Universidade Federal do Pampa

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PROSPECÇÃO E TRANSFERIBILIDADE DE MARCADORES EST-SSR USADOS PARA ANÁLISES FILOGENÉTICAS EM *Poa annua* L.

Dissertação de Mestrado

São Gabriel

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Dissertação apresentada ao programa de Pós-graduação *Strito Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para a obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Dr. Antonio Batista Pereira

Co-orientador: Dr. Filipe de Carvalho Victoria

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Área de concentração: Ecologia e Sistemática

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"Descobri como é bom chegar quando se tem paciência. E para se chegar onde quer que seja, aprendi que não é preciso dominar a força, mas a razão. É preciso antes de mais nada querer."

Amyr Klink

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RESUMO

Poa annua L. é a única espécie invasora de plantas com flores que obteve sucesso reprodutivo na Antártica, constituindo uma ameaça para as espécies nativas desse ecossistema. A hipótese da origem e colonização dessa gramínea nesse ambiente extremo é a de que as plantas pioneiras teriam vindo da Polônia, porém não é descartada a possibilidade de mútiplos eventos de introdução e diferentes fontes de distribuição. A disponibilidade de dados de sequências expressas (EST) tem facilitado o desenvolvimento de marcadores microssatélites (SSR) que podem ser utilizados como ferramentas para estudos populacionais em diferentes níveis, fluxo gênico, níveis de parentesco e informações sobre padrões filogeográficos. O objetivo desse trabalho foi desenvolver marcadores microssatélites a partir de sequências de regiões expressas da família *Poaceae*, testar o potencial de transferência em *P. annua* e utilizar esses marcadores para análise filogeográfica de *P. annua*, a fim de esclarecer a origem e colonização dessa espécie na Antártica. A prospecção de marcadores microssatélites foi desenvolvida com ferramentas de bioinformática, através de análises *in sílico* SSR em banco de dados EST para família *Poaceae,* disponíveis no Genbank (NCBI). Foram utilizados os programas CAP3 e SSRLocator para prospecção dos marcadores microssatélites. Uma pesquisa de Termos Gene Ontology (GO) foi realizada no banco de dados de sequências ESTs para avaliar associações entre *locus* SSR e processos biológicos, componentes celulares e função molecular de genes conhecidos, utilizando os programas Blast2GO e Revigo. O teste de transferência dos *primers* e análise molecular de *P. annua* foram conduzidos através da Reação em Cadeia da Polimerase (PCR). Foram prospectadas uma lista de 568 pares de *primers*, destes foram sintetizados 28 marcadores microssatélites para a transferência em *P. annua*. 68% dos marcadores EST-SSR tiveram potencial de transferência para esta espécie. A análise sugere que as amostras da Antártica são diferentes das amostras do Chile, Brasil, Irlanda e Argentina. Além disso, foram encontrados 613 transcritos divididos em 302 famílias gênicas. Com esta análise, foi possível desenvolver ferramentas moleculares para a análise genética com *P. annua* e outras espécies de gramíneas, mapear os motivos mais frequentes e funções dos genes em cada *locus* SSR, e sugerir que os diásporos de *P. annua* encontrados na Antártica podem ter vindos de fontes distintas das populações da America do Sul.

Palavras-chave: Antártica, *Primers,* Microssatélites, Gramínea, Espécies invasoras.

ABSTRACT

Poa annua L. is the only invasive species of flowering plants that reached reproductive success in Antarctica, posing a threat to native species of this ecosystem.The hypothesis of the origin and colonization of grass in this extreme environment is the pioneer plants would have come from Poland, but it is not ruled out event of multiple introduction and different sources of distribution. Recent increase in the availability of expressed sequence data (EST) has facilitated the development of microsatellite markers (SSR) can be used as tools for population studies at different levels, gene flow, relationship of levels and patterns phylogeographical information. The objective of this study was to develop microsatellite markers from expressed sequence regions of the *Poaceae* family, test the potential transfer in *P. annua* and use these markers for phylogeographic analysis of *P. annua* in order to clarify the origin and colonization of this species in Antarctica. The prospect of microsatellite markers was developed with bioinformatics tools, through an analysis *in silico* SSR in EST database to *Poaceae* family, available in Genbank (NCBI). Were used the CAP3 and *SSRLocator* programs for prospecting of microsatellite markers. A Search terms Gene Ontology (GO) were performed in ESTs sequences database to evaluate associations between SSR *locus* and biological processes, cellular components and molecular function of known genes, using the Blast2GO and Revigo programs. The transfer test of *primers* and molecular analysis of *P. annua* was conducted by *Polymerase Chain Reaction* (PCR). Were prospected a list of 568 primer pairs, these were synthesized 28 microsatellite markers for the transfer in *P. annua*. 68% of EST-SSR markers have potential transfer for this species. The analysis suggests that the samples from Antarctica are different from samples from Chile, Brazil, Argentina and Ireland. In addition, they found 613 transcripts divided into 302 genic families. With this analysis, it was possible to develop molecular tools for genetic analysis with *P. annua* and other grass species, mapping the most frequent motifs and functions of genes in each SSR *locus*, and suggest that the introduction of *P. annua* found in Antarctica may have come from sources other than South American populations..

Keywords: Antarctica, *Primers*, Microsatellite, Grass, Invasive species.

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1 INTRODUÇÃO

Antártica é o continente mais frio, ventoso e seco do planeta (Convey, 2011). São consideradas todas as terras e gelo localizadas abaixo do paralelo 60°S (Ugolini & Bockheim, 2007). Pode ser subdividido biogeograficamente em zonas latitudinais que correspondem a regiões climáticas distintas (Ochyra, 1998). Segundo sistema apresentado por Greene (1964) e detalhado por Lewis-Smith (1984) a Antártica é classificada em Zona Subantártica, Antártica continental e Antártica Marítima.

A Antártica Marítima compreende a costa oeste da Península Antártica e os arquipélagos e ilhas abaixo do paralelo 55°S. Possui clima oceânico úmido frio e temperaturas médias mensais superiores a 0° nos meses de verão (Ochyra, 1998; Ochyra et al., 2008). Fatores ambientais, tais como temperatura baixa, água líquida disponível, regime de luz altamente sazonal e radiação UV-B elevada, com a combinação de isolamento geográfico, condições ambientais extremas e acessibilidade limitada restringiram tanto o impacto antrópico como as consequências desses impactos (Frenot et al., 2005; Cowan, et al, 2011). Além disso, a Corrente Circumpolar Antártica e os prevalecentes padrões de circulação atmosférica formavam uma forte barreira para transporte passivo de potenciais colonizadores (Barnes et al 2006;. Hughes et al 2010).

As atividades humanas estão agora reduzindo o isolamento biológico da Antártica (Tin et al. 2009). Com o aumento da atividade humana, o impacto sobre os ecossistemas terrestres tornou-se cada vez mais visível e é especialmente refletido por mudanças na vegetação e distribuição geográfica de várias espécies (Olech, 1996). A macroflora é constituída por grupos de plantas baixas, com predominância de musgos e líquens, com algumas espécies de hepáticas e três espécies de plantas com flores *Colobanthus quitensis* (Kunth) Bartl. *Deschampsia antarctica* E. Desv. (nativas) e *Poa annua* L. (invasora) (Convey, 2001; Øvstedal & Lewis Smith, 2001).

Poa annua L. é um híbrido alotetraplóide (2n=28) (Nannfeldt, 1937; Warwick, 1979.) derivado do cruzamento entre *Poa infirma e Poa supina (*2n=14) (Tutin, 1952). É uma das espécies invasoras mais amplamente distribuídas em todo o mundo. Possui origem européia e cresce em regiões polares, bem como equatoriais (Fenner, 1985). *Poa annua* L. (*Poaceae*) é a única espécie invasora de plantas com flores que obteve sucesso reprodutivo na Antártica (Chwedorzewska et. al., 2014). A grande capacidade de colonização de *P. annua* em regiões

Antárticas e Subantárticas pode estar associada a sua condição allotetraplóide e a plasticidade fenotípica dessa espécie (Frenot et. al., 1999). Outras características que permitem a sua adaptação na Antártica estão relacionadas com a grande variabilidade genética e epigenética (Chwedorzewska & Bednarek, 2012) e a combinação de diferentes sistemas reprodutivos constituem outro traço importante que favorece a adaptação *P. annua* (Koshy 1969).

Essa espécie anual cresce e se reproduz rapidamente, principalmente através de sementes que retém sua viabilidade por muitos anos, produzindo até 20.000 sementes em uma temporada (Hutchinson e Seymour 1982). É uma espécie autógama, com 0-15% de cruzamento, dependendo das condições ambientais (Ellis, 1973). Apomixia também foi observada em alguns indivíduos (Johnson et al. 1993). *Poa annua* é uma espécie extremamente variável, apresentando formas anuais (Grime 1979) ou bianuais (Tutin 1957; Warwick 1979) como também tipos perenes (Lush 1989; Till-Bottraud et al. 1990; Frenot and Gloaguen 1994). É comumente associada com habitats antrópicos, mas também pode ser encontrado em habitats naturais nas ilhas antárticas e subantárticas (Frenot et al 2005;. Chwedorzewska 2009; Molina-Montenegro et al 2015).

Poa annua, foi registrada nas ilhas subantárticas South Georgia, Marion, Prince Edward, Crozet, Kerguelen, Heard, Macquarie (Frenot, *et. al*, 2005); Ilha Decepction (Skottsberg, 1954; Longton, 1966); Estação Polonesa Henry Arctowski em 1985 (Olech, 1996); Estação Gabriel González Videla na Baía Paradise (Molina-Montenegro et al. 2015).

Na Estação Polonesa Henry Arctowski, situada na Ilha Rei Jorge, Baía do Almirantado, Olech (1996) monitorou o aparecimento e propagação de *P. annua* no período de 1985 a 1992. O primeiro registro dessa espécie foi no verão de 1985/86 com poucos indivíduos ocorrendo próximo ao local onde era realizada a limpeza dos calçados. Foi observado um aumento significativo da densidade (sete localidades) e abundância até 1989, após houve uma drástica redução da população, sendo encontrada em apenas uma localidade. Em 1991/92 foi registrado um aumento repentino do número de indivíduos na população de *P. annua* em vários pontos (cerca de 30 localiesda) formando um tapete denso.

A expansão e o aumento das populações de *P.annua* foram evidenciados no verão austral de 2006/2007 em comunidades de tundra e em 2008/2009 há cerca 1,5 km da Estação Polonesa em área de ASPA (Antarctic Specially Protected Area) 128, com aproximadamente 70 indivíduos ocupando uma área de (100m²) (Olech & Chwedorzewska, 2008; 2011). Apesar de diversas pesquisas realizadas com essa planta na Antártica (Frenot et al., 1999;2005;

Chwedorzewska et. al., 2013; 2014; Molina-Montenegro et. al., 2014) a origem dessa planta nesse continente não é totalmente esclarecida.

A hipótese para a entrada de *P. annua* na Estação Polonesa Henry Arctowski, teria sido através de diásporos originados da Polônia, transportados para a estação em 1978 junto com solo não esterilizado (Chwedorzewska et. al., 2014). Pesquisas realizadas de 2008-2010 também evidenciaram a presença de espiguetas de *Poa annua* em calçados, roupas e equipamentos de poloneses a cada expedição Antártica, sendo assim a atividade humana como principal vetor da introdução dessa espécie (Chwedorzewska et. al., 2013). Porém devido a Estação Polonesa receber elevado número de turistas e pesquisadores todo ano e cargas contendo materiais frescos de outros países como o Chile e Argentina não pode ser descartado múltiplos eventos e diversas fontes de introdução dessa espécie (Molina-Montenegro et. al., 2014; Chwedorzewska et. al., 2014).

A filogeografia é uma área de estudo relacionada com os princípios e processos que governam as distribuições geográficas das linhagens genealógicas, principalmente de nível intraespecífico (Avise, 1998), baseada em análises moleculares para inferir acerca da evolução de populações. Estas permitem concluir em relação às sequências de colonização, diversificação e extinção de linhagens gênicas em determinadas regiões (Lanteri & Confalonieri, 2002).

Uma abordagem filogeográfica pode ser realizada através do uso de marcadores moleculares microssatélites. Esses são ideais para estudos populacionais, trazendo informações sobre padrões filogeográficos, pois contribui para responder a questões como o grau de mistura genética entre as populações e diferentes níveis de parentesco e fluxo gênico (Turchetto-Zolet et. al., 2013). Esses marcadores denominados Microssatelites ou simples sequências repetidas (SSR) consistem em repetições em tandem de DNA, compostas de 1-6 pares de bases (Field and Wills 1996). São caracterizados pela sua hipervariabilidade, abundância, reprodutibilidade, herança mendeliana e natureza codominante (Scott, 2000).

Entre os métodos para síntese de marcadores SSR está o método tradicional de desenvolvimento de SSR genômicos, incluindo a construção de bibliotecas genômicas, isolamento e sequenciamento de clones que contenham SSRs positivos, seguido desenho e teste de *primers* flanqueadores, consistindo em uma técnica demorada, trabalhosa e cara (Li, 2008). No entanto, com a disponibilidade de grandes quantidades de sequências de regiões expressas (ESTs) e outros dados de sequência de DNA, o desenvolvimento de SSR através de

ferramentas de bioinformática tornou-se uma eficiente opção de baixo custo para muitas espécies de plantas (Saha et. al., 2004).

Como os marcadores EST-SSR são derivados a partir de regiões transcritas de DNA, espera-se que sejam mais conservados e menos polimórficos em relação aos SSR genômicos (Scott et al., 2000, 2001). Por outro lado SSRs derivados de regiões expressas possuem maior taxa de transferência e maior probabilidade de ser funcionalmente associada com as diferenças na expressão de genes do que SSRs derivados de DNA genômico. (Ayers et al., 1997). Além disso, são rapidamente obtidos por triagem eletrônica, estão presentes em genes na regiões do genoma, e são normalmente abundantes (Scott, 2001). A hipótese é que esses marcadores prospectados a partir de regiões expressas da família *Poaceae* tenham potencial de transferência e polimorfismo suficiente para a utilização como ferramentas moleculares para desenvolvimento de análises filogeográficas em *P.annua.*

1.1 Objetivo geral

Desenvolver marcadores microssatélites a partir de sequências de regiões expressas da família *Poaceae* e utilizar esses marcadores para análise filogeográfica de *Poa annua* L., a fim de esclarecer a origem e dispersão dessa espécie na Antártica.

1.2 Objetivos específicos

1. Buscar os motivos SSR mais frequentes no banco de dados de *Poaceae*;
2. Analisar Termos Gene Ontology (GO) para avaliar associações entre *locus* SSR e processos biológicos, componentes celulares e função molecular de genes conhecidos;
3. Testar o potencial de transferência desses marcadores microssatélites em *Poa annua;*
4. Otimizar os protocolos de extração de DNA e amplificação, se necessário;
5. Usar os marcadores sintetizados como ferramenta para análise filogeográfica com amostras de *P. annua* proveniente de diferentes locais (Antártica, Argentina, Brasil, Chile e Irlanda).

2 ARTIGO: Prospecting and Transferability of EST-SSRS Markers Used for Phylogenetic Analysis in P*oa annua* L.

(Artigo submetido para a revista *Plant Molecular Biology,* conforme normas da revista)

Prospecting and Transferability of EST-SSR Markers Used for Phylogenetic Analysis in P*oa annua* L.

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2.1 Abstract Recent increase in the availability of expressed sequence tag (EST) data has facilitated the development of microsatellite or simple sequence repeat (SSR) markers in a number of plant species groups. The aim of this work was to prospect microsatellite markers on expressed regions (EST-SSR) of the family *Poaceae* and test its potential transfer in differents populations of *Poa annua* L. for use in molecular analyzes about origin and colonization this specie in Antartic. This research was performed using bioinformatic tools, through of *in silico* analysis of in SSR databases of 272.632 ESTs regions available in Genbank (NCBI). A search terms Gene Ontology (GO) was performed in ESTs sequences to evaluate associations between SSR *loci* and biological processes, cellular components and molecular function of known genes. The transferability test and analysis molecular in *P. annua* was conducted by Polimersase Chain Reaction (PCR). Was prospected a list of 568 primer pairs, these were synthesized 28 microsatellite markers for transfer in *P. annua.* 68% of EST-SSR markers have potential transfer to *P. annua*. The analysis suggests that the Antarctic samples of *P. annua* are dissimilar from samples of Chile, Brazil, Ireland and Argentina. Furthermore, were found 613 transcripts divided into 302 gene families.With this analysis it was possible develop molecular tools for genetic analysis with *P. annua* and other grass species,to map the most frequent motifs and gene functions in each SSR locus found and suggest that diasporas of *P. annua* found in Antarctica have from Poland.

Keywords Antarctic · *Primers* · Microssatellites · Grass · Invasive Species

2.2 Introduction

Poa annua L. is an Europeian origin plant (Fenner 1985), allotetraploid (4n= 28), derived of cross between *Poa infirma* Kunth and *Poa supina* Schrader (Nannfeldt 1937; Tutin 1952, 1957), occurring both in natural habitats and in cropping systems where it is considered a invasive species (Mengistu et al. 2000). This species has adapted to a broad range of weather conditions, from hot deserts to cold polar regions (Darmency and Gasquez 1981; Frenot et al. 2001).

In Antarctic this species was recorded in Polish Henry Arctowski Station, located in (King George Island, Admiralty Bay), from the summer of 1985 (Olech 1996). The hypothesis for input *P. annua* in station, would have been through human activity, where diasporas originating from Poland, were transported to the station in 1978 in unsterilized soil (Chwedorzewska et al. 2013; 2014). But because high flow of tourists and researchers every year in Antarctica and receiving cargoes containing fresh materials from other countries such as Chile and Argentina, multiple events and diverse sources of introduction of this species can not be discarded (Molina-Montenegro et al. 2014; Chwedorzewska et al. 2014).

Research shows that in recent years this invasive species is increasing in areas of occurrences and numbers of individuals, posing a threat to native species of the Antarctic ecosystem (Olech and Chwedorzewska, 2008, 2011; Chwedorzewska and Bednarek 2012). The competitive effect of *P. annua* with two native species of Antarctic showed reduced growth, low photosynthetic performance and decrease biomass *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) and *Deschampsia Antarctica* E. Desv. (*Poaceae*) (Molina-Montenegro et al. 2012). The large capacity of colonization and competition of *P. annua* in Antarctic and Sub-Antarctic regions may be associated with their allotetraploid condition, combination of different reproductive systems, phenotypic plasticity, genetic variation and epigenetic (Frenot et. al. 1999; Chwedorzewska and Bednarek 2012; Koshy 1969).

Analysis of morphological variation and genetic variability *P. annua* were performed using biochemical and molecular markers: Isoenzimes (Frenot1999), RAPD (*Random Amplified Polymorphic DNA*) (Mengistu et al. 2000), AFLP (*Amplified fragment length polymorphism*) (Chwedorzewska 2008; Chwedorzewska and Bednarek 2012) and ISSR (*Inter-Simple Sequence Repeat*) (Carsonet et al. 2007), but these molecular markers are dominant, AFLP has high cost requiring several steps and reagents for synthesis (Ferreira and Grattapaglia 1998) and RAPD molecular markers have low reproducibility (Faleiro et al. 2004e). With this development informative and easy to use tools become necessary for molecular analysis of the origin, colonization and adaptability of this invasive species in Antarctica.

Microsatellites markers or simple sequence repeats (SSRs) are tandemly repeated tracts of DNA composed of 1–6 base pair (bp) long units (Field and Wills 1996). They are ideal as molecular markers because of the codominant inheritance, relative abundance, multi-allelic nature, extensive genome coverage, high reproducibility, and simple detection (Powell et al. 1996). Sequences containing conserved regions of a gene flanking a hypervariable region such as EST-SSRs are most useful for designing *primers* that work across two or more species (Kantetet al. 2002). EST-SSRs are superior to other markers, due to their higher levels of transferability and the fact that they provide better estimates of genetic diversity, they can be developed from EST databases at no cost and unlike of genomic SSRs, they may be used across a number of related species (Gupta et al. 2003).

The aim of this study was to prospect microsatellite markers expressed regions (EST-SSR) of the family Pooaceae, to test its potential transfer in Antarctic populations of *P. annua*, and to use this markers as a tools for phylogentic analysis in order to clarify origin of this species in Antarctic.

2.3 Materials and Methods

In Silico analysis

The work was developed with help of bioinformatic tools, through of *in silico* analysis in SSR databases of ESTs regions registered for *Poaceae* species. The object of the study was defined by the presence of ESTs data available in Genbank (NCBI) closer to *P. annua*, such few molecular information are available in the public databases. The total database were 272,632 ESTs.

A preprocessing was done with CAP3 program (Huang and Madan 1999) to eliminate the redundancy in the database. A GC content perl script were used in order to evaluate the contigs number, base pairs and the percentage of guanine and cytosine in all EST database, and after in the data without redundancy (Victoria et al. 2011).

The search of SSR motifs in the EST database chosed for this study were done using the SSRLocator software (Maia et al. 2008), investigating the presence of repetitive elements in tandem (SSR), as well the occurrence, frequency and types of motifs and amino acids occurring at each SSR *loci*. The analysis was performed following the search parameters for repetitive elements in class I ($\geq$ 20 bp) described as more efficient molecular markers (Temnykh et al. 2001).

The SSR searh results were annotated 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 the databases microsatellite EST-SSRs more frequent found to Pooaceae was subjected to Blast2GO software (Conesa et al. 2005) and ran against the GO annotated sequences, and the obtained hits were compiled. This analysis was performed using the entire sequence as well as a non-redundant sequence containing the most frequent motifs for *Poaceae*.

Was performed a search for terms Gene Ontology (GO) in database of expressed regions (EST) of *Poaceae* on TRAPID (Bel et al. 2013) using as reference notes (GO) database PLAZA 2.5. Transcripts containing the GO terms associated have been exported to REVIGO (Supek et al. 2011) and visualized in treemaps that cluster ontologies with high semantic similarity. It was used as the reference genome of *Oryza sativa* and p-values 10^{-5}.

Samples Colletion

The colletion of *P. annua* specimens were conducted in five locations, representing disjunct population of Antarctic, South America and Europe. The sampling of Antarctica were conducted during the austral summer of 2014/2015, in the Brazilian Antarctic Expedition XXXIII (2014-2015) near the vicinity of the Polish Antarctic Station Henry, at King George Island (62º09’33.2”S 58º28’24.5”W). The samples of South America were collected in São Gabriel, Brazil (30º34’09.59”S 54º32’55.68”W), in port of Ushuaia, Argentina (54º80’79.7”S 68º30.4’176”W) and in Punta Arenas, Chile (53º16’19.8”S 70º90’10.3”W). The European sample were collected in Maynooth, Co. Kildare, Ireland (53º38’57.34”S 6º59'57.59"W). The samples were transported to the

laboratory of Center os Antarctic Plants Studies (UNIPAMPA/São Gabriel/Brazil) in frozen zipper bags for the maintenance of the genetic material.

Entire plants samples were deposited in the Bruno Edgar Irgang herbarium (HBEI- Universidade Federal do Pampa – Campus São Gabriel), under the vouchers numbers HBEI-1249 (Antarctica), HBEI-1250 (Argentina), HBEI-1251 (Brazil), HBEI-1252 (Chile) and HBEI-1253 (Ireland).

DNA Extraction, Amplification and Electrophoresis

DNA was extracted of five samples (Antarctica, Brazil, Ireland, Argentina and Chile) with about 100 mg of frozen tissue, using PureLink® Genomic Plant DNA Purification Kit, following the manufacturer protocol. DNA was quantified using NanoVue™ Plus Spectrophotometer.

The PCR are conducted in two steps. The first for transferability of prospected markers, PCR amplification was used in Eppendorf Mastercycler DNA Engine Thermall Cycler PCR, with an initial denaturation at 95 ◦C for 2min, 35 cycles of 1min at 95 ◦C, 30s at 52 ◦C, and 2min at 72 ◦C, and a final elongation for 5 min at 72◦C. A single annealing temperature was employed to accommodate all *primers* tested in this study.

For phylogenetic analyzes, the PCR amplification was also utilized Eppendorf Mastercycler DNA Engine Thermall Cycler PCR, however, with na initial desnaturation at 95ºC for 5min, 35 cycles of 30s at 94ºC, variations in temperature annealing of 1ºC on each cycle, starting with 62ºC ending in 52ºC each lasting 1min, and 30s at 72ºC, and a final elongation for 10min at 72ºC.

Were used twenty markers which had the best results, named as SSRPOA_2, SSRPOA_3, SSRPOA_4, SSRPOA_5, SSRPOA_6, SSRPOA_7, SSRPOA_8, SSRPOA_11, SSRPOA_12, SSRPOA_13, SSRPOA_14, SSRPOA_17, SSRPOA_20, SSRPOA_21, SSRPOA_23, SSRPOA_24, SSRPOA_25, SSRPOA_26, SSRPOA_27, and SSRPOA_28.

Reactions were carried out in 15µL volumes containing 7 µL de GoTaq® Green Master Mix (Promega) containing (*Taq* DNA polymerase, dNTPs and MgCl2), 2,5 µL water, 3 µL DNA and 1,25 µL of each primer pair.

PCR amplicons were examined on 3% agarose gels (1X TBE buffer), using 2 µL 50 bp DNA Ladder (Invitrogen), 5 µL PCR product and 2 µL GelRed™. Amplified products gels were viewed and photographed using transiluminador L. Pix (Loccus Biotechnology). Primers with potential transferability for *Poa annua* were selected by the presence of amplified fragments.

Analysis of datas

The sizes of the alleles each *loci* were determined for markers of DNA (100bp). From the images of the 3% agarose gels were evaluated the presence (1) and default (0) of each alleles by *loci*, in each acess, using the sistem “Numerical Taxonomic and Multivariate Analysis System” (NTSYS) – version 2.1 (Rohlf 2000), where in the similarity matrix was gerated by the coeficiente of “Jaccard” (Jaccard 1901). One dendogram was built by the method of grouping by the arithmetic average “Unweighted Pair Group Method with Aritmetic Average” (UPMGA). We calculated the cophenetic correlation coefficient (CCC) between the array of genetic similarities and the matrix of cophenetic values in order to verify the cluster consistency.

2.4 Results

Distribution of EST-SSRs and Marker Prospection

A database was analyzed containing 272,632 contigs. After eliminated the redundancy in CAP3 program, were tabulated 50.496 contigs in ESTs database to *Poaceae* 28,510.064 base pairs, being 54% of Guanine and Cytosine.

Were founds in the database 1804 motifs where 3.6% of EST sequences contained microsatellites. The distribution of occurrences detected by SSRLocator (Fig. 1) was consisted of 198 dimers, 827 trimers, 121 tetramer, 136 pentamer, 398 hexamer, 105 heptamer, 6 octamer, 12 nonamer and 1 decamer, corresponding to rates of 10.98%, 45.84%, 6.71%, 7.54%, 22.06%, 5.82%, 0.33%, 0.67%, 0.05%, respectively. Among the di-nucleotide repeat motifs present in *Poaceae* database AG/CT were most abundant and AT/TA was the least abundant motif. The most abundant tri-nucleotide repeat motif was CCG/GGC in all database of *Poaceae*. The hexa-nucleotide was the second more frequent with 398 repeat motifs, being most common CCCCTC, CCACCG and CCCCGC.

The Amino acids (Fig. 2) more frequently presents in SSR locus of databases were: Proline (Pro) (20%), arginine (Arg) (17%) and Alanine (Ala) (15%) and least common: aspartic acid (Asn) (0,4%) and Tyrosine (Tyr) (0.2%).

A list of 568 primer pairs were obtained from the SSRLocator software and to validate microsatellite markers obtained from *Poaceae* database, we synthesized 28 primer pairs using the 9 most common motifs, as follow: CCG (20%), CGC (15%), GCC (11%), AG (11%), GGC (10%), CGG (9%), GCG (9%), CT (8%) and GCA (7%). The prospected *primers* possessed size between 20 to 22 base pairs and about 23 to 55% Guanine and Cytosine in each marker. The annealing temperature ranged from 48.64 to 54.48 °C. The data to each EST-SSR marker (Sequence 5' to 3' Forward and Reverse , %GC, primer length, annelling temperature and motif repeat) are specified in (Supplementary File 1).

Transferibility of EST-SSRs Marker

The analysis of amplicons by the presence or absence of bands showed 68% of EST-SSR markersprospected from the *Poaceae* family database have potential transfer *Poa annua*, with high level of polymorphism at microsatellite *loci* (fig. 3), where 29% was 2 to 5 alleles by *loci* (*primers* SSRPOA_4, SSRPOA_8, SSRPOA_11, SSRPOA_13, SSRPOA_14, SSRPOA_17, SSRPOA_22, SSRPOA_27) and 39% over 5 alleles (*primers* SSRPOA_5, SSRPOA_6, SSRPOA_7, SSRPOA_12, SSRPOA_15, SSRPOA_20, SSRPOA_21, SSRPOA_23, SSRPOA_24, SSRPOA_25, SSRPOA_26*).* The remaining 14% of the markers did not amplify (*primers* SSRPOA_1, SSRPOA_9, SSRPOA_10, SSRPOA_18), and 18% showed monomorphic bands (*primers* SSRPOA_2, SSRPOA_3, SSRPOA_16, SSRPOA_19 and SSRPOA_28).

The markers were reproducible presenting the same pattern of bands in all analyzes for samples from Brazil and Antarctica. The expected size of the products to *Poaceae* microsatellite l*oci* (Supplementary File 2) were small, ranging from 106-279 base pairs, but 78% of amplicons seen in *Poa annua* ranging from 100 to 1600 base pairs.

Gene Ontology (GO) assignments

The fig. 4 and 5 are illustrating annotations Gene Ontology (GO) for the most common motifs *Poaceae* sequences and SSR *loci* used in the prospection of microsatellite markers respectively. As biological processes (BP) most SSR *loci* were found involved in Cellular metabolic process (15%), organic substance metabolic process (15%), primary metabolic process (14%) and process biosynthetic. Comparing with *Poaceae* database the genes had similar functions.

The Molecular Function (MF) SSR motifs found occurs more frequently associated with gene expression Organic cyclic compound binding (18%), heterocyclic compound binding (18%) and ion binding (13%). The analysis Cellular component (CC) showed that (30%) of genes are active in Cell part, (27%) membrane-bounded organelle and (11%) membrane part.

The analyzes performed in TRAPID of the motifs most representative in *Poaceae* database were similar to the species *Brachypodium distachyon* (L.) P. Beauv. (33.2%), *Zea mays L.*(11.4%), *Oryza sativa* ssp. japonica Portères (11.1%) *Sorghum bicolor* (L.) Moench (10.2%) *Oryza sativa* ssp. Indica Portères (8.1%). The data analyzed were found 613 transcripts divided into 302 gene families. The TreeMaps from REVIGO shown in Supplementary Files (3, 4 and 5) illustrate the terms Gene Ontolgy for gene families related with molecular function, biological processes and cellular components of these regions. Each rectangle is a cluster representative; larger rectangles represent 'superclusters' including related terms. The size of the rectangles reflects the p-value.

The data visualized in REVIGO showed that in relation the molecular function, most number of ESTs belonged gene family imidazole glycerol-phosphate synthase activity (including genes related with prenyl transferase activity, transferase activity, transferring one-carbon groups), Succinate dehydrogenase activity (peroxidase activity) and Poly (ADP-ribose) glycohydrolase activity (with gene to thiolester hydrolase activity, serine hydrolase activity, serine-type peptidase activity, chitinase activity and motor activity).

For gene families involved in Cellular Component stand out Part Cytosolic and Protein-DNA complex. Gene Ontology terms for Biological Process evidenced most ESTs related to two gene families: Metabolism lipoprotein and Cell wall macromolecule metabolism. Gene Ontology terms for metabolism lipoprotein includes genes related to RNA modification, pseudouridine synthesis, post-translational protein modification protein lipidation, protein folding, mRNA metabolism, Peptidyl-amino acid modification, translational initiation, protein ubiquitination, RNA splicing tRNA metabolism. Already gene family Cell wall macromolecule metabolism is related with genes to amine metabolism, tetrapyrrole metabolism, DNA replication, DNA conformation change, DNA-dependent DNA replication, branched-chain amin acid metabolism, aminoglycam metabolism, cellular amine metabolism, nucleobase metabolism and nucleoside triphosphate biosynthesis.

Phylogeographical analysis of *Poa annua* L samples.

The twenty pairs of *primers* used for microsatellite analysis in 5 samples of *P. annua* generated a total of 85 amplified alleles fragments ranging from 1 to 5 alleles per primer, with size of 100 to 1600 base pairs.

The dendrogram obtained from the data similarity matrix by agglomerative method UPGMA (Fig. 6) showed low similarity for 5 samples of *P. annua* about (0.08). The sample of Antarctica was the furthest in relation Chile, Brazil, Argentina and Ireland, which was an isolated group compared to samples from other

locations. *P. annua* from Brazil and Ireland showed greater similarity each other (0.23). The grouping is consistent with high-value cophenetic correlation coefficient (CCC) r= 0.93, once values above 0.8 indicate good representation of the distances (Bussad et al. 1990).

2.5 Discussion

Distribution of EST-SSRs and Marker Prospection

The frequency of SSR motif in the *Poaceae* EST database were 3.6%. Similar frequency 3.1% were record nonredundant EST-SSRs in genotypes of *Lolium perenne* (Asp et al. 2007), 3.2% in related species de *Poaceae* (Kantet et al. 2002) and 2.52% SSRs in the ESTs sequences from wheat, barley, maize,rice, and sorghum (Li et al. 2008)

The di-nucleotide AG/CT and AT/TA was the most and least abundant repeat motif in rice, soybean, maize and wheat (Morgante et al. 2002). AG and AT motifs were the di-nucleotide repeat with higher and lower frequency, respectively in EST sequences barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), oats (*Avena sativa* L.), rye (*Secale cereale* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolour* (L.) Moench) and wheat (*Triticum aestivum* L.) (Varshney et al. 2002). The tri-nucleotide CCG/GGC was he motif most frequent in expressed sequence tags from barley (*Hordeum vulgare*), maize (*Zea mays* L.), Rice (*Oryza sativa* L.), sorghum *(S. bicolor*) (Kantet et al. 2002). The repetitions of the trinucleotide CCG/CGG were abundant in monocots and are related to a high GC content in this plant group (Morgante et al. 2002). Also the presence of CCG motif repeats in the 5'-UTR of ribosomal protein genes of *Zea mays* is related with fertilization regulation (Dresselhaus et al. 1999).

The hexamer motifs showed a high abundance in the database (22,06%). The high frequency of these complex motifs might be a result of DNA polymerase slippage due to strands mispairing, during DNA replication of long repeat sequences (Chistiakov et al. 2006).

The Amino acids more frequently presents in SSR locus of *Poaceae* databases agree with (Victoria et al. 2011), where (Pro), (Arg) and (Ala) are among the predominant amino acid in flowering plants. The high occurrence of Proline Amino acid 20% may be related to tri-nucleotide motif most abundant, because this amino acid is coded by CCG (Nicot et al. 2004). In many plant species accumulate proline may be associated with reproduction and also as an adaptive response to adverse conditions, such as salt stress conditions (Mergulhão et al. 2002).

Transferibility of EST-SSRs Marker

A high percentage (68%) of the transferibility can be indicating a high level of sequence conservation among these species (Saha et al. 2004). Similar results for the *Poaceae* family is reported by Varshney et al. (2005) where potential amplification of 165 EST-SSR markers mapped to barley in wheat, rye and rice, found a transferability rate of (78,2%), (75,2%) and (42,4%) respectively. Li et al. (2008) observed the effective primer pairs in wheat (69.93%), rice (53.57%), maize (63.22%), cotton (49.93%), and soybean (56.27%). Eighty two EST-SSR markers developed in barley to potential use in variability and phylogenetic analysis of the *Hordeum chilense* Roem. & Schult. genome, detected level polymorphism of (26%) (Castillo et al. 2008).

Similar results were also observed for other species. In barley, SSR *loci* amplified from both EST and total genome were compared, demonstrating that amplification of larger-size alleles are actually more frequent than those of expected size (Thiel et al. 2003). Another possibility for the size observed to be higher than expected is that ESTs sequences used for the prospecting of the markers were from different species of *P. annua*.

The high level of conservation of many EST-SSR *primers* across distantly related grass species indicates the potential of these markers to be useful in different grass species (Rouf Mian et al. 2005). Castillo et al. (2008) also report the possibility of a higher ratio of transference of EST-SSRs markers and the potential uses of this markers for comparative mapping between species of monocots, demonstrating a high degree of transferability and polymorphism these markers. As the EST-SSR markers are derived from transcribed regions of DNA, they are expected to have less tolerance for mutation and have a higher rate of transferability than genomic SSR markers (Scott et al. 2000).

Gene Ontology (GO) assignments

The results this work show that SSR *loci* have no genes with specific functions being representing the database of the most frequent motifs for *Poaceae*. Similar results were found for *Oryza rufipogon* Griff. (Tian et al. 2015), *Oryza officinalis* Wall. ex G. Watt (Bao et al. 2015), *Spartina maritima* (Curtis) Fernald and *Spartina alterniflora* Loisel. (Carvalho et a. 2013) where GO terms for metabolic process, cellular process, cell, cell part and binding were the most representative functions of genes. Nishiyama et al. (2003) suggest that genes that are involved in protein metabolism and biosynthesis are well conserved between mosses and vascular plants.

Wang et al. (2014) found in the nuclear genome of *Popolus* to biological processes, more related terms to cellular process (25.21%) and metabolic process (22.65%), suggesting a high degree of basic metabolic activity in the regulation of nuclear genes in photosynthesis.

The gene associations visualized in the REVIGO found in the EST database for molecular function clustering three groups representative. The first Imidazole glycerol-phosphate synthase that is a metabolic enzyme catalyzer da reaction for biosynthesis of histidine (Chaudhuri et al. 2001). The amin acid histidine play an important role in regulation of biosynthesis of other unrelated amino acids, in chelation and transport of metal ions, and in plant reproduction and growth (Stepansky and Leustek 2006). The second Succinate dehydrogenase activity is an enzime located in the inner membrane of mitochondria and its activity is connected with the operation of the electron transport chain. Research carried out with *Arabidopsis thaliana* (L.) Heynh. showed that the succinate dehydrogenase expression is inhibited by phytochrome It can be considered an important mechanism for the regulation of the mitochondrial respiration in the light (Popov et al. 2010). The third Poly (ADP-ribose) glycohydrolase activity that plays a role in regulating in *Arabidopsis* circadian rhythm and in the photoperiod-dependent transition from vegetative growth to flowering (Panda et al. 2002).

The ESTs sequences involving the Cellular Component are related with Protein-DNA complex and Part Cytosolic (involve peroxisome, endoplasmic reticulum, mitochondria plastid, chloroplast) organelles responsible respectively for primary and secondary metabolism, development and responses to abiotic and biotic stresses (Hu et al. 2012); protein synthesis and lipid biosynthesis (Galili et al. 1998); respiration (Jacoby et al. 2012); and photosynthetic process (Nakayama and Archibald 2012).

Biological Process of lipoprotein metabolism has many GO terms associated with protein synthesis processes as: protein lipidation, protein folding, mRNA metabolism, translational initiation, RNA splicing and tRNA metabolism. The other genes are involved in post-transcriptional processes like RNA modification or pseudouridine synthesis and protein ubiquitination. Pseudouridine is the enzyme responsible for post-transcriptional modifications of RNA (Hamma and Ferré-D'Amaré 2006); protein ubiquitination is fundamental regulatory post-translational modifications controlling intracellular signalling events (Komander 2009). The post-translational protein modification is associated with activation of the immune complexes adaptation of plants to environmental changes (Piquerez et al. 2014).

The genic family (cell wall macromolecule metabolism) is related with chemical reactions and pathways involving macromolecules forming, or destined to form, part of cell wall as, DNA conformation change, branched-chain amin acid metabolism, aminoglycam metabolism, nucleobase metabolism and nucleoside triphosphate biosynthesis amine metabolism and tetrapyrrole metabolism, this last, involved in light harvesting and light perception, electron-transfer reactions, and as co-factors for key enzymes and sensory proteins (Busch and Montgomery 2015).

In addition to conserved genes involved in metabolic process and biosynthesis, 11% of the most frequents motifs presented putative genes, demonstrating the potential of these markers to differentiate organisms. The high level of transferability and genetic information because these markers are associated with putative gene or know functions, become useful tools, low cost and highly polymorphic for genome exploration and genetic analysis (Garcia et al. 2011).

Phylogeographical analysis of samples de *Poa annua* L.

Samples of *P. annua* have low similarity between sites, this may be related due to Morphological variability (Ellis et al. 1971; Darmency and Gasquez 1983; Wu et al. 1987), genetic diversity (Mengistu et al. 2000) genetic and epigenetic variation (Chwedorzewska and Bednarek 2012), phenotypic plasticity (Frenot et al. 1999) and allotetraploid condition of this species (Nannfeldt 1937; Tutin 1952, 1957). Furthermore *P. annua* is well known to reproduce by outbreeding, inbreeding or apomixis according to the environmental conditions, determining the genetic structure of plant populations (Darmency and Gasquez 1983).

According Chwedorzewska et al. (2014) the origin of this invasive species in Antarctica has been through from Poland diasporas, or loads of fresh material from Chile or Argentina, but the microsatellite data showed a dissimilarity among sample of Antarctica compared with Chile and Argentina, which suggests that individuals present in the Polish Henry Arctowski station probably have been transported from distinct geographical location from those which the samples used in the present research were obtained.

2.6 Conclusion

With this analysis it was possible to map the most frequent microsatellite and gene functions related to EST-SRR regions in *Poaceae*, where most genes are associated with cellular metabolic processes, photosynthesis, respiration and protein synthesis. The results also confirmed the higher level amplification of EST-SSR p*rimers* in *P. annua* with 68% of transferability and high polymorphism for discrimination of individuals, can be used as tools to molecular analysis of genetic diversity, phylogeographic patterns and

population studies of different levels with *P. annua* and other species of monocots phylogenetically related. The phylogeographic analysis showed low similarity between the sample Antarctic with samples from other locations, route of researchers who go to the Antarctic expeditions, suggesting that *P. annua* found in Polish Henryk Arctowski station may have actually come from Poland, however, additional phylogeographic studies comparing the Antarctic populations with the Poland populations should be conducted to clarify the origin and colonization of this species in Antarctica.

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2.9 Figures

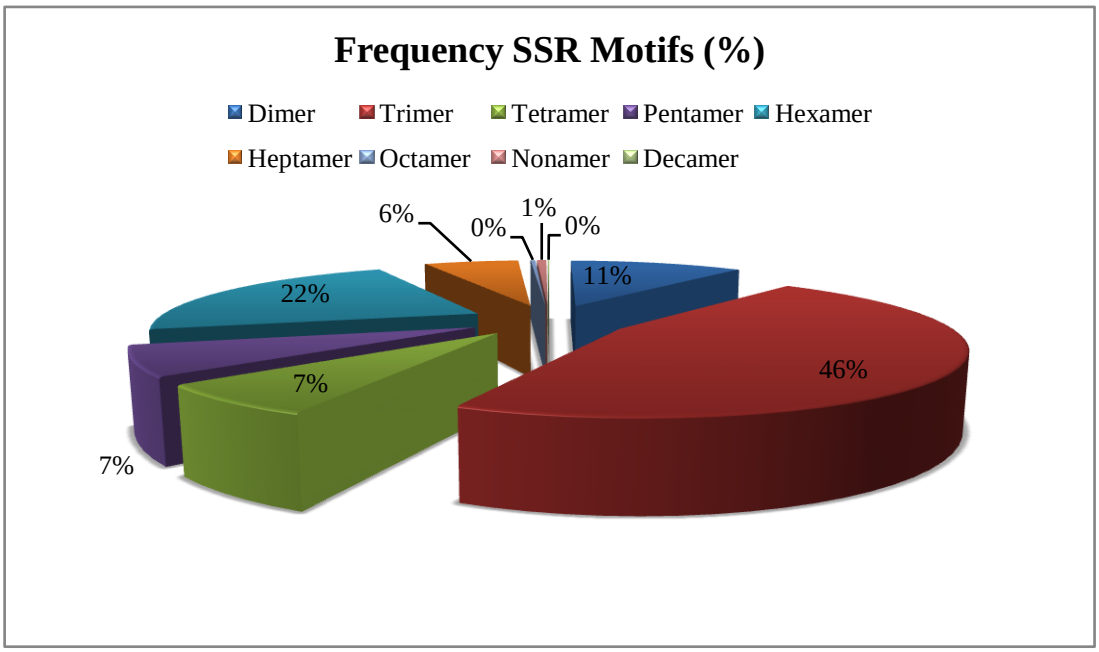

Fig. 1 Frequency (%) of the most common SSR motifs in *Poaceae* EST database. Data of SSRLocator software (Maia et al. 2008).

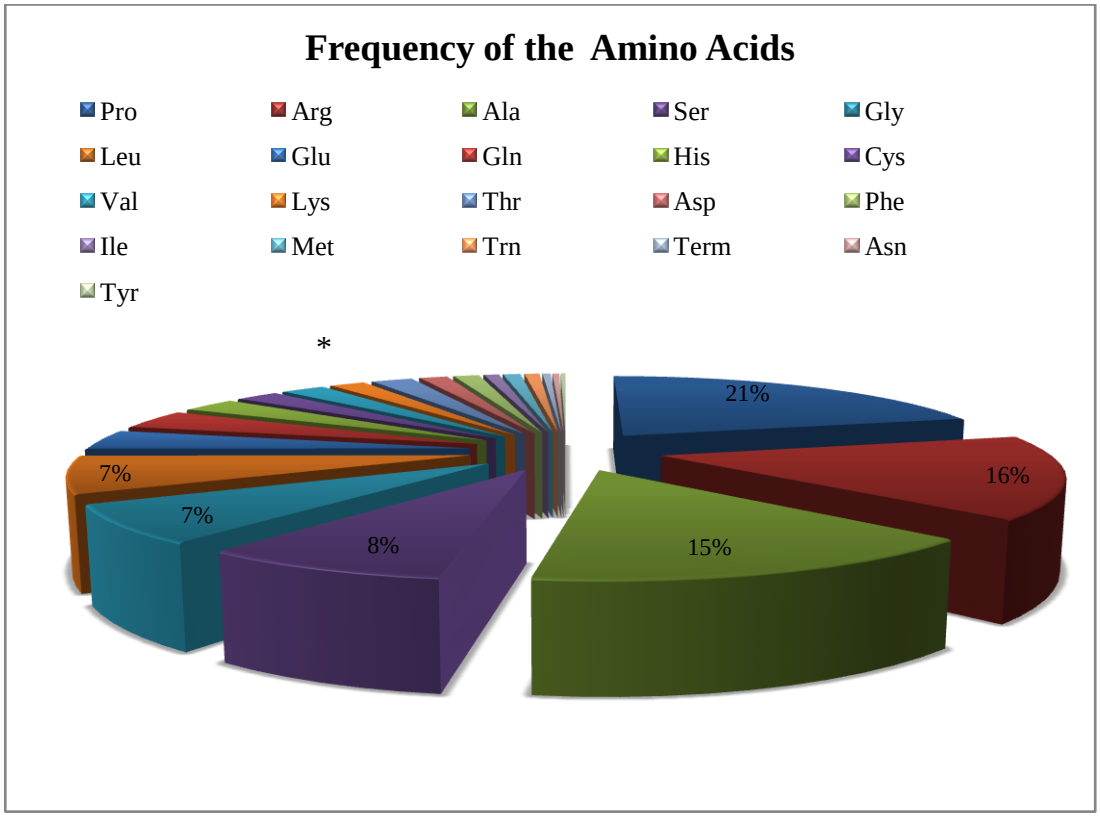

Fig. 2 Frequency of the amino acids in *Poaceae* EST database.**(Pro)** – Proline; **(Leu)** – Leucine; **(Val)** – Valine; **(Ile)** – Isoleucine; **(Tyr)** – Tyrosine; **(Arg)** – Arginine; **(Glu)** – Glutamine; **(Lys)** – Lysine; **(Met)** – Methionine; **(Ala)** – Alanine;**(Gln)** – Glutamic acid; **(Thr)** – Threonine; **(Trn)**–Tryptophan; **(Ser)** – Serine; **(His)** – Histidine; **(Asp)** – Aspartic acid; **(Gly)** - Glycine; **(Cys)** – Cysteine; **(Phe)** – Phenylalanine; **(Asn)** – Asparagine. (*) Amino acids with frequency of 1 to 4%.

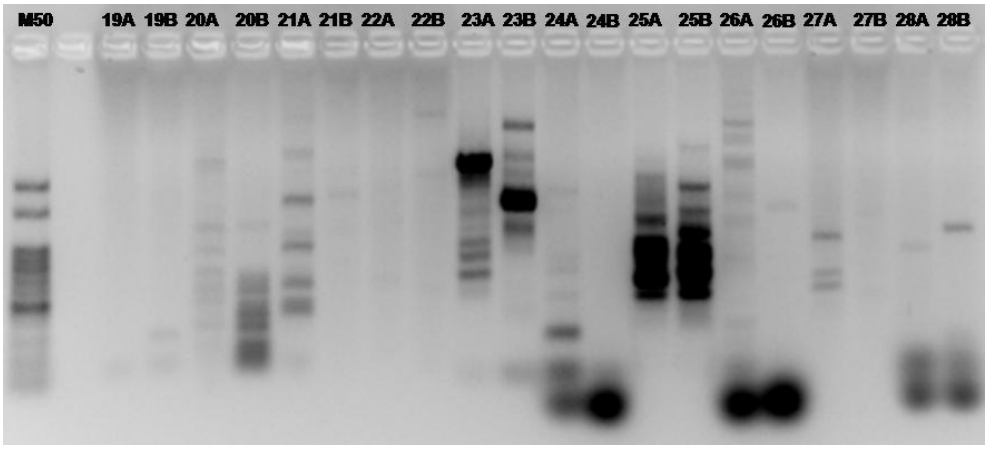

Fig. 3 Product separated in 3% agarose gel showing polymorphisms in *loci* ESTs; **(M50)** - DNA ladder marker 50 base pairs; The number in each *loci* corresponding to number microsatellite marker tested; **(A)** - Sample Antarctica; **(B)** - Sample Brazil.

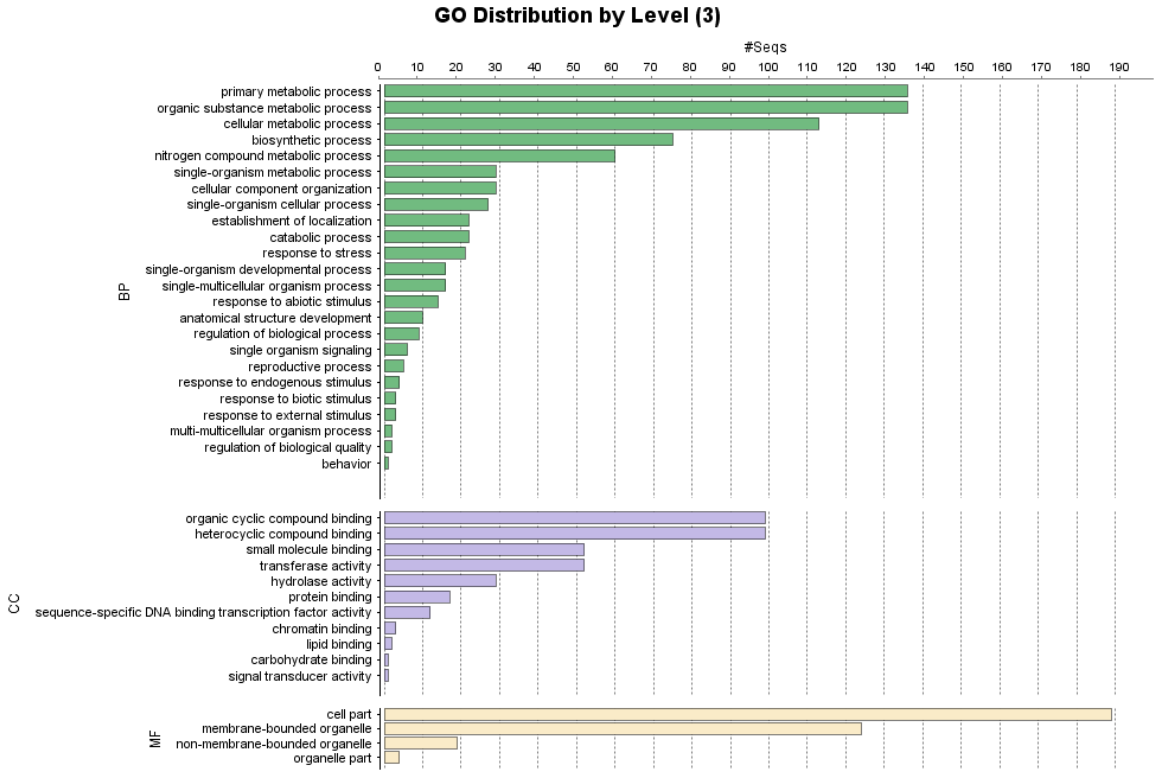

Fig. 4 Results Blast2GO program regarding GO distribuition - **(BP)** biological process; **(CC)** cellular component; **(MF)** molecular function.Motifs more frequent.

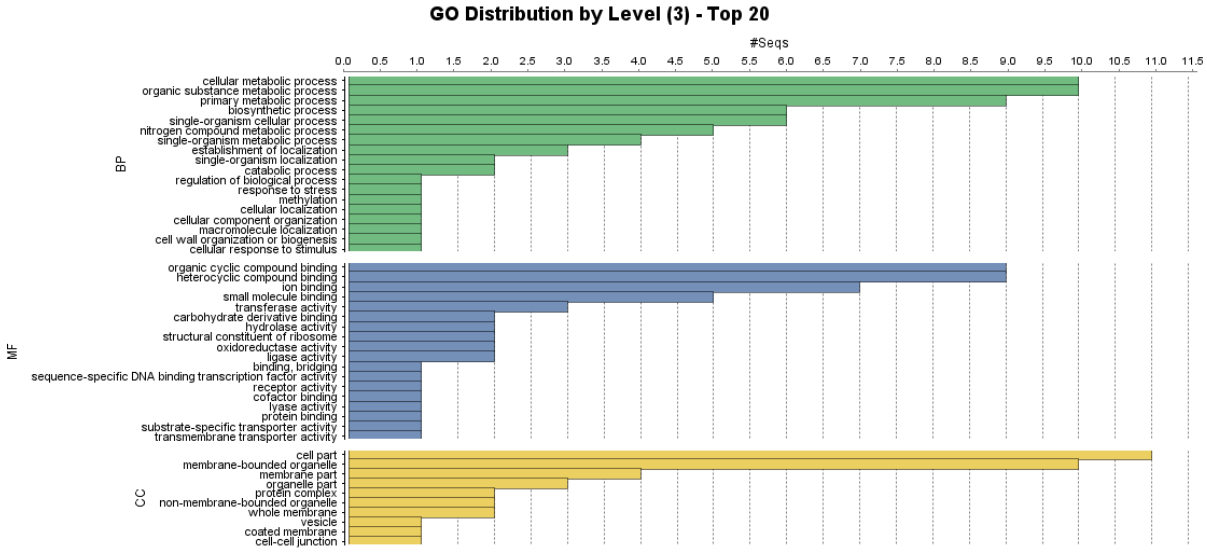

Fig. 5 Results Blast2GO program regarding GO distribuition - **(BP)** biological process; **(CC)** cellular component; **(MF)** molecular function. Database of ESTs sequences used to design *primers.*

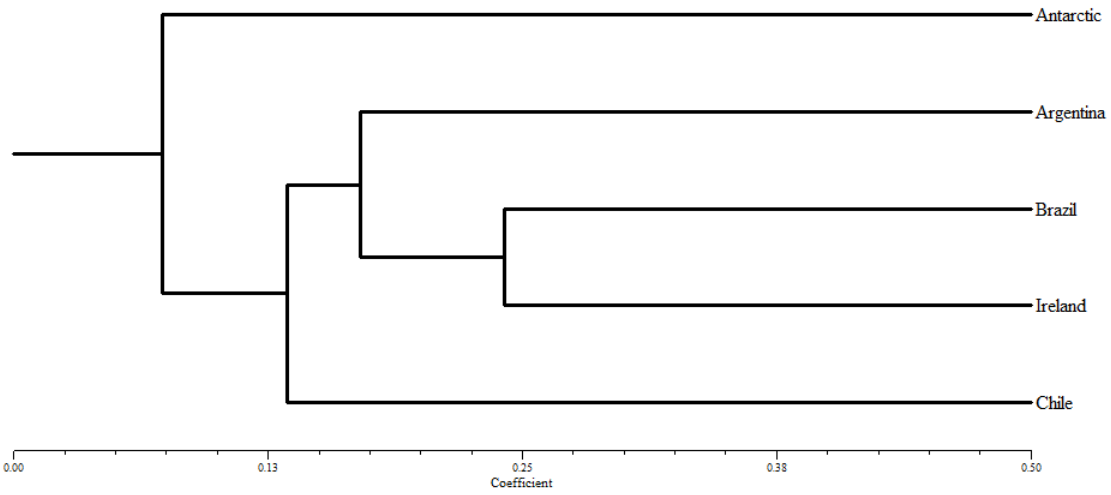

Fig. 6 Microssatelite (SSR) marker unwigthed pair group method with arithmetic means (UPGMA) dendrogram resulting from analysis of samples of *P. annua* from Antarctica, Argentina, Brazil, Chile and Ireland using the complement of the similarity índex of Jaccard (1901) based 85 alleles fragments of 20 *primers* pairs as a measure of genetic distance. The value of the cophenetic correlation coefficient (CCC) (r) = 0.93 (Bussad et al. 1990).

3 CONSIDERAÇÕES FINAIS

Com essse trabalho foram desenvolvidos 28 marcadores microssátelites com 68% de transferibilidade e alta variação polimórfica, permitindo a discriminação de indivíduos. Devido o alto nível de polimorfismo e conservação das regiões flanqueadoras das sequências EST-SSR, estes marcadores podem ser usados como ferramentas para análises de diversidade genética, padrões filogeográficos e estudos populacionais de diferentes níveis com *P. annua* e outras espécies de monocotiledôneas filogeneticamente relacionadas.

Também foi possível mapear os microssátelites mais frequentes e as funções dos genes relacionados às sequencias EST-SSR do banco de dados de *Poaceae*, onde a maioria dos genes estão associados com processos metabólicos celulares, fotossíntese, respiração e síntese protéica.

A análise filogeográfica demonstrou baixa similaridade da amostra proveniente da Antártica quando comparada com amostras da Argentina, Brasil, Chile e Irlanda, rotas locais de pesquisadores que realizam trabalhos científicos nesse continente. Com essa baixa similaridade (0.07) é possível que os indivíduos de *P. annua* encontrados na Estação Polonesa Henryk Arctowski tenham vindos de fontes distintas da América do Sul, porém estudos filogeográficos complementares comparando as populações da Antártica com populações da Pôlonia devem ser realizados para esclarecer a origem e colonização dessa espécie na Antártica.

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5 ANEXOS

Anexo 1: Carta de Sumissão do artigo - revista *Plant Molecular Biology*

Plant Molecular Biology

Prospecting and Transferability of EST-SSR Markers Used for Phylogeographical Analysis in Poa annua L.

–Manuscript Draft–

Manuscript Number:		
Full Title:	Prospecting and Transferability of EST-SSR Markers Used for Phylogeographical Analysis in Poa annua L.	
Article Type:	Original Article	
Keywords:	Antarctic; Primers; Microssatellites; Grass; Invasive Species.	
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Funding Information:	National Council for Research and Development - CNPq (574018/2008)	Not applicable
	Carlos Chagas Research Support Foundation of the State of Rio de Janeiro (FAPERJ (E-26/170.023/2008)	Not applicable
Abstract:	Recent increase in the availability of expressed sequence tag (EST) data has facilitated the development of microsatellite or simple sequence repeat (SSR) markers in a number of plant species groups. The aim of this work was to prospect microsatellite markers on expressed regions (EST-SSR) of the family Poaceae and test its potential transfer in differents populations of Poa annua L. for use in molecular analyzes about origin and colonization this specie in Antartic. This research was performed using bioinformatic tools, through of in silico analysis of in SSR databases of 272.632 ESTs regions available in Genbank (NCBI). A search terms Gene Ontology (GO) was performed in ESTs sequences to evaluate associations between SSR loci and biological processes, cellular components and molecular function of known genes. The transferability test and analysis molecular in P. annua was conducted by Polimersase Chain Reaction (PCR). Was prospected a list of 568 primer pairs, these were synthesized 28 microsatellite markers for transfer in P. annua. 68% of EST-SSR markers have potential transfer to P. annua. The analysis suggests that the Antarctic samples of P. annua are dissimilar from samples of Chile, Brazil, Ireland and Argentina. Furthermore, were found 613 transcripts divided into 302 gene families.With	

	this analysis it was possible develop molecular tools for genetic analysis with P. annua and other grass species,to map the most frequent motifs and gene functions in each SSR locus found and suggest that diasporas of P. annua found in Antarctica have from Poland.
Suggested Reviewers:	Lubomir Kovacik, PhD Professor, Commenius University kovacik@fns.uniba.sk
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	Michael Stech Naturalis Biodiversity Center michael.stech@naturalis.nl
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Supplementary File 1 - SSR *primers* developed from *Poaceae* EST database. For each SSR, Sequence 5' to 3' Forward and Reverse, % guanine and cytosine (GC), primer length, annelling temperature (TM) repeat unit, are shown. This dates were calculated using *SSRLocator* software (Maia et al. 2008).

Primer	**Name**	**Sequence (5' to 3')**	**%GC**	**Primer Length**	**TM (ºC)**	**Motif repeat**
1	SSRPOA_1F	GTTCATTCATCATTCGTATC	35	20	50,2	(CCG)7
	SSRPOA_1R	TATATAATTATCATCATTTCCG	23	22	49,29	
2	SSRPOA_2F	TCAAACAGTACATTCTCTATCA	32	22	50,11	(CCG)7
	SSRPOA_2R	TCATGTTGCTCTACTACGTG	45	20	52,26	
3	SSRPOA_3F	GGAGCTAAAGCTAATTGTACT	38	21	50,29	(CCG)7
	SSRPOA_3R	ATAATGGCTTCTCACCATAG	40	20	51,92	
4	SSRPOA_4F	GAAGATCTCGTTGTACCAGT	45	20	51,65	(CCG)9
	SSRPOA_4R	ATGGTATACAGAACAGATCG	40	20	49,93	
5	SSRPOA_5F	CTTTCGTTTCTCTTGTTGCT	40	20	54,91	(CGC)7
	SSRPOA_5R	CTACTCTTTTCCTTGATGTAG	38	21	48,64	
6	SSRPOA_6F	AAAATTGAGGTCAAAAGAAG	30	20	51,32	(CGC)10
	SSRPOA_6R	GATTCCAGTAGTAGAGGTAGC	48	21	50,04	
7	SSRPOA_7F	GTGTCTGGGAATCCTAATCT	45	20	53,11	(CGC)7
	SSRPOA_7R	CTCGTAGTCGTAGGAGAGGT	55	20	53,65	
8	SSRPOA_8F	ACCTCTTGTATGATTCTTAGC	38	21	50,49	(CGC)8
	SSRPOA_8R	CTGACTCTCACCATCCTTAC	50	20	52,03	
9	SSRPOA_9F	GCCAAATTTTTATTGTTTTT	20	20	51,6	(GCC)7
	SSRPOA_9R	GATGCTGGATGACATAGTTA	40	20	50,93	

10	SSRPOA_10F	GTTGAGAAATTTCCTATTCTT	29	21	49,73	(GCC)7
	SSRPOA_10R	TCTTCTAGAGTTATCCATCAGT	36	22	50,03	
11	SSRPOA_11F	GTCAACTCCAAGATCCTCTC	50	20	53,66	(GCC)7
	SSRPOA_11R	CCTTGATGTTGAGGTAGTTG	45	20	53,15	
12	SSRPOA_12F	TATATATCCTAGATTTCTTGCC	32	22	50,12	(AG)14
	SSRPOA_12R	ACCTAATAACTGCTTCACAC	40	20	49,41	
13	SSRPOA_13F	CTAGCCTTCTCTTCTTCTCT	45	20	49,88	(AG)13
	SSRPOA_13R	CTATATCTCTGTGCAGGTTC	45	20	49,79	
14	SSRPOA_14F	GAATCCGAGAGAATAGAAAG	40	20	50,81	(AG)18
	SSRPOA_14R	ATGTATCTTTTCATGACAGC	35	20	50,04	
15	SSRPOA_15F	ATGACTGTATATGGTGAAGATT	32	22	50,45	(AG)23
	SSRPOA_15R	GTCTCTCTTGGTCTTGTTGT	45	20	51,59	
16	SSRPOA_16F	AATAGAGTAATTATTAACACCGA	26	23	49,94	(GCC)7
	SSRPOA_16R	CATGCTCCATGTCAAGATAG	45	20	54,13	
17	SSRPOA_17F	GTGAAGATTGATTCTGGTCT	40	20	51,48	(CGG)7
	SSRPOA_17R	ATCTTCACTGTCGGAGTCTA	45	20	52,22	
18	SSRPOA_18F	CTTCTTGATTCGCTTCTGTT	40	20	54,82	(CGG)7
	SSRPOA_18R	ATGATGTTCACATCTGTTGG	40	20	54,07	
19	SSRPOA_19F	AAAACAAGAGTTTATATTCAGC	27	22	50,24	(CGG)8
	SSRPOA_19R	CACCTCGACTACTACTTCTG	50	20	49,9	

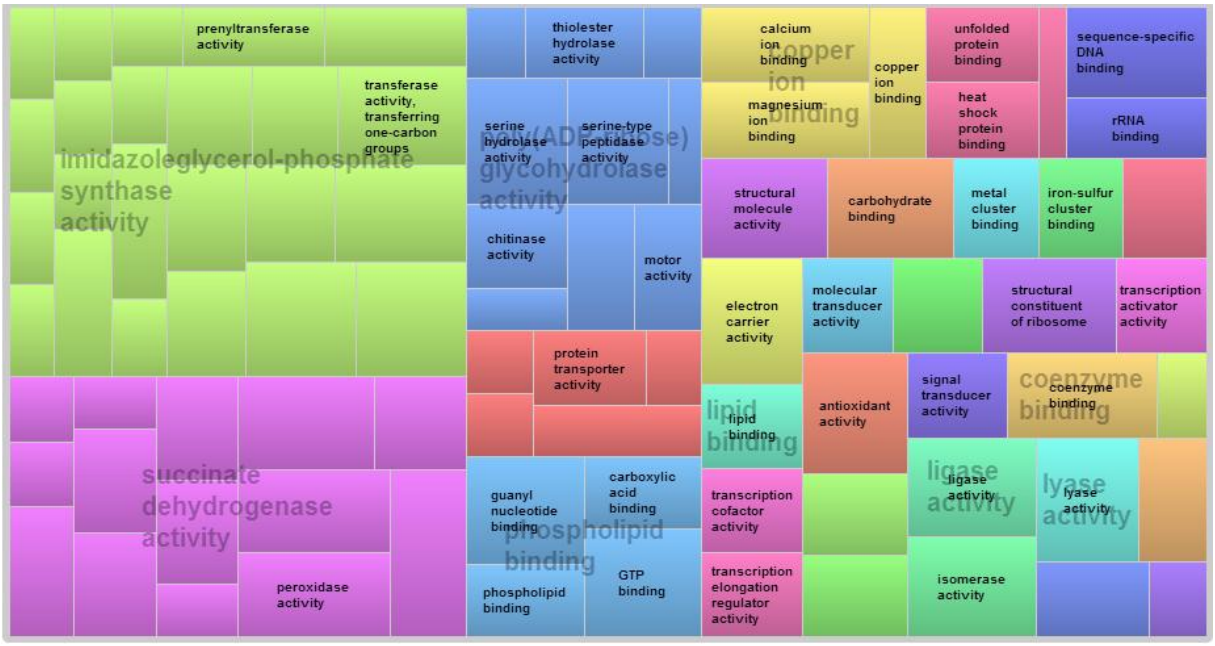
20	SSRPOA_20F	GTGATTAACTCGTCCACATC	45	20	52,81	(GCG)7
	SSRPOA_20R	CAGACTCTTTTCTATCCTCTC	43	21	49,98	
21	SSRPOA_21F	GATTTCACCGACATTTTCTT	35	20	54,26	(GCG)8
	SSRPOA_21R	CTTCTTCTGTTTTCTCTTGG	40	20	51,83	
22	SSRPOA_22F	TTCAGAGAAACTACCAACAC	40	20	50,11	(GCG)7
	SSRPOA_22R	TTCTATCTTCCTACTTGGTTAC	36	22	49,93	
23	SSRPOA_23F	TTCCACTTTCATTGTATCTC	35	20	50,03	(CT)11
	SSRPOA_23R	GAGTATAGTCGCGTGAAGAG	50	20	52,74	
24	SSRPOA_24F	CTTATCCCTCTCCTTCCTTT	45	20	54,23	(CT)11
	SSRPOA_24R	GTTTCCCTGGTGAAATAAGA	40	20	54,27	
25	SSRPOA_25F	CTATACTAGTCAGTTCGGTTTC	41	22	50,37	(CT)11
	SSRPOA_25R	ACATACACAGAGAGAGAGAGAG	45	22	50,22	
26	SSRPOA_26F	TGACTCTGAGCAGACAGAGA	50	20	54,48	(GCA)7
	SSRPOA_26R	AATTGCCTTGTACACATGAT	35	20	52,95	
27	SSRPOA_27F	CTGCAGAGAGCAAAGAAGTA	45	20	53,55	(GCA)8
	SSRPOA_27R	AAGTTTCAATCTAAGCATAGTC	32	22	50,08	
28	SSRPOA_28F	TATACTATGGCCTTGATGTT	35	20	49,89	(GCA)7
	SSRPOA_28R	GTGTTAGCACAAATGTAACTT	33	21	49,75	

Supplementary File 2 – Data of presence and ausence of polymorphi bands in agarose gel 3% to size of the amplicons expected and observed for 28 EST-SRR markers amplified in *Poa annu*a samples from Brazil and Antarctica. The amplicons were separated by different size, minimum of 50 to 250 base pairs; greater than 250 to 500 base pair and fragments with amplicons larger than 500 base pairs.

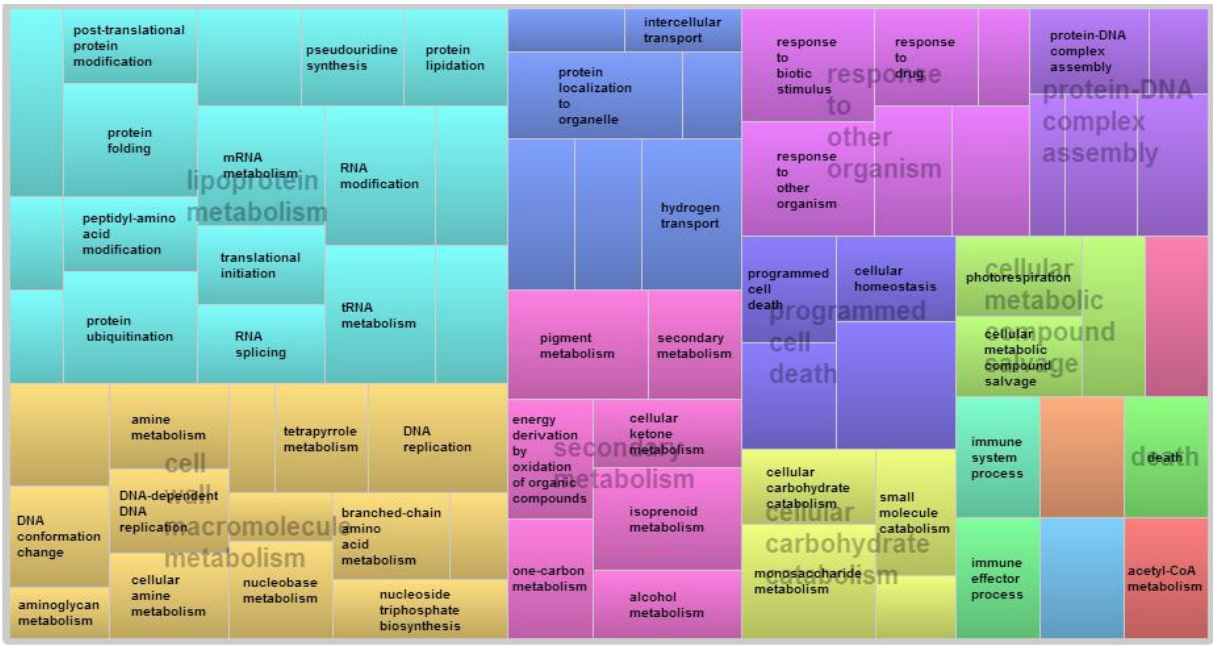
	Amplicons Size						
Marker	50-250 (bp)		>250-500(bp)		>500(bp)		Expected product size (bp)
	Antarctic	Brazil	Antarctic	Brazil	Antarctic	Brazil	
SSRPOA_1	0	0	0	0	0	0	267
SSRPOA_2	0	0	1	0	0	0	248
SSRPOA_3	0	0	0	0	1	0	211
SSRPOA_4	0	0	1	1	1	0	239
SSRPOA_5	0	0	1	1	1	0	254
SSRPOA_6	1	0	1	0	1	0	241
SSRPOA_7	0	0	1	1	1	1	278
SSRPOA_8	0	0	1	0	1	0	226
SSRPOA_9	0	0	0	0	0	0	267
SSRPOA_10	0	0	0	0	0	0	236
SSRPOA_11	0	1	1	1	1	0	226
SSRPOA_12	1	0	0	0	1	0	261
SSRPOA_13	0	0	1	1	1	1	217
SSRPOA_14	0	0	1	0	1	0	232
SSRPOA_15	1	1	1	1	1	0	201
SSRPOA_16	1	0	0	0	0	0	266
SSRPOA_17	0	0	0	1	1	1	260
SSRPOA_18	0	0	0	0	0	0	260
SSRPOA_19	0	1	0	0	0	0	230
SSRPOA_20	1	1	1	1	1	1	209
SSRPOA_21	1	0	1	0	1	1	240
SSRPOA_22	0	0	1	1	0	1	199
SSRPOA_23	0	0	1	0	1	1	231
SSRPOA_24	1	0	1	0	1	0	106

SSRPOA_25	0	0	1	1	1	1	163
SSRPOA_26	1	0	1	0	1	1	227
SSRPOA_27	0	0	1	0	1	1	145
SSRPOA_28	0	0	1	0	0	1	279

Supplementary File3: TreeMap visualization obtained by REVIGO analysis of the summary of GO Cellular component, using p-values 10^{-5}.



Supplementary File4: TreeMap visualization obtained by REVIGO analysis of the summary of GO Molecular Function process, using p-values 10^{-5}.



Supplementary File5: TreeMap visualization obtained by REVIGO analysis of the summary of GO Biological process, using p-values 10^{-5}.