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The impact of genome defense on mobile elements in *Microbotryum*

Louise J. Johnson · Tatiana Giraud · Ryan Anderson · Michael E. Hood

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Abstract Repeat induced point mutation (RIP), a mechanism causing hypermutation of repetitive DNA sequences in fungi, has been described as a 'genome defense' which functions to inactivate mobile elements and inhibit their deleterious effects on genome stability. Here we address the interactions between RIP and transposable elements in the *Microbotryum violaceum* species complex. Ten strains of *M. violaceum*, most of which belong to different species of the fungus, were all found to contain intragenomic populations of *copia*-like retrotransposons. Intragenomic DNA sequence variation among the *copia*-like elements was analyzed for evidence of RIP. Among species with RIP, there was no significant correlation between the frequency of RIP-induced mutations and inferred transposition rate based on diversity. Two strains of *M. violaceum*, from two different plant species but belonging to the same fungal lineage, contained *copia*-like elements with very low diversity, as would result from a high transposition rate, and these were also unique in showing no evidence of the hypermutation patterns indicative of the RIP genome defense. In this species, evidence of RIP was also absent from a Class II *helitron*-like transposable element. However, unexpectedly the absolute repetitive element load was lower than in other strains.

Keywords Anther smut · Ustilago violacea · Helitron · Retrotransposons · Copy number variation

L. J. Johnson (✉)
School of Biological Sciences, University of Reading, Reading RG6 6AS, UK
e-mail: l.j.johnson@reading.ac.uk

T. Giraud
Ecologie, Systématique et Evolution, Centre National de la Recherche Scientifique, CNRS, 91405 Orsay cedex, France

T. Giraud
Université Paris-Sud, 91405 Orsay cedex, France

R. Anderson
Department of Biology, University of Virginia, Charlottesville, VA 22904, USA

M. E. Hood
Department of Biology, Amherst College, Amherst, MA 01002, USA

Introduction

Mobile genetic elements, such as Class I retrotransposons and Class II transposons, are abundant in almost all eukaryotic genomes, and profoundly influence genome evolution (Hurst 1992; Kidwell and Lisch 2001). Mobile elements cause insertional mutations, and they also impose on their hosts the burden of replicating extraneous DNA and the transcription or protein expression associated with the element. Furthermore, repetitive elements dispersed across the genome increase the risk of ectopic recombination, which can result in large-scale sequence deletions or duplications. Because of these deleterious effects, mobile elements are often considered genomic parasites, spreading by virtue of their non-Mendelian transmission (Nee and Maynard Smith 1990). As with conventional host-parasite systems, many organisms have evolved ways to defend their genomes against this attack, either by suppressing the replication of mobile elements or by ameliorating their harmful effects (reviewed by Johnson 2007). RNA interference and cytosine methylation are familiar examples of genome defenses, although these processes also have vital roles as mechanisms of gene regulation (Agrawal et al. 2003; Kidwell and Lisch 2001).

Repeat-induced point mutation (RIP) is a remarkable mechanism whereby certain fungi detect and hypermutate repetitive DNA sequences using specialized molecular machinery (reviewed by Selker 2002; Galagan and Selker 2004). Most studies of RIP focus on *Neurospora crassa*, in which it was first discovered, although other fungi, including many model systems, employ RIP-like processes. These include *Aspergillus nidulans* (Clutterbuck 2004), *Podospora anserina* (Hamann et al. 2000), *Magnaporthe grisea* (Ikeda et al. 2002), *Fusarium oxysporum* (Daboussi et al. 2002), and *Colletotrichum cereale* (Crouch et al. 2008). Duplicated sequences appear to interact in a pairwise manner with the RIP machinery, which induces transition mutations of cytosine residues at particular di- or tri-nucleotide combinations. RIP therefore leaves a detectable signature on repetitive DNA sequences, although the specificity of target site varies between fungal taxa. Different sites may be mutated in each of the paired sequences, such that sequence similarity between the two copies is decreased. In this way RIP reduces the risk of ectopic recombination, in addition to causing loss-of-function mutations that disable duplicated genes. As RIP has no other known role in the genome, it is regarded as a clear-cut example of a host defense system against transposable elements (Daboussi and Capy 2003). Many species with the RIP defense, including *Neurospora crassa*, have a very low complement of repetitive DNA, due to hypermutation and elimination of repeated sequences in the past. A paucity of duplicated genes may potentially constrain adaptive evolution in such species (Galagan and Selker 2004).

Microbotryum violaceum provides an opportunity to observe the action of the defense mechanism upon natural intragenomic populations of transposable elements. The *M. violaceum* species complex (Le Gac et al. 2007; Lutz et al. 2008) is a group of parasitic fungi infecting many plant hosts of the family Caryophyllaceae, and was the first basidiomycete where sequences of repetitive elements were found to have mutation patterns indicative of a RIP-like process (Hood et al. 2005). The genome of *M. violaceum* has been estimated from low-coverage shotgun sequencing to contain at least 15% interspersed repetitive DNA, with 8% consisting of retrotransposons, predominantly *copia*-like elements, and 2% as the unusual rolling-circle Class II *helitron* elements (Hood 2005). Some of these elements appear to be active based on studies of mutation rates (Garber and Ruddat 1994, 1998, 2000, 2002; Hood et al. 2005), and studies of gene expression (Yockteng et al. 2007). It is unknown to what extent RIP-competent lineages of *M. violaceum* have, like *Neurospora* (Galagan and Selker 2004), a "frozen genome" and dearth of recent gene duplications.

Here we describe the DNA sequence variation of mobile elements within the genomes of ten *M. violaceum* strains, belonging to at least five different sibling species. Sequence diversity in mobile elements can give an estimation of transposition rate; we exclude RIP target sites from this analysis as these sites are by definition subject to hypermutation. We also examine DNA sequence variation at RIP target sites to quantify the intensity of the RIP mutation signature in different fungal species, in order to determine whether estimates of element activity and the intensity of RIP were negatively correlated, as might be expected.

Materials and methods

Fungal strains

This study made use of a collection of strains, belonging to different species of the *M. violaceum* species complex, isolated from host plants of the Caryophyllaceae in Europe and North America. Le Gac et al. (2007) indeed showed that *M. violaceum* encompasses several species, specific to one or a few related plants, that are evolutionarily independent and should be considered as separate species. The generic name *Microbotryum* is used here to refer to this species complex as taxonomic revisions are currently ongoing elsewhere (see Lutz et al. 2008). The geographic origin of lineages used in this study and the year of isolation, as well as their species affiliations according to Le Gac et al. (2007) and Lutz et al. (2008), are listed in Table 1. Species affiliations were confirmed by both the host-of-origin and by DNA sequence of the nuclear gamma-tubulin gene, and strain names throughout the text include an abbreviation indicating the host-of-origin. We avoided subjecting any strain to repeated rounds of growth in the laboratory; cultures were stored frozen for the vast majority of the time since their date of collection.

PCR and cloning of transposable element sequences

To sample DNA sequence variation within the *M. violaceum* genomes, PCR primers based on a conserved region of the integrase gene of the *copia*-like element (as in Hood et al. 2005) were used to amplify from genomic DNA of each fungal strain. Amplicons were then cloned using the TA Cloning Kit (Invitrogen), and 33–45 clones per lineage were sequenced using dye-termination methods (Applied Biosystems). Sequences were aligned by eye, and a 350 bp region of *copia*-like element was used in the analyses below, excluding amplification primers (NCBI accession numbers EF413182–EF413583).

Nucleotide diversity and Fst values were calculated using DNASp (Rozas et al. 2003) and neighbor-joining trees built using MEGA (Kumar et al. 2004). For the ITS

Table 1 Origins of *Microbotryum* strains and results on intragenomics of *copia*-like retrotransposons

Host plant	Location	Year	Species (Le Gac)[a]	Species (Lutz)[b]	Strain	*copia*-like sequences	Nonsense mutants	Nucleotide diversity, all sites	Nucleotide diversity, non-RIP sites	RIP intensity
Silene latifolia	Lamole, Italy	2001	MvSl	*M. lychnidis-dioicae*	Sa01 Lamole A2	42 (33)	7 (6)	2.64% (2.68%)	1.19% (1.17%)	0.248
S. acaulis	Lac de Puy Vachier, France	2002	MvSa	*M. silenes-acaulis*	FR02 5.2 A2D	39 (23)	8 (5)	3.50% (3.68%)	1.91% (1.93%)	0.197
S. vulgaris	St. Johann im Walde, Austria	2002	MvSv2	*M. silenes-inflatae*	Johann 2	41 (18)	24 (10)	2.73% (3.43%)	1.75% (2.23%)	0.298
Dianthus alpinus	Orsiera Rocciavre, Italy	2002	MvDsp1 or 2	*M. dianthorum*	FR02-25-2	39 (25)	2 (2)	2.88% (3.85%)	2.98% (3.18%)	0.193
S. paradoxa	Lamole, Italy	2004	N/A	N/A	IT04 14p1	41 (28)	4 (2)	3.22% (3.72%)	2.34% (2.86%)	0.199
Petrorhagia saxifraga	St. Johann im Walde, Austria	2003	MvDsp 1 or 2	*M. dianthorum*	AT03 2 Col B	43 (22)	0 (0)	11.00% (11.18%)	3.45% (3.88%)	0.188
Saponaria ocymoides	Cesana Tor, Italy	2002	MvSoff	*M. saponariae*	A1T (IT02 19.1 Tet2T)	33 (7)	1 (1)	0.28% (0.73%)	0.15% (0.54%)	0.002
Sa. officinalis	Borro, Italy	2002	MvSoff	*M. saponariae*	IT02 1-2A A1	35 (9)	3 (3)	0.33% (0.82%)	0.18% (0.81%)	0
S. parryi	Olympic Peninsula, WA, US	2003	N/A	N/A	#11 10/03	45 (13)	0 (0)	4.40% (4.47%)	3.91% (3.75%)	0.156
S. virginica	Floyd, VA, US	2001	MvSvir&Caro	N/A	RT 8.1.2. 4/9/01 A1	44 (10)	43 (9)	0.78% (3.66%)	0.74% (3.14%)	0.218

Numbers in parentheses are the results for when identical sequences within each dataset are excluded from the analyses

[a] Species designations from Le Gac et al. (2007) are used throughout the text

[b] Species designations are from Lutz et al. (2008)

phylogeny, the fungus found infecting *Polygonum bistortum* is used as an outgroup because it was shown that samples from this host form a distinct clade exclusive to *M. violaceum* causing anther-smut disease on the Caryophyllaceae (Almaraz et al. 2002). To determine the significance of differences in intra-genomic diversity of *copia*-like elements, datasets of the same size as those measured in the *M. violaceum* lineages from *Saponaria* spp. were generated by randomly sampling the sequences obtained from the RIP-competent *M. violaceum* lineages. Pairwise nucleotide diversity for *copia*-like elements was then calculated, excluding RIP target sites, as determined by Hood et al. (2005); i.e. the tri-nucleotide TCG (or its reverse complement CGA).

PCR primers designed to amplify the Class II helitron element from two fungal species (*Microbotryum* from *S. latifolia* and *Sa. ocymoides*) were similarly used to obtain cloned sequences for comparison (Hel F: TGTGCAGAT CAATCCCAATG; Hel R: GCTAGCAGGAAAAACACA TGC) (accession numbers EF419835–EF419884).

Quantifying RIP

For each strain of *M. violaceum*, RIP scores were calculated as the proportion of all C to T mutation sites in the alignment that are attributable to RIP because they occurred in the TCG (or its reverse complement CGA) trinucleotide target site. RIP scores can therefore range from 0 to 1; a score of 1 would indicate that only RIP changes, and no other C to T mutations are seen in the alignment, whereas a score of 0 would indicate no C to T mutations at RIP target sites. Note that RIP intensity is calculated per variable site rather than per sequence, to ensure that TTG or CGA trinucleotides shared due to common ancestry do not affect the score; for this reason, RIP scores based on all sequences and only on unique sequences would be identical.

Transposable elements in genomic survey sequencing

Five of the *Microbotryum* strains were subjected to low coverage shotgun genome sequencing; including the *Microbotryum* species isolated from *S. latifolia*, *Sa. ocymoides*, *S. paradoxa*, *S. virginica*, and *S. acaulis*. Genomic DNA samples were submitted to the Genomic Core Facility at Purdue University, where mechanically-sheared libraries were prepared and end processed using an ABI 3730XL sequencer (accession numbers GS594340–GS597455). Genomic fragments were compared against the DNA and protein databases available through the National Center for Biotechnology Information (NCBI) using the BLASTn and BLASTx programs (www.ncbi.nlm.nih.gov/BLAST/) as in Hood (2005). The following categories were distinguished

based on BLAST hit annotations: retrotransposon-related (i.e. retrotransposons, reverse transcriptases, gag or pol genes, polyproteins, or retrovirus sequences), helicases, and Class II Transposase. The cut-off for significance of BLAST hits was an E-value of 10^{-4}. Retrotransposon-related sequences were then compared to known *copia*-like, gypsy-like, and non-LTR sequences from *M. violaceum* (Hood 2005) for further classification. The proportion of the genome made up by transposable elements was then estimated by dividing the number of sequences identified by the total number of fragments analyzed.

Results

For sequences of cloned *copia*-like element for each of the 10 strains of *Microbotryum*, RIP intensity (i.e. C to T mutations occurring at RIP target sites), and nucleotide diversity (excluding the TCG/CGA RIP target sites) were calculated (Table 1). All strains of *Microbotryum* except those isolated from the host genus *Saponaria* (i.e. belonging to the species MvSoff or *M. saponariae*) showed a large excess of TCG to TTG (or the reciprocal CGA to CAA) mutations in the *copia*-like element alignments, indicative of a RIP-like process. No other trinucleotide showed a similar excess. An association between repetitive elements and the excess of TCG to TTG mutations was confirmed by a lack of any such pattern in a comparison of the single-copy γ-tubulin among lineages of *M. violaceum* (data not shown; see Hood et al. 2005).

There was no overall significant relationship between RIP intensity and nucleotide diversity; however, even if RIP target sites are excluded, intragenomic nucleotide diversity in *copia*-like element sequences was considerably lower in the two strains lacking RIP, from *Sa. ocymoides* and *Sa. officinalis*, than in other *M. violaceum* lineages (Fig. 1a). These two strains, both from host plants in the genus *Saponaria*, appear to be closely related (Fig. 2a) and therefore probably represent a lack of RIP shared by common descent.

The significance of differences in intra-genomic nucleotide diversity of *copia*-like elements among *Microbotryum* strains was assessed by randomization. For all 16 comparisons between the two lineages on *Saponaria* spp. and the other eight species that appear to give evidence of RIP activity, less than 5 of 1000 random samples of the RIPed element population were less diverse than the population without RIP. The only exception was the strain from *S. virginica* (denoted as MvSvir&caro in Le Gac et al. 2007) which showed no significant difference in nucleotide diversity from either *Microbotryum* strain from *Saponaria* hosts (365 of 1000 in the strain from *Sa. officinalis*, 374 of 1000 in the strain from *Sa. ocymoides*). Similar results were obtained using DNA cloned sequences of the Class II

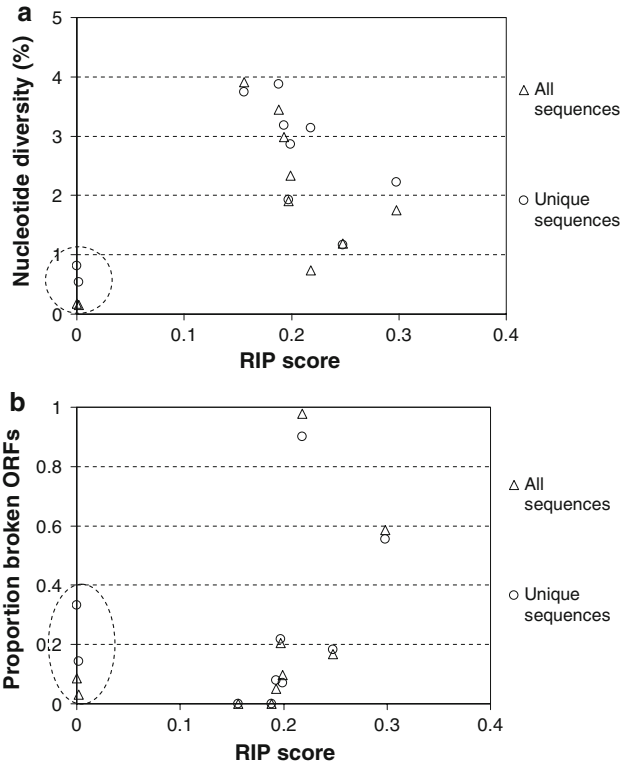

Fig. 1 RIP intensity and element activity in *Microbotryum*. **a** Nucleotide diversity excluding RIP target sites, plotted against RIP intensity (see Materials and methods); **b** Proportion of sequences with stop codons in the sequenced region of the *copia*-like integrase gene. *Open circles* are figures for all sequences, *triangles* are figures with identical sequences removed, equivalent to the numbers in parentheses in Table 1. *Dashed circles* enclose datapoints from *Microbotryum* from *Saponaria*

helitron elements in *Microbotryum*, where the pairwise nucleotide diversity in *Microbotryum* from *Saponaria* was significantly lower than in *Microbotryum* from *S. latifolia*: 0.002 versus 0.030 (from 22 and 28 sequences, respectively). Also, evidence of RIP-induced mutations was absent from *helitron* sequences in *Microbotryum* from *Saponaria* in contrast to those of *Microbotryum* from *S. latifolia*, the RIP scores being 0.001 and 0.080, respectively.

Few of the *copia*-like sequences from *Microbotryum* from *Saponaria* contained in-frame stop codons (3 of 35 in the strain from *Sa. officinalis* and 1 of 33 in the strain from *Sa. ocymoides*), whereas over half of the sequences contained in-frame stop codons in the lineage with highest RIP intensity, *Microbotryum* from *S. vulgaris* (Table 1). Overall, however, there was no significant relationship between broken reading frames and RIP intensity (Fig. 1b). In addition, the populations of *copia*-like elements in *Microbotryum* from the species *Sa. officinalis* and *Sa. ocymoides* were very similar to each other based upon Fst values, although these populations were radically different in composition from all other lineages (Fig. 2b).

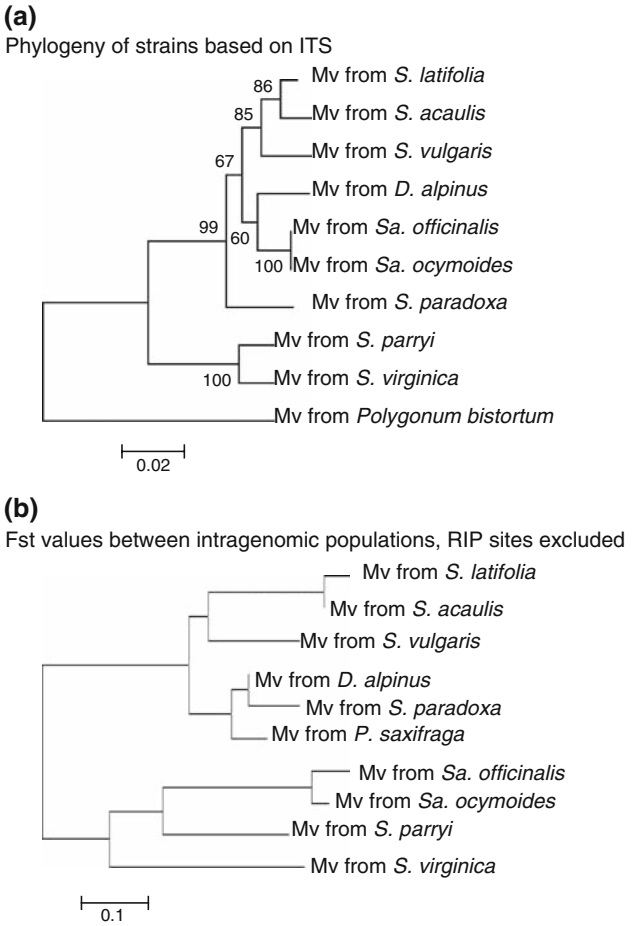

Fig. 2 Phylogeny of strains and divergence between intragenomic populations. **a** Neighbor-joining tree of ITS sequences from the fungal strains, with support scores from 1,000 bootstrap replications, showing changes per base pair. **b** Dendrogram of Fst values between intragenomic populations of *copia*-like elements

The estimated transposable element composition of five *M. violaceum* strains varied from 5 to 12% (Table 2), with the lowest proportion of transposable elements being found in the *Microbotryum* strain from *Sa. ocymoides*. A range of element types was found in the genome of each strain, including gypsy-like and non-LTR element, but the largest contribution was by *copia*-like retrotransposons. The estimated copy numbers of the two most frequent classes of element, *copia*-like retrotransposons and *Helitrons*, are both lowest in *Microbotryum* from *Saponaria* and highest in *Microbotryum* from *S. virginica*.

Discussion

Both the intensity of RIP-induced mutations and *copia*-like element diversity show wide variation within the *M. violaceum* species complex. Species that show evidence of RIP mutations can nevertheless contain groups of highly similar *copia*-like and *helitron* element sequences that lack stop codons. Transposable elements are also known to be actively transcribed in the *Microbotryum* species from *S. latifolia*, as observed in the study of expressed sequences (Yockteng et al. 2007). Both of these observations suggest that, in *Microbotryum*, thriving intragenomic populations of mobile elements may coexist with active genome defense. Subtle genome defense systems which are compatible with mobile element activity (e.g. telomeric silencing in *Drosophila*; Josse et al. 2007) can still be powerful forces in genome evolution. Such systems may be far more common than we currently recognize, and many of them, unlike RIP, will not be readily detectable from sequence data. The apparent substantial variation in RIP intensity between *Microbotryum* species strengthens this system as a valuable model for the study of genome defense. Further studies are warranted that assess RIP activity via experimental manipulations, in particular genetic engineering, to confirm the existence of variable levels of RIP activity. An approach of this type has been successfully employed in *Magnaporthe grisea* (Ikeda et al. 2002), but its use in *Microbotryum* must await the development of reliable transformation techniques.

The activity of elements inferred from sequence diversity across *Microbotryum* species does not neatly correlate

Table 2 Transposable elements found in genomic survey sequences of *Microbotryum* species

	Mv from *Sa. ocymoides*	*Mv* from *S. acaulis*	*Mv* from *S. latifolia*	*Mv* from *S. paradoxa*	*Mv* from *S. virginica*
Copia-like	14	19	21	31	42
Gypsy-like	3	1	0	10	3
Non-LTR	2	1	4	2	2
Helitrons-like	5	14	9	8	19
Class II transposase	0	0	0	3	0
Unclassified TE	21	3	14	9	13
GSS sequences	695	653	450	609	709
Proportion TE	5%	7%	8%	12%	11%

with RIP intensity as one might expect. Several factors may be responsible for this. Firstly, the PCR primers used to analyze the *copia*-like element were based on a region of *copia* integrase that was conserved in the intragenomic population within the *Microbotryum* species from *S. latifolia* (Hood et al. 2005). As other species could have a different consensus sequence, it is possible that these primers amplify only a subset of *copia* retrotransposons in other species. However, one would in that case expect elements from MvSl to appear more diverse than any other species; in fact, they are intermediate in their diversity.

Secondly, as well as being highly mobile within a single genome, *copia*-like elements belong to a group that appears to be frequently transferred between species (Flavell 1999). It is therefore possible that fungal hybridization, even between otherwise genetically distinct lineages, may sporadically introduce large numbers of functional elements into any particular *Microbotryum* species. There may also be intraspecific variation in copy number, which can persist in the face of low migration (Deceliere et al. 2005). Perhaps most intriguingly, efficient RIP defense may be evolutionarily unstable and tend to deteriorate when most elements are disabled. This could lead to a cycling process of element accumulation and inactivation, comparable to the cycles of invasion and deletion seen in homing endonucleases in yeast (Goddard and Burt 1999).

It is clear that there has not been a constant rate of element turnover during the course of lineage divergence in the *Microbotryum* species complex. This is evidenced by the large differences in estimated whole-genome copy number of various mobile element types, and by the discrepancies between the fungal phylogeny based upon nuclear gene sequence data and the dendrogram describing the similarity of intragenomic populations of copia-like elements. The high Fst values between TE copies from closely related fungal lineages, for example, between the strains from *S. virginica* and from *S. parryi*, suggests rapid element turnover in some species. In particular, *copia* populations in the *Microbotryum* species from *Saponaria* are highly differentiated from those in their close relatives, suggesting rapid changes in the composition of element populations in this lineage.

Microbotryum strains from *Saponaria* are, in fact, unusual in several ways. They lack evidence of the RIP genome defense based on sequence analysis, and showed the lowest intragenomic nucleotide diversity. A similar pattern was found for transposable elements with very different mechanisms of replication. Retrotransposable elements such as the *copia*-like elements in *Microbotryum* proliferate by an RNA-based retrotransposition method (Flavell et al. 1997), whereas *Helitron* elements employ a very dissimilar, DNA-based transposition method. Both element types show a similarly low diversity and lack of RIP evidence in *Microbotryum* from *Saponaria*. This species also has the lowest estimated total genomic proportion of mobile elements. One possible explanation for these observations is a recent uptick in their transpositional activity which has not yet led to a discernible effect on copy number. This would be consistent with an early stage of mobile element accumulation in the cycle proposed above, although further work would be needed to substantiate this interpretation. An alternative is that *Microbotryum* from *Saponaria* has evolved another effective genome defense system, such as epigenetic regulation through DNA methylation or RNAi. Future experimental work could potentially distinguish between competing explanations by measuring transcription rates of individual transposable element copies, or, as suggested above, by directly introducing naïve transposable elements into *Microbotryum* from *Saponaria* and resequencing.

The low outcrossing rate in *Microbotryum* (Giraud et al. 2005, 2008) should disfavor the persistence of transposable elements that are individually extremely harmful to the fungal lineage (Bestor 1999). However, the total cost of all elements in highly infested genomes may nevertheless be high, and it is in such cases of high element density that a genome defense will have the greatest selective benefit. Further theoretical and experimental work is needed to clarify the evolutionary dynamics that allow genome defenses to coexist with intragenomic communities of molecular parasites, which may help explain the unexpected results observed in this study. Genome defense systems have provided indispensable tools for molecular biology, from restriction-modification systems to RNA interference, and including the use of RIP as a gene knockout mechanism. A rigorous evolutionary understanding of genome defense may similarly enrich our understanding of the forces determining genome size, structure and stability.

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