In silico analysis of *Pinus* L. chloroplast DNA to microsatellites regionsAnálise *in silico* das regiões microssatélites de DNA cloroplastidial de *Pinus* L.**Michele Moro¹, Fernanda Bortolanza Pereira^{1*}, Jaire Alves Ferreira Filho²,
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and Evandro Vagner Tambarussi³****Abstract**

The *Pinus* genus covers a wide variety of widely cultivated species due to adaptability, high growth and wood quality. Molecular markers have been used for many genetic analyses, and among them, the microsatellite markers (SSR) have several applications and can be found in chloroplast genome (cpDNA) as well as in nuclear genomes (ntDNA). The chloroplast microsatellites markers (cpSSR) can be used for gene flow analysis, identification of hybrids, clones, paternity tests, genetic diversity studies, phylogenetic analysis, among others. The work aimed to characterize the cpSSRs of *Pinus* species with cpDNA sequenced and deposited in NCBI (National Center for Biotechnology Information). In the twenty species of *Pinus* spp. studied, 1.542 cpSSRs were identified, with 86,45% of mononucleotide type, and the less frequent the penta- (1.10%) and hexanucleotide (1.04%) types. Predominated cpSSRs in non-coding regions (intergenic). The results indicate presence of a wide range of cpSSR for *Pinus* spp., which can subsidize breeding programs of interesting species.

Keywords: cpSSR; cpDNA; genetical improvement; forest improvement

Resumo

O gênero *Pinus* compreende uma ampla variedade de espécies cultivadas devido à adaptabilidade, elevado crescimento e qualidade de madeira. Marcadores moleculares têm sido usados para diversas análises genéticas, e entre eles, os marcadores microssatélites (SSR) apresentam várias aplicações e podem ser encontrados no genoma cloroplastidial (cpDNA) bem como no genoma nuclear (ntDNA). Os marcadores microssatélites cloroplastidiais (cpSSR) podem ser usados para análises de fluxo gênico, identificação de híbridos, clones, testes de paternidade, estudos de diversidade genética, análises filogenéticas, entre outros. O trabalho objetivou caracterizar os cpSSRs de espécies de *Pinus* com cpDNA sequenciado e depositado no NCBI (*National Center for Biotechnology Information*). Nas vinte espécies de *Pinus* estudadas, foram identificados 1.542 cpSSRs, com 86,45% do tipo mononucleotídico, e em menor frequência dos tipos penta- (1,10%) e hexanucleotídico (1,04%). Os cpSSRs predominaram em regiões não codificantes (intergênicas). Os resultados indicam presença de uma ampla gama de cpSSRs para *Pinus* spp., que podem subsidiar programas de melhoramento das espécies de interesse.

Palavras-chave: cpSSR; cpDNA; melhoramento genético; melhoramento florestal

INTRODUCTION

Pinus spp. is one of the 11 genera in Pinaceae Family and comprises 111 species. It is a Gymnosperm's group that belongs to Pinophyta Division, also called coniferous. The conifers cover various woody plant species, among the world most robust and ancient (GERNANDT et al., 2011), with origin center in North Hemisphere (PRICE et al., 2000). The *Pinus* species are ecologically and economically important due to the high productivity, broad adaptation to climate adversities and multiple uses as lumber, resins, fibers, woody chips, cellulose, among others (MACEDO et al., 2015; MISSIO et al., 2015; SEBBENN et al., 1994; SHIMIZU, 2008; VITALE; MIRANDA et al., 2010).

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Molecular data base are important tools for genetic studies due to easily and fast access as well as availability of genomes to scientific community (MACHADO, 2012). Nowadays, the National Center for Biotechnology Information (NCBI) has the biggest public database available, counting on with 127 million sequences, approximately, which makes viable many researches *in silico* (MACHADO, 2012; MELOTTO-PASSARIN et al., 2011; TAMBARUSSI et al., 2009). Many plastid genomes are available at public database *GenBank*, which facilitates the development and selection of cpDNA molecular markers to genetic studies and forest breeding program.

The number of available in DNA database as *GenBank* is in constantly expansion especially due to sequencing methodologies development. More than 800 complete chloroplast genomes of terrestrial plants were deposited until December 2016 in NCBI (BARRET et al., 2016; ZENG et al., 2014). The most representative families with complete cpDNA available are Chrysobalanaceae, followed to Myrtaceae, Arecaceae and Pinaceae (personal communication).

Molecular markers make viable to characterization of large number of genotypes with relatively fast and simple procedures (BERED et al., 1997), and have been used to various genetic analysis, as hybrid and clone identification, gene flow studies (FERREIRA; GRATTAPAGLIA, 1998), determination of strategies for the conservation of endangered populations (BUCCI et al., 1997), population genetics studies, paternity tests, forensic science, demographic process, germplasm characterization (KURT et al., 2012; OLIVEIRA et al., 2006; SANTOS et al., 2011), among others.

The chloroplast microsatellite markers (cpSSRs) are found in chloroplast genomes and can be applied in breeding programs (ANDRADE et al., 2018; MCKINNON et al., 2010; TAMBARUSSI et al., 2009) and to vegetal systematics to infer about phylogenetic relations in different taxa (DREW et al., 2014; MELOTTO-PASSARIN et al., 2011; RAJENDRAKUMAR et al., 2007; TAMBARUSSI et al., 2009; ZHANG et al., 2016). The abundance of these repetitive regions is highly variable among taxa, which is useful for distinguishing individuals (OLIVEIRA et al., 2006). However, the identification of these regions demands on knowledge of genome sequences (MORRIS, 2016).

Thus, with the present research we aimed to characterize all chloroplast DNA of *Pinus* species available in NCBI database and determine which and how many cpSSR regions exist and how they are distributed throughout the cpDNA in this gender.

MATERIAL AND METHODS

We selected all the cpDNA complete sequences of *Pinus* species available in NCBI database. Only the *Pinus longaeva* D. K. Bailey (NC_011157) did not present available sequence.

All the 20 cpDNAs were processed with FastPCR 6.5.40 software (KALENDAR et al., 2009), to identify the cpSSR regions. The repetitive sequences observed in the genomes were classified as cpSSR when presented motif repetition ≥ 8 to mononucleotides, ≥ 6 to dinucleotides and ≥ 3 to tri-, tetra-, penta- and hexanucleotides. We still classified the cpSSR according its presence in coding (genic) or non-coding (intergenic) regions. The types of cpSSR were considered according to Oliveira et al. (2006).

RESULTS AND DISCUSSION

The *Pinus* spp. cpDNAs presented different sizes, varying between 115,576 bp for *P. strobus* to 121,530 bp for *P. taeda* (Table 1).

The size range observed in cpDNAs of closer species can be attributed to variations in intergenic regions length, that can be associated to species evolution process (XIAO-MING et al., 2017). In terrestrial plants, the cpDNA varies between $\approx 120,000$ and $\approx 160,000$ bp (PALMER, 1995). For Gymnosperm, the size of cpDNA the chloroplast genome is significantly lower than for Angiosperm (XIAO-MING et al., 2017) and, for *Eucalyptus* spp. and another superior genus, cpDNAs with around 160,000 bp are commonly observed (EGUILUZ et al., 2017; LI et al., 2017; PAIVA et al., 2011; SAINA et al., 2018; STEANE et al., 2005).

Table 1. Accession number of *Pinus* spp. with available cpDNA from GenBank.**Tabela 1.** Número de acesso de *Pinus* spp. com cpDNA disponível no GenBank.

Species	Plastome size (pb)	Accession number
<i>Pinus armandii</i> Franch.	117.265	NC_029847
<i>Pinus bungeana</i> Zucc. ex Endl.	117.861	NC_028421
<i>Pinus contorta</i> Douglas ex Loudon	120.438	NC_011153
<i>Pinus gerardiana</i> Wall. ex D.Don	117.618	NC_011154
<i>Pinus greggii</i> Engelm. ex Parl.	120.501	NC_035947
<i>Pinus jaliscana</i> Perez de la Rosa	120.715	NC_035948
<i>Pinus koraiensis</i> Siebold & Zucc.	117.190	NC_004677
<i>Pinus krempfii</i> Lecomte	116.989	NC_011155
<i>Pinus lambertiana</i> Douglas	117.239	NC_011156
<i>Pinus massoniana</i> Lamb.	119.739	NC_021439
<i>Pinus monophylla</i> Torr. & Frem.	116.479	NC_011158
<i>Pinus nelsonii</i> Shaw	116.834	NC_011159
<i>Pinus oocarpa</i> Schiede ex Schltdl.	120.596	NC_036949
<i>Pinus sibirica</i> Du Tour	116.635	NC_028552
<i>Pinus strobus</i> L.	115.576	NC_026302
<i>Pinus sylvestris</i> L.	119.758	NC_035069
<i>Pinus tabuliformis</i> Carriere	119.646	NC_028531
<i>Pinus taiwanensis</i> Hayata	119.741	NC_027415
<i>Pinus thunbergii</i> Parl.	119.707	NC_001631
<i>Pinus taeda</i> L.	121.530	NC_021440

Regarding the cpSSR regions identified, the majority were classified as mononucleotides. In 31 *Eucalyptus* species the total number of cpSSRs varied between 71 for *Eucalyptus melliodora* A. Cunn and 135 for *Eucalyptus aromaphloia* L.D.Pryor J.H.Willis, superior than we observed to *Pinus* spp. but with predominance of mononucleotides too (ANDRADE et al., 2018). George et al. (2015) also observed a wide range of SSRs in many superior species, featured for 270 cpSSRs for *P. koraiensis* and 693 cpSSRs for *Welwitschia mirabilis* Hook. F., with predominance of mononucleotide type.

We found 1,542 cpSSRs in the 20 studied *Pinus* species, 56.15% of them in non-coding regions (Table 2). The most part of cpSSRs were classified as mononucleotides microsatellites (1,333), but we also found 53 dinucleotides, 61 tri-, 62 tetra-, 17 penta- and 16 hexanucleotides repeats. The total number of cpSSR observed for each species varies between 66 for *P. contorta* and 84 for *P. strobus* and *P. lambertiana*.

We found the majority of cpSSRs in non-coding regions, which was observed to other botanical families, with *in silico* analysis, as Brassicaceae (GANDHI et al., 2010) and Myrtaceae (ANDRADE et al., 2018). According to Hancock (1995), in general, the microsatellite markers are found in non-coding regions of genome, due to the neutrality of evolutive process in these regions, which favoured the accumulation of tandem repetitive regions, as well as due to the slippage in DNA replication, which occurs specially in short motifs riches of adenine and thymine. Other researches with species of Poaceae, Fabaceae (SASKI et al., 2005) and Solanaceae (DANIELL et al., 2006) corroborates with what we found on the present research.

Usually, in *Pinus* species the dinucleotides repetitions are much more abundant than tri- or tetranucleotides (SMITH et al., 1994), which we did not observed, once the total cpSSRs among these types were similar, with 4.02% of tetra-, 3.96% of tri- and 3.44% of dinucleotides repeats. In cereal species, the trinucleotides SSR are more frequent (54-78%), followed by dinucleotides (17.1-40.4%) and tetranucleotides repeats (3-6%) (VARSHNEY et al., 2012). Andrade et al. (2018) found only 0.48% of di- and 3.57% of trinucleotides repeats for species of *Eucalyptus* genera.

Table 2. Total and frequency (%) of genic and intergenic cpSSRs based on motif size, for each species.**Tabela 2.** Número total e frequência (%) de cpSSRs gênicos e intergênicos baseado no tamanho da sequência de repetição, para cada espécie.

Species	Mono			Di			Tri			Tetra			Penta			Hexa			Total
	Intergenic (%)	Genic (%)	Intergenic (%)	Intergenic (%)	Genic (%)	Intergenic (%)	Intergenic (%)	Genic (%)	Intergenic (%)	Intergenic (%)	Genic (%)	Intergenic (%)	Intergenic (%)	Genic (%)	Intergenic (%)	Intergenic (%)	Genic (%)	Intergenic (%)	
<i>P. armandii</i>	42.6 (29)	57.3 (39)	0 (0)	0 (0)	100 (4)	0 (0)	0 (0)	0 (0)	25 (1)	75 (3)	0 (0)	100 (1)	0 (0)	100 (1)	100 (1)	0 (0)	0 (0)	0 (0)	78
<i>P. bungeana</i>	52.8 (38)	47.2 (34)	100 (2)	0 (0)	0 (0)	33.3 (1)	66.7 (2)	0 (0)	0 (0)	100 (1)	0 (0)	100 (2)	100 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	80
<i>P. contorta</i>	63.2 (36)	36.8 (21)	100 (3)	0 (0)	0 (0)	0 (0)	100 (2)	0 (0)	66.7 (2)	33.3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	66
<i>P. gerardiana</i>	58.8 (40)	41.2 (28)	66.7 (2)	33.3 (1)	33.3 (1)	100 (1)	100 (1)	0 (0)	0 (0)	100 (2)	0 (0)	100 (1)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	75
<i>P. greggi</i>	56.7 (38)	43.3 (29)	100 (2)	0 (0)	0 (0)	75 (3)	25 (1)	66.7 (2)	66.7 (2)	33.3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	76
<i>P. jaliscana</i>	64.8 (46)	35.2 (25)	100 (2)	0 (0)	0 (0)	100 (3)	0 (0)	66.7 (2)	66.7 (2)	33.3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	80
<i>P. koraiensis</i>	33.8 (22)	66.1 (43)	66.7 (2)	33.3 (1)	33.3 (1)	33.3 (1)	66.7 (2)	0 (0)	0 (0)	100 (3)	0 (0)	100 (2)	100 (2)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	77
<i>P. krempfii</i>	59.4 (41)	40.6 (28)	100 (3)	0 (0)	0 (0)	50 (2)	50 (2)	60 (3)	60 (3)	40 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	82
<i>P. lambertiana</i>	58.3 (42)	41.7 (30)	75 (3)	25 (1)	25 (1)	50 (1)	50 (1)	33.3 (1)	33.3 (1)	66.7 (2)	0 (0)	100 (2)	100 (2)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	84
<i>P. massoniana</i>	60.6 (37)	39.3 (24)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	100 (2)	100 (2)	0 (0)	0 (0)	100 (4)	100 (4)	0 (0)	0 (0)	100 (1)	0 (0)	0 (0)	69
<i>P. monophylla</i>	57.6 (38)	42.4 (28)	100 (3)	0 (0)	0 (0)	50 (2)	50 (2)	33.3 (1)	33.3 (1)	66.7 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	50 (1)	50 (1)	0 (0)	78
<i>P. nelsonii</i>	54.5 (30)	45.4 (25)	100 (4)	0 (0)	0 (0)	40 (2)	40 (2)	60 (3)	50 (1)	50 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	100 (1)	0 (0)	67
<i>P. oocarpa</i>	57.6 (38)	42.4 (28)	100 (2)	0 (0)	0 (0)	50 (4)	50 (4)	50 (4)	50 (1)	50 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	79
<i>P. sibirica</i>	54.3 (38)	45.7 (32)	75 (3)	25 (1)	25 (1)	50 (2)	50 (2)	50 (2)	66.7 (2)	33.3 (1)	0 (0)	100 (1)	100 (1)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	83
<i>P. strobus</i>	54.9 (39)	45.1 (32)	50 (2)	50 (2)	50 (2)	50 (2)	50 (2)	50 (2)	50 (1)	50 (1)	0 (0)	100 (2)	100 (2)	0 (0)	0 (0)	100 (1)	0 (0)	0 (0)	84
<i>P. sylvestris</i>	62.3 (43)	37.7 (26)	100 (3)	0 (0)	0 (0)	100 (1)	100 (1)	33.3 (1)	33.3 (1)	66.7 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	76
<i>P. tabulaformis</i>	58.5 (38)	41.5 (27)	100 (2)	0 (0)	0 (0)	60 (4)	60 (4)	40 (1)	60 (3)	40 (2)	0 (0)	50 (1)	50 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	79
<i>P. taeda</i>	59.4 (38)	40.6 (26)	100 (1)	0 (0)	0 (0)	33.3 (2)	33.3 (2)	66.7 (1)	33.3 (1)	66.7 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	100 (1)	0 (0)	72
<i>P. taiwanensis</i>	54.4 (37)	45.6 (31)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	50 (3)	50 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	0 (0)	76
<i>P. thunbergii</i>	44.9 (31)	55.1 (38)	100 (2)	0 (0)	0 (0)	100 (5)	0 (0)	50 (2)	50 (2)	50 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)	0 (0)	81
Total per region and type	55.44(739)	44.56(594)	81.13(43)	18.87(10)	44.26(2)	55.74(34)	44.26(2)	46.77(29)	53.23(33)	88.23(15)	11.77(2)	37.5(6)	62.5(10)	1542					
Total	86.45(1333)	3.44(53)	3.96(61)	4.02(62)	1.10(17)	1.04(16)													

CONCLUSION

The species with the most part of identified cpSSRs were *P. strobus* and *P. lambertiana*, and the species with the minor part were *P. contorta*.

A wide range of cpSSRs were identified in the studied species, with predominance of mononucleotide microsatellites in non-coding regions.

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