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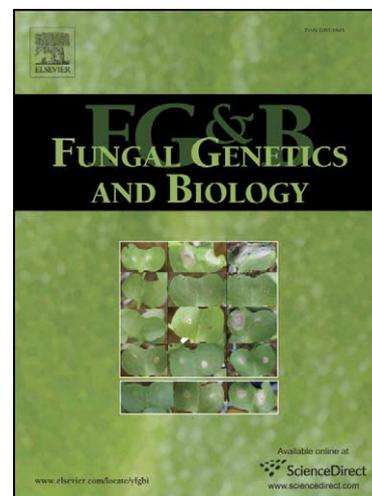
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Challenges of microsatellite isolation in fungi

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1 **Abstract**

2 Although they represent powerful genetic markers in many fields of biology,
3 microsatellites have been isolated in few fungal species. The aim of this study was to assess
4 whether obtaining microsatellite markers with an acceptable level of polymorphism is
5 generally harder from fungi than in other organisms. We therefore surveyed the number,
6 nature and polymorphism level of published microsatellite markers in fungi from the literature
7 and from our own data on seventeen fungal microsatellite-enriched libraries, and in five other
8 phylogroups (angiosperms, insects, fishes, birds and mammals). Fungal microsatellites indeed
9 appeared both harder to isolate and to exhibit lower polymorphism than in other organisms.
10 This appeared to be due, at least in part, to genomic specificities, such as scarcity and
11 shortness of fungal microsatellite loci. A correlation was observed between mean repeat
12 number and mean allele number in the published fungal microsatellite loci. The cross-species
13 transferability of fungal microsatellites also appeared lower than in other phylogroups.
14 However, microsatellites have been useful in some fungal species. Thus, the considerable
15 advantages of these markers make their development worthwhile, and this study provides
16 some guidelines for their isolation.

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1 **Introduction**

2 Microsatellite loci, short tandemly repeated motifs of 1-6 bases, also known as simple
3 sequence repeats (SSR), are widely used as genetic markers because of their ubiquity, ease to
4 score, co-dominance, reproducibility, assumed neutrality and high level of polymorphism
5 (Jarne and Lagoda, 1996). They have proved to be invaluable in many fields of biology, from
6 genome mapping to forensics, paternity testing and population genetics (Jarne and Lagoda,
7 1996; Luikart et al., 2003). Their interest to biologists goes beyond their high polymorphism:
8 when one can assume a model for their evolution, taking into account the number of repeats
9 allows inference of kin relationships among alleles, and they can thus be developed as
10 powerful tools for inferring evolutionary and demographic parameters (Cornuet et al., 1999;
11 Luikart et al., 2003; Michalakis and Excoffier, 1996). The major drawback of microsatellite
12 loci is that they often need to be isolated *de novo* from each species, which can be time-
13 consuming and expensive. Cross-amplification, i.e. amplification of loci from a species other
14 than the one in which they were cloned, is generally possible only among species of the same
15 genera, and even in this case the percentage of cross-amplification is low (Rossetto, 2001).
16 Furthermore, cross-amplification often generates null alleles which can bias genetic analyses
17 (Hardy et al., 2003). In species for which no microsatellite markers for related species are
18 available or cross-amplifiable, recently developed techniques, especially those involving
19 enrichment of genomic DNA in microsatellites (Zane et al., 2002), have rendered the step of
20 microsatellite isolation less laborious and more likely to succeed. However, the task of
21 developing a working primer set from an enriched library can also represent a significant
22 workload (Squirrell et al., 2003).

23 Microsatellites have been isolated across a wide range of taxonomic groups, but
24 surprisingly little in fungi (Zane et al., 2002; Fig. 1). The low number of population
25 geneticists interested in fungi compared to other organisms certainly explains this rarity, in

1 addition to a preference for anonymous markers such as random amplified polymorphic DNA
2 markers (RAPD), amplified fragment length polymorphisms (AFLP) and inter-simple
3 sequence repeats (ISSR). These markers have only two alleles per locus, but they are easy to
4 develop in large numbers without the fastidious step of building a genomic library, and they
5 generally yield enough polymorphism to differentiate individuals within populations.
6 However, for some fungal species, their lack of species specificity can represent a serious
7 problem. For instance in fungal pathogens, DNA of the focal species can be difficult to isolate
8 from those of the host and of hyperparasites (e.g. mycoparasites; Kiss, 1998), thus requiring
9 an *in vitro* isolation step. Another drawback of AFLP, RAPD and ISSR is dominance, which
10 prevents the detection of heterozygotes in diploid species. Finally, even for haploid species
11 convenient for *in vitro* culture, the problem of anonymity remains, which can introduce
12 serious bias in population genetic studies. Indeed, different alleles from a single locus cannot
13 be easily recognized and markers with occasional non Mendelian behaviour, such as
14 transposable elements, are frequently amplified by these techniques. Amplification problems,
15 such as null alleles are easy to detect in microsatellites, and as minute amounts of template are
16 required, culture can be bypassed.

17 In addition to the low number of population studies on fungi and the preference for
18 anonymous markers, some peculiar biological and genomic traits of fungi may have limited
19 the number of polymorphic microsatellite loci isolated from genomic libraries. First,
20 pathogens which are the most extensively studied species within fungi, have demographic and
21 reproductive traits promoting a low genetic diversity. Crop or human pathogens have for
22 instance often experienced recent bottlenecks, through geographical introduction (Engelbrecht
23 et al., 2004; Milgroom et al., 1992; Rivas et al., 2004) or host shifts (Mackenzie et al., 2001;
24 Paraskevis et al., 2003; Tobler et al., 2003), which can drastically reduce intraspecific genetic
25 diversity. Furthermore, some specific life history traits of fungal pathogens, such as frequent

1 asexual reproduction and recurrent bottlenecks in epidemic cycles, associated with low winter
2 survival and/or selective sweeps following new virulence attributes, are also likely to result in
3 low level of genetic diversity (Goodwin et al., 1994; Guérin and Le Cam, 2004; Hovmøller et
4 al., 2002). Second, fungal genomes may exhibit some peculiarities. Several recent papers
5 have examined the nature and abundance of microsatellites in published partial or complete
6 fungal genomes (Field and Wills, 1998; Karaoglu et al., 2005; Lim et al., 2004).
7 Microsatellites indeed appeared less abundant in these fungal genomes than in other
8 organisms (Morgante et al., 2002; Tóth et al., 2000), had different most abundant motifs
9 (Morgante et al., 2002; Tóth et al., 2000) and long loci were under-represented (Karaoglu et
10 al., 2005; Lim et al., 2004). Lim et al. (2004) reported that ca. 90% of microsatellite loci in 14
11 fungal genomes had low numbers of repeats, i.e. below eight. Long microsatellites, with high
12 numbers of perfect repeats, are more likely than short ones to be polymorphic because of a
13 higher rate of DNA replication slippage or unequal crossing-overs. Several studies have
14 indeed shown that the number of repeats is a good predictor of the level of variability in other
15 organisms (e.g. Brinkmann et al., 1998; Goldstein and Clark, 1995; Thuillet et al., 2002;
16 Vigouroux et al., 2002; Wierdl et al., 1997). If this correlation holds in fungi, most of their
17 microsatellites are expected to exhibit a low polymorphism. Furthermore, most of the
18 microsatellite loci detected in the published fungal genomes (94%) are mononucleotide
19 repeats (Lim et al., 2004) that are seldom used in population genetics because of difficulties in
20 scoring alleles separated by single base pairs. The shortness of microsatellite loci in fungi,
21 their weak representation in the genomes, the low abundance of useful motifs, together with
22 the small size of fungal genomes (between 10 and 40 Mb), may limit the ability to find
23 numerous polymorphic microsatellite, even when a genomic library is available.

24 It is not entirely clear, however, whether the genomic and biological specificities listed
25 above are the major factors limiting the development of microsatellite markers in fungi. First,

1 the conclusions drawn from these genomic studies are limited by the low number of complete
2 fungal genomes available, especially as a huge variability has been found among closely
3 related species in the number and nature of microsatellites (Ellegren, 2004; Karaoglu et al.,
4 2005; Lim et al., 2004). A survey of microsatellite development studies in different fungal
5 species would allow determination whether microsatellites are indeed generally difficult to
6 isolate and are particularly short. Second, another important limitation of genome analyses is
7 the lack of polymorphism assessment, which is the most valuable information for population
8 geneticists. Estimations of demographic or genetic parameters are indeed more powerful with
9 more polymorphic loci (e. g. Paetkau et al., 2004). If, for instance, microsatellite loci with
10 short repeats are reasonably polymorphic in fungi, their predominance in the genomes would
11 not be a problem for the development of useful markers. Comparing the degree of
12 polymorphism of microsatellites in fungi and in other organisms, and assessing whether the
13 correlation between the number of alleles and the number of repeats holds in fungi, are
14 therefore essential for determining whether attempts to develop microsatellites in this
15 kingdom are worthwhile given the investment required.

16 The aims of this paper were therefore to assess the yield of microsatellites from
17 enriched libraries in fungi and to compare the polymorphism of isolated fungal microsatellites
18 to that of other organisms, in order to determine whether obtaining microsatellite markers
19 with an acceptable level of polymorphism is generally harder in fungi than in other
20 organisms. The specific objectives of this paper were thus to (1) assess the yield of our own
21 seventeen microsatellite-enriched libraries, through the different steps, to identify which steps
22 limited the isolation of polymorphic loci; our data are free from publication bias, whereas
23 failures to develop polymorphic markers are rarely published; (2) estimate the general yield of
24 published microsatellite development in fungi; (3) evaluate the possibility of cross-
25 transferability of microsatellites among fungal species, which may represent an alternative to

1 the fastidious development of a genomic library; (4) assess whether there is a correlation
2 between length and allele number among fungal microsatellites; (5) compare the nature, in
3 particular the size, of fungal microsatellites and their level of polymorphism to those of other
4 groups of organisms. In this study, we considered fungal species *sensus lato*, i.e. including
5 Oomycota, because these organisms share similarities with true fungi in their morphology and
6 life cycles, and many are also responsible for destructive plant diseases (Tyler, 2001).

7
8

9 **Material and methods**

10 *Enriched libraries*

11 The methods used to isolate our microsatellite loci were adapted from two protocols
12 using oligoprobes for the enrichment of genomic libraries. The principle of both methods is
13 the hybridization of restricted genomic DNA on microsatellite oligoprobes, followed by the
14 washing of the non-hybridized genomic fragments. The first protocol, adapted from Edwards
15 et al. (1996), uses membranes on which microsatellite oligoprobes are fixed. The second
16 method is very similar, but uses streptavidin-coated magnetic beads on which biotin-labelled
17 microsatellite oligoprobes are linked. Genomic fragments containing microsatellites hybridize
18 with the oligoprobes, whose biotin links to the streptavidin of the magnetic beads. A magnet
19 therefore allows retention of mostly DNA fragments with microsatellite loci (Kijas et al.,
20 1994). The first method, with a membrane, was used for the species *Cryphonectria parasitica*
21 (Breuillin et al., 2006), *Erysiphe alphitoides* (unpublished data) and *Melampsora larici-*
22 *populina* (Barrès et al., 2006). The bead method was used in addition for *E. alphitoides* and
23 *M. larici-populina*, and for the 14 other species: *Erysiphe necator* (unpublished data),
24 *Fusarium culmorum* (Giraud et al., 2002b), *Fusarium poae* (unpublished data), *Magnaporthe*
25 *grisea* (Kaye et al., 2003), *Microbotryum violaceum* (Giraud et al., 2002a), *Microcyclus ulei*

1 (Le Guen et al., 2004), *Mycosphaerella eumusae* (unpublished data), *Mycosphaerella fijiensis*
2 (unpublished), *Mycosphaerella musicola* (unpublished data), *Penicillium camembertii*
3 (unpublished data), *Penicillium roqueforti* (unpublished data), *Plasmopara viticola* (Delmotte
4 et al., 2006), *Puccinia triticina* (Duan et al., 2003) and *Puccinia striiformis fsp tritici*
5 (Enjalbert et al., 2002). For some species, several libraries had to be produced because of the
6 poor yield of the first one(s).

7 Twelve out of 17 libraries (70%) were enriched for dinucleotide loci using $(AC/TG)_n$
8 and $(AG/TC)_n$ oligoprobes, with $n= 10$ or 15 (Table 1). The five other libraries were
9 enriched using only $(AC)_{10}$. These two dinucleotide motifs were chosen for enrichment
10 because they had generally been reported as the most frequent in complete fungal genomes
11 (e.g. Lim et al., 2004). Ten libraries were also enriched with trinucleotide motifs (mainly
12 $(AAG)_{10}$; Table 1).

13 For each of our enriched libraries, we recorded: (1) the percentage of positive clones,
14 (2) the percentage of redundant sequences, i.e. of identical sequences, (3) the percentage of
15 contaminant clones, i.e. with a significant BLAST value towards a sequence from another
16 species, (4) the percentage of unique sequences, excluding contaminants, with a microsatellite
17 locus (tandemly repeated motifs of 1-6 bases with at least 5 pure repeats, according to the
18 most common definition; Ashley and Dow, 1994 ; Lim et al., 2004), (5) the percentage of
19 unique sequences, excluding contaminants, with a microsatellite locus and suitable flanking
20 regions, (6) the percentage of sequences yielding loci with a clear amplification, (7) the
21 percentage of sequences yielding polymorphic loci at the intra-population level and (8) the
22 percentage of sequences yielding polymorphic loci at the largest measured scale (from inter-
23 population to inter-continental levels, or between populations from different host species). All
24 the above percentages were estimated as ratios over the number of inserts correctly
25 sequenced, except the percentage of positive clones, which was estimated over the total

1 number of clones with inserts. When several libraries had been built for one species, the
2 average yield was taken for each step. In addition, we recorded for each polymorphic locus:
3 (1) The base composition of the microsatellite motif (2) its perfection (a locus was considered
4 as imperfect if the tandem repeats were interrupted or if several different tandem repeats with
5 more than five repeats each were amplified as a single locus), and (3) the number of tandem
6 repeats (for imperfect loci, number of repeats of the longest perfect microsatellite). The
7 number of repeats was recorded from the sequenced fragment obtained in the library. Finally,
8 we estimated genetic diversity of microsatellite loci as the number of alleles at the largest
9 scale (spatially or inter-hosts). We also recorded the sample size used for assessing
10 polymorphism.

11

12 *Literature search and data extraction*

13 To find studies reporting the development of microsatellite markers in fungi, we
14 searched the bibliographic data bases Web of Knowledge (<http://isi4.newisiknowledge.com/>)
15 and Pubmed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) from January 1985 to June 2006
16 for all papers with "microsat* and fung* and (isol* or clon* or characteri*)" and "SSR and
17 fung* and (isol* or clon* or characteri*)" in the title, keyword or abstract. We included all
18 data from all papers to which we had access, regardless of the method of microsatellite
19 isolation, except that we kept a single study per species and only the studies having isolated at
20 least two polymorphic dinucleotide loci for comparison between species (see below). For
21 each locus, we recorded the following information when available (1) the length and base
22 composition of the motif, (2) its perfection, (3) the number of repeats of the longest perfect
23 microsatellite (4) the sample size used for assessing polymorphism, (5) the number of alleles.
24 We also recorded the number of loci for which primers could be designed, the percentage of
25 scorable loci and the percentage of polymorphic loci per species, when available.

1 In addition, cross-species transferability of microsatellite markers in fungi was
2 evaluated from published studies and our own data. We kept 20 studies for which one source
3 species could be clearly identified and data on cross-species transferability was available
4 within a genus, data at lower or higher taxonomic levels being scarce in fungi. In total, 24
5 source species, 88 target species and 302 primer pairs were tested across these studies. For
6 each target species, a primer pair was considered as transferable when a PCR product of
7 expected size was obtained in at least one individual. We computed the transferability as the
8 mean percentage of loci that were transferable to other species.

9 To compare the yield and polymorphism of microsatellite development in fungi to
10 those of other organisms, we searched issues of Molecular Ecology Notes from March 2001
11 to June 2005 for studies reporting isolation of microsatellites in angiosperms, insects, fish
12 (restricted here to Actinopterygii), birds and mammals. These different phylogenetic groups
13 ('phylogroups' hereafter) were chosen to span a wide range of living species and to include at
14 least 50 studies, i.e. a number similar to that of published studies in fungi. We counted the
15 number of primer notes for each phylogroup, and for the 50 most recent, we recorded for each
16 polymorphic dinucleotide locus with a minimum of 5 repeats, the same items as for the fungal
17 bibliographic data above. A few studies had to be discarded because they reported less than
18 two polymorphic dinucleotide markers. The complete dataset included 2,923 microsatellite
19 loci.

21 ***Data analyses***

22 Using a Mann-Withney's test performed with Statistica 6.0 (Statsoft 2001), we
23 compared 1) the yield of the different steps of microsatellite isolation, 2) the mean repeat
24 number, and 3) the mean allele number per locus and per species, between our dataset and the
25 published studies on other fungi. For the two latter comparisons, only dinucleotides (the most

1 isolated motifs in libraries) were retained to remove any possible effect of the length of the
2 motif on allelic diversity.

3 To analyse the effect of the phylogroup (i.e. fungi, angiosperms, fishes, insects, birds
4 or mammals) on the mean repeat number and on the mean allele number per species,
5 unpublished studies on fungi were discarded and only polymorphic dinucleotide loci were
6 retained, in order to have data similar to the other phylogroups. The phylogroup effect was
7 tested using an analysis of variance, with the GLM procedure of the SAS software (SAS
8 Institute, SAS Publishing, Cary, NC). Variables were Log-transformed for the residuals to
9 reach normality. Pairwise mean comparisons among phylogroups were performed using
10 Student-Newman-Keuls tests (SNK; Means option in GLM, SAS software). For the mean
11 number of alleles per species, we retained only the studies with a minimum of 14 genotyped
12 individuals to reduce the bias of a too small sample size.

13 The effects of the imperfection, the motif (CA/GT vs GA/CT, the other dinucleotides
14 being too rarely isolated), the number of repeats, the sample size and the species on allele
15 numbers were assessed using a generalized linear model (GENMOD procedure of SAS),
16 assuming a Poisson distribution and a log-link function. Because the “allele” variable was
17 over-dispersed, a scaling parameter was calculated to improve the fit to the Poisson
18 distribution. Full models were first fitted including all factors and all interactions, and then
19 simplified by sequential removal of the least significant highest-order interaction term,
20 retaining significant interactions and all main effects, even when non-significant.

21

22

23 **Results**

24

25 *Yield of our 17 fungal microsatellite-enriched libraries*

1 In preliminary experiments, we tried to clone fungal microsatellites without
2 enrichment in two species (*P. striiformis fsp triti* and *P. triticina*). The yield was so low (ca.
3 0.5% of positive clones) that enrichment appeared unavoidable. In our enriched libraries
4 where the clones were screened for the presence of microsatellites, the mean percentage of
5 positive clones (\pm SE) was 20.2 % (\pm 5.2). Five libraries had more than 30% of positive
6 clones and four had fewer than 6 %.

7 After the cloning step, several problems were met due to the method of enrichment.
8 First, a non negligible number of redundant clones were recovered in all experiments,
9 probably due to the two PCR steps required for enrichment: the average number of sequences
10 identical to previous sequences had a mean (\pm SE) of 26.2 % (\pm 5.1). Second, in three
11 laboratories, contamination by foreign DNA occurred in six species (*P. roqueforti*, *P.*
12 *camemberti*, *F. poae*, *P. viticola*, *M. larici-populina* and *C. parasitica*) and could reach up to
13 69% of the sequences. These contaminant sequences were easily identified: they were
14 repeated several times in the libraries, blasted significantly to sequences in public databases,
15 and/or were sequenced in the previous enrichments performed in the same laboratory. Third,
16 problems in sequencing were met in most of the libraries, in several different laboratories,
17 using either DNA extracted from clones or PCR products purified with various commercial
18 kits. The failure of sequencing reactions seemed to be specific to our adaptors, *Mlu*I (Edwards
19 et al., 1996), that may adopt a particular 3D structure when linked into vectors from the Topo
20 TA Invitrogen kit, impeding sequencing reactions. The problems in sequencing may also be
21 due to the presence of identical adaptors at each end of the insert. Proper sequences could
22 only be obtained using a particular protocol of PCR product purification, using PEG
23 (Rosenthal et al., 1993). Other studies have used adaptors encompassing a restriction site to
24 avoid this problem (Armour et al., 1994; Tenzer et al., 1999).

1 In our 17 enriched libraries, the average percentage (\pm SE) of unique sequences,
2 excluding contaminants, having a microsatellite locus of at least five perfect repeats
3 (Appendix 1) was only 55.4 % (\pm 4.6) of the correctly sequenced inserts. Among those, the
4 percentage of useful sequences consistently and sharply decreased along the different steps of
5 the experiment (Fig. 2). The mean (\pm SE) of the number of loci eventually polymorphic at the
6 intra-population scale was only of 9.6 % (\pm 2.5). One of the most critical steps was the
7 suitability of the sequences for primer design (mean \pm SE of 56.9 % \pm 6.2 of unique
8 sequences with a microsatellite), due to flanking regions with unsuitable base composition or
9 length, or to microsatellites with too few perfect repeats. The percentage of amplified loci
10 among those suitable for primer design was generally high (mean \pm SE of 68% \pm 8.2),
11 although it was very low in some species. In *E. alphitoides*, *E. necator*, *F. poae*, *M. larici-*
12 *populina*, and *P. viticola*, less than 45 % of the loci retained for primer design could actually
13 be amplified (Appendix 1). The second most important source of attrition was the level of
14 polymorphism obtained from amplifiable loci : the percentage of sequences that eventually
15 yielded polymorphic loci ranged from 0 to 50 % of the initial number of sequenced clones,
16 with only five species above 20% at the inter-population level (Appendix 1) and a mean (\pm
17 SE) of 17.2 % (\pm 3.5) (Fig. 2). The intra-population level of polymorphism was even lower,
18 with a mean (\pm SE) of 9.6 % (\pm 2.7) of polymorphic loci (Fig. 2). In four species (the two
19 *Puccinia* spp. and the two *Penicillium* spp.), no polymorphic loci at all could eventually be
20 recovered at the intra-population scale (Appendix 1).

21 It was not possible to test here if the method of enrichment (membrane versus beads)
22 or the length of oligoprobes impacted on the yield of libraries, because the number of studies
23 was too low and because microsatellite isolation was performed using different methods in
24 too few species (Table 1). However, regardless of the method, the general trend was a poor
25 yield of enriched libraries in fungi. Furthermore, in the species for which different methods

1 were used (*M. larici-populina* and *E. alphitoides*), similar results were observed (data not
2 shown), suggesting a lack of protocol effect. There was also no indication that the libraries
3 enriched for both di- and trinucleotides had a better yield than the libraries enriched only for
4 dinucleotides (means of 17% (\pm 4.9) and 14% (\pm 4.3) of polymorphic loci isolated,
5 respectively). For four out of the ten species enriched with a trinucleotide oligoprobe, no
6 polymorphic trinucleotide loci could be isolated, and for three of them a single polymorphic
7 locus was recovered (data not shown). The mean percentage of polymorphic loci at the largest
8 scale seemed to be slightly higher in the five genomic libraries enriched using only (AC)₁₀
9 than in those enriched using both (AC/TG)_n and (AG/TC)_n (means of 25 % and 12%
10 respectively; Appendix 1), but there were too few studies to test this difference given the large
11 species effect (see below).

12

13 ***Comparison between our dataset and the published studies in fungi***

14 We compared the yield of our enriched libraries with that of the published studies in
15 other fungi to detect possible publication bias or specificities in our data. We collected data on
16 microsatellite isolation from 37 fungal species from the literature (Table 1). Among these, 14
17 % used non-enriched libraries and 43% used beads or membranes to enrich libraries. The
18 other methods of microsatellite isolation were based on ISSR (Burgess et al., 2001), FIASCO
19 (Zane et al., 2002), anchored PCR (Zane et al., 2002), and or searches in EST libraries
20 (Appendix 2). Despite the diversity of the methods of microsatellite isolation, the proportion
21 of polymorphic loci relative to the loci tested in fungal species was not significantly different
22 in the literature (mean \pm SE of 49.7 \pm 5.0 % among the sequences for which primers were
23 designed, Appendix 2) and in our dataset (53.2 \pm 7.4 %; Mann-Whitney's test, Z = -0.43, P =
24 0.67).

1 As in our libraries, most of the polymorphic loci in published studies on fungi were
2 dinucleotides (69% and 88% for our data). Considering only the dinucleotide loci, the mean
3 number of repeats per locus and per species was similar in our dataset and in the published
4 studies (11.1 ± 0.7 vs 11.9 ± 0.8 ; Mann-Whitney's test, $Z = 0.11$, $P = 0.92$). In the 22 studies
5 in which libraries were enriched for both the dinucleotides $(AC/GT)_n$ and $(AG/CT)_n$,
6 consistently more polymorphic microsatellites were isolated with AC repeats than with AG
7 repeats, regardless of the method of enrichment (5.3 ± 1.3 versus 2.8 ± 0.8 loci per species in
8 the published studies and 8.7 ± 2.5 versus 2.3 ± 0.7 in our data, for $(AC/GT)_n$ and $(AG/CT)_n$
9 loci respectively).

10

11 ***Cross-species transferability of microsatellite markers between fungal species***

12 Cross-species transferability of microsatellite primer pairs in fungi was estimated
13 based on 24 studies from the literature and our own data. Only 34 % of the 1045
14 species/primer pair combinations tested within genera were successful in amplifying bands of
15 the expected size. Neither homology, polymorphism nor presence of null alleles in the
16 transferred microsatellite markers were generally assessed.

17

18 ***Comparison of fungal microsatellites with those of other organisms***

19 Only 53% of the published loci were dinucleotides in birds, against ca. 70 % in fungi
20 and fish and more than 80 % in plants, mammals and insects. More than 34% of the published
21 loci were tetranucleotides in birds, against less than 5% in fungi, insects and plants.

22 Comparison of the 46 published studies on fungal microsatellite development with at
23 least two dinucleotide polymorphic loci and the 50 published Primer Notes on microsatellites
24 isolated from angiosperms, birds, mammals, fish and insects, found no significant difference
25 in mean number of dinucleotide polymorphic loci per species among the phylogroups

1 (Kruskal-Wallis' test, $H(5,292) = 8.1$, $P = 0.15$). The mean number of polymorphic loci per
2 species was not the lowest in fungi, with a mean \pm SE of 9.3 ± 1.0 ; the other phylogroups
3 ranging from 8.5 ± 0.8 (insects) to 13.6 ± 3.4 (fish).

4 The number of repeats per dinucleotide locus and per species had a significantly lower
5 mean in fungi (11.8 ± 0.7) than in all the other phylogroups except birds (13.2 ± 0.6). The
6 means of the other phylogroups ranged from 15.3 (insects) to 17.4 (mammals and fish)
7 repeats per locus per species (Fig. 3A). The mean number of alleles per species also had a
8 significantly lower mean in fungi (5.4 ± 0.4) than in all the other phylogroups (Fig. 3B). All
9 these phylogroups had similar means of number of alleles per species, ca. 8 alleles, except
10 fish that had a significantly higher mean (11.6 ± 1.2 ; Fig. 3B).

12 *Factors affecting the diversity of dinucleotide microsatellites*

13 In fungi, the correlation between the mean repeat number and the mean allele number
14 per species was marginally significant ($r=0.28$, $P=0.06$, Fig. 4). The short loci indeed had a
15 very low level of polymorphism, whereas long loci had a larger range of allele numbers, their
16 polymorphism being high for some but still low for others. The mean repeat number and the
17 mean allele number per species were significantly correlated in birds ($r=0.72$, $P<0.001$),
18 insects ($r=0.40$, $P=0.007$) and fish ($r=0.32$, $P=0.03$), but not in mammals ($r=0.07$, $P=0.63$) or
19 in angiosperms ($r=0.19$, $P=0.19$). However, GENMOD analyses on the complete dataset with
20 all loci and taking into account the effects of the other parameters (see below) found the effect
21 of the number of repeats on the number of alleles significant in all the phylogroups, the
22 regression coefficients being consistently lowest in mammals and angiosperms.

23 A generalized analysis of variance was performed to further investigate which
24 parameters influenced the diversity of individual microsatellite dinucleotide loci in fungi,
25 among motif (considering only AC and AG), imperfection, repeat number, sample size and

1 species (Table 2). The main source of variation affecting the number of alleles of
2 microsatellites was the differences among species. A significant effect of the number of
3 repeats was also detected, whereas sample size, imperfection and motif were not significant.

4

5

6 **Discussion**

7

8 Consistent problems were met when isolating microsatellite loci from different fungal
9 species. First, the yield of enriched libraries (percentage of positive clones) was low, mostly
10 less than 30%. This percentage is at the lower limit of what has been obtained from other
11 groups of organisms using the same protocols of microsatellite enrichment, which usually
12 leads to 20% - 90 % of positive clones (Zane et al., 2002). Second, the attrition along the
13 different steps from positive clones to polymorphic loci was very high (mean \pm SE of 83.8 %
14 \pm 3.2). This attrition level is similar to that found in plants, which are known to be recalcitrant
15 species for isolating microsatellite markers (Squirrell et al., 2003). For both fungi and plants,
16 the percentage of loci suitable for primer design and the percentage of polymorphic loci seem
17 to be the two most critical steps in microsatellite development. The low percentage of loci
18 suitable for primer design, both in our data and the literature, is partly due to the choice of
19 discarding loci with less than eight perfect repeats. The reason for disregarding them was that
20 very low polymorphism, if any, is generally expected for such short microsatellites. The low
21 polymorphism of short loci observed in fungi, and the positive correlation between number of
22 repeats and number of alleles in fungal species, validated this choice. The final mean
23 percentage of the initial non redundant sequences containing a polymorphic microsatellite at
24 the intra-population level was less than 10%. In several fungal species, no polymorphic loci at

1 all could be found polymorphic at the within-population level, precluding any estimation of
2 population genetics parameters, such as mode of reproduction or level of gene flow.

3

4 In addition to the established redundancy of sequences in enriched libraries (Squirrell
5 et al., 2003), several of our libraries had high rates of contaminant sequences and/or a low
6 success of primer amplification; in particular in *P. viticola*, *M. larici-populina*, *E.*
7 *alphitoides*, *E. necator*, *P. roqueforti*, *P. camemberti*, and *F. poae*. In the cases of *P.*
8 *roqueforti*, *P. camemberti* and *F. poae*, contamination was clearly due to the enrichment
9 protocol, involving several PCR steps, because contaminant sequences were from *M.*
10 *violaceum* which had previously been enriched for microsatellites in the same laboratory.
11 Contaminations by the host DNA, bacteria or saprophytic fungi may explain the lack of
12 amplification with tested primer pairs for the obligate pathogens *P. viticola*, *E. alphitoides*, *E.*
13 *necator* and *M. larici-populina*. The contamination problems may be exaggerated in obligate
14 pathogens by the small quantity of DNA that can be obtained after cultivation on their host,
15 which favours the amplification of foreign DNA during the enrichment steps.

16 Published studies on microsatellite development from fungi experienced the same
17 difficulties as our work, regardless of the method used. The yield of libraries was consistently
18 low, both in terms of quantity and quality of loci isolated, with a few exceptions. It is likely
19 that many attempts to develop microsatellite in other fungal species have never been
20 published because the number of polymorphic loci isolated is too low, as was the case for our
21 libraries: only nine of our 17 microsatellite developments have been published, five of the
22 eight unpublished libraries having yielded each fewer than three loci polymorphic at the intra-
23 population level. The proportion of polymorphic loci relative to the number of loci tested and
24 the mean number of alleles per locus in fungi may therefore be yet lower than suggested by
25 this survey.

1 Specific difficulties in developing fungal microsatellites were further revealed when
2 comparing microsatellites to those isolated in other organisms. The mean number of loci
3 isolated from fungi was similar to that of birds or insects, but they were significantly shorter
4 in fungi than in other phylogroups, except birds. This difference was even stronger when
5 considering all published microsatellites, i.e. with fewer than five repeats, which were not
6 included in our study according to our microsatellite definition. These very short
7 microsatellites represented almost 15 % of all the published loci in fungi, against less than 5%
8 in the other phylogroups. Despite a preference for loci with the highest numbers of repeats,
9 generally assumed to be the most polymorphic, the scarcity of long microsatellites in the
10 fungal libraries has certainly constrained the choice towards very short microsatellites. The
11 methods used to isolate microsatellites in fungal species may have affected the statistics of the
12 cloned loci, in particular their length. However, most of the methods used in fungi were also
13 used in the other phylogroups (Zane et al., 2002); enrichments with beads or membranes
14 being prominent in all the groups. Despite the noise that the different methods of isolation
15 may have introduced, the present literature survey based on more than 2200 dinucleotide loci
16 and 250 species showed that microsatellite loci isolated from fungal species had both
17 significantly fewer repeats and fewer alleles than those from the other phylogroups examined.

18 In addition, our study showed that there is little to expect from cross-species
19 transferability of microsatellites in fungi. Within genera, only 34% of the loci tested could be
20 transferred, which appears much lower than in animals or plants. In plants for instance, 76.4
21 % of 1800 species/primer combinations tested within genera were successful (Rossetto,
22 2001). This discrepancy may be due to higher levels of sequence divergence between fungal
23 species within genera in comparison to animals and plants. In fact, the percentage of protein
24 identity between three fungal species belonging to the genus *Aspergillus* has been found to be
25 comparable to that between mammals and fish (i.e. 66 to 70%; Galagan et al., 2005). This

1 lower transferability in fungi compared to other organisms suggests that the need to build a
2 specific library for each species to be studied is even more crucial in fungi.

3

4 A first explanation for the low number and the lack of diversity of microsatellite loci
5 in fungi is linked to the genomic specificities of these organisms. A general low density of
6 microsatellites has been found in the complete fungal genomes available (Morgante et al.,
7 2002; Tóth et al., 2000) as well as a rarity of long motifs (Lim et al., 2004; Karaoglu et al.,
8 2005). Both the low density and the shortness of microsatellites should lower their propensity
9 to hybridize on the probes used for enrichment. Furthermore, the positive correlation between
10 microsatellite polymorphism and number of repeats in perfect tandem was shown to hold in
11 fungi. Therefore, the general shortness of microsatellites in fungal genomes should not only
12 impact on the number of isolated loci, but also, and more importantly, on their level of
13 polymorphism. So far, we do not have any strongly supported explanation for the low density
14 and the low level of repeat number observed in fungal microsatellites. It should, however,
15 only depend on genomic processes limiting the number and the size of microsatellites but not
16 on other fungal specificities such as life history traits. Measures of mutation rates, greatly
17 facilitated in fungi by their asexual reproduction and rapid generation times, would be
18 valuable for assessing the existence of an efficient mismatching repair system in fungi that
19 limits expansion of microsatellites, as suggested by Karaoglu et al. (2005).

20 Restricted sampling design may also be invoked to explain the low polymorphism of
21 fungal microsatellites. However, we recorded the results of fungal diversity from the largest
22 spatial scale tested in each study, we retained only the studies with more than 14 strains, and
23 the sampling scheme was often chosen to maximise diversity by testing the loci on
24 populations that were as different as possible, i. e. from different countries or continents, or
25 from different host species. There was no indication that the sample scale in fungi was less

1 representative of species diversity than that in the other phylogroups. The analysis of variance
2 furthermore confirmed that the effect of sampling size on diversity was not significant in our
3 survey.

4 In addition to genomic characteristics, i.e. shortness of loci, the lower polymorphism
5 observed in fungal microsatellites compared to those in other phylogroups may be due to
6 some specificities of their life-history traits or history. For instance, the species in which long
7 microsatellites (i.e. higher than 12 perfect repeats in average) have been found but that
8 exhibited a mean diversity lower than five alleles per locus were generally pathogens on few
9 cultivated plants, or were recently introduced (e.g. *Ascochyta rabei*, *Cryphonectria parasitica*,
10 *Magnaporthe grisea*, *Microcyclus ulei*, *Mycosphaerella fijiensis*, *M. musicola*, *Paecilomyces*
11 *fumosoroseus*, *Phytophthora infestans*). These results are consistent with reduced diversity in
12 these species being due to bottlenecks following introductions and/or recent host shifts, as
13 may be the case in many studied fungi. However, additional analyses are required because the
14 diversity in the life-history traits and in the population history of the species precluded
15 detailed analyses testing for their effects on microsatellite polymorphism.

16
17 Although the overall yield of microsatellite development was generally low in fungi,
18 microsatellites have been isolated and have proven to be polymorphic within populations in a
19 few species. In fact, the main factor explaining the level of polymorphism among fungal
20 species was the species identity. We could not identify clear factors explaining the high
21 polymorphism in certain species. The most polymorphic of our 17 species (*C. parasitica*, *F.*
22 *culmorum*, *M. violaceum* and *M. mycosphaerella*) were not necessarily those having the
23 highest numbers of sequenced clones, tested primer, of individuals sampled, or largest spatial
24 scales sampled, nor did they have any obvious peculiar history or reproductive mode. In *M.*
25 *violaceum*, the diversity was certainly over-estimated because of the existence of cryptic

1 sibling species (Giraud et al. 2002a), but it does not seem to be the case in the other above
2 species. We could not find any obvious factors explaining the level of diversity per species in
3 the literature survey of fungal microsatellites either. Because there is no apparent rule, a good
4 strategy when deciding whether a microsatellite library is worth constructing may therefore be
5 to first evaluate the polymorphism of the species using other markers, such as AFLP, ISSR or
6 RAPD. Such methods do not require the investment that microsatellites do for development
7 and may serve as a preliminary test for the level of polymorphism in the focal species. For
8 instance, our own AFLP analyses conducted in parallel to the microsatellite ones on five
9 fungal species (Lopez-Villavicencio et al. 2005; Enjalbert et al. 2005; unpublished data)
10 indicated that when two individuals presented on average less than one band difference per
11 AFLP profile (using 2 selective bases on each primer), polymorphism was difficult to find
12 with microsatellites. We advocate that if AFLP on a given sample yields less than 2 %
13 difference between individuals per profile (i.e., one band difference on a profile with fifty
14 bands), microsatellites probably will not be polymorphic, and building enriched microsatellite
15 libraries may just be a waste of time and money. More efficient alternatives should then be
16 sought. For instance, polymorphism can be looked for in fragments generated by RAPD or
17 ISSR and specific primers can be developed such as sequence characterised amplified regions
18 (SCAR). Relative to a microsatellite library, this method is a rapid screen of putative
19 polymorphic loci. In addition, the rapid development of genomics may offer a new route for
20 efficiently developing powerful markers in fungi. In particular, expressed sequence tags or
21 extensive genomic libraries provide interesting material for finding long microsatellites (Kaye
22 et al. 2003; Breuillin et al. 2006) or other markers, such as single nucleotide polymorphism
23 (SNPs).

24

1 If microsatellite development is to be performed, the present study provides some
2 guidelines. First, this survey points to the necessity of using enriched protocols for isolation of
3 microsatellite markers. Our rare attempts to directly clone microsatellites from total DNA
4 failed and 86% of the published microsatellite fungal isolations were performed using
5 enriched protocols or searches in databases. It was not possible to test the effect of the
6 methods of enrichment on the yield of libraries and on the polymorphism of the loci isolated,
7 although the rare studies using several methods on a single species gave no indication of any
8 differences. However, we found enrichment with beads more convenient than with
9 membranes. Furthermore, it is worth noting that the ISSR method, more specifically used in
10 fungi, seemed to preferentially isolate short microsatellites, but comparisons of several
11 methods of isolation on the same species are required to provide compelling evidence for such
12 a bias. Second, regarding the choice of motif for enrichment, AG appears the most frequent in
13 the published fungal genomes (Lim et al., 2004). In contrast, we found that in the studies in
14 which libraries were enriched for both the dinucleotides AC and AG, consistently more
15 polymorphic microsatellites were isolated with AC repeats than with AG repeats. However,
16 the analyses of fungal genomes also underlined a high variability among species regarding the
17 most frequent motifs, with several genomes with a low density of AG motif and higher levels
18 of AT (Lim et al., 2004). The AT motif should, however, be avoided as self-complementarity
19 makes this motif difficult to amplify, and the number of repeats is generally low (Karaoglu et
20 al., 2005). As it is difficult to decide *a priori* which motifs are the most abundant in a given
21 species, the best strategy may be to enrich for both the AC and AG motifs in order to increase
22 the probability to recover numerous and long loci. In addition, although our libraries did not
23 yield many useful trinucleotide markers, enriching libraries for trinucleotides may be worth
24 trying. Such motifs indeed represented 24% of all the polymorphic published loci in fungi.
25 Third, our analyses confirmed that, after species identity, the best predictor of polymorphism

1 was the length of the longest perfect microsatellite. For developing polymorphic
2 microsatellites, loci with the highest number of perfect repetitions should therefore be the best
3 candidates. However, we found that it may be hard in many fungal species to isolate several
4 microsatellite loci with more than eight repeats. Many loci with five to seven repetitions were
5 polymorphic within populations in some fungal species, and those may thus be worth testing.

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- 20

1

2

3 **Figure legends**

4

5

6

7 **Figure 1.** Number of primer notes published in Molecular Ecology Notes between March
8 2001 and June 2005 (black bars) on fungi, angiosperms, fish, birds, mammals and insects. For
9 fungi, microsatellite isolation reports found in other journals are represented in grey.

10

11 **Figure 2.** Change in the average percentage of useful sequences along the different steps of
12 microsatellite isolation for 17 fungal microsatellite-enriched libraries. Percentages are given
13 for each step relative to the initial number of inserts correctly sequenced. Maximum and
14 minimum values are given by the vertical lines and dashed lines indicate limits of the first and
15 the third quartiles. See Appendix 1 for details on the libraries.

16

17 **Figure 3.** Boxplots of the number of repeats (A), and of the number of alleles (B) for fungi,
18 angiosperms, fishes, birds, mammals and insects. Statistics are represented on the means per
19 species for all primer notes published in Molecular Ecology Notes between March 2001 and
20 June 2006 (and published in all journals for fungi). Boxes indicate quartiles, dark squares
21 means and vertical traits minimal and maximal values. Different letters indicate significantly
22 different groups of means in a SNK pairwise comparison test ($P < 0.05$).

23

1 **Figure 4.** Mean number of alleles per species plotted against mean number of repeats (of the
2 longest pure tandem repeat) for all available data on fungal dinucleotide microsatellites. The
3 regression line is drawn ($r=0.28$, $P=0.06$).

4

ACCEPTED MANUSCRIPT

Table 1. Fungal species for which the authors built microsatellite-enriched libraries, with their characteristics.

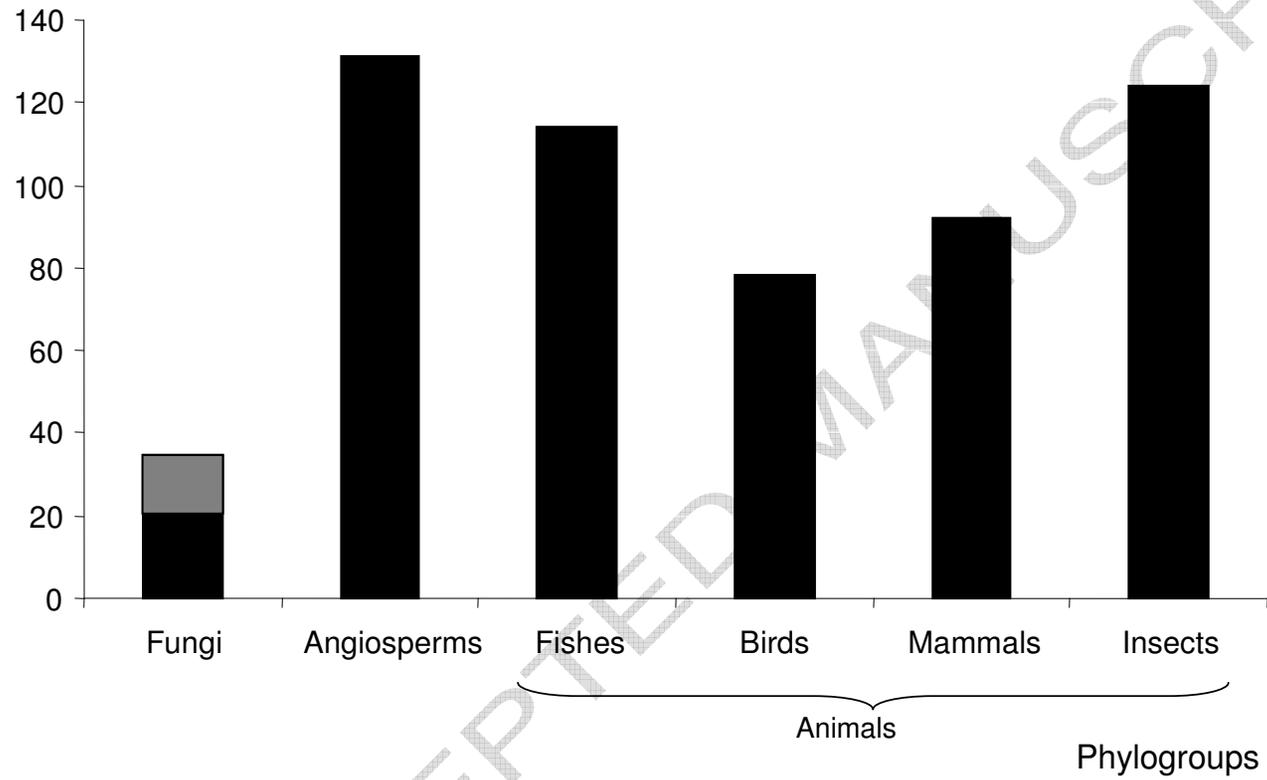
| Species | Order | Method of enrichment | Motif for enriched libraries | Number of screened individuals |
|--|---------------|-------------------------|---|--------------------------------|
| <i>Cryphonectria parasitica</i> | Ascomycota | membrane | (AC) ₁₅ (AG) ₁₅ (AAG) ₁₀ | 113 |
| <i>Erysiphe alphitoides</i> (2 libraries) | Ascomycota | Membrane beads | (AC) ₁₅ (AG) ₁₅ (AAG) ₁₀ (AC) ₁₅ (AG) ₁₅ | 8 |
| <i>Erysiphe necator</i> (2 libraries) | Ascomycota | beads | (TC) ₁₀ (TG) ₁₀ | 15 |
| <i>Fusarium culmorum</i> | Ascomycota | beads | (AC) ₁₀ (AAG) ₁₀ | 20 |
| <i>Fusarium poe</i> | Ascomycota | beads | (AC) ₁₀ | 20 |
| <i>Magnaporthe grisea</i> | Ascomycota | beads | (TC) ₁₅ (AC) ₁₅ | 6 |
| <i>Melampsora larici-populina</i> (5 libraries) | Basidiomycota | membrane beads beads | (AC) ₁₅ (AG) ₁₅ (AAG) ₁₀ (AC) ₁₅ (AG) ₁₅ (TC) ₁₀ (TG) ₁₀ | 30 |
| <i>Microbotryum violaceum</i> (3 libraries) | Basidiomycota | beads | (AC) ₁₀ (AAG) ₁₀ | 30 |
| <i>Mycosphaerella musicola</i> | Ascomycota | beads | (TC) ₁₅ (AC) ₁₅ | 15 |
| <i>Mycosphaerella fijiensis</i> | Ascomycota | beads | (TC) ₁₅ (AC) ₁₅ | 15 |
| <i>Mycosphaerella eumusae</i> | Ascomycota | beads | (TC) ₁₅ (AC) ₁₅ | 15 |
| <i>Mycrocyclus ulei</i> | Ascomycota | beads | (TC) ₁₅ (AC) ₁₅ | 16 |
| <i>Penicillium roqueforti</i> (4 libraries) | Ascomycota | beads | (AC) ₁₀ (AAG) ₁₀ (CAC) ₁₀ (GGA) ₁₀ | 5 |
| <i>Penicillium camembertii</i> | Ascomycota | beads | (AC) ₁₀ (AAG) ₁₀ | 5 |
| <i>Plasmopara viticola</i> | Oomycota | beads | (TC) ₁₀ (TG) ₁₀ (GAA) ₁₀ (TAA) ₁₀ | 100 |
| <i>Puccinia striiformis fsp tritici</i> | Basidiomycota | beads | (AC) ₁₀ (AG) ₁₀ (AAC) ₁₀ (AAG) ₁₀ | 96 |
| <i>Puccinia triticina</i> | Basidiomycota | beads | (AC) ₁₀ (AG) ₁₀ (AAC) ₁₀ (AAG) ₁₀ | 15 |

Table 2. Results of the GENMOD analysis testing for an effect of repeat number, imperfection and motif of the loci, species and sample size on the allele number of microsatellites ($R^2=0.49$).

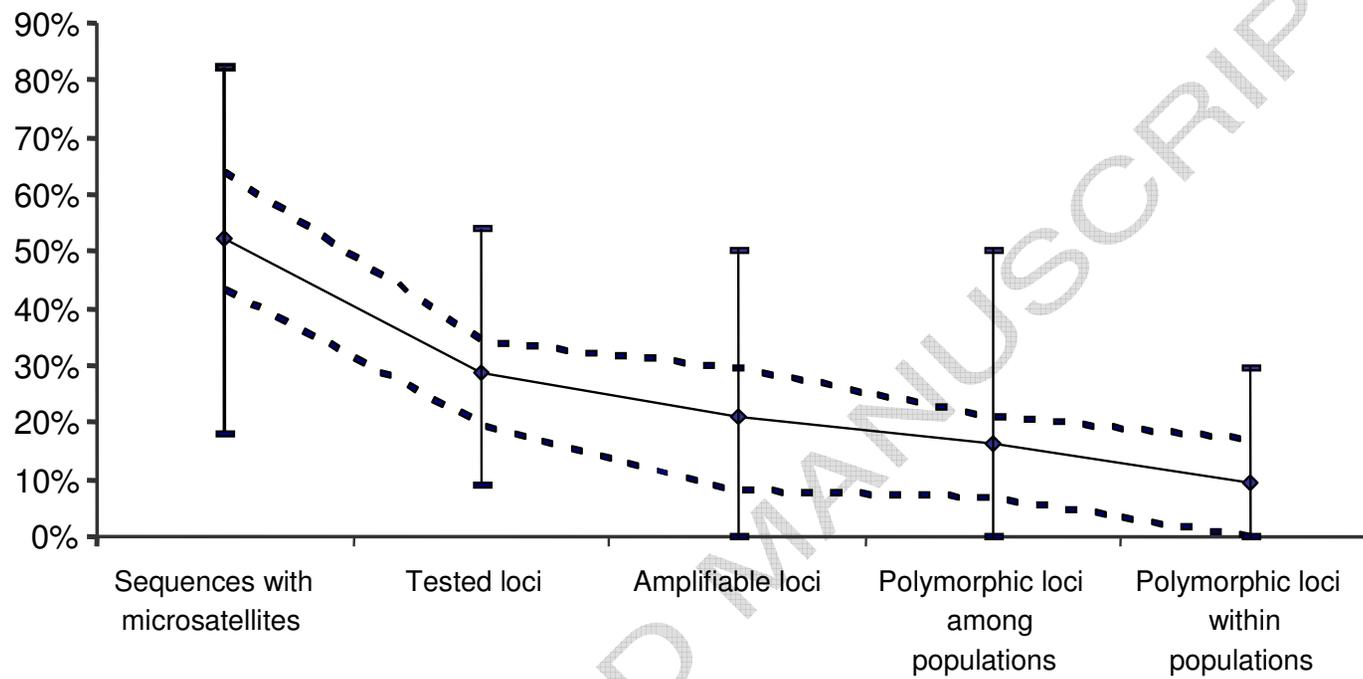
| Source | D. F. | Chi Square | P |
|-------------------|-------|------------|---------|
| Number of repeats | 1 | 25.34 | <0.0001 |
| Imperfection | 1 | 1.59 | 0.2076 |
| motif | 1 | 0.48 | 0.4888 |
| Species | 40 | 223.39 | <0.0001 |
| Sample size | 1 | 0.49 | 0.4828 |

ACCEPTED MANUSCRIPT

Number of published papers



Dutech et al. Figure 1



Dutech et al. Figure 2

Number of repeats

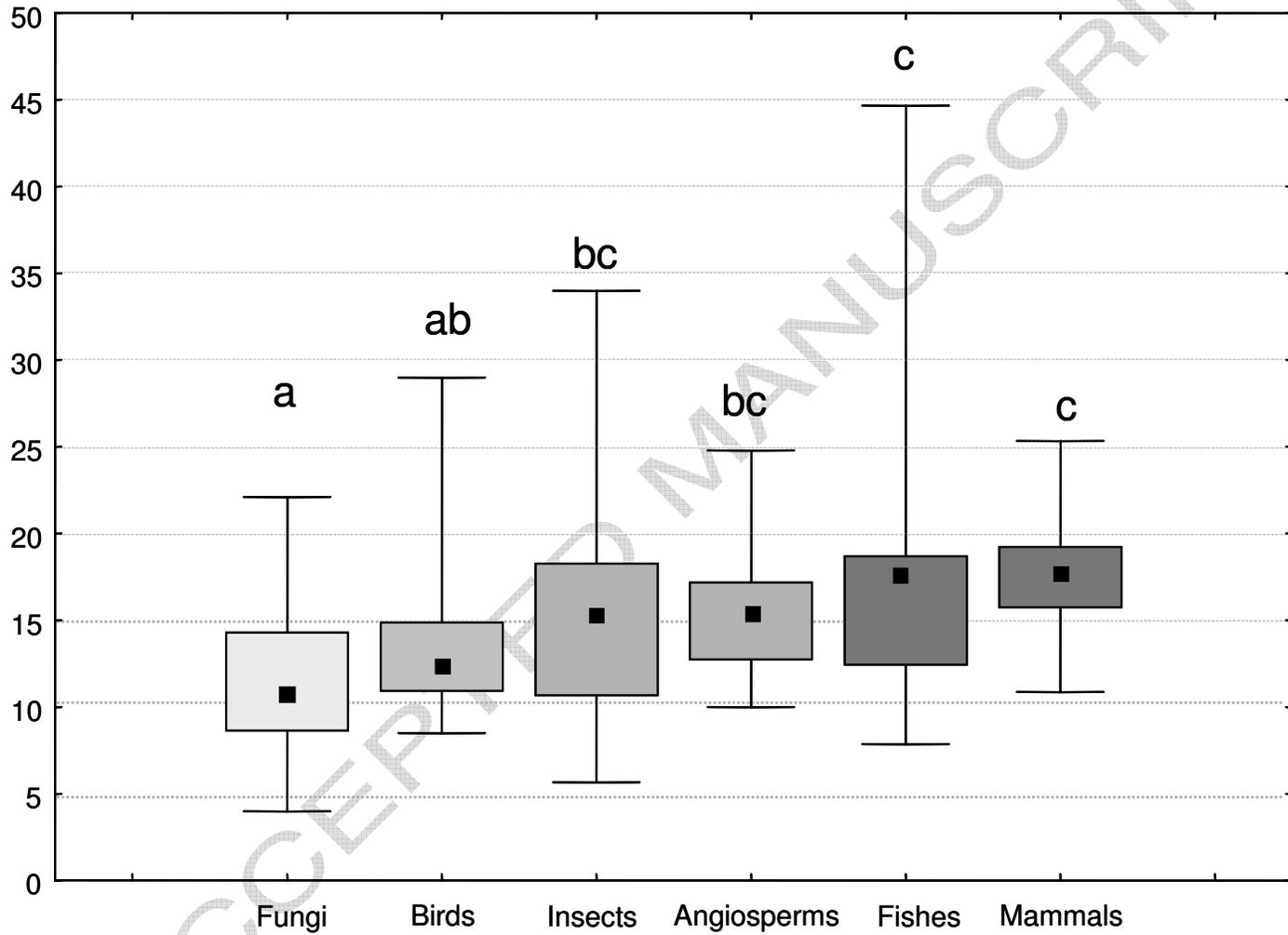


Figure 3A Dutech et al.

Number of alleles

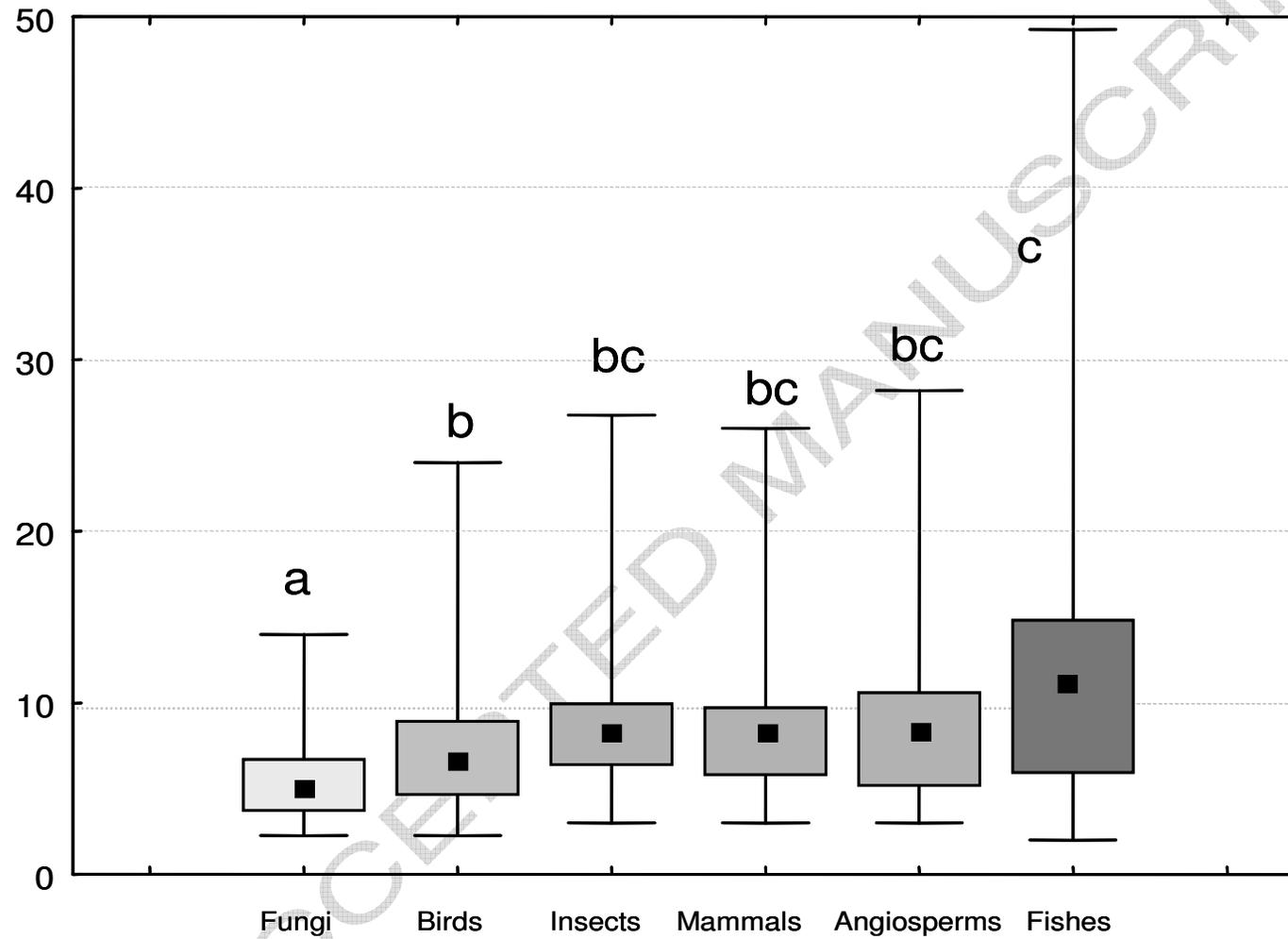
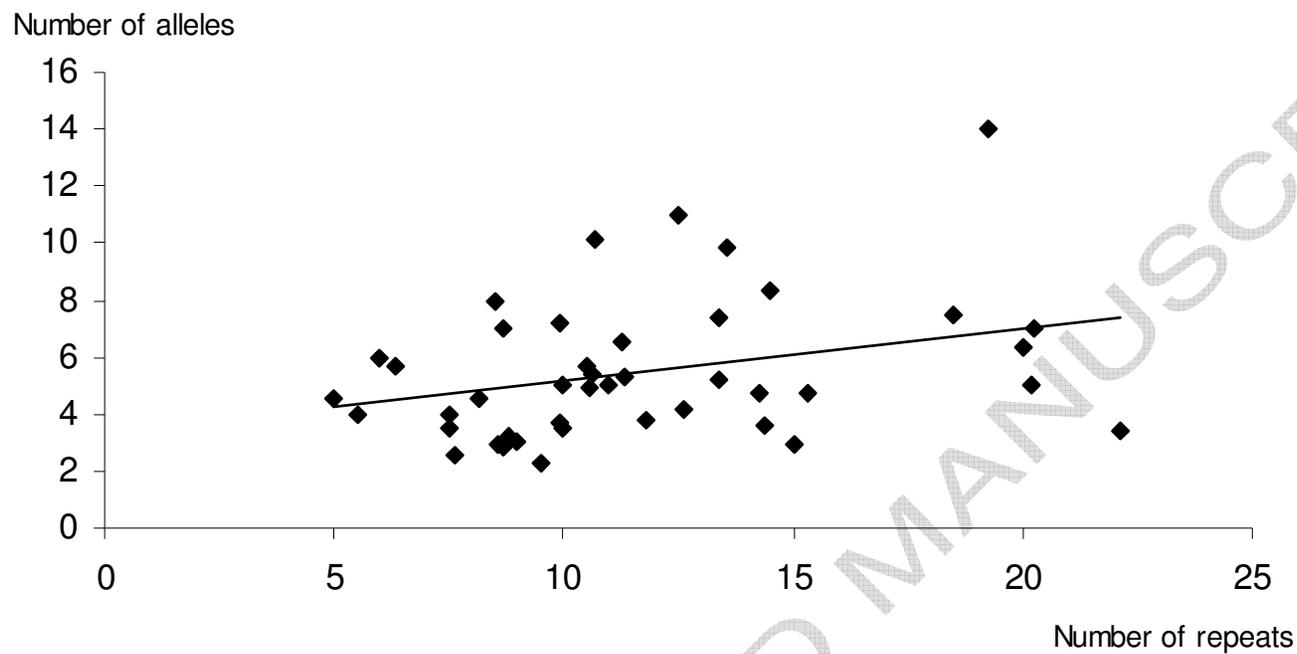


Figure 3B Dutech et al.



Dutech et al. Figure 4

Appendix 1. Proportion of useful loci along the different steps of microsatellite development in 17 fungal species. The percentages are relative to the initial number of sequenced clones.

| Species | Number of sequenced clones | Percentage of unique sequences with a microsatellite | Percentage of sequences suitable for primer design | Percentage of sequences yielding correct amplification | Percentage of sequences yielding polymorphic loci at the largest scale | Percentage of sequences yielding intra-population polymorphic loci |
|---|----------------------------|--|--|--|--|--|
| <i>Cryphonectria parasitica</i> | 58 | 44.8 | 29.3 | 27.6 | 17.2 | 17.2 |
| <i>Fusarium culmorum</i> | 33 | 57.6 | 27.3 | 27.3 | 24.2 | 23.7 |
| <i>Fusarium poae</i> | 137 | 63.5 | 14.6 | 1.5 | 1.5 | 1.5 |
| <i>Erysiphe alphitoides</i> | 110 | 19.1 | 9.1 | 0.0 | 0.0 | 0.0 |
| <i>Erysiphe necator</i> | 96 | 79.2 | 18.8 | 8.3 | 3.1 | 2.1 |
| <i>Magnaporthe grisea</i> | 96 | 46.9 | 20.8 | 10.4 | 10.4 | 10.4 |
| <i>Melampsora larici-populina</i> | 208 | 17.8 | 17.8 | 7.2 | 6.7 | 6.3 |
| <i>Microbotryum violaceum</i> | 143 | 42.0 | 30.8 | 30.8 | 30.8 | 13.0 |
| <i>Microcyclus ulei</i> | 52 | 46.2 | 26.9 | 25.0 | 21.2 | 21.2 |
| <i>Mycosphaerella eumusae</i> | 58 | 63.8 | 32.8 | 19.0 | 15.5 | 15.5 |
| <i>Mycosphaerella fijiensis</i> | 111 | 46.8 | 43.2 | 29.7 | 29.7 | 29.7 |
| <i>Mycosphaerella musicola</i> | 64 | 53.1 | 34.4 | 18.8 | 18.8 | 18.8 |
| <i>Penicillium camembertii</i> | 24 | 66.7 | 54.2 | 50.0 | 50.0 | 0.0 |
| <i>Penicillium roqueforti</i> | 23 | 43.5 | 43.5 | 39.1 | 17.4 | 0.0 |
| <i>Plasmopara viticola</i> | 186 | 37.1 | 19.9 | 4.8 | 3.8 | 3.8 |
| <i>Puccinia striiformis fsp tritici</i> | 79 | 75.9 | 35.4 | 30.4 | 15.2 | 0.0 |
| <i>Puccinia triticina</i> | 130 | 82.3 | 27.7 | 23.8 | 9.2 | 0.0 |
| Mean | | 52.1 ± 4.7 | 28.6 ± 3.9 | 20.8 ± 3.5 | 16.2 ± 3.2 | 9.6 ± 2.5 |

Appendix 2. Isolation and characterization of microsatellite loci in fungal species

OUR DATA

| Species | Method of library enrichment | Nb of tested primers | Percentage of scorable loci | Percentage of polymorphic loci | Nb of loci used for analyses ⁽¹⁾ | Nb of repeats ⁽²⁾ | Nb of alleles ⁽²⁾ | Nb of strains tested | References |
|---|---------------------------------|----------------------|-----------------------------|--------------------------------|---|------------------------------|--------------------------------|----------------------|--|
| <i>Cryphonectria parasitica</i> | Membrane and EST ⁽³⁾ | 17 | 94 | 59 | 8 | 13.3 | 4.9 | 113 | Breuillin <i>et al.</i> (2006) |
| <i>Erysiphe necator</i> | Beads | 18 | 44 | 11 | DA | DA | DA | DA | Unpublished |
| <i>Fusarium culmorum</i> | Beads | 9 | 100 | 89 | 7 | 13.0 | 6.1 | 20 | Giraud <i>et al.</i> (2002a) |
| <i>Fusarium poae</i> | Beads | 20 | 10 | 10 | DA | DA | DA | DA | Unpublished |
| <i>Magnaporthe grisea</i> | Beads and EST ⁽³⁾ | 19 | 50 | 30 | 24 | 16.7 | 2.9 | 6 | Kaye <i>et al.</i> (2003) and unpublished |
| <i>Melampsora larici-populina</i> | Beads and membrane | 37 | 41 | 35 | 12 | 9.7 | 4.9 | 30 | Barrès <i>et al.</i> (2005) |
| <i>Microbotryum violaceum</i> | Beads | 44 | 100 | 100 | 44 | 11.4 | 5.4 | 30 | Giraud <i>et al.</i> (2002b) |
| <i>Microcyclus ulei</i> | Beads | 14 | 93 | 93 | 11 | 15.0 | 2.9 | 16 | Le Guen <i>et al.</i> (2004) and unpublished |
| <i>Mycosphaerella eumusae</i> | Beads | 19 | 58 | 47 | 9 | 8.6 | 2.9 | 15 | Unpublished |
| <i>Mycosphaerella fijiensis</i> | Beads | 48 | 69 | 69 | 33 | 10.2 | 4.6 | 15 | Unpublished |
| <i>Mycosphaerella musicola</i> | Beads | 19 | 63 | 63 | 12 | 10.2 | 4.1 | 15 | Unpublished |
| <i>Penicillium camembertii</i> | Beads | 13 | 100 | 92 | 11 | 12.2 | 2.6 | 5 | Unpublished |
| <i>Penicillium roqueforti</i> | Beads | 19 | 84 | 37 | 4 | 11.8 | 3.5 | 5 | Unpublished |
| <i>Plasmopara viticola</i> | Beads | 15 | 40 | 40 | 6 | 8.2 | 4.5 | 100 | Delmotte <i>et al.</i> (2006) |
| <i>Puccinia striiformis fsp tritici</i> | Beads | 28 | 86 | 43 | 11 | 7.3 | 2.5 | 96 | Enjalbert <i>et al.</i> (2002) |
| <i>Puccinia triticina</i> | Beads | 36 | 86 | 33 | 12 | 8.1 | 3.2 | 15 | Duan <i>et al.</i> (2003) |
| Mean | | 23.4 ± 3.0 | 69.9 ± 7.0 | 53.2 ± 7.4 | 14.6 ± 2.9 | 11.1 ± 0.7 | 4.1 ± 0.3⁽⁴⁾ | 44.4 ± 12.1 | |

LITERATURE DATA

| Species | Method of library enrichment | Nb of tested primers | Percentage of scorable loci | Percentage of polymorphic loci | Nb of published loci ⁽¹⁾ | Nb of repeats ⁽²⁾ | Nb of alleles ⁽²⁾ | Nb of strains tested | References |
|------------------------------------|------------------------------|----------------------|-----------------------------|--------------------------------|-------------------------------------|------------------------------|------------------------------|----------------------|-------------------------------------|
| <i>Alternaria brassicicola</i> | Beads | 21 | 81 | 57 | 12 | 8.7 | 2.9 | 46 | Avenot <i>et al.</i> (2005) |
| <i>Armillaria ostoyae</i> | Membrane | 12 | 100 | 100 | 12 | 9.9 | 7.2 | 23 | Langrell <i>et al.</i> (2001) |
| <i>Ascochyta rabiei</i> | Non enriched | 37 | 70 | 54 | 20 | 12.6 | 4.2 | 22 | Geistlinger <i>et al.</i> (2000) |
| <i>Aspergillus flavus</i> | GenBank ⁽⁵⁾ | NA | NA | NA | 6 | 13.4 | 7.4 | 20 | Tran-Dinh & Carter (2000) |
| <i>Aspergillus parasiticus</i> | GenBank | NA | NA | NA | 6 | 13.4 | 5.2 | 15 | Tran-Dinh & Carter (2000) |
| <i>Aspergillus fumigatus</i> | Non enriched | 8 | 100 | 50 | 4 | 19.3 | 14.0 | 100 | Bart-Delabesse <i>et al.</i> (1998) |
| <i>Beauveria brongniartii</i> | Beads | 11 | 91 | 91 | 10 | 11.8 | 3.8 | 96 | Enkerli <i>et al.</i> (2001) |
| <i>Beauveria bassiana</i> | Beads | NR | NR | NR | 8 | 20.0 | 6.3 | 24 | Rehner & Buckley (2003) |
| <i>Botryosphaeria parva</i> | ISSR ⁽⁶⁾ | 20 | NR | 35 | 5 | 9.0 | 3.0 | 82 | Slippers <i>et al.</i> (2004) |
| <i>Botryosphaeria rhodina</i> | ISSR | 19 | 68 | 42 | 5 | 5.0 | 4.0 | 9 | Burgess <i>et al.</i> (2003) |
| <i>Coccidioides immitis</i> | Non enriched | 9 | 100 | 100 | 9 | 13.6 | 9.9 | 25 | Fisher <i>et al.</i> (1999) |
| <i>Cryphonectria eucalypti</i> | FIASCO ⁽⁷⁾ | NR | NR | NR | 8 | 20.3 | 7.0 | 20 | Nakabonge <i>et al.</i> (2005) |
| <i>Cylindrocladium parasiticum</i> | ISSR | 25 | NR | 48 | 8 | 7.5 | 3.5 | 17 | Wright <i>et al.</i> (2006) |
| <i>Dothistroma pini</i> | Anchored and EST | 9 | 78 | 33 | 4 | 6.7 | 2.7 | 12 | Ganley & Bradshaw (2001) |
| <i>Fusarium oxysporum</i> | ISSR | NR | NR | NR | 9 | 12.5 | 11 | 64 | Bogale <i>et al.</i> (2005) |
| <i>Heterobasidion annosum</i> | Beads | NR | NR | NR | 5 | 11.3 | 6.5 | 19 | Johannesson & Stenlid (2004) |
| <i>Laccaria bicolor</i> | Beads | 150 | 26 | 5 | 7 | 8.7 | 7.0 | 12 | Jany <i>et al.</i> (2006) |
| <i>Leptosphaeria maculans</i> | Non enriched and EST | 36 | 94 | 19 | 6 | 9.5 | 2.3 | 14 | Hayden <i>et al.</i> (2004) |
| <i>Lobaria pulmonaria</i> | Beads | 21 | 57 | 52 | 11 | 14.5 | 8.3 | 30 | Walser <i>et al.</i> (2003) |
| <i>Melampsora lini</i> | FIASCO | 41 | 83 | 27 | 10 | 7.5 | 4 | 102 | Barett & Brubaker (2006) |
| <i>Metarhizium anisopliae</i> | Beads | 14 | 100 | 100 | 8 | 10.6 | 5.4 | 34 | Enkerli <i>et al.</i> (2005) |
| <i>Mycosphaerella fijiensis</i> | Beads | 19 | 58 | 58 | 10 | 20.2 | 5.0 | 29 | Neu <i>et al.</i> (1999) |
| <i>Mycosphaerella musicola</i> | Beads | 48 | NR | 54 | 24 | 22.1 | 3.4 | 24 | Molina <i>et al.</i> (2001) |
| <i>Mycosphaerella populorum</i> | EST | 10 | NR | 50 | 3 | 11 | 5 | 77 | Feau <i>et al.</i> (2006) |
| <i>Paecilomyces fumosoroseus</i> | Beads | 9 | 100 | 89 | 8 | 14.4 | 3.6 | 26 | Dalleau-Clouet <i>et al.</i> (2005) |

| | | | | | | | | | |
|---------------------------------|----------------------|-----|-----|----|----|------|------|-----|-------------------------------|
| <i>Phaeosphaeria nodorum</i> | EST | 73 | NR | 12 | 11 | 6.0 | 6.0 | 121 | Stukenbrock et al. (2005) |
| <i>Phytophthora cinnamomi</i> | Membrane | 10 | 40 | 40 | 4 | 20.5 | 5.3 | 5 | Dobrowolski et al. (2002) |
| <i>Phytophthora ramorum</i> | Beads | 24 | 58 | 29 | 7 | 10 | 3.5 | 26 | Prospero et al. (2004) |
| <i>Phytophthora infestans</i> | Non enriched and EST | 108 | NR | 11 | 13 | 15.3 | 4.7 | 90 | Lees et al. 2006 |
| <i>Pisolithus microcarpus</i> | RAPD-PCR | 25 | NR | 24 | 4 | 5.5 | 4.0 | 30 | Hitchcock et al. 2006 |
| <i>Pisolithus spp.</i> | ISSR | 8 | 100 | 75 | 3 | 5.0 | 4.5 | 39 | Kanchanaprayudh et al. (2002) |
| <i>Pleurotus eryngii</i> | Beads | 6 | 83 | 83 | 5 | 10.0 | 5.0 | 22 | Della Rosa et al. (2004) |
| <i>Russula brevipes</i> | Enriched | 26 | 23 | 23 | 5 | 6.3 | 5.7 | 27 | Bergemann et al. (2005) |
| <i>Sclerotinia sclerotiorum</i> | Membrane | 70 | NR | 36 | 17 | 9.9 | 3.7 | 44 | Sirjusingh & Kohn (2001) |
| <i>Sphaeropsis sapinea</i> | ISSR | 22 | 82 | 68 | 3 | 8.5 | 8.0 | 40 | Burgess et al. (2001) |
| <i>Tuber magnatum</i> | Beads | 23 | 91 | 35 | 8 | 18.5 | 7.5 | 370 | Rubini et al. (2004) |
| <i>Venturia inaequalis</i> | Beads | 52 | 40 | 40 | 17 | 10.7 | 10.1 | 44 | Guérin et al. (2004) |

Mean **31.2 ± 5.8** **75.0 ± 5.3** **49.7 ± 5.0** **8.6 ± 0.8** **11.9 ± 0.8** **5.8 ± 0.4⁽⁴⁾** **52.2 ± 10.8**

NA: Not applicable

NR: Not reported

⁽¹⁾ Number of polymorphic loci with a minimum of five repeats

⁽²⁾ Only dinucleotide loci considered

⁽³⁾ Loci isolated from an expressed sequence tags (EST) library

⁽⁴⁾ Loci isolated from genomic data available at <http://www.ncbi.nlm.nih.gov/>

⁽³⁾ Loci isolated from an enriched library using an intergenic simple sequence repeats (ISSR) protocol (Burgess et al., 2001)

⁽⁴⁾ Loci isolated from an enriched library using an anchored protocol (Fisher et al., 1996)

⁽⁵⁾

⁽⁶⁾ Loci isolated from an enriched library using a FIASCO protocol (Zane et al., 2002)

⁽⁷⁾ Mean estimated using only the studies with at least 14 haplotypes.

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