**In silico** comparative analysis of SSR markers in plants

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**Abstract**

**Background:** The adverse environmental conditions impose extreme limitation to growth and plant development, restricting the genetic potential and reflecting on plant yield losses. The progress obtained by classic plant breeding methods aiming at increasing abiotic stress tolerances have not been enough to cope with increasing food demands. New target genes need to be identified to reach this goal, which requires extensive studies of the related biological mechanisms. Comparative analyses in ancestral plant groups can help to elucidate yet unclear biological processes.

**Results:** In this study, we surveyed the occurrence patterns of expressed sequence tag-derived microsatellite markers for model plants. A total of 13,133 SSR markers were discovered using the SSRLocator software in non-redundant EST databases made for all eleven species chosen for this study. The dimer motifs are more frequent in lower plant species, such as green algae and mosses, and the trimer motifs are more frequent for the majority of higher plant groups, such as monocots and dicots. With this *in silico* study we confirm several microsatellite plant survey results made with available bioinformatics tools.

**Conclusions:** The comparative studies of EST-SSR markers among all plant lineages is well suited for plant evolution studies as well as for future studies of transferability of molecular markers.

**Background**

In agriculture, productivity is affected by environmental conditions such as drought, salinity, high radiation and extreme temperatures faced by plants during their life cycle, that impose severe limitations to the growth and propagation, restricting their genetic potential and, ultimately, reflecting yield losses of agricultural crops. Although, advances have been achieved through classical breeding, further progress is needed to increase abiotic stress tolerance in cultivated plants. New gene targets need to be identified in order to reach these goals, requiring extensive studies concerning the biological processes related to abiotic stresses. Comparative analysis between primitive and related groups of cultivated species may shed some light on the understanding of these processes.

Microsatellites or SSRs (Simple Sequence Repeats) are sequences in which one or few bases are tandemly repeated, ranging from 1-6 base pair (bp) long units. They are ubiquitous in prokaryotes and eukaryotes, present even in the smallest bacterial genomes [1-3]. Variations in SSR regions originate mostly from errors during the replication process, frequently DNA Polymerase slippage. These errors generate base pair insertions or deletions, resulting, respectively, in larger or smaller regions [4]. SSR assessments in the human genome have shown that many diseases are caused by mutation in these sequences [5]. The genomic abundance of microsatellites, and their ability to associate with many phenotypes, make this class of molecular markers a powerful tool for diverse application in plant genetics. The identification of microsatellite markers derived from EST (or cDNAs), and described as functional markers, represents an even more useful possibility for these markers when compared to those based on assessing anonymous regions [6-8]. EST-SSRs offer some advantages over other genomic DNA-based markers, such as detecting the variation in the expressed portion of the genome, giving a “perfect”marker-trait association; they can be developed from EST databases.
at no cost and unlike genomic SSRs, they may be used across a number of related species [9].

Many studies indicate UTRs as being more abundant in microsatellites than CDS regions [10]. In a study of micro- and minisatellite distribution in UTR and CDS regions using the Unigene database for several higher plants groups, higher occurrence of these elements in coding regions were found for all the studied species [11]. Disagreements between earlier reports and the later, reflect a deficiency in annotation when translated and non-translated fractions are separated in the Unigene transcript database. Dimer repeats were also frequent in CDS regions, which could be due to the fact that the Unigene database contains predominantly EST clusters. Therefore, there is a tendency for under-representing the UTR regions in the annotated sequences [11].

The characterization of tandem repeats and their variation within and between different plant families, could facilitate their use as genetic markers and consequently allow plant-breeding strategies that focus on the transfer of markers from model to orphan species to be applied. EST-SSR also have a higher probability of being in linkage disequilibrium with genes/QTLs controlling economic traits, making them more useful in studies involving marker-trait association, QTL mapping and genetic diversity analysis [9].

On model organisms, microsatellites have been reported to correspond to 0.85% of Arabidopsis thaliana (L.) Heynh, 0.37% of maize (Zea mays L.), 3.21% of tiger puffer (Takifugu rubripes Temminck & Schlegel), 0.21% of the nematode Caenorhabditis elegans Maupas and 0.30% of yeast (Saccharomyces cerevisiae Meyer ex. E.C. Hansen) genomes [10]. Moreover, they constitute 3.00% of the human genome [12]. All kinds of repeated element motifs, excluding trimers and hexamers, are significantly less frequent in the coding sequences when compared to intergenic DNA stretches of A. thaliana, Z. mays, Oryza sativa subsp japonica S. Kato (rice), Glycine max (L.) Merr. (soybean) and Triticum aestivum L. (wheat) [10].

Close to 48.67% of repeat elements found in many species are formed by dimer motifs. In Picea abies (L.) H. Karst. (Norway spruce), for example, the dimer occurrence is 20 times more frequent in clones originating from intergenic regions vs. transcript regions [13]. Approximately 14% of protein translated sequences (CDS - coding sequences) contain repetitive DNA regions, and this phenomenon is 3 folds more frequent in eukaryotes than prokaryotes [14]. Clustering studies showing microsatellite occurrence in distinct protein families (non-homologous) from either prokaryotic or eukaryotic genomes, indicate that the origins of these loci occurred after eukaryotic evolution [14-16]. The highest and lowest repeat counts were found in rodents and C. elegans, respectively [3].

In plant species, some reports have described the levels of occurrence of microsatellites associated to transcribed regions [7,8,10,11,17-22]. However, some comparative and/or descriptive approaches, still can offer new perspectives on the features of these markers. Furthermore, frequently new groups of plant species have their genome sequenced, enabling the reassessment of databases using new sequences, representing divergent evolutionary groups and/or with different genetic models.

The online platforms for nucleotide, protein and transcript (ESTs) databases available for the majority of species are relatively small when compared with model species, e.g Physcomitrella patens (Hedw.) Bruch & Schimp., O. sativa and A. thaliana. Since the protocols for the isolation of repetitive element loci, such as microsatellites, require intensive labour and can be expensive, the exploitation of these elements in silico on databases of model plants and their respective transfer to orphan species, is a potentially fruitful strategy.

In this study we present our results on the SSR survey for the development of plant SSR markers. The survey was based on clustered non-redundant EST data, their classification, characterization and comparative analysis in eleven phylogenetically distant plant species including two green algae, a hepatic, two mosses, two fern, two gymnosperms, a monocot and a dicot.

Results and Discussion

We analysed 560,360 virtual transcripts with the SSRLocator software (Table 1). The species with most abundant records in Genbank was Arabidopsis thaliana with 224,496 virtual transcripts (40%), followed by Oryza sativa with 121,635 (21.7%), Physcomitrella patens with 79,537 (14.19%), Pinus taeda with 58,522 (10.44%) and Chlamydomonas reinhardtii with 40,525 (7.2%).

The remaining species added up to 11.7% of virtual transcripts analysed. When total genome sizes are compared for the model plants included in this analysis, the virtual transcripts of P. patens (511 Mb) represent 0.01% of genome size. For O. sativa (389 Mb) and A. thaliana (109.2 Mb) the ESTs analysed represent 0.02% and 0.18%, respectively, of the genome. The highest average bp count per EST sequence was found for Selaginella spp. (924 bp) followed by M. polymorpha (777 bp), C. reinhardtii (775 bp) and P. taeda (760 bp). The lower average bp per sequence was found for G. gnemon (563 bp) and A. capillus-veneris (580 bp). For the model plants, A. thaliana showed the lowest average bp count (321 bp), with P. patens and O. sativa presenting similar bp counts (737 and 755 bp, respectively). Shorter observed sequences could be an indication of
incomplete representation of genes, but one must keep in mind that average gene sizes could vary among species, i.e., rice fl-cDNAs (1,747 bp) are 14% longer than Arabidopsis fl-cDNAs (1,532 bp) (TAIR 9 and RIKEN, accessed in 12.2.2010). The overall bp counts are very similar to those found by other authors [23].

The frequency of SSR per EST database was higher (4.66%) in Selaginella spp virtual transcripts (Table 2). For model plants, 3.57% and 0.84% SSRs/EST were found for O. sativa and A. thaliana, respectively.

The average motif length, excluding compound SSRs, was 27.03 bp. Mesostigma EST database shows the longest SSR average size with 34.13 bp, and the shortest size was found for Marchantia polymorpha with 22.56 bp mean size. The SSR size for model plants was similar. For P. patens, O. sativa and A. thaliana, average sizes of 24.2, 23.4 and 26.5 bp were found, respectively. A total 1,106 EST sequences contained more than one SSR. Among the species, O. sativa and P. patens are on the extremes of the distribution with 37.34% and 3.46% of virtual transcripts containing one or more microsatellites. However, Adiantum capillus-veneris EST database contained the highest percentage of transcripts displaying more than one SSR (20.86%) based on the database size. Similar results were found in our group [11], using the Unigene database for grasses and other allies. In the same study, rice was shown to have the highest frequency of ESTs containing more than one SSR (11.28%). In the present study, a similar value was found for rice (10.20%). These small differences could be due to different redundancy reduction parameters used in Unigene species database and CAP3 default settings. Other reports for higher plants [19,20,24-26], showed different ranges, but never higher than 2-3 fold. The variations encountered in different reports are related to the strategy employed by investigators (software, repeat number and motif type) [11]. The results for each species, regarding the percentage of SSRs found per EST database size are shown on Table 2.

### Table 1 EST database size and Overall occurrence of SSR, percentages and average length motifs per species

| Species                      | EST database count | sbp | Average pg count per EST | GC Content % |
|------------------------------|--------------------|-----|--------------------------|--------------|
| Chlamydomonas reinhardtii    | 40,525             | 31,388,333 | 775                      | 57.22        |
| Mesostigma vinde             | 6,401              | 4,273,634   | 668                      | 51.36        |
| Marchantia polymorpha        | 10,086             | 7,836,025   | 777                      | 54.75        |
| Syntrichia ruralis           | 7,114              | 4,764,692   | 670                      | 49.20        |
| Physcomitrella patens        | 79,537             | 58,636,814  | 737                      | 47.60        |
| Selaginella spp.             | 19,830             | 18,318,250  | 924                      | 51.38        |
| Adiantum capillus-veneris    | 16,138             | 9,363,530   | 580                      | 45.97        |
| Gnetum gnemon                | 6,076              | 3,420,021   | 563                      | 44.33        |
| Pinus taeda                  | 58,522             | 44,467,932  | 760                      | 43.64        |
| Oryza sativa                 | 121,635            | 91,859,132  | 755                      | 47.52        |
| Arabidopsis thaliana         | 224,496            | 72,013,660  | 321                      | 41.10        |

### Table 2 EST database size and Overall occurrences of SSRs, percentages and average length motifs per species

| Species                      | Number of SSR loc | SSR/EST database (%) | Average motif length (bp) | EST sequences with SSRs (%) | N. of seq. containing more than one SSR (%) | Single SSRs | Compound SSRs |
|------------------------------|-------------------|----------------------|---------------------------|-----------------------------|--------------------------------------------|-------------|--------------|
| Chlamydomonas reinhardtii    | 980               | 2.41                 | 33.21                     | 886 (2.19)                  | 94 (9.78)                                  | 899         | 81           |
| Mesostigma vinde             | 81                | 1.26                 | 34.12                     | 73 (1.14)                   | 8 (0.87)                                  | 73          | 8            |
| Marchantia polymorpha        | 437               | 4.33                 | 22.56                     | 436 (4.32)                  | 1 (0.52)                                  | 425         | 12           |
| Syntrichia ruralis           | 190               | 2.67                 | 23.84                     | 149 (2.09)                  | 41 (10.09)                                | 189         | 1            |
| Physcomitrella patens        | 2753              | 3.46                 | 24.20                     | 2577 (3.24)                 | 176 (6.6)                                 | 2670        | 83           |
| Selaginella spp.             | 968               | 4.66                 | 23.71                     | 868 (4.38)                  | 100 (11.13)                               | 927         | 41           |
| Adiantum capillus-veneris    | 749               | 4.64                 | 31.14                     | 599 (3.71)                  | 150 (20.86)                               | 624         | 125          |
| Gnetum gnemon                | 212               | 3.48                 | 23.62                     | 195 (3.21)                  | 17 (8.45)                                 | 203         | 9            |
| Pinus taeda                  | 568               | 0.97                 | 30.89                     | 530 (0.91)                  | 38 (6.85)                                 | 539         | 29           |
| Oryza sativa                 | 4347              | 3.57                 | 23.44                     | 3934 (3.23)                 | 413 (10.19)                               | 4199        | 148          |
| Arabidopsis thaliana         | 1890              | 0.84                 | 26.52                     | 1822 (0.81)                 | 68 (3.62)                                 | 1837        | 53           |
The microsatellite survey using SSRLocator showed that 13,133 SSRs were available as potential marker loci. From those, 12,585 loci were found in single formation and only 590 were found in compound formation. The fern *A. capillus-veneris* showed the highest percentage (20%) of compound SSR loci. When compared with other available SSR marker search tools, similar results were found. Using MISA software, a total of 13,861 SSRs were available as potential marker loci, being 13,172 SSRs single and 689 compound SSRs for all studied species. *Adiantum* EST database showed the highest percentage of SSR in compound formation (15.55%). This trend does not hold for the majority of lower plants. *P. patens*, for example, presented few EST-SSRs in compound formation (3.57%) and possibly the fern lower database size is masking the results. When it is compared with the majority of plant groups, *P. taeda* is the only species showing a high percentage of compound SSRs (5.81%), corroborating other studies which report that compound and imperfect tandem repeats are most common in pines [27-29].

A total of 3,723 EST-SSRs were found in *P. patens* database using the MISA software [23]. The SSRLocator analysis resulted in 2,839 SSR for this species. When the same non-redundant databases were run in other bioinformatics tools, the results were similar to MISA. Using the SciKoko package [30] combined with MISA, Sputinik and Modified scripts, it was possible to narrow SSR results to a 2-fold range variation.

The search for repetitive elements in EST databases of the eleven taxa listed above enabled the comparison of patterns of occurrence of these elements in lower and higher plants (Figure 1). In some species such as *C. reinhardtii*, *Mesostigma viride* and bryophytes, we found that dimer (NN) microsatellites are more common when compared to higher plants (Figure 2). The trimer (NNN) microsatellites are predominant in higher plants (See additional files), in agreement with other SSR survey studies [6,10,11,21] supporting the relative distribution of motifs in these plant groups. However, gymnosperm species showed the lowest SSR occurrence within the derived plant groups. *Pinus* and *Gnetum* results indicate low SSR frequencies as intrinsic characteristics of gymnosperms, such as suggested by other results obtained with distinct methods [10,23,28,29]. The patterns of occurrence of dimers and trimers found in the EST databases of the selected species are shown on Additional files 1 and 2, respectively.

The average GC-content in the 11 datasets was 48.55%. Significantly increased GC-contents were detected for the green algae *Chlamydomonas* (57.22%) and *Mesostigma* (51.36%), for the moss *Syntrichia ruralis* (54.75%) and the fern moss *Sellaginella* spp. (51.38%). These results are in agreement with other genomic comparative analyses of a wide range of plant groups, where the lower groups presented the higher contents [23,31,32]. The remaining species showed similar results (Table 1).

**Dimer and Trimer most frequent motifs**

For algae species, the most frequent dimer motifs were AC/GT and CA/TG (Figure 2). For example, in *C. reinhardtii*, from 548 dimer occurrences, 199 AC/GT and 233 CA/TG motifs were found. The predominant trimer motifs found were GCC/GGC, GCC/GGC and GCC/GGC with 55, 46 and 39 occurrences in 263 trimers found for algae species. For nonvascular plants, the predominant dimer motifs were AG/CT (239/1,049), AT/AT (226/1,049) and GA/TG (340/1,049), as found for *P. patens*. For mosses, the most

![Figure 1 SSR motifs occurrences by plant group studied](image)
frequent trimers found within the studied species were GCA/TGC, AAG/CTT and AGC/GCT. For vascular plants, the most frequent motifs were AG/CT and GA/TC. In *O. sativa*, 246 (43%) and 191 (33%) occurrences for these motifs were found, respectively, in a total of 578 dimer occurrences. The GC/GC was only detected in *C. reinhardtii*. There has been a report on the abundance of GC elements in *Chlamydomonas* genome libraries [33].

For the other species this motif has not been reported in high frequencies [10,11,23,28,34].

Among trimer motifs, there was a predominance of AAG/CTT, AGA/TCT, GGA/TCC and GAA/TTC in higher plants. In lower plants, the motifs GCA/TGC and CAG/CTG were predominant. The trimer motif CCG/CGG is predominant in the algae *C. reinhardtii* and the model moss *P. patens*, and could reflect the high GC content in these two species. However, this relationship does not hold for the other cryptogams analysed. The increased CCG/CGG frequency has been described earlier for grasses and has been related to a high GC-content [10]. In this context, the CCG/CGG increase in *Chlamydomonas* and *P. patens* could reflect the high GC content in these two species. However, this relationship does not hold for the other cryptogams analysed. The increased CCG/CGG frequency has been described earlier for grasses and has been related to a high GC-content [10]. In this context, the CCG/CGG increase in *Chlamydomonas* and *P. patens* was consistent, but, a previous study reported that it can not be taken as a rule, since higher GC values were found for other lower groups with low CCG/CGG contents [23]. For rice CCG/CGG is the predominant motif and its content appears to be high in the members of the grass family [11,21].

Comparing all plant groups selected for this *in silico* study, the most frequent dimer motifs found were AG/CT and GA/TC, occurring for all plant species. The most frequent trimers were AAG/CTT and GCA/TGC occurring in the 11 studied species.

**Tetramers, Pentamers and Hexamers**

Tetramer and pentamer motifs were rare for all studied species except for *M. viride*. This algae showed the higher frequencies in loci formed by motifs longer than three nucleotides with 36.95% of tetramer and 19.56% of pentamer motifs. Although these results are in agreement with other study [23], it is difficult to state that this is a rule for this species, since the EST database size for *Mesostigma* is the smallest one available among the studied databases. In general, tetramer and pentamer motifs predominantly found for *Oryza*, *Physcomitrella* and *Selaginella* where CATC/GATG, CTCC/GGAG, GATC/GATC, TGCT/AGCA (Additional file 4) and CTTCT/AGAAG, GGAGA/TCTCC, GGCAG/CTGCC, TCTCG/CGAGA and TGCTG/CAGCA (Additional file 5) and these were the most frequent motifs, at least for two out of three of these species.

Hexamer motifs were predominant in novel taxa such as gymnosperms and flowering plants [3,21,35]. *P. taeda* and *G. gnemom* showed the highest frequency (26.95%) of these motifs, but none of the hexamer motifs found in *Gnetum* and *Pinus* were found in common with other plant EST databases. However, one can not state the absence of hexamer motif patterns in plant groups, since in Bryophytes there is a possibility of patterns occurring within closely related groups. For *P. patens* and *M. polymorpha* the AGCAGG/AGCAGG, AGCTGG/CCAGGT, CAGCAA/TTGCTG and TGGTGC/GCA

**Figure 2** Predominant loci containing dinucleotide microsatellites motifs per species.
Regarding biological processes, the majority of SSR loci found were involved with metabolic (32.17%) and cellular (31.02%) processes (Figure 3). Comparing all P. patens genome sequences with Gene Ontology assignment and those containing SSRs (Figure 4), there was a concentration of SSRs in metabolic process genes. Biological adhesion, rhythmic processes, growth and cell killing processes had the lowest SSR contents among the P. patens transcripts. Similar results were found comparing P. patens and A. thaliana EST libraries [37]. This author suggested that genes that are involved in protein metabolism and biosynthesis are well conserved between mosses and vascular plants. These patterns were confirmed for mosses using Syntrichia ruralis and P. patens transcript databases, respectively [38,39]. For cellular components (Figure 5) the majority of SSRs found are related to intracellular component gene sequences (52.52%) and membrane elements (12.15%). This ontology levels were reported as the majority of GO assignments in for P. patens annotated sequences [39]. Currently, more than half of cellular component GO annotations for P. patens genome [32] are related with membrane structure (Figure 6). Our results show the enrichment of SSR occurrence mainly for genes related to this structural level. The whole genome molecular function assignment level in Gene Ontology revealed a predominance of binding genes (80.51%), suggesting these are representatively higher in P. patens genome (Figure 7). However, when EST sequences containing SSRs are assessed with the Gene Ontology assigned molecular function (Figure 8), a relative increase of other functions is revealed. Sequences associated with binding decrease (42.81%), and those related to catalytic activity (33.76%), and structural molecule activity (10.80%) increase. These findings agree to the expectations concerning the cellular function and are consistent with ratios observed for rice, Arabidopsis, and for the bryophytes Syntrichia ruralis and P. patens [32,38-41]. The higher occurrence of SSR loci in this ontology level indicate a good potential for using these molecular markers to saturate pathways associated to those functions described above.

**Predicted coding for SSR loci**

The predicted amino acid content for the SSR loci detected in the eleven species studied is shown in Figure 9. The amino acids arginine (Arg), alanine (Ala) and Serine (Ser) were predominant for all species. Alanine was predominant for the majority of cryptogams, ranging from 14.85% to 29.7%. Exceptions were observed for Adiantum, Mesostigma and Physcomitrella, in which serine (Ser), glutamic acid (Glu) and leucine (Leu) were the predominant amino acid (up to 17%). Serine (up to 11%) was predominant for fern species and for Gnetum.

### Table 3 Distribution of Blast hits for *Physcomitrella patens* SSR loci sequences against several taxa with GO assignment

| Taxa                      | Best Hits (%) |
|---------------------------|---------------|
| *Physcomitrella patens*   | 26.90         |
| Oryza sativa              | 10.89         |
| Vitis vinifera            | 10.80         |
| Arabidopsis thaliana      | 9.00          |
| Populus trichocarpa       | 8.60          |
| Zea mays                  | 7.18          |
| Picea sitchensis          | 5.60          |
| Ricinus communis          | 4.80          |
| Glycine max               | 3.90          |
| Sorghum bicolor           | 3.90          |
| Medicago truncatula       | 1.48          |
| Nicotiana tabacum         | 0.75          |
| Solanum tuberosum         | 0.63          |
| Micromonas pusilla        | 0.56          |
| Micromonas sp.            | 0.55          |
| Chlamydomonas reinhardtii | 0.48          |
| Triticum aestivum         | 0.47          |
| Solanum lycopersicum      | 0.46          |
| Elaeis guineensis         | 0.41          |
| Hordeum vulgare           | 0.40          |
| Ostreococcus lucimarus    | 0.39          |
| Ostreococcus tauri        | 0.35          |
| Cyanophyceae sp.          | 0.29          |
| Pisum sativum             | 0.28          |
| Brassica rapa             | 0.28          |
| Spinacia oleracea         | 0.25          |
| Gossypium hirsutum        | 0.21          |
| Pinus contorta            | 0.21          |

CCA motifs occur in both species (Additional file 6). Based on plastid molecular data, Marchantiophyta and Bryophyta originated about 450 Mya [36] and it is possible that some repeats are conserved for recently formed groups, but it would be necessary to include others species in further analyses to confirm this hypothesis. For the other SSR types (7, 8, 9 and 10 repeats) frequencies were very low (less than 2 occurrences per motif) and were not further characterized.

**Physcomitrella patens** SSR loci versus Gene Ontology assignments

For the 4,909 SSR loci found for P. patens EST sequences, 1,750 had GO assignments. More than 25% of these hits were exclusive to P. patens. However, up to 70% of SSR loci were found as conserved across the moss and the higher plant species O. sativa, Vitis vinifera L. and A. thaliana. On Table 3, the distribution of the best Blast hits is presented.
**Figure 3** Distribution of *Physcomitrella patens* SSR loci within sequences of known biological processes in Gene Ontology.

**Figure 4** Distribution of *Physcomitrella patens* genome sequences with Gene Ontology assignments into biological processes. (Data: Rensing et al., 2008).
Figure 5 Distribution of *Physcomitrella patens* SSR loci within sequences of known cellular component in Gene Ontology.

Figure 6 Distribution of *Physcomitrella patens* genome sequences with Gene Ontology assignments into cellular component. (Data: Rensing et al., 2008).
and Arabidopsis, Pinus and Oryza showed arginine as the predominant amino acid (10.46% and 23.31%, respectively). Tyrosine (Tyr), asparagine (Asp), aspartic acid (Asn) were the amino acids found at lower frequencies among SSR loci for all species and were practically absent in the algae species surveyed. In bryophytes, methionine was only found in Physcomitrella, but at a small frequency (1.7%). For all higher plant species databases used in this survey, arginine, alanine, serine, glutamic acid, proline (Pro) and leucine were among the

Figure 7 Distribution of Physcomitrella patens SSR loci within sequences of known molecular function in Gene Ontology.

Figure 8 Distribution of Physcomitrella patens genome sequences with Gene Ontology assignments into molecular function. (Data: Rensing et al., 2008).
predominant amino acids, agreeing with previous reports for flowering plants [11,3,22,42-45]. No reports were found for amino acid distribution in SSR loci in lower plants.

The small EST databases available for some species did not seem to have hampered the results, since the predicted loci distribution found were consistent within the taxonomic groups. The absence of a relationship between genome size and tandem repeat loci content were reported based in grass genome studies [11], where large genomes such as sugarcane (Saccharum officinarum L.), maize and wheat did not present higher frequencies of SSR loci.

**Relationship of Codon-bias with EST-SSR motif occurrences**

The high GC-content in some EST-SSR motifs found in the present study can be a result of a codon usage preference by plant species. When we compare the codon usage for the model species included in this study (Chlamydomonas reinhardtii, Physcomitrella patens, Oryza sativa and Arabidopsis thaliana) the occurrence of some repeat motifs are reflected in codon-bias known for each species. Higher frequencies of GC were found in the first and third codon position for all four species. However, for the basal plant (C. reinhardtii), the preference for GC3 was much higher than the other three species. The first (GC1) and the third (GC3) codon position reached 64.8% and 86.21% of the occurrences, respectively. For rice, GC1 and GC3 frequencies were 58.19% and 61.6%, respectively. For the other model plants, the occurrences at GC3 were lower than the occurrences in GC1, i.e., for Physcomitrella patens and Arabidopsis thaliana, GC1 (55.49% and 50.84%, respectively) and GC3 (54.6% and 42.4%, respectively) values were found. When one associates these codon usage values with the SSR motif frequencies found, a striking result is obtained for C. reinhardtii and rice. In the first, the most frequent motifs were GCA/TGC, CAG/CTG and GCC/GGC and could be explained by the GC1s and GC3s codon preference. In rice the CCG/CGG predominant motif could also be a reflection of GC3s codon preference. For Arabidopsis, the most frequent motif found in this study (GAA/TTC) is also the most preferred codon used by this species (GAA) with 34.3% of the occurrences. It also reflects the GC1 preference in the codon usage in this species. In the model moss species the most frequent motifs do not show a relationship with the GC codon usage (Figure 10). Despite the similarities in average codon bias between P. patens and Arabidopsis thaliana, the distribution pattern is different, with 15% of moss genes being unbiased [46]. An association between the frequency of microsatellite motifs and codon usage could explain the occurrences found in P. patens. For example, the most representative motifs GCA/TGC, AAG/CTT and AGC/GCT are also found among the most used codons GCA, AAG and AGC (20.7%, 33.6% and 15%, respectively).

The width of the GC3 distribution in flowering plants was found to be a result of variation in the levels of
directional mutation pressure or selection against mutational biases. Likewise, the low frequency of GC2 occurrences is a result of a strong selective pressure against peptide substitution. The balance between these forces could be shaping the distribution of EST-SSR by means of codon usage preference [47].

**Positive and negative selection sites in EST-SSR across species**

SSRs represent hyper mutable loci subject to reversible changes in their length [8]. Significant differences in SSR representations exist even among closely related species, suggesting that SSR abundance may change relatively rapidly during evolution [48]. To infer about the selection pressures (dN/dS ratio) on EST-SSR found for the 11 species chosen for this work, we used the common most frequent motif in all species (AAG/CTT and GCA/TGC). The dN-dS test revealed few negatively selected sites in the triplets for each EST-SSR (Additional file 7). The positive selection in SSR based sequence was reported in other studies [8,49-51]. More than 50% of sites for both motifs analyzed across species were under a positive selection (dN/dS > 1), suggesting a weak selection pressure on these EST-SSR motifs, as was reported for other species [52,53]. The occurrence of selective sweeps or background selection in ancestral lineages [54] cannot be discarded, however it could not be tested with the present data.

**In silico transferability of EST-SSR across species**

Across-species transferability of EST-SSRs is greater than genomic SSRs, as they originate from expressed regions and therefore they are more conserved across a number of related species [6].

The virtual PCR shows a lower transferability of *Chlamydomonas reinhardtii* EST-SSR for most of the
plant species tested. The best results were found for *Adiantum* and *Arabidopsis*, where successful rates of positive EST-SSR amplicons derived from algae were 26% and 9%, respectively. When EST-SSR primers designed from *Arabidopsis* were used against other species, again low transferability rates were found, being the best positive cases found in *Physcomitrella, Pinus* and rice with amplification rates of 1.04%, 1.20% and 1.90%. The summary of in silico PCR results can be accessed in the Additional files section of this article. Some reports suggest that SSR markers have higher transferability rates when used between closely related species [6,22,55]. In this work virtual PCR amplification did follow the same trend.

For the positive EST-SSRs found for the in silico transfer, ten sets of *Physcomitrella* EST-SSR primers were used to illustrate the transferability results using an electronic tool [56] to simulate gel electrophoresis (Figure 11). For the three tested EST-databases only two primers amplified a single locus in each species (SSR9 and SSR10). In the other sets 2, 3 and even 4 virtual amplicons were observed (Additional file 8). For *Chlamydomonas*, 70% of the tested primers resulted in one amplicon and 10% each resulted in 2, 3 or 4 amplifications. However, only 20% of amplicons obtained in this algae species are related to the EST-SSR sequence, suggesting that the majority of designed EST-SSR primers act as degenerate when applied to *Chlamydomonas*. For rice, 30%, 40% and 10% of tested primers resulted in one, two or three amplifications, respectively. In *Arabidopsis* 40%, 40% and 20% of tested primers results in one, two or three amplifications, respectively. For both flowering plants, 50% of tested primers amplified moss EST-SSR homologue sequences, showing a high rate of success for transferability across species. These results agree with other studies where the transfer success rates decrease with the increasing evolutionary distance [55,57-60]. The use of this molecular marker across distant taxonomical groups are not impossible, however our findings confirm that only a few retain their EST-SSR homologue sequences, making this effort hardly worthwhile [61].

**Conclusions**

These results make it possible to create strategies for transferring molecular markers based on microsatellites from model to orphan species. Microsatellites were found in all species studied and variable transfer rates were found as a function of genetic distance among taxa. The motifs found are influenced by species codon usage preference. The two most common motifs among the eleven species are under a positive selection pressure. Primers generating one amplicon in the genome of origin may generate multiple amplicons in other taxa and only a few retain their original targeting sequence. The similarities between the
results here presented and other initiatives using similar bioinformatics Perl scripts, such as MISA [23], support SSRLocator as a useful tool for SSR survey analyses.

Methods

An exploratory in silico analysis of SSRs was made in ESTs databases of 11 taxa, as follows: two unicellular green algae (Chlamydomonas reinhardtii Dang, Mesostigma viride Lauterborn.), three bryophytes s. l. [Marchantia polymorpha L., Physcomitrella patens and Syntricha ruralis (Hedw.) Weber & Mohr], two ferns (Selaginella spp. and Adiantum capillus-veneris L.), two gymnosperms (Gnetum gnemon L. and Pinus taeda L.) and two flowering plants, a monocot (Oryza sativa) and a dicot (Arabidopsis thaliana). These species were chosen because the amount of available ESTs data in Genbank (NCBI). As these databases may have redundancy, we used the program CAP3 [62] for MacOSX, to construct contigs with the sequences and get non-redundant sequences for each database following the default settings.

Taxa data were loaded into the software SSRLocator [63], to investigate the presence of tandem repetitive elements (SSRs). The analysis was performed following the search parameters for repetitive elements in class I (≥ 20 bp) described as more efficient molecular markers [17]. Data resulting from in silico analyses were assessed for occurrence patterns in chosen taxa databases. The same analysis was performed using MISA script http://pgrc.ipk-gatersleben.de/misa/ software to search for SSR occurrences per contig. Several instructions in the algorithm used in SSRLocator resemble those from MISA [19] and SSRIT [17]. However, additional instructions have been inserted in SSRLocator’s code. Instead of allowing the overlap of a few nucleotides when two SSRs are adjacent to each other and one of them is shorter than the minimum size for a given class as found in MISA and SSRIT, a module written in Delphi language records the data and eliminates such overlaps. For GC content, Perl scripts were used and the results were stored in text files (.txt) for later comparative analyses.

For the predicted amino acid contents in the SSR loci, an additional routine script was written in the SSRLocator software. This script determined which amino acids were coded by trimer, hexamer and nonamer motifs found in the EST database analysed [63].

To validate the frequencies obtained using the SSRLocator software, the Physcomitrella patens EST database was chosen.

This database was run with other SSR search scripts and softwares, such as MISA [19] and SPUTINIK [64], running in SCIROKO package [30], MINE SSR http://www.genome.clemson.edu/resources/online_tools/ssr, SSRIT following the SSR categories defined above [17]. The results were exported into Microsoft Excel spreadsheets (MacOSX-Office 2008) and respectively grouped by taxon.

A codon-bias for the model plants included in this research (Chlamydomonas reinhardtii, Physcomitrella patens, Oryza sativa and Arabidopsis thaliana) was made comparing with the preferencial codon table for each species available at http://www.kazusa.or.jp/codon/. The sequences containing EST-SSR for Physcomitrella patens was submitted to CodonO server [65] to confirm the preferential codon usage compared with the know codon table for this species. To investigate the selective pressure on the triplets on the EST-SSR which occurs in all studied species a dN-dS statistics [66] was used to verify the synonymous and non-synonymous substitutions in the preferential codons nearby the repeats chosen using the molecular phylogenetics package MEGA4 [67].

The Physcomitrella patens SSR results were run through a Gene Ontology (GO) assignment database in order to assess associations between SSR loci and biological processes, cellular components and molecular function of known genes. A fasta file with all EST-SSRs found in P. patens was subjected to Blast2GO software and ran against the GO annotated sequences, and the obtained hits were compiled.

To verify the potential transferability of this molecular markers we have tested in silico all EST-SSR found for the plant ancestral lineage, and for the derivative plant group, represented here by the green algae Chlamydomonas reinhardtii and Arabidopsis thaliana, across the others species EST database used for the present SSR survey. Electronic PCR [68] was used to verify the transferability of EST-SSRs across studied species. The positive results found were used to simulate a gel electrophoresis with aid of SIMGEL.exe included in the SPCR package [56] using the Physcomitrella patens EST-SSR sequences to design primers and Chlamydomonas, rice and Arabidopsis as templates. The virtual amplicons resulted for each primer set tested across species were aligned to verify the homology between the amplicons.

Additional material

Additional file 1: Patterns of occurrence for dimer SSR motifs in percentage.

Additional file 2: Patterns of occurrence for trimer SSR motifs in percentage.

Additional file 3: Predominant trinucleotide microsatellites motifs loci occurrences per species.

Additional file 4: Predominant tetramers microsatellites motifs loci occurrences per species.

Additional file 5: Predominant pentamers microsatellites motifs loci occurrences per species.

Additional file 6: Predominant hexamers microsatellites motifs loci occurrences per species.
Additional file 7: dN/dS table for the common most frequent motifs for 11 species tested EST databases.  
Additional file 8: Eletronical PCR results table.

Acknowledgements
We would like to thank the Developmental Center of Technology (CDTec/UFFP) for the support to the first author. This work was supported by the National Council for Scientific and Technological Development CNPq (process # 480938/2009-1 and 475122/2007-0).

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Authors’ contributions
FCV carried out all in silico studies, including the SSR survey, the electronic PCR and the sequence alignment for selective sites mining and drafted the manuscript. LCM created the SSR script used and participated in the design of the study. ACO conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Received: 10 July 2010 Accepted: 19 January 2011  
Published: 19 January 2011

References
1. Morgante M, Olivieri AM: PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 1993, 3(1):175-182.
2. Jurka J, Pethiyagoda C: Simple repetitive DNA sequences from Primates: Compilation and analysis. Journal of Molecular Evolution 1994, 40:120-126.
3. Tóth G, Gáspári Z, Jurka J: Microsatellites in different eukaryotic genomes: survey and analysis. Genome Research 2000, 10:967-981.
4. Iyer RR, Pluciennik A, Rosche WA, Sinder RR, Wells RD: DNA polymerase III proofreading mutants enhance the expansion and deletion of triplet repeat sequence in Escherichia coli. Journal of Biological Chemistry 2000, 275(3):2174-2184.
5. Mirkin SM: DNA structures, repetitive expansions and human hereditary disorders. Current Opinion in Structural Biology 2006, 16(3):351-358.
6. Vashnevsky RK, Graner A, Sorells ME: Genic microsatellite markers in plants: features and applications. Trends in Biotechnology 2005, 23(1):48-55.
7. Vashnevsky RK, Hoisington DA, Tyagi AK: Advances in cereal genomics and applications in crop breeding. Trends in Biotechnology 2006, 24(11):490-499.
8. Kashy Y, King DG: Simple sequence repeats as advantageous mutators in evolution. Trends in Genetics 2006, 22:253-259.
9. Gupta PK, Rustgi S, Sharma S, Singh R, Kumar N, Balyan HS: Transferable EST-SSR markers for the study of polymorphism and diversity in bread wheat. Molecular Genetics and Genomics 2003, 270:315-323.
10. Morgante M, Hanafey M, Powell W: Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. Nature Genetics 2002, 32(2):194-200.
11. Maia LC, Souza VQ, Kopp MM, Carvalho FF, Oliveira AC: Tandem repeat distribution of gene transcripts in three plant families. Genetics and Molecular Biology 2009, 32(4):1-12.
12. Subramanian S, Mishra RK, Singh L: Genome-wide analysis of microsatellite repeats in humans: their abundance and density in specific genomic regions. Genome Biology 2003, 4(2):R13.
13. Li YC, Korol AB, Fahima T, Beiles A, Nevo E: Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular Ecology 2002, 11:2453-2465.
14. Marcotte EM, Pellegrini M, Yeates TO, Eisenberg D: A census of protein repeats. Journal of Molecular Biology 1999, 293:151.
15. Kashy Y, King D, Soller M: Simple sequence repeats as a source of quantitative genetic variation. Trends in genetics 1997, 13:74-78.
16. Wren JD, Forgacs E, Fondon JW III, Pertsemlidis A, Cheng SY, Gallardo T, Williams RS, Shohet RV, Minna JD, Garner HR: Repeat polymorphisms within gene regions: phenotypic and evolutionary implications. American Journal of Human Genetics 2000, 67:345-356.
17. Tennen Y, De Clerck G, Lukashova A, Lipovich L, Cartinholir S, McCouch S: Computational and experimental analysis of microsatellites in rice (Oryza sativa L): frequency, length variation, transposon associations, and genetic marker potential. Genome Research 2001, 118(1):1441-52.
18. McCouch SR, Teytelman L, Xu Y, et al.: Development and mapping of 2240 new SSR markers for rice (Oryza sativa L.). DNA research 2002, 9(6):199-207.
19. Thiel T, Michael A, Vashnevsky RK, Graner A: Exploiting EST databases for the development of cDNA derived microsatellite markers in barley (Hordeum vulgare L.). Theoretical and Applied Genetics 2003, 1-6:411-422.
20. Nicot N, Chiquet V, Gandon B, Amihart L, Legaz F, Leray P, Bernard M, Sourdille P: Study of simple sequence repeat (SSR) markers from wheat expressed sequence tags (ESTs). Theoretical and Applied Genetics 2004, 1-9(4):808-5.
21. Lavezzoni M, Zhang L: Distinct patterns of SSR distribution in the Arabidopsis thaliana and rice genomes. Genome Biology 2006, 7(14):3.
22. Zhang L, Yuan D, Yu S, Li Z, Cao Y, Miao Z, Qian H, Tang K: Preference of simple sequence repeats in coding and non coding regions of Arabidopsis thaliana. Bioinformatics 2004, 20:1081-1086.
23. von Steppkeberg MV, Rensing SA, Rokski K: Identification of gene mosaic SSR markers and a comparative analysis of twenty-four algal and plant gene indices reveal species-specific bias rather than group-specific characteristics of microsatellites. BMC Plant Biology 2006, 6(5).
24. Cordeiro GM, Casu R, McIntyre CL, Manners JM, Henry RJ: Microsatellite markers from sugarcane (Saccharum spp.) ESTs cross transferable to erianthus and sorghum. Plant science 2001, 16(6):1115-123.
25. Kaniety RV, La Rota M, Matthews DE, Sorells ME: Data mining for simple sequence repeats in expressed sequence tags from barley, maize, rice, sorghum and wheat. Plant molecular biology 2002, 48(3-4):5-11.
26. Asp T, Frei UK, Didion T, Nielsen KK, Lubberstedt T: Frequency, type, and distribution of EST-SSRs from three genotypes of Lollum perenne, and their conservation across orthologous sequences of Festuca arundinacea, Brachypodium distachyon, and Oryza sativa. BMC plant biology 2007, 12(7):9.
27. Echt CS, May-Marquardt P, Hesl M, Zahorchak R: Characterization of microsatellite markers in eastern white pine. Genome 1996, 39:1102-1108.
28. Echt CS, May-Marquardt P: Survey of microsatellite DNA in pine. Genome 1997, 40:9-17.
29. Fisher PJ, Gardner RC, Richardson TE: Single locus microsatellites isolated using Sanchored PCR. Nucleic Acids Research 1996, 24:4369-4372.
30. Koffler R, Schlottterer C, Leitly T, Sciliroko: A new tool for whole genome microsatellite search and investigation. Bioinformatics 2007, 23:1683-1685.
31. Qiu Y-L, Lee J, Bernasconi-Quadroni B, Soltes D, et al: The earliest Angiosperms: Evidence from mitochondrial, palstid and nuclear genomes. Nature 1999, 402:404-407.
32. Rensing SA, Lang D, Zimmer AD, et al: The Physcomitrella genome reveals insights into the conquest of land by plants. Science 2008, 319:64-69.
33. Wakurakhw W, Muller FW, Beck C, F: Two GC-rich elements of Chlamydomonas reinhardtii with complex arrangements of directly repeated sequences motifs. Plant Molecular Biology 1992, 18:143-146.
34. Yashoda R, Sumathi R, Cheshian P, Kavitha S, Ghosh M: Eucalyptus microsatellites mined in silico: survey and evaluation. Journal of Genetics 2008, 87(1):21-25.
35. Jiang D, Zhong GY, Hong QB: Analysis of microsatellites in citrus unigenes. Acta genetica Sinica 2006, 33(4):345-353.
36. Magallon S, Hili KW: Land plants (Embryophyta). In: The Timetree of Life. Edited by: S. B. Hedges, S. Kumar. Oxford, University Press, 2009:133-137.
37. Nishiyama T, Fujita T, Shin-I T, Seki M, Nomizu H, Uchiyama I, Kamiya A, Carninci P, Hayashizaki Y, Shinozaki K, Kohara Y, Hasebe M: Comparative genomics of Physcomitrella patens gametophytic transcriptome and Arabidopsis thaliana: Implication for land plant evolution. PNAS 2003, 100(13):8007-8012.
38. Oliver MI, Dowed SE, Zaragoza J, Muagat SA, Payton PR: The rehydration transcriptome of the desiccation-tolerant bryophyte Tortula ruralis: Transcript classification and analysis. BMC Genomics 2004, 5:89.
39. Lang D, Essinger J, Rokski R, Rosing SA: Representation and High-Quality Annotation of the Physcomitrella patens Transcriptome Demonstrates a High Proportion of Proteins Involved in Metabolism in Mosses. Plant Biology 2005, 7:238-250.

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Victoria et al. BMC Plant Biology 2011, 11:15 http://www.biomedcentral.com/1471-2229/11/15
ler: In silico analysis on frequency and distribution of microsatellites in ESTs of some cereal species. Cell Mol Biol Lett 2002, 7:537-546.

51. Varshney RK, Theil T, Stein N, Langridge P, Graner A: In silico analysis on frequency and distribution of microsatellites in ESTs of some cereal species. Cell Mol Biol Lett 2002, 7:217-223.

52. Ellis JR, Burke JM: EST-SSRs as a resource for population genetic analyses. Heredity 2007, 99:123-124.

53. Yatabe Y, Kane NC, Scotti-Saintagne C, Rieseberg LH: RAMPANT gene exchange across a strong reproductive barrier between the annual sunflowers, Helianthus annuus and H. petiolaris. Genetics 2007, 175:1883-1893.

54. Wright SI, Gaut BS: Molecular population genetics and the search for adaptive evolution in plants. Molecular Biology and Evolution 2005, 22(3):506-519.

55. Chapman MA, Hvala J, Strever J, et al: Development, polymorphism, and cross-taxon utility of EST-SSR markers from safflower (Carthamus tinctorius L). Theoretical and Applied Genetics 2009, 120:85-91.

56. Cao Y, Wang L, Xu K, Kou C, Zhang Y, Wei G, He J, Wang Y, Zhao L: Information theory-based algorithm for in silico prediction of PCR products with whole genomic sequences as templates. BMC bioinformatics 2005, 6:190.

57. Brondani C, Rangel PHN, Borba TCO, Brondani RPV: Transferability of microsatellite and sequence tagged site markers in Oryza species. Hereditas 2003, 138:187-192.

58. Castillo A, Budak H, Varshney RK, Dorado G, Graner A, Hernandez P: Transferability and polymorphism of barley EST-SSR markers used for phylogenetic analysis in Hordeum chilense. BMC plant biology 2008, 8:97.

59. Yodav OP, Mitchell SE, Fulton TM, Kresovich S: Tranferring molecular markers from sorghum, rice and other cereals to pearl millet and identifying polymorphic markers. Journal of SAT Agricultural Research 2008, 61:4.

60. Zeid M, Yu JK, Goldowitz I, Denton VE, et al: Cross-amplification of EST-derived markers among 16 grass species. Field Crops Research 2010, 118:33-35.

61. Barbára T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C: Cross-species transfer of nuclear microsatellites markers: potential and limitations. Molecular Ecology 2007, 16:3759-3767.

62. Huang X, Madan A: CAP3: A DNA sequence assembly program. Genome Research 1999, 9:688-877.

63. Maia LC, Palmieri DA, Souza VQ, Kopp MM, Carvalho FF, Oliveira AC: SSR Locator: Tool for Simple Sequence Repeat Discovery Integrated with Primer Design and PCR Simulation. International Journal of Plant Genomics 2008, Article ID 412696, 9 pages.

64. Abajian C, SPUTNIK. 1994 [http://espresosoftware.com/sputnik/index.html].

65. Angellotti MC, Bhuyan SB, Chen G, Wan X-F: CodonO: codon usage bias analysis within and across genomes. Nucleic Acids Research 2007, 35:W132-W136.

66. Yang Z, Bielawski JP: Statistical methods for detecting molecular adaptation. Trends in Ecology and Evolution 2000, 12:496-503.

67. Tamura K, Dudley J, Nei M, Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 2007, 24:1596-1599.

68. Schuler GD: Sequence mapping by eletronic PCR. Genome Research 1997, 7(5):541-550.