2.5 | $(TC)_{28}(TG)_{40}$ | 50 |

[a] Locus 1207 was not used further in this study.
[b] Forward primer.
[c] Reverse primer.
[d] Annealing temperature.
[e] Sequence length sum of 3′ and 5′ flanking regions.

sequenced using BigDye Terminator RR mix (Applied Biosystems, Foster City, CA) and an ABI Prism 377 DNA sequencer (Perkin-Elmer, Norwalk, CT). Seventy-nine unique microsatellite (~30% of inserts checked) sequences and flanking regions were obtained (GenBank accession nos. DQ118782–DQ118860). The most common motif found was GT/AC, although other dinucleotides, as well as more complex microsatellites, were also identified. Of the 15 sequences selected for designing primers, six primer pairs failed to amplify any reproducible products, six loci resulted in monomorphic patterns, and three polymorphic loci were obtained (Table 2). Two loci (1407N and 3314) were chosen for further use because of the clarity of the amplified alleles, taking into consideration that reliability of any scored allele is crucial in a mutational study. Locus 1407N is based on a perfect microsatellite sequence (TC/GA), and locus 3313 amplifies alleles of a compound dinucleotide microsatellite (two immediately adjacent repeats of different motifs: TC/GA and TG/CA). Some alleles in a few individuals were sequenced via cloning for both loci to check for interruptions or changes in repeat structure and possible indels in flanking regions (see supplemental data at http://www.genetics.org/supplemental/ for sequences of some alleles at the two loci genotyped for *R. carpaticola* apomictic lineages. Note that, for locus 1407N, in the supplemental data the genotyped region does not include the entire flanking region presented here.).

**Microsatellite genotyping:** All individuals were genotyped using 5′ fluorescently labeled forward primers. PCR was performed separately for each primer pair in 6-μl volumes with 1.1× ReddyMix PCR Master Mix (ABGene), 1.5 pmol of each primer, and 4–5 ng of DNA. PCR amplification was carried out in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems) using the following cycling conditions: for the first cycle, 95° 4.5 min, $T_a$ (see Table 2) − 2° 1 min, 72° 1 min; for the following 40 cycles, 95° 1 min, $T_a$ 1 min, 72° 1 min, followed by a final elongation (at 72°) of 10 min. The fluorescence-labeled microsatellite fragments were separated on a 5% denaturing polyacrylamide gel with an internal size standard [GeneScan-500 (ROX), PE Applied Biosystems] on an automated sequencer (ABI 377, Perkin-Elmer). Raw data were collected, manually scored, and exported as allele size using ABI Prism GeneScan analysis software (PE Applied Biosystems) and Genographer (version 1.1.6, Montana State University 1998; http://hordeum.msu.montana.edu/genographer/). The allele lengths were further converted to a number of repeat units, discarding the known flanking sequence length. For the compound microsatellite, the number of repeat units is reported as a sum for the two adjacent repeat regions.

**Identification of mutations:** The genotype of each individual was confirmed by at least two independent repeats (starting from DNA extracts) until all putative genotyping or binning errors were removed. To facilitate the correct estimation of the evolutionary pathways that interconnect different alleles, the microsatellite alleles were classified by size, with the first allele of each individual assigned to the first class, etc. (Figure 1), at the level of each AFLP clone. Because, within each lineage, the genomes screened have not experienced any recombination after their most recent common ancestor (Paun *et al.* 2006a), each lineage can be thought of as a mutation accumulation line. The alleles visible today within a lineage and a class must be homologous; therefore they must all be derived by mutations from a single ancestral allele that existed in the past. We follow the assumption of general population genetics theory that the most frequent allele usually reflects the state of the coalescent one (Watterson and Guess 1977; Udupa *et al.* 2004). Therefore, the alleles not identical in state to the most frequent are interpreted as mutations or novel size variants (NSV). Overall, we examined 537 NSV from 1449 alleles scored in two loci. For the identification of the magnitude of mutations, we usually assumed the smallest mutational change in allele size to be most likely (Brohede *et al.* 2002; Beck *et al.* 2003; Ellegren 2004), but in one case we also interpreted our data by minimizing the number of mutation events involving larger jumps: in locus 3313, lineage 4, individual 20 (Figure 1), we preferred to consider a loss of 27 repeats of the allele in class 4 and a gain of 1 repeat in class 3, rather than the alternative, where two multiple-step losses occur, 1 of 14 repeats in class 4 and 1 of 12 repeats in class 3. Because dinucleotide microsatellites are assumed to be selectively neutral (*e.g.*, Ellegren 2000a, 2004; Schlötterer 2000), we considered the frequency of a NSV in a class to reflect the frequency of the mutations that produced them. The reliable detection of mutations and their frequency also depends on random sampling of individuals within each clone and on the use of "young" clonal lineages, in which recurrent mutations occur at low frequencies.

**Analysis of mutation data:** Using SPAGeDi v.1.1 (Hardy and Vekemans 2002), we calculated the number of alleles and mean allele size for each clone and locus, together with the frequencies of all different alleles in the total sample on which the median allele size was inferred. We then divided the putative mutations into two groups: "single-step" mutations, which differ by 1–2 units from the most frequent allele and are considered to be mainly the result of polymerase slippage (Levinson and Gutman 1987), and "multi-step" mutations, which differ by >2 units from the parental allele and are

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**TABLE 3**

**Summary of microsatellite data in 178 individuals at two loci**

| | Microsatellite locus | |
|---|---|---|
| | 1407N | 3313 |
| Allele size range (no. of repeats) | 5–68 | 35–99 |
| Mean allele size (no. of repeats) | 33.5 | 60.8 |
| No. of alleles | 56 | 59 |
| Average no. of alleles/ individual (SD[a]) | 4.50 (1.26) | 3.71 (0.93) |
| $H_o$[b] | 1 | 1 |
| Total no. of alleles scored | 788 | 661 |
| Total no. of NSV[c] scored (%) | 225 (28.6) | 312 (47.2) |

[a] Standard deviation.
[b] Observed heterozygosity.
[c] Novel size variants.

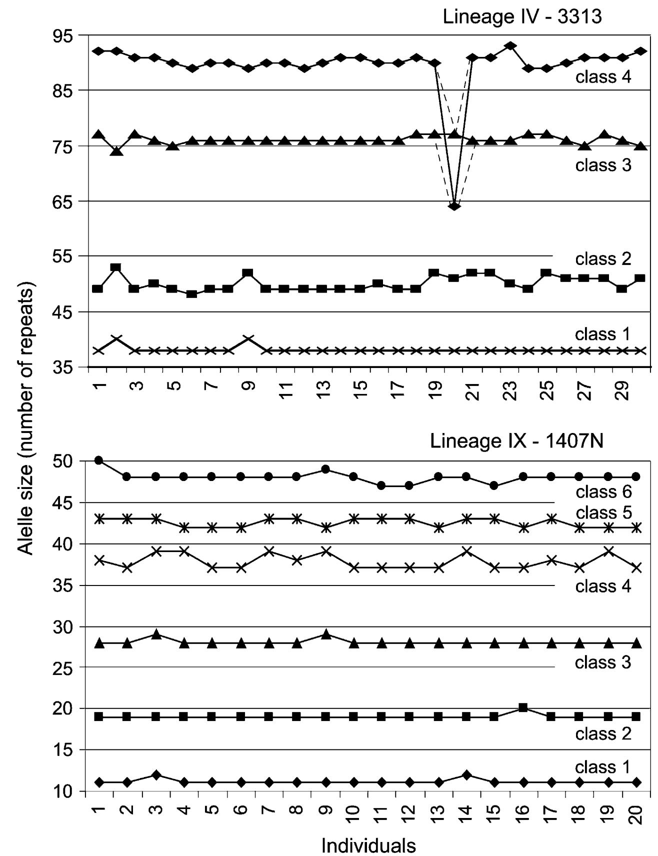


Figure 1.—Examples of classes of microsatellite alleles in asexual lineages: lineage IV for locus 3313 and lineage IX for locus 1407N. Allele size is given in number of repeat units. The dotted line indicates an alternative mutational change (see text).

probably the result of other processes [*e.g.*, mitotic crossings over, (Birky 1996) or illegitimate recombination (Devos *et al.* 2002)]. While it is possible that some of these NSVs could be the result of multiple mutations, we define the change in length from one size to another as a single mutation, irrespective of the number of repeat unit changes involved. Hence, our approach is probably biased toward overestimation of the frequency of large-size mutation events. If so, in a random sample, we expect the overestimation to be proportional in all classes and groups and equally distributed between losses and gains of repeats. However, the lack of some intermediate mutational steps gives evidence that these assumptions might be generally correct.

Statistical analyses were performed with the software SPSS version 10.0.5 (SPSS, Chicago). Within each lineage we tested for correlations of allele size changes across pairs of classes, using Pearson two-tailed comparisons and adjusting the statistical significance with Bonferroni correction (Shaffer 1995). Pooling data from all classes and lineages, we again used Pearson two-tailed correlations to analyze the distribution of expansion mutations, contractions, and constant ancestral-type alleles on allele length (given as number of repeats). When the same size class was found in different lineages, an average of the frequencies of the different values was calculated and used in further analysis. The frequency of constant ancestral-type alleles is the complement to the mutability of a particular progenitor allele. Finally, a $\chi^2$-test was applied to test whether expansions and contractions of microsatellite alleles occur at equal frequencies (1) for all mutations, (2) for single-step mutations, and (3) for multi-step ones.

## RESULTS

**Overall variation:** In the 178 individuals analyzed, the two loci used in this study were highly polymorphic (56 and 59 alleles in locus 1407N and 3313, respectively; Table 3), facilitating the representation of many intermediate steps between minimum and maximum allele sizes and the accurate identification of NSV (Figure 1). Consistent with the hexaploid level of the investigated individuals (Paun *et al.* 2006a), a banding pattern with between 2 and 6 alleles detected per individual was apparent at each microsatellite locus (average 4.50 and 3.71 alleles/individual/locus for 1407N and 3313, respectively; Table 3). Of the 788 and 661 alleles scored, 28.6 and 47.2% were interpreted as NSV for loci 1407N and 3313, respectively. Performing 156 Pearson two-tailed correlations of allele size change across size classes (within each lineage each pair of classes was considered just once), only 12 were found significant at the 0.05 level and two at the 0.01 level. Applying a Bonferroni correction to these multiple comparisons, the level of significance decreased to 0.00032 and all correlations become not significant at a level corresponding to $\alpha = 0.05$ for single comparisons.

**Magnitude and directionality of mutations:** Dividing the alleles within each lineage into classes allowed us to specifically identify all NSV as either expansions or contractions. At both loci, the difference between the overall number of additions and deletions was not significant (117 *vs.* 108 for locus 1407N and 168 *vs.* 144 for 3313; Table 4); we rejected, therefore, the hypothesis of a general trend for microsatellite growth in our data. Next, we examined separately for each locus whether gains and losses observed in two different categories (*i.e.*, single step and multi-step) occurred asymmetrically. We found a significant asymmetrical distribution

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TABLE 4

**Results of $\chi^2$ tests comparing contractions and expansions**

| Category | Locus | Contractions | Expansions | $\chi^2$ | d.f. | *P* |
|---|---|---|---|---|---|---|
| Overall | 1407N | 108 | 117 | 0.36 | 1 | 0.549 |
| | 3313 | 144 | 168 | 1.85 | 1 | 0.174 |
| Single step[a] | 1407N | 92 | 111 | 1.78 | 1 | 0.182 |
| | 3313 | 134 | 141 | 0.18 | 1 | 0.673 |
| Multi-step[b] | 1407N | 16 | 6 | 4.55 | 1 | 0.033 |
| | 3313 | 10 | 27 | 7.81 | 1 | 0.005 |

[a] The NSVs differing by one to two repeat units from the putative coalescent.
[b] The alleles differing by more than two repeat units from the putative coalescent.

for multi-step mutations only, in favor of expansions at locus 3313 and of contractions at locus 1407N (Table 4). The average absolute change per NSV scored was 1.6 repeat units for locus 1407N and 1.58 units for locus 3313. However, per NSV scored, there is an overall mean addition of just 0.09 units at locus 3313 and an overall mean loss of 0.12 units at locus 1407N. The distribution of the NSV according to the magnitude of mutations is shown in Figure 2. The majority of the NSV (1407N, 90.2%; 3313, 88.1%; Table 4) differed from the putative coalescent by an increase or decrease of just 1 or 2 repeat units.

**Length-dependent mutational bias:** At both loci, we found a negative correlation between allele length (repeat number) and frequency of ancestral-type alleles ($P < 0.005$; Table 5) in accordance with a positive correlation between allele length and mutability (Figure 3). Furthermore, the frequency of contractions was strongly correlated with allele length for both loci ($P < 0.005$; Table 5), with the longest allele losing repeats with an average frequency of ~40% (Figure 4). In contrast, at both loci, allele length did not influence the frequency of expansions, which have a constant trend of occurrence over the entire allelic length distribution (Table 5 and Figure 4). Analyzing the distributions of contractions and expansions separately for the single-step and multi-step mutations, we found slightly different trends at least for contractions involving >2 units (Figure 5).

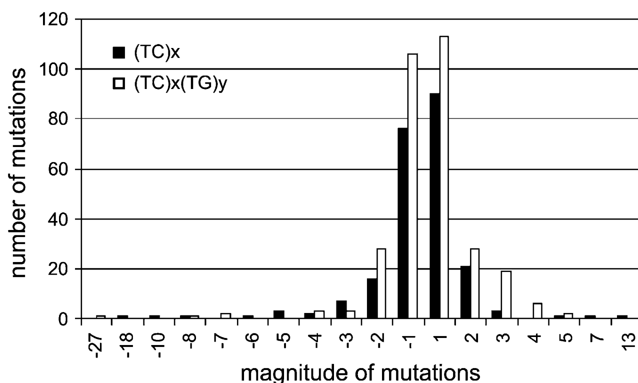


FIGURE 2.—The distribution of the mutation magnitude according to number of mutations. Solid bars are given for locus 1407N, open bars for 3313. Note that the *x*-axis is not continuous.

## DISCUSSION

**Diversity and heterozygosity:** Although there is a significant correlation between the genetic patterns detected with AFLPs and SSRs (Paun *et al.* 2006a), a marked difference in the resolution of the two markers is obvious. Due to the high levels of polymorphism, two microsatellite loci were sufficient to detect divergent "clone mates" (Mes *et al.* 2002) within the apomictic *R. carpaticola* lineages identified by isozymes and AFLPs (Hörandl and Greilhuber 2002; Paun *et al.* 2006a), with almost as many multilocus genotypes as individuals. This relates to the high mutation rate at microsatellite loci ($10^{-2}$–$10^{-6}$; Schlötterer 2000). The equation of genotypes with clones, *i.e.*, plants that have undergone only asexual reproduction since their most recent common ancestor, is appropriate only if the frequency of sexual reproduction is higher than the mutation rate of the marker (Brookfield 1992). Therefore, individuals that belong to a single asexual lineage may nevertheless be genetically different due to accumulation of mutations (Mes *et al.* 2002). However, clonality is also indicated in microsatellite data by the lack of allelic segregation (consistent number of alleles per individual plus obvious allelic classes within one lineage) and the slightly lower genotypic diversity compared with closely related sexual populations (see Paun *et al.* 2006a).

The amplified alleles confirmed the maximal level of heterozygosity (*i.e.*, $H_o$ always 1, Table 3). An important effect of heterozygosity generally found in apomicts (Gornall 1999; Hörandl and Paun 2006) is that a considerable part of the overall allelic diversity remains within each individual of an apomictic lineage. Apomicts may therefore have an advantage over sexuals in overbalance inbreeding depression when founding populations with a single or few individuals. Additionally, heterozygosity in connection with polyploidy is probably important for buffering deleterious mutations, otherwise

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**TABLE 5**

**Results of Pearson two-tailed correlation tests for frequency of expansions, ancestral-type alleles, and contractions *vs.* number of repeats**

| | Locus | | | | | |
|---|---|---|---|---|---|---|
| | 1407N[a] | | | 3313[b] | | |
| Correlations | Expansions | Constant | Contractions | Expansions | Constant | Contractions |
| Pearson *r* | 0.041 | −0.791 | 0.779 | 0.065 | −0.703 | 0.686 |
| *P* | 0.817 | 0.000 | 0.000 | 0.743 | 0.000 | 0.000 |

[a] $N = 34$.
[b] $N = 28$.

usually recessive, that are expected to accumulate in apomictic plants (Maynard Smith 1978; Richards 1997). Mechanisms that have been proposed to lead to increased heterozygosity in asexual individuals include hybrid origin and mutational divergence of alleles (Gornall 1999). Indeed, the apomictic group of *R. carpaticola* is of hybrid origin (Hörandl and Greilhuber 2002; Paun *et al.* 2006b), thus partly explaining the observed levels of heterozygosity. In addition, in asexual lineages, the different alleles of each individual at a locus evolve independently and accumulate different mutations leading to increased heterozygosity over a short timescale, a process that starts from the moment that sexual reproduction is lost (Birky 1996). Consequently, we suspect that the cases of missing alleles per individual corresponding to hexaploidy must relate to null alleles rather than to more copies of visible ones. Otherwise, at least some individuals in each lineage would be expected to accumulate mutations in a visible spectrum of amplification. Due to asexual reproduction, and thereby failing to meet Hardy–Weinberg equilibrium, the presence of null alleles in these populations is difficult to prove statistically. Nevertheless, by sequencing alleles randomly (supplemental data at http://www.genetics.org/supplemental/ for sequences of some alleles at the two loci genotyped for *R. carpaticola*

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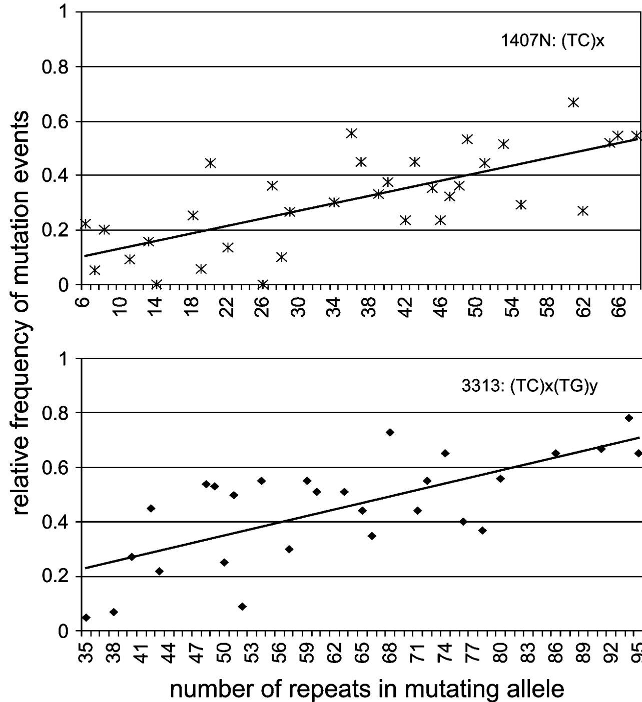


Figure 3.—The distribution of mutability by number of repeats at two dinucleotide microsatellite loci. The mutability is here defined as the tendency to mutate and is calculated as 1 minus the frequency of putative ancestral, nonmutant alleles. Note that although the *x*- axis is continuous, not all possible allele lengths are sampled.

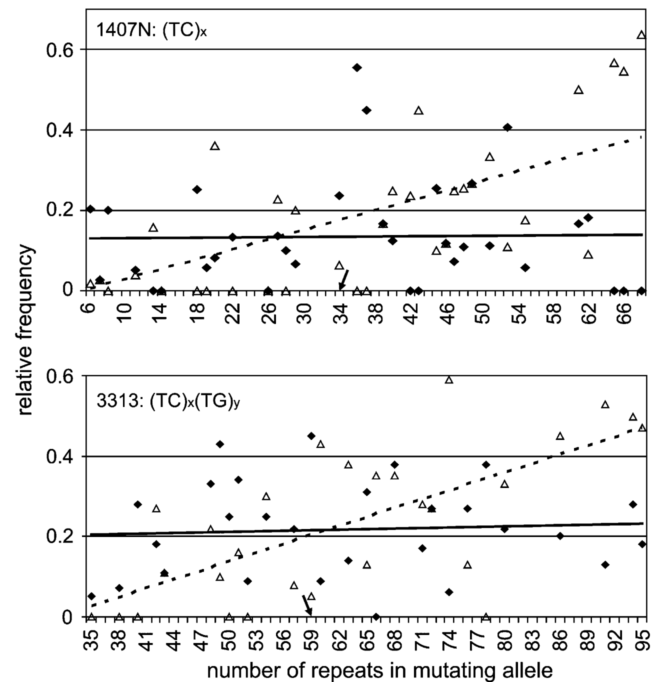


Figure 4.—The frequency distribution of expansions (solid diamond and solid line) and contractions (open triangles and dotted line) by number of repeats at two microsatellite loci. Solid arrows indicate estimated median allele size. Note that although the *x*-axis is continuous, not all possible allele lengths are sampled.

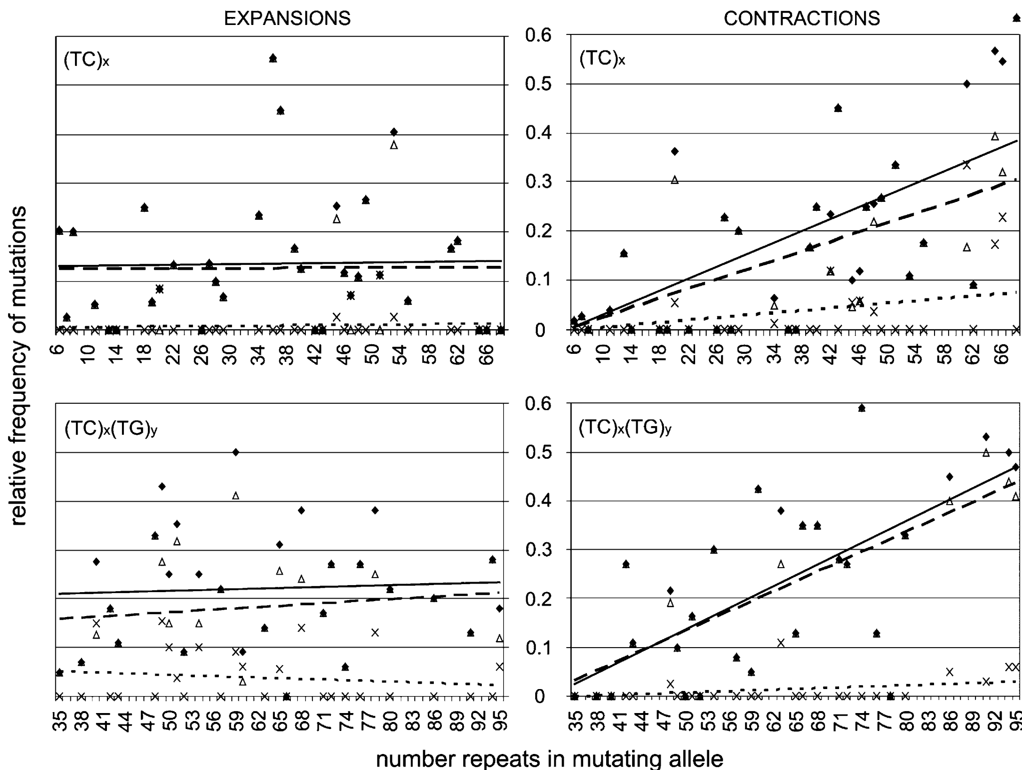


Figure 5.—The frequency distribution of expansions and contractions by number of repeats at two microsatellite loci, overall (solid diamond and solid line) and by magnitude of mutation: 1–2 units change (single-step: open triangles and dashed line) and >2 repeats (multi-step: ×'s and dotted line). Note that although the *x*-axis is continuous, not all possible allele lengths are sampled.

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apomictic lineages. Note that, for locus 1407N, in the supplemental data the genotyped region does not include the entire flanking region presented here.) we could identify alleles not considered in the fingerprinting method due to lengths <50 bp (*i.e.*, null alleles). However, dealing primarily with repeat number rather than with base-pair length between primer pairs, our results are based on the present classes of alleles and are not altered in any way by such null alleles.

**Estimation of the mutational trend:** There have been conflicting assertions of trends in the mutational process of microsatellites toward expansions over contractions (see Introduction). The somatic mutations of asexual lineages of *R. carpaticola* show no significant asymmetry in overall gains and losses of repeats at both microsatellite loci. Moreover, the very low average number of repeat units changed per mutation event in both loci indicates that these microsatellites approach a state of equilibrium. This symmetry is consistent with expectations of a simple stepwise mutation model (*e.g.*, Valdes *et al.* 1993; Eisen 1999). Indeed, mutations of microsatellites involve mainly slipped-strand mispairing events leading, with equal overall probability, to insertions or deletions of usually one repeat unit during mitotic cell divisions (Levinson and Gutman 1987; Eisen 1999; Ellegren 2000a; Schlötterer 2000). Changes of 1 or 2 repeat units dominate in our data, but we also found 10–12% of the NSVs to be different from the putative coalescent by >2 repeat units. This pattern of changes, common to both loci, better fits expectations of a two-phase model of microsatellite evolution (*e.g.*, Di Rienzo *et al.* 1998), which is more likely to result in irregular, multimodal distributions. A direct test of our data for multimodal distributions of repeat alleles would be inconclusive due to asexual reproduction and the relatively recent reduction of allelic variation experienced by our populations (Paun *et al.* 2006a). As a result, the irregular distribution of alleles visible in our data (not shown) is very likely to be caused by conservation throughout the bottleneck of few alleles that now have increased frequencies (see also Valdes *et al.* 1993; Dettman and Taylor 2004).

One unusual feature of our data is the presence of rather large multi-step mutation events (up to 27 repeats; Figure 2), in contrast to other studies (*e.g.*, changes of up to 10 repeat units in Huang *et al.* 2002 or up to 18 repeats in Beck *et al.* 2003). This pattern might be also a consequence of undetected multiple mutation events. On the other hand, because of the lack of some intermediate steps (Figure 2), the relatively recent origin of these asexual lineages (Paun *et al.* 2006a,b) and similar percentage of large jumps found in direct studies (*e.g.*, Xu *et al.* 2000; Udupa and Baum 2001; Brohede *et al.* 2002; Vigouroux *et al.* 2002), such multiple mutations might be infrequent. A possible influence of polyploidy by increasing the span of mutations needs further studies. Our results are more surprising in the absence of recombination and, consequently, of unequal crossings over in our lineages, which have previously been proposed as a major mechanism for multi-step mutations (*e.g.*, Wierdl *et al.* 1997), although criticized recently (Eisen 1999; Schlötterer

2000; Ellegren 2000a, 2004). Other processes have also been shown to relate to larger jump changes in repetitive sequences, such as mitotic crossings over (Birky 1996), noncrossover gene conversion (meiotic, but also mitotic; Richard and Pâques 2000), and illegitimate recombination (Devos *et al.* 2002). These processes seem more probable, as it has been demonstrated that strains with and without a functional recombination system behave in similar ways (see Schlötterer 2000), and also in the light of *R. carpaticola* lineages, each of them most probably completely free of recombination (Paun *et al.* 2006a). Furthermore, although the genomic microsatellite distributions seem to be associated with sites of recombination, most tests for a correlation between microsatellite mutability and recombination rate have failed to find such an effect (see Ellegren 2004).

The distribution of NSVs in the lineages analyzed (Figure 3) agrees with an increase in mutability with the length of the microsatellite, reflecting a positive correlation of mutation rate with progenitor allele size seen also in many other organisms (*e.g.*, Dettman and Taylor 2004; Thuillet *et al.* 2004; see also Ellegren 2004). An increase in mutability with allele length is plausible because longer microsatellites should, intuitively, give more opportunity for slippage (Ellegren 2000a,b). Brohede *et al.* (2002) calculated an increase of mutation rate with allele size, at ∼0.1%/repeat unit over the observed range of allele sizes. The trend in our data is even more visible: the mean mutability increased from ∼10–20% for the smallest allele to 50–70% for the longest one (Figure 3). This corresponds to an increase of mutability with allele size, at ∼0.65–0.83%/repeat unit, although this cannot be directly compared with mutation rate.

The directionality of microsatellite mutation has been the focus of much controversy (*e.g.*, Ellegren 2000a, 2004; Schlötterer 2000). Our results indicate a size-dependent mutational bias, in which long alleles are biased toward contraction, whereas short alleles are biased toward expansion. Indeed, within both loci, there is a strong, statistically significant positive relationship between the frequency of contracting mutations and allele length, whereas the frequency of expansions is constant over the entire allelic distribution (Figure 4). Therefore, mutations show a nonrandom distribution among alleles within loci, with alleles close to the median size having an almost equal frequency of expansions and contractions and being nearly free of any bias. A consequence of these findings is that homoplasy affects medium-sized alleles with a higher probability than extreme alleles in the range, supporting previous results (Queney *et al.* 2001). As the within-locus polymorphism positively correlates with the allele length, this relationship is caused only by the influence of contraction mutations. A downward bias for long alleles has been found also in other studies (*e.g.*, in barn swallows, Primmer *et al.* 1996; in yeast, Wierdl *et al.* 1997; in humans, Ellegren 2000b; in Drosophila, Harr and Schlötterer 2000); few others reported simultaneously the bias at short alleles for increase in size (*e.g.*, in chickpea, Udupa and Baum 2001; in humans, Huang *et al.* 2002). However, to our knowledge, only one study has previously found such a pattern with constant expansion frequency but increasing contraction rate with allele length, based on data acquired from 273 human tetranucleotide loci (Xu *et al.* 2000). The authors state that such a size-dependent mutation mechanism will result at equilibrium in a bell-shaped distribution of alleles, with the modal allele at the microsatellite length where the rate of expansion equals that of contraction ("critical length"; Xu *et al.* 2000). This gives a very elegant explanation for stationary distribution of alleles (*e.g.*, Garza *et al.* 1995; Queney *et al.* 2001) and of the generally observed length constraints on microsatellites (*i.e.*, the absence of very long alleles). Alternative explanations range from assuming that microsatellites beyond a certain length collapse (Levinson and Gutman 1987) to bias toward larger deletions at long alleles (Ellegren 2000b; Vigouroux *et al.* 2002), selection against long alleles (*e.g.*, Garza *et al.* 1995), and counteracting point mutations that stabilize long repetitive strands (*e.g.*, Thuillet *et al.* 2004).

Considering this kind of microsatellite dynamics, the median length for a locus at equilibrium should correspond with the critical length at which the mutation spectrum changes. For locus 1407N, the median value (34 repeats) is over the critical size (27 repeats) (Figure 4). Regarding the critical length, at this locus there are more alleles biased toward contractions (Figure 4). Moreover, we find significantly more multistep contractions than expansions at this locus (Table 4): there is a net loss of 0.12 repeat units with each change, and the frequency of long deletions (*i.e.*, >2 units) is approximately two times higher than in locus 3313 (Figure 4). In contrast, for locus 3313 (Figure 4), median length (59 repeats) is under the critical length (61 repeats). In this case, the number of NSV resulting from contractions is slightly lower than that of NSVs resulting from expansions (144/168). This locus shows an addition of 0.09 repeat units with each NSV scored, the median length is biased toward expansions (Figure 4), and the expansions of >2 units are significantly more common than contractions (Table 4). Therefore, it is clear that even if the two loci are close to an equilibrium state, the overall bias at these loci is a dynamic one.

**Microsatellites in evolutionary and population studies:** Such dynamic bias toward an equilibrium distribution of alleles around a target length—which is probably not only species specific (Harr and Schlötterer 2000) but also dependent on base composition (Eckert *et al.* 2002)—type of microsatellite, etc., could provide an explanation for the high levels of variation in

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microsatellite length distribution and in biases among different loci, organisms, and studies. Other explanations for contrasting results are the random selection in some studies of relatively young microsatellites, which had not reached the complete allelic distribution (and still experience an overall bias toward expansion), or the pooling of scarce data from many variable loci with different target lengths.

Given the complexities of the DNA replication and mismatch repair system (Eisen 1999), the intrinsic mechanisms that account for the types of bias observed are not yet understood. Our observations show that evolutionary processes leading to length polymorphism at microsatellite loci do not follow a simple stepwise model (Ellegren 2000a, 2004; Brohede *et al.* 2002) and most probably also involve other processes, aside from polymerase slippage. To improve and derive new models of mutation necessary for the accurate use of microsatellites, especially for reconstructing processes in populations, the entire range of factors influencing their genesis and evolution must be considered. Some authors have incorporated details of microsatellite behavior in models of evolution (*e.g.*, Rose and Falush 1998; Calabrese and Durrett 2003; Lai and Sun 2003), but these models need to be further improved and should be included in genetic distance calculations and analysis software.

**Implications for evolution of clonal lineages:** Sexual populations undergoing bottlenecks or founder events will usually conserve a few common alleles by random drift and will show lower allelic variation than conspecific populations that have not undergone a bottleneck (Hartl and Clark 1997). Although mostly heterozygotes, clonal organisms such as apomictic plants are expected to exhibit low evolutionary responsiveness and to require a longer time than sexually reproducing outcrossing plants for recovering genetic variation after founding populations with single individuals (Chapman *et al.* 2000). We have demonstrated here that within asexual lineages of *R. carpaticola* extensive allelic variation can be generated via mutations, which have been shown to be an important source of clonal variation at noncoding, but also coding, sites (*e.g.*, King and Schaal 1990; King 1993). Furthermore, in accord with other studies (Wilson *et al.* 1999, 2003), the mutational processes in microsatellite evolution in *R. carpaticola* apomictic lineages are not necessarily random. The microsatellite loci studied approach a dynamic evolutionary equilibrium and do not show mutations out of a normal register (equivalent to deleterious mutations). Our data provide direct evidence of evolution within wild asexual lineages in which microsatellite loci regenerate their genetic variability and reach equilibrium of allelic distribution against a background lacking the jumbling effect of recombination and segregation.

Chenuil *et al.* (1997) report an uncommon bias toward fewer and shorter dinucleotide microsatellites in tetraploid sexual *Barbus* sp. compared to its diploid relatives. Weetman *et al.* (2002) found a strong tendency toward deleting repeats at seven microsatellite loci in triploid asexual *P. antipodarum* in the United Kingdom. Both studies hypothesized that polyploidy in these lineages may be associated with this mutation bias toward deletions. Although being apomicts and allohexaploids, the asexual lineages analyzed in this study do not show such patterns. In addition, we find comparable allelic distributions when comparing these lineages with their sexual relatives, diploids as well as tetraploids (Paun *et al.* 2006a,b; O. Paun and E. Hörandl, unpublished results). Our results indicate that the mutational behavior of microsatellites in asexual lineages of *R. carpaticola* has similarities to that of a broad range of organisms, including sexuals and diploids. We observe a dynamic balance in the mutational process and a directional bias in microsatellite alleles such that: (1) mutations occur at a higher frequency in the larger alleles at a locus; (2) the frequency of contractions, but not of expansions, increases with allele size; (3) the overall frequency of contractions is not significantly different from that of expansions; and (4) mutations predominantly, but not exclusively, involve the gain or loss of 1 or two repeat units. Our study is among the very few dealing with patterns of microsatellite evolution in plants and in polyploids and the first one to characterize individual loci in such detail. In plants, the soma and germline are one and the same (*e.g.*, Fagerström *et al.* 1998); nevertheless, the observed trends at microsatellite loci in *R. carpaticola* asexual lineages support the view that somatic and germline mutations share the same fundamental characteristics (Sturzeneker *et al.* 2000) and indicate that they constitute useful models for studying the mechanisms that generate microsatellite variability in natural populations.

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