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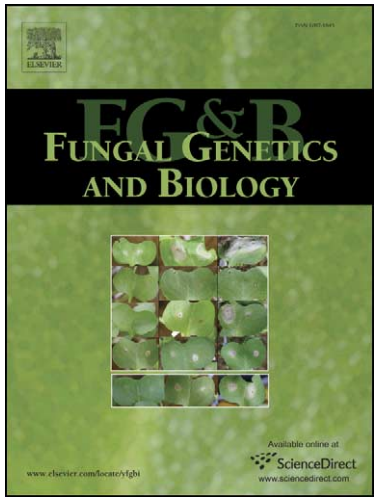

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Challenges of microsatellite isolation in fungi

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Material and methods

Enriched libraries

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

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

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

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

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

Literature search and data extraction

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

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

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

Data analyses

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

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

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

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

Results

Yield of our 17 fungal microsatellite-enriched libraries

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

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

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

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

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

Comparison between our dataset and the published studies in fungi

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

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

Cross-species transferability of microsatellite markers between fungal species

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

Comparison of fungal microsatellites with those of other organisms

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

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

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

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

Factors affecting the diversity of dinucleotide microsatellites

In fungi, the correlation between the mean repeat number and the mean allele number per species was marginally significant (r=0.28, P=0.06, Fig. 4). The short loci indeed had a very low level of polymorphism, whereas long loci had a larger range of allele numbers, their polymorphism being high for some but still low for others. The mean repeat number and the mean allele number per species were significantly correlated in birds (r=0.72, P<0.001), 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 in angiosperms (r=0.19, P=0.19). However, GENMOD analyses on the complete dataset with all loci and taking into account the effects of the other parameters (see below) found the effect of the number of repeats on the number of alleles significant in all the phylogroups, the regression coefficients being consistently lowest in mammals and angiosperms.

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Figure legends

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

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

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

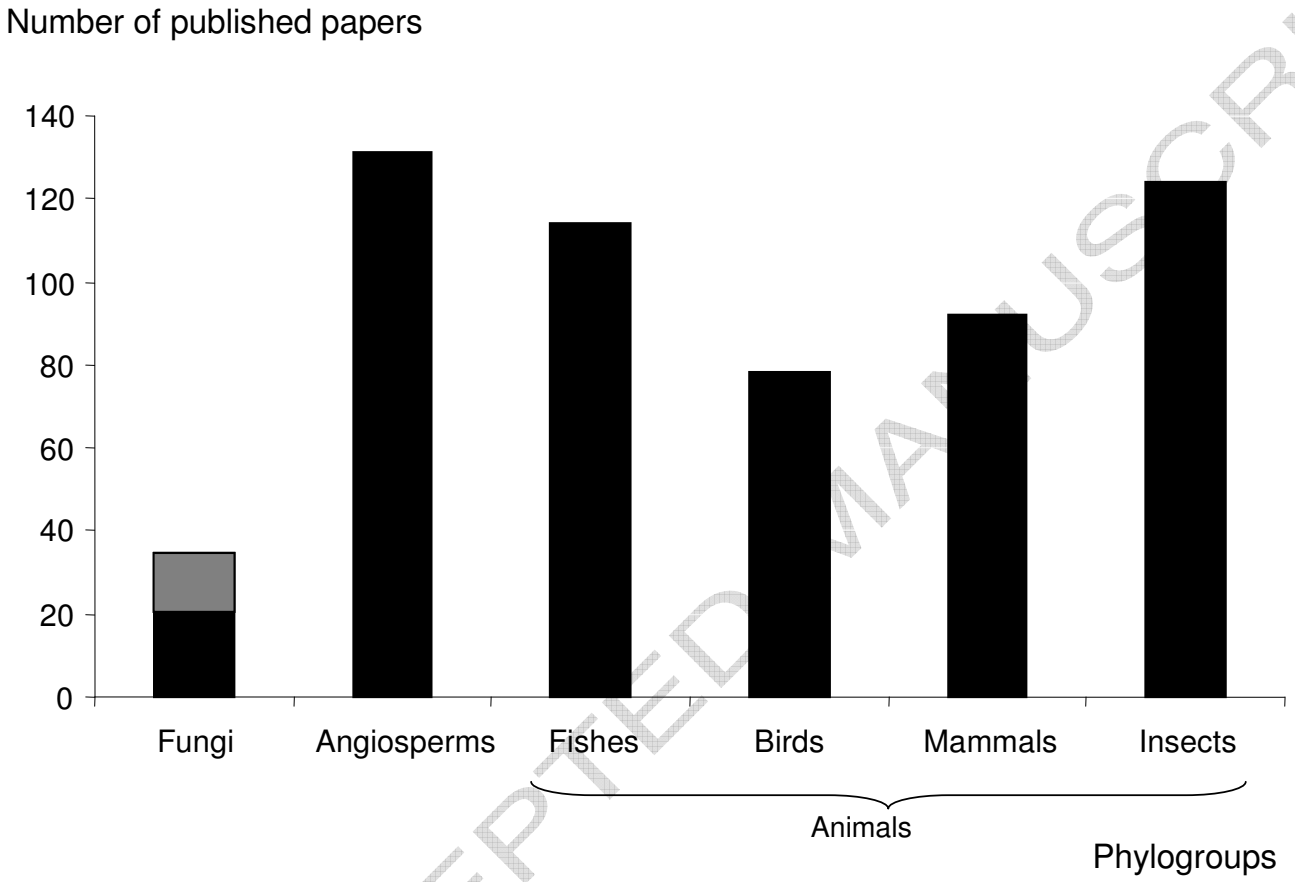
Figure 4. Mean number of alleles per species plotted against mean number of repeats (of the longest pure tandem repeat) for all available data on fungal dinucleotide microsatellites. The regression line is drawn (r=0.28, P=0.06).

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

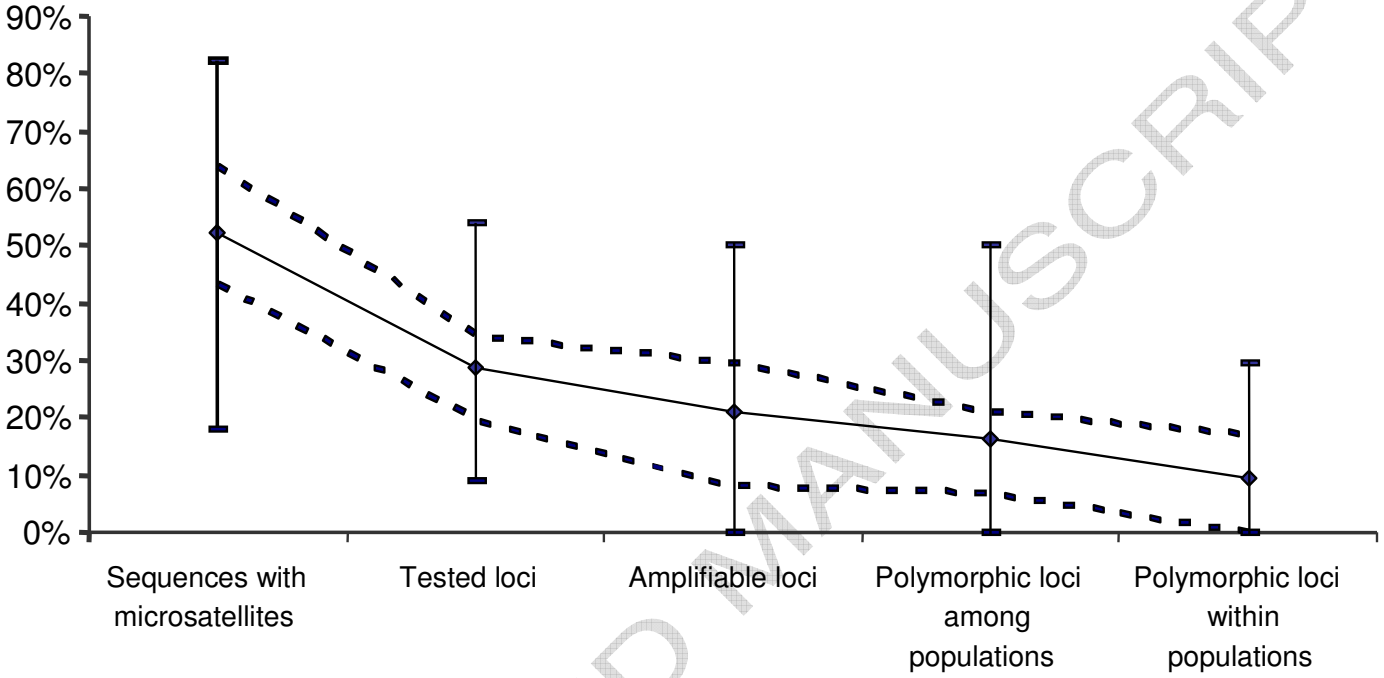
Species	Order	Method of enrichment	Motif for enriched libraries	Number of screened individuals
Cryphonectria parasitica	Ascomycota	membrane	$(AC)_{15}$ $(AG)_{15}$ $(AAG)_{10}$	113
Erysiphe alphitoides (2 libraries)	Ascomycota	Membrane beads	$(AC)_{15}$ $(AG)_{15}$ $(AAG)_{10}$ $(AC)_{15}$ $(AG)_{15}$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



Dutech et al. Figure 1



Dutech et al. Figure 2

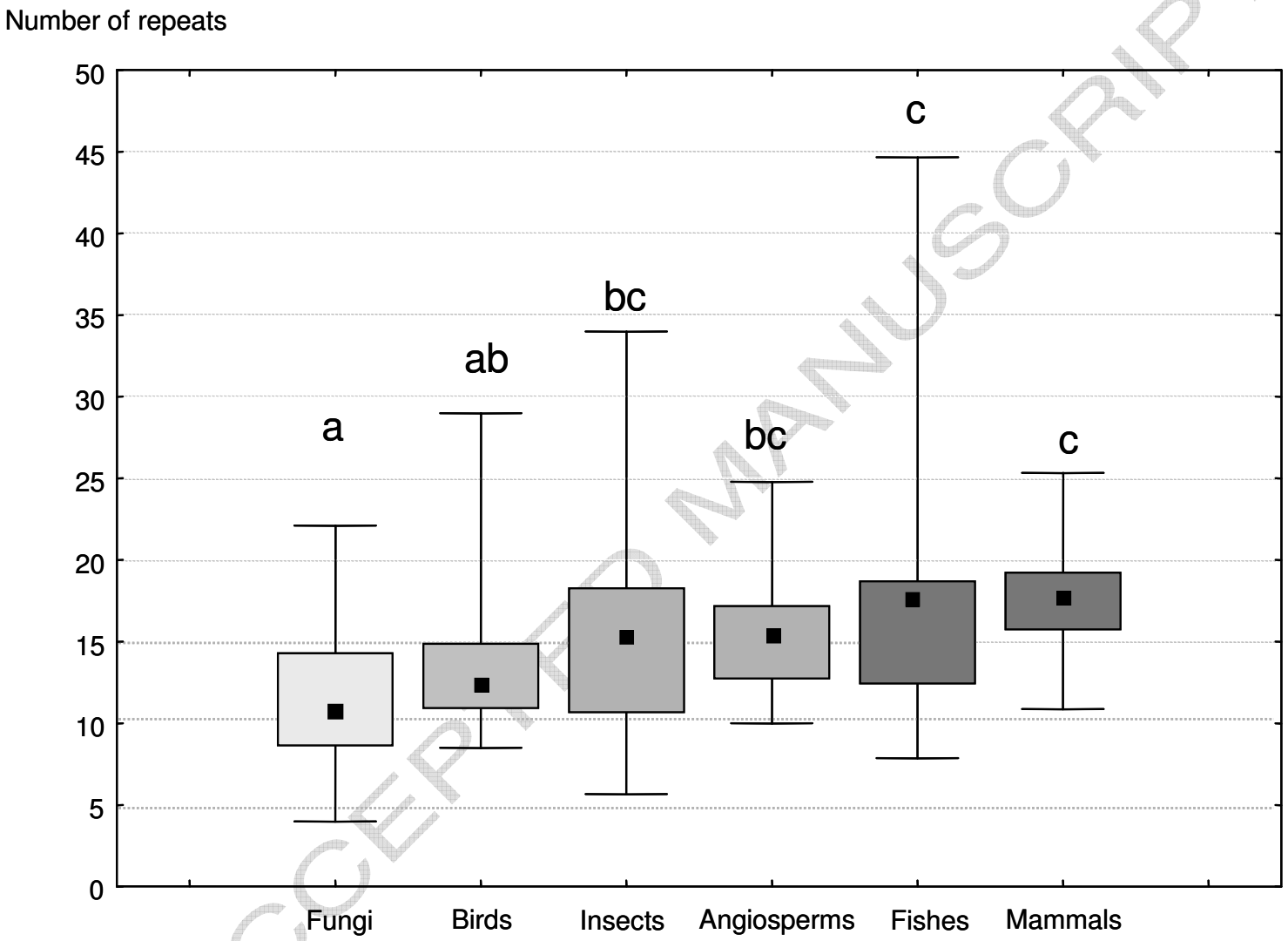


Figure 3A Dutech et al.

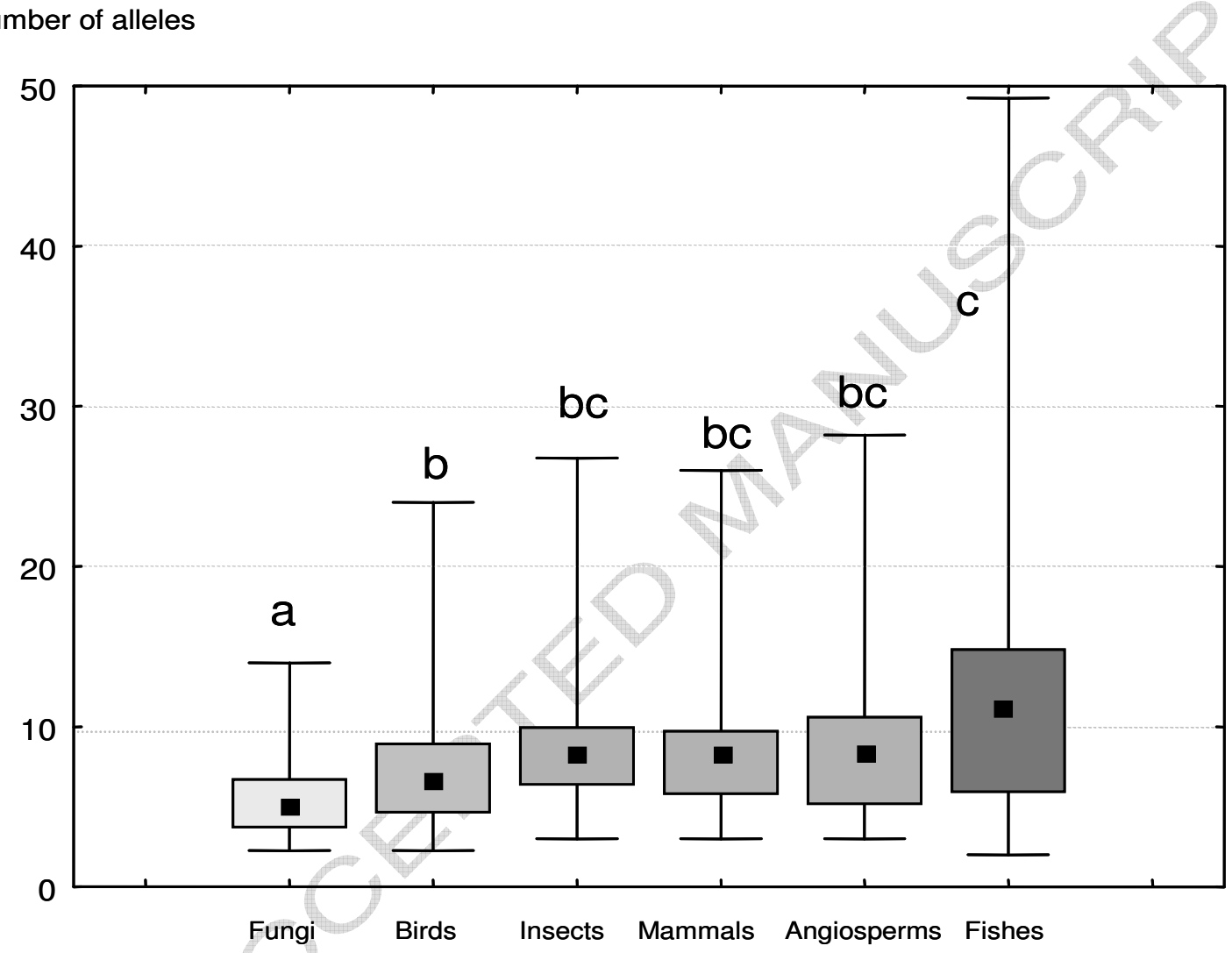
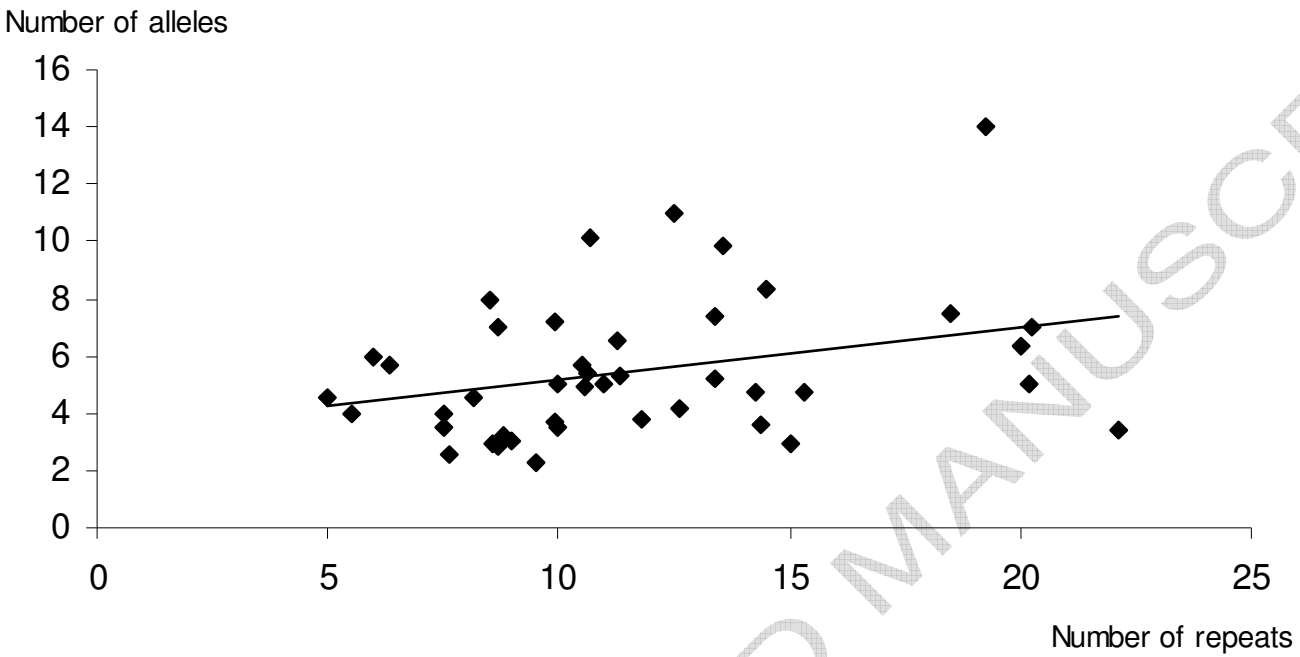


Figure 3B Dutech et al.



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
Cryphonectria parasitica	58	44.8	29.3	27.6	17.2	17.2
Fusarium culmorum	33	57.6	27.3	27.3	24.2	23.7
Fusarium poae	137	63.5	14.6	1.5	1.5	1.5
Erysiphe alphitoides	110	19.1	9.1	0.0	0.0	0.0
Erysiphe necator	96	79.2	18.8	8.3	3.1	2.1
Magnaporthe grisea	96	46.9	20.8	10.4	10.4	10.4
Melampsora larici-populina	208	17.8	17.8	7.2	6.7	6.3
Microbotryum violaceum	143	42.0	30.8	30.8	30.8	13.0
Microcyclus ulei	52	46.2	26.9	25.0	21.2	21.2
Mycosphaerella eumusae	58	63.8	32.8	19.0	15.5	15.5
Mycosphaerella fijiensis	111	46.8	43.2	29.7	29.7	29.7
Mycosphaerella musicola	64	53.1	34.4	18.8	18.8	18.8
Penicillium camembertii	24	66.7	54.2	50.0	50.0	0.0
Penicillium roqueforti	23	43.5	43.5	39.1	17.4	0.0
Plasmopara viticola	186	37.1	19.9	4.8	3.8	3.8
Puccinia striiformis fsp tritici	79	75.9	35.4	30.4	15.2	0.0
Puccinia triticina	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
Cryphonectria parasitica	Membrane and EST[3]	17	94	59	8	13.3	4.9	113	Breuillin *et al.* (2006)
Erysiphe necator	Beads	18	44	11	DA	DA	DA	DA	Unpublished
Fusarium culmorum	Beads	9	100	89	7	13.0	6.1	20	Giraud *et al.* (2002a)
Fusarium poae	Beads	20	10	10	DA	DA	DA	DA	Unpublished
Magnaporthe grisea	Beads and EST[3]	19	50	30	24	16.7	2.9	6	Kaye *et al.* (2003) and unpublished
Melampsora larici-populina	Beads and membrane	37	41	35	12	9.7	4.9	30	Barrès *et al.* (2005)
Microbotryum violaceum	Beads	44	100	100	44	11.4	5.4	30	Giraud *et al.* (2002b)
Microcyclus ulei	Beads	14	93	93	11	15.0	2.9	16	Le Guen *et al.* (2004) and unpublished
Mycosphaerella eumusae	Beads	19	58	47	9	8.6	2.9	15	Unpublished
Mycosphaerella fijiensis	Beads	48	69	69	33	10.2	4.6	15	Unpublished
Mycosphaerella musicola	Beads	19	63	63	12	10.2	4.1	15	Unpublished
Penicillium camembertii	Beads	13	100	92	11	12.2	2.6	5	Unpublished
Penicillium roqueforti	Beads	19	84	37	4	11.8	3.5	5	Unpublished
Plasmopara viticola	Beads	15	40	40	6	8.2	4.5	100	Delmotte *et al.* (2006)
Puccinia striiformis fsp tritici	Beads	28	86	43	11	7.3	2.5	96	Enjalbert *et al.* (2002)
Puccinia triticina	Beads	36	86	33	12	8.1	3.2	15	Duan *et al.* (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
Alternaria brassicicola	Beads	21	81	57	12	8.7	2.9	46	Avenot *et al.* (2005)
Armillaria ostoyae	Membrane	12	100	100	12	9.9	7.2	23	Langrell *et al.* (2001)
Ascochyta rabiei	Non enriched	37	70	54	20	12.6	4.2	22	Geistlinger *et al.* (2000)
Aspergillus flavus	GenBank[5]	NA	NA	NA	6	13.4	7.4	20	Tran-Dinh & Carter (2000)
Aspergillus parasiticus	GenBank	NA	NA	NA	6	13.4	5.2	15	Tran-Dinh & Carter (2000)
Aspergillus fumigatus	Non enriched	8	100	50	4	19.3	14.0	100	Bart-Delabesse *et al.* (1998)
Beauveria brongniartii	Beads	11	91	91	10	11.8	3.8	96	Enkerli *et al.* (2001)
Beauveria bassiana	Beads	NR	NR	NR	8	20.0	6.3	24	Rehner & Buckley (2003)
Botryosphaeria parva	ISSR[6]	20	NR	35	5	9.0	3.0	82	Slippers *et al.* (2004)
Botryosphaeria rhodina	ISSR	19	68	42	5	5.0	4.0	9	Burgess *et al.* (2003)
Coccidioides immitis	Non enriched	9	100	100	9	13.6	9.9	25	Fisher *et al.* (1999)
Cryphonectria eucalypti	FIASCO[7]	NR	NR	NR	8	20.3	7.0	20	Nakabonge *et al.* (2005)
Cylindrocladium parasiticum	ISSR	25	NR	48	8	7.5	3.5	17	Wright et al. (2006)
Dothistroma pini	Anchored and EST	9	78	33	4	6.7	2.7	12	Ganley & Bradshaw (2001)
Fusarium oxysporum	ISSR	NR	NR	NR	9	12.5	11	64	Bogale et al. (2005)
Heterobasidion annosum	Beads	NR	NR	NR	5	11.3	6.5	19	Johannesson & Stenlid (2004)
Laccaria bicolor	Beads	150	26	5	7	8.7	7.0	12	Jany et al. (2006)
Leptosphaeria maculans	Non enriched and EST	36	94	19	6	9.5	2.3	14	Hayden *et al.* (2004)
Lobaria pulmonaria	Beads	21	57	52	11	14.5	8.3	30	Walser *et al.* (2003)
Melampsora lini	FIASCO	41	83	27	10	7.5	4	102	Barett & Brubaker (2006)
Metarhizium anisopliae	Beads	14	100	100	8	10.6	5.4	34	Enkerli *et al.* (2005)
Mycosphaerella fijiensis	Beads	19	58	58	10	20.2	5.0	29	Neu *et al.* (1999)
Mycosphaerella musicola	Beads	48	NR	54	24	22.1	3.4	24	Molina *et al.* (2001)
Mycosphaerella populorum	EST	10	NR	50	3	11	5	77	Feau et al. (2006)
Paecilomyces fumosoroseus	Beads	9	100	89	8	14.4	3.6	26	Dalleau-Clouet *et al.* (2005)

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