Communication

Genetic Markers for Species Conservation and Timber Tracking: Development of Microsatellite Primers for the Tropical African Tree Species *Prioria balsamifera* and *Prioria oxyphylla*

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Received: 30 September 2019; Accepted: 13 November 2019; Published: 15 November 2019

Abstract: *Research Highlights:* Two novel sets of polymorphic microsatellite markers were developed for *Prioria balsamifera* and *Prioria oxyphylla* through high-throughput sequencing. Validation in two populations of each species proved the utility of the developed primers to estimate genetic diversity at population level. *Background and Objectives: Prioria balsamifera* and *Prioria oxyphylla* are tropical tree species from Central Africa. They produce a high-quality, multi-purpose timber that is of great interest to the international market. *Prioria balsamifera* has been included as 'endangered' on the IUCN Red List of Threatened Species. In order to set up adequate management plans and facilitate timber tracking, knowledge on the genetic diversity at population level is needed. Therefore, we aim to develop microsatellite markers that can be used for species conservation, forensics, plant breeding and population genetics studies. *Materials and Methods:* Genomic DNA of *P. balsamifera* and *P. oxyphylla* was sequenced on an Illumina NextSeq platform (Illumina Inc., San Diego, CA, USA), generating 829,421 and 772,018 paired-end reads that contained 7148 and 7004 microsatellite sequences, respectively. The QDD-pipeline was used to design primers, which were tested for amplification in two populations of each species. Cross-species amplification was tested in all seven African *Prioria* species. *Results:* For *P. balsamifera*, 16 polymorphic microsatellite markers were developed and combined in three multiplexes. Inbreeding appeared to be absent but genetic diversity was low in both populations. For *P. oxyphylla*, 15 polymorphic microsatellite markers were developed and combined in three multiplexes. Genetic diversity was low in both populations and estimated null allele frequencies were high for multiple loci. Cross-species amplification tests demonstrated the occurrence of conserved loci that amplified for most of the African *Prioria* species. *Conclusions:* The microsatellite markers prove to be useful for estimating genetic diversity at population level. These novel markers can be used to study gene flow and spatial genetic structure in *Prioria* species, which is needed to set up proper conservation guidelines and to prevent genetic erosion.

Keywords: African rainforest; SSR; population genetics; timber tracking; species identification; Fabaceae; Detarioideae; DNA fingerprinting; high-throughput sequencing

1. Introduction

The genus *Prioria* Griseb. (Fabaceae, subfamily Detarioideae [1]) comprises 14 tree species that occupy various habitats in tropical regions [2]. Multiple species of the genus are of national economic importance and four are listed as 'vulnerable' or 'endangered' on the IUCN Red List of Threatened Species [3]. Until the taxonomic revision by Breteler in 1999 [2], the name *Prioria* was only used for the American species (*Prioria copaifera*). However, based on morphological evidence, the genera *Kingiodendron* from Asia and the Pacific region (six species) and *Gossweilerodendron* and *Oxystigma* from Africa (7 species) were lumped into the genus *Prioria* [2].

In Central Africa, *Prioria balsamifera* (Verm.) Breteler (syn. *Gossweilerodendron balsamiferum*) and *Prioria oxyphylla* (Harms) Breteler (syn. *Pterygopodium oxyphyllum* and *Oxystigma oxyphyllum*) are of great interest for the national and international market, since both tree species produce a high-quality multi-purpose timber. The wood of *Prioria balsamifera*, traded as 'tola' or 'agba', is used for construction, flooring, joinery, ship building, interior trim, furniture, veneer and plywood [4]. In riverine areas, the boles are traditionally used to make canoes [5]. Furthermore, the sapwood resin can be applied to protect furniture from wood parasites [5] or is used as illuminant [4]. Since the wood of *P. balsamifera* resembles that of African mahogany (*Entandrophragma* and *Khaya* spp.), it has been traded as a substitute. Also, *P. balsamifera* logs are sometimes mixed with those of *P. joveri*, which is classified as 'vulnerable' on the IUCN Red List of Threatened Species [4]. *Prioria balsamifera* can be found in lowland semi-deciduous and evergreen forests from southeast Nigeria to Cabinda (Angola) and the Democratic Republic of Congo (DR Congo) [4]. Although *P. balsamifera* is shade tolerant and natural generation can be abundant, it has a scattered forest distribution—often occurring in small groups of a few trees—caused by openings in the canopy that needed to reach maturity [4]. *Prioria oxyphylla* occurs scattered in lowland rainforests from southeast Nigeria and Central African Republic to Cabinda and DR Congo [6]. Although *Prioria oxyphylla* produces timber similar in quality to that of *P. balsamifera*, only small amounts are traded on the international market [6]. The wood is known as 'tchitola' and is used for light construction works, flooring, joinery, furniture, plywood, and veneer. The latter wood product is used as a substitute for veneer of walnut (*Juglans regia*) and jatoba or guapinol (*Hymenaea courbaril*) in Europe and the United States [6].

Both *P. balsamifera* and *P. oxyphylla* populations have suffered from heavy exploitation and habitat loss or degradation [4,6]. Therefore, *P. balsamifera* has been listed as 'endangered' on the IUCN Red List. Furthermore, the Food and Agriculture Organization of the United Nations (FAO) recommends protection of the genetic material in order to set up planting programmes in the future [3]. *Prioria oxyphylla* is not listed on the IUCN Red List but despite its large distribution area, its occurrence is scattered and is uncommon in many regions of its distribution range [6]. Consequently, the species may be susceptible to genetic erosion, so characterization of its genetic variation and monitoring of its populations is highly recommended. Additionally, knowledge on natural regeneration, gene flow and genetic diversity is needed to design adequate management plans which ensure a sustainable production and harvest, especially since the volume of timber available for the international market appears to be limited [6]. Similar issues occur for *P. balsamifera*. Despite the commercial importance of *Prioria* timber for the international market, there has been no attention for genetic research and silviculture for future planting programmes [4].

In order to obtain more insights into the genetic diversity at population level, microsatellite markers have proven to be a valuable tool. Microsatellites, also referred to as simple sequence repeats (SSRs) or short tandem repeats (STRs), are short repetitive regions (1 to 6 bp) in the genome that mainly occur in non-coding DNA [7]. Because of their high levels of polymorphism, co-dominance and reproducibility, microsatellites have proven their utility in many research areas such as plant breeding, forensics, species conservation, population genetics, phylogeography and species delimitation [8–13].

Given this broad range of applications, the development of microsatellites can be considered as very beneficial for *P. balsamifera* and *P. oxyphylla*. For example, by applying them in population genetics studies, information can be gained on the genetic composition of both species, as well as on the

amount of gene flow between populations. This knowledge is essential to prevent genetic erosion and to make proper assessments for conservation management. Moreover, *P. balsamifera* and *P. oxyphylla* may be suitable for commercial plantations. For this, information about the intraspecific diversity is of great importance, as it optimises breeding programmes by enabling the selection of superior parent trees. Properly managed plantations or production forests can reduce the pressure on wild stands and increase the timber availability for the international market. Additionally, microsatellites provide a useful tool for timber tracking based on DNA fingerprinting [10,14,15]. Since illegal logging causes degradation and loss of forests worldwide, and trade in illegal timber and wood products distorts global markets, unfalsifiable methods to identify the origin of timber, such as DNA fingerprinting, are extremely valuable. Moreover, since microsatellite loci are usually short fragments (<500 bp), amplification from degraded DNA, often found in logs or processed wood, is possible.

To address the listed issues, we aim to develop a novel set of microsatellite markers for *Prioria balsamifera* and *Prioria oxyphylla* using high-throughput sequencing data. These markers will be validated in two populations of each species in order to assess their applicability. Additionally, cross-species amplification will be tested in all seven African *Prioria* species.

2. Materials and Methods

2.1. Microsatellite Primer Development

DNA was isolated from silica-dried leaves of *Prioria balsamifera* (corresponding voucher: BR0000013003845 [16]) and *Prioria oxyphylla* (corresponding voucher: BR0000013007126 [17]), using a cetyltrimethylammonium bromide (CTAB) protocol [18] with an additional sorbitol washing step. Subsequently, the extracted DNA was used to develop a non-enriched genomic library for each species using a modified version of the protocol in [19], described in full by Tosso et al. [20]. Genomic libraries were sent for sequencing on an Illumina NextSeq platform at GIGA (Liège, Belgium). The sequencing run generated 829,421 paired-end 150 bp reads for *P. balsamifera* and 772,018 paired-end 150 bp reads for *P. oxyphylla*.

Reads were merged with FLASH v1.2.11 (Center for Computational Biology, Baltimore, MD, USA) [21] and the QDD pipeline [22] was used to identify microsatellite loci, for which suitable primers were developed with the implemented Primer3 algorithm [23] using the parameters described in [24]. For *P. balsamifera*, 7148 microsatellite sequences with primers were generated, from which the 24 best primer pairs, forward and reverse, were selected following the criteria described in [22]. All chosen microsatellite loci contained 8 to 15 di- or trinucleotide repeats. For *P. oxyphylla*, 7004 microsatellite sequences with primers were generated and as for *P. balsamifera*, the 24 best primer pairs were selected. Here, all chosen microsatellite loci contained 8 to 23 di- or trinucleotide repeats.

First, the two sets of the 24 selected primer pairs were tested individually on three individuals of the corresponding species. The products used for the amplification reaction and the PCR conditions were the same as in [25]. PCR products were visualized with a QIAxcel Advanced system (QIAGEN, Venlo, Netherlands) to check whether amplification was successful.

Second, the primers that showed amplification in at least one of the three samples were reamplified and genotyped to assess their level of polymorphism and to check the readability of the peak pattern. The amplification protocol with low-cost M13-like fluorescent labelling and the PCR conditions are described in [25]. In this study, four universal sequences (Q1–Q4, Table 1) and fluorescent dyes (6-FAM, NED, VIC and PET respectively) (Applied Biosystems, Foster City, CA, USA) were used; the annealing temperature during the first 25 repeated cycles was set to 55 °C. Genotyping was done on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), with 0.8 µL PCR product (3 to 4 samples were pooled), 10 µL Hi-Di Formamide (Applied Biosystems) and 0.3 µL MapMarker 500 labelled with DY-632 (Eurogentec, Liège, Belgium). The resulting electropherograms were analysed using the Microsatellite Plugin 1.4.6 in Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand).

Table 1. Characterization of polymorphic microsatellite markers isolated from *Prioria balsamifera* (16 loci) and *Prioria oxyphylla* (15 loci).

Locus	Primer Sequences (5′-3′) [1]	Labelled Primer	Repeat Motif [2]	Allele Size Range (bp)	GenBank Accession No.
Prioria balsamifera					
Multiplex 1					
PriB07	F: CACTGCTTAGAGCGATGCTCAGGGCAAGATGAATAATG R: AAAGGAACCACCGATGAATA	Q3-VIC	$(TC)_8$	144–147	MN648900
PriB19	F: CACTGCTTAGAGCGATGCTCTGAATTATTATCAGCCACTTC R: GGCGTTTCTTAATTTGGTTT	Q3-VIC	$(TC)_8$	198–207	MN648909
PriB23	F: CACTGCTTAGAGCGATGCTAATATGGAGTCATCGCTTCC R: GCATTCGGACAGAGGGAG	Q3-VIC	$(GA)_9$	243–254	MN648912
PriB10	F: TAGGAGTGCAGCAAGCATTTTGCATCTAAAGTTTGAGGG R: TAATGGAGCTTATGCTTTGG	Q2-NED	$(AG)_9$	146–149	MN648902
PriB22	F: TAGGAGTGCAGCAAGCATAACGGACCGGTACTTACAGA R: GCTTAGACAAATGTTAGAATCACC	Q2-NED	$(TG)_9$	203–211	MN648911
PriB04	F: CTAGTTATTGCTCAGCGGTAATATGCTTGGAATGGATGG R: ATTACTCCTTGGCGCAGTC	Q4-PET	$(GA)_8$	113–142	MN648899
Multiplex 2					
PriB13	F: TGTAAAACGACGGCCAGTTGTTTACTAACTTGCAGAATCCA R: CAGTAAGGATGGCTCTCCC	Q1-6-FAM	$(CT)_{12}$	139–159	MN648905
PriB15	F: CACTGCTTAGAGCGATGCCAAGTCTACGCCAAATGGTC R: GCGTTTAAACATCAATTGGAC	Q3-VIC	$(TC)_{12}$	147–167	MN648907
PriB02	F: TAGGAGTGCAGCAAGCATTGCGTGTACATGTGTATCTCC R: AGACACCCAACTTTCAATGAT	Q2-NED	$(GA)_{10}$	95–116	MN648897
PriB14	F: TAGGAGTGCAGCAAGCATGGGAAGACAAACAAGAGTCAG R: TGACCTAAAGAATAAGACATCCC	Q2-NED	$(CT)_9$	153–157	MN648906
PriB12	F: CTAGTTATTGCTCAGCGGTAAATTTGCCCTCCCTTACAT R: TGACTACAAAGCATATGAATAGAAA	Q4-PET	$(GT)_{12}$	136–159	MN648904
Multiplex 3					
PriB03	F: CACTGCTTAGAGCGATGCATCGGTGAGTACATCGAACC R: GCAGTTCAAGTTAGTTTGTGC	Q3-VIC	$(AC)_8$	106–112	MN648898
PriB11	F: CACTGCTTAGAGCGATGCCAATAGAATGATGGTCAAGAGC R: CTTCCAGAGAAACCCACCT	Q3-VIC	$(CT)_8$	144–160	MN648903
PriB18	F: TAGGAGTGCAGCAAGCATAGAGTCGTTGTGAGCTGTGA R: AGTGACACGCGTTCAAATAC	Q2-NED	$(AG)_{14}$	168–202	MN648908
PriB08	F: CTAGTTATTGCTCAGCGGTATATTGCAGCAGAGACACCA R: AGTTTCGCTCTTCTTACCGA	Q4-PET	$(AG)_8$	147–167	MN648901
PriB20	F: CTAGTTATTGCTCAGCGGTTGTGTTGCAAGAACGATAGTC R: ACAAGACTCTAAATTCCAAGACA	Q4-PET	$(GA)_{10}$	196–223	MN648910

Table 1. *Cont.*

Locus	Primer Sequences (5′-3′) [1]	Labelled Primer	Repeat Motif [2]	Allele Size Range (bp)	GenBank Accession No.
Prioria oxyphylla					
Multiplex 1					
PriO03	F: CACTGCTTAGAGCGATGCGAGAAGTGGTCTCCAACCAT R: CCTGAAGTCGAGAGGAGTGT	Q3-VIC	$(TC)_{8}$	103–117	MN648914
PriO23	F: CACTGCTTAGAGCGATGCTTACGCTATTTACTTTGCCGT R: GCGTTTATGTGAAGCATTTG	Q3-VIC	$(AG)_{9}$	196–213	MN648926
PriO18	F: TAGGAGTGCAGCAAGCATAGGAGGTCCCGGATAATCTA R: TAGGGACATGAGCAGTAGCA	Q2-NED	$(TTG)_{8}$	168–185	MN648922
PriO04	F: CTAGTTATTGCTCAGCGGTTACATGCACCGTTTGAGTGT R: CTGCGTTTGAAGTGGAAAT	Q4-PET	$(CT)_{11}$	115–120	MN648915
Multiplex 2					
PriO13	F: TGTAAAACGACGGCCAGTTGGAAATCATTCAATCTCCC R: ATCCATCCCTCCTCGTTG	Q1-6-FAM	$(GCA)_{8}$	151–162	MN648919
PriO19	F: CACTGCTTAGAGCGATGCGAAATTCTGTGGTAGTGGTGG R: GAGCTATCAATATCACCAAACG	Q3-VIC	$(TTC)_{8}$	182–227	MN648923
PriO10	F: TAGGAGTGCAGCAAGCATAACCCTCTCACTTCCATCTTT R: GAAGGCCTAAGTAATTATCAACC	Q2-NED	$(TC)_{12}$	128–151	MN648918
PriO22	F: TAGGAGTGCAGCAAGCATGTGAGCTGGAACGCAAGT R: TACGTCCAATCCTTCTAGTCA	Q2-NED	$(TC)_{11}$	165–203	MN648925
PriO16	F: CTAGTTATTGCTCAGCGGTTGTCGGAGCCAATCTATTCT R: CAGCAAATTTCACCACTTCA	Q4-PET	$(AG)_{11}$	169–189	MN648921
PriO24	F: CTAGTTATTGCTCAGCGGTGTTGCACGGAGGAAATACAT R: TGTAGAATAAGATAAGGTTGCCA	Q4-PET	$(AG)_{10}$	250–259	MN648927
Multiplex 3					
PriO01	F: TGTAAAACGACGGCCAGTTGCATAGTGCTACTCCTCACA R: GGGATGGTCACTACCATAGTT	Q1-6-FAM	$(AG)_{8}$	94–115	MN648913
PriO07	F: CACTGCTTAGAGCGATGCTCATCTATCAAGTAAGAGGTGGA R: TCTCCACTTGAATGTAAATGCT	Q3-VIC	$(AC)_{11}$	128–144	MN648917
PriO15	F: CACTGCTTAGAGCGATGCTTTGATAGAGATCAATGGCG R: CAAACTGATACCAAATTAAGTGAA	Q3-VIC	$(TTG)_{9}$	155–182	MN648920
PriO06	F: TAGGAGTGCAGCAAGCATGCACAAGAGGCTATGGAGTT R: AACCGTTGGAATTCTCGTTA	Q2-NED	$(AG)_{9}$	111–135	MN648916
PriO20	F: CTAGTTATTGCTCAGCGGTTCACCAGGATGTATAACATTGC R: GTATATTGCGTGAACATACCCA	Q4-PET	$(TC)_{10}$	193–213	MN648924

[1] The universal linkers (Q1–Q4) attached to the sequence of the forward primers are underlined. [2] The number of repeats found in the sample used for the primer development. Corresponds to the GenBank accession number.

Third, unreadable and monomorphic loci were excluded, while the remaining loci were combined in various multiplexes per species using Multiplex Manager 1.2 [26] to reduce costs and hands-on time in the lab. Amplification of the different primer combinations was tested in seven samples per species with the Type-it Microsatellite PCR kit (QIAGEN) using the amplification volumes and PCR conditions described in [25], except that the annealing temperature during the first 25 repeated cycles was at 56 °C instead of 57 °C.

Cross-species amplification was tested with the final *P. balsamifera* and *P. oxyphylla* multiplexes on the other five African *Prioria* species: *P. buchholzii, P. msoo, P. gilbertii, P. mannii* and *P. joveri*.

The microsatellite containing sequences obtained through high-throughput sequencing and used for the primer development were deposited on GenBank (Table 1).

2.2. Microsatellite Characterization and Preliminary Population Genetics Analyses

The newly developed primer sets were validated for population genetics with 65 individuals of *P. balsamifera* and 53 individuals of *P. oxyphylla*, collected at two locations in the DR Congo, the Luki Biosphere Reserve (5°41′42.4″S, 13°11′05.5″E) and the Yangambi Biosphere Reserve (0°48′01.2″N, 24°28′59.5″E) (Figure S1). For both species and populations, a reference voucher was deposited at the herbarium of Meise Botanic Garden, Belgium (barcodes *Prioria balsamifera*: BR0000013572778 (Luki) and BR0000013277444 (Yangambi); barcodes *Prioria oxyphylla*: BR0000015225634V (Luki) and BR0000013007126 (Yangambi)).

Extractions were made from leaves (silica-dried or herbarium) and cambium using a cetyltrimethylammonium bromide (CTAB) protocol [18] with an additional sorbitol washing step. The PCR and genotyping conditions were the same as those in the third step of the primer development (see Section 2.1). The resulting electropherograms were analysed using the Microsatellite Plugin 1.4.6 in Geneious 9.1.8 (Biomatters Ltd.).

The number of alleles per locus (N_A), observed (*Ho*) and expected (*He*) heterozygosity, Weir and Cockerham's fixation index (F_{IS}) and the deviation from Hardy-Weinberg Equilibrium (HWE) were calculated with SPAGeDi 1.5d [27]. The Jackknife method in INEST v2.2 [28] was used to estimate the expected frequency of null alleles. Polymorphism Information Content (PIC) was calculated using CERVUS 3.0.7 (Field Genetics Ltd., London, UK) [29].

3. Results and Discussion

3.1. Microsatellite Primer Development

For *Prioria balsamifera*, 23 out of the 24 primer pairs selected for testing in the laboratory could be amplified in at least one of three samples. These 23 pairs were then reamplified and genotyped, resulting in 16 useful polymorphic primer pairs, which were combined in three multiplexes (Table 1) and used for all subsequent analyses. Two out of the seven removed primer pairs (Table S1) appeared monomorphic in the seven samples used for testing, while another four pairs failed to amplify, probably due to problems with the incorporation of the fluorescently labelled Q-tails. A last primer pair showed polymorphism and successfully amplified in simplex, but amplification was unsuccessful in all multiplex combinations that were tested.

For *Prioria oxyphylla*, 16 out of 24 primer pairs amplified in at least one of three samples. Reamplification and genotyping of these 16 pairs yielded 15 readable polymorphic primer pairs, which were combined in three multiplexes (Table 1). The pair that was removed showed multiple peaks and stutters, which made it impossible to infer allele sizes and to define bins.

3.2. Population Genetics in Prioria balsamifera

The final three microsatellite multiplexes containing 16 primers for *P. balsamifera* were tested for population genetic analyses in 65 individuals originating from Luki (*n* = 34) and Yangambi (*n* = 31). Microsatellite loci showed one to nine alleles per locus in both populations combined, with an average

of 4.310 alleles per locus (Table 2). Although loci PriB4 and PriB14 appeared to be monomorphic in individuals from Luki and Yangambi, multiple alleles were detected when testing for polymorphism during primer development. Consequently, both primers could be informative in genetic studies that consider a larger part of the species' distribution area.

Table 2. Genetic diversity indices for two populations of *Prioria balsamifera* and *Prioria oxyphylla* using the 16 and 15 newly developed microsatellite markers.

	PIC	N_a	N_a	H_o	H_e	F_{IS}	***Null***	N_a	H_o	H_e	F_{IS}	***Null***
Prioria balsamifera			Luki (n = 34)					Yangambi (n = 31)				
PriB07	0.292	2	2	0.412	0.500	0.179	0.055	1	0.000	0.000	*na*	*na*
PriB19	0.044	2	2	0.088	0.086	−0.031	0.000	1	0.000	0.000	*na*	*na*
PriB23	0.372	4	2	0.559	0.507	−0.104	0.000	4	0.258	0.237	−0.088	0.000
PriB10	0.336	2	2	0.382	0.314	−0.222	0.000	2	0.483	0.503	0.042	0.013
PriB22	0.197	3	3	0.382	0.372	−0.029	0.000	1	0.000	0.000	*na*	*na*
PriB04	0.000	1	1	0.000	0.000	*na*	*na*	1	0.000	0.000	*na*	*na*
PriB13	0.705	7	6	0.706	0.684	−0.032	0.000	5	0.548	0.535	−0.025	0.000
PriB15	0.584	6	5	0.735	0.613	−0.204	0.000	3	0.613	0.524	−0.173	0.000
PriB02	0.651	9	5	0.441	0.446	0.010	0.002	6	0.516	0.749	0.314 *	0.125
PriB14	0.000	1	1	0.000	0.000	*na*	*na*	1	0.000	0.000	*na*	*na*
PriB12	0.737	7	4	0.500	0.741	0.328 *	0.141	4	0.655	0.649	−0.009	0.018
PriB03	0.404	3	3	0.529	0.551	0.040	0.045	2	0.419	0.455	0.080	0.021
PriB11	0.529	6	5	0.647	0.544	−0.194	0.000	3	0.516	0.539	0.044	0.007
PriB18	0.316	7	3	0.147	0.140	−0.051	0.000	7	0.226	0.507	0.558 *	0.209
PriB08	0.146	3	3	0.324	0.280	−0.160	0.000	1	0.000	0.000	*na*	*na*
PriB20	0.518	6	5	0.735	0.608	−0.213	0.000	4	0.138	0.134	−0.032	0.000
Multilocus average	0.364	4.313	3.250	0.412	0.399	−0.032	0.017	2.880	0.273	0.302	0.097	0.037
Prioria oxyphylla			Luki (n = 21)					Yangambi (n = 32)				
PriO03	0.504	6	5	0.714	0.688	−0.040	0.000	4	0.250	0.308	0.191	0.048
PriO23	0.597	4	1	0.000	0.000	*na*	*na*	3	0.071	0.505	0.863 *	0.748
PriO18	0.309	3	3	0.154	0.465	0.678 *	0.486	1	0.000	0.000	*na*	*na*
PriO04	0.367	2	1	0.000	0.000	*na*	*na*	1	0.000	0.000	*na*	*na*
PriO13	0.593	4	4	0.524	0.515	−0.019	0.000	3	0.625	0.659	0.052	0.010
PriO19	0.720	10	4	0.429	0.484	0.118	0.015	8	0.563	0.640	0.123	0.058
PriO10	0.679	6	3	0.158	0.457	0.660*	0.348	5	0.130	0.621	0.794 *	0.550
PriO22	0.606	5	3	0.111	0.307	0.652	0.451	3	0.438	0.538	0.189	0.092
PriO16	0.136	2	1	0.000	0.000	*na*	*na*	2	0.000	0.239	1.000 *	0.000
PriO24	0.416	4	4	0.421	0.467	0.100	0.065	3	0.000	0.420	1.000 *	0.000
PriO01	0.706	6	5	0.476	0.621	0.238	0.091	4	0.625	0.646	0.033	0.000
PriO07	0.754	8	4	0.167	0.557	0.707 *	0.415	8	0.615	0.810	0.244 *	0.133

Table 2. *Cont.*

	PIC	N_a	N_a	H_o	H_e	F_{IS}	*Null*	N_a	H_o	H_e	F_{IS}	*Null*
Prio15	0.589	5	4	0.000	0.788	1.000 *	0.000	2	0.000	0.515	1.000 *	0.000
PriO06	0.782	10	6	0.762	0.731	−0.044	0.013	8	0.750	0.750	0.000	0.000
PriO20	0.706	7	2	0.000	0.356	1.000	0.000	6	0.261	0.667	0.614 *	0.424
Multilocus average	0.564	5.467	3.330	0.261	0.429	0.407	0.157	4.070	0.289	0.488	0.416	0.159

PIC polymorphism information content, *n* number of individuals analysed, N_a number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} fixation index following Weir and Cockerham, *Null* null allele frequency estimated in INEST v2.2 [28], *na* not available, * indicates significant departures from HWE ($p < 0.05$).

The observed and expected heterozygosity ranged from 0 to 0.735 (average *Ho* = 0.412) and 0 to 0.741 (average *He* = 0.399) in Luki, respectively. In Yangambi, *Ho* ranged from 0 to 0.655 (average = 0.273) and *He* from 0 to 0.749 (average = 0.302) (Table 2). Deviation from HWE occurred at one locus (PriB12) in the Luki population and two loci (PriB02 and PriB18) in the Yangambi population. These loci were associated with higher null allele frequencies (0.141, 0.125 and 0.209, respectively). Although inbreeding appeared to be absent, genetic diversity was low in both *P. balsamifera* populations. Hence, genetic erosion appears to be a real risk and efforts should be made to protect *P. balsamifera* genetic resources. PIC values ranged from 0 to 0.737, with an average of 0.364. Thus, most of the developed microsatellite markers could be useful for parentage analyses and setting up breeding programmes for the endangered *P. balsamifera*.

3.3. Population Genetics in *Prioria oxyphylla*

For *P. oxyphylla*, the final three microsatellite multiplexes containing 15 primers were validated in 53 individuals collected in Luki (*n* = 21) and Yangambi (*n* = 32). Microsatellite loci showed 2 to 10 alleles per locus in both populations combined, with an average of 5.467 alleles per locus (Table 2). Interestingly, locus PriO4 showed only two alleles, with each allele being specific to either Luki or Yangambi. So, based on the allele observed for this locus only, the origin of an individual could be determined when considering trees from Luki and Yangambi.

Ho ranged from 0 to 0.762 (average = 0.261) and *He* from 0 to 0.788 (average = 0.429) in Luki, and similarly in Yangambi, *Ho* ranged from 0 to 0.750 (average = 0.289) and *He* from 0 to 0.810 (average = 0.488) (Table 2). Multiple loci deviated from HWE in both Luki and Yangambi (4 and 7 respectively). All these loci showed null alleles. Surprisingly, various loci were characterized by high levels of missing genotypes. Only the sample used for the primer development showed successful amplification for all 15 loci. PIC values ranged from 0.136 to 0.782 with an average of 0.564. Hence, the developed markers could be a useful tool for parentage studies and for setting up breeding programmes.

3.4. Microsatellite Cross-Species Amplification in African *Prioria*

The cross-species amplification tests showed that multiple loci from both microsatellite sets are conserved across species (Table 3), as various loci were successfully amplified in the different African *Prioria* species. Therefore, the primers developed in this study could be of great value for genetic studies focused on other *Prioria* species as well. Since *P. oxyphylla* and *P. buchholzii* are closely related sister-taxa [30], cross-species amplification appeared to be highly successful. The low amplification success in *P. joveri* (both primer sets) and *P. balsamifera* (*P. oxyphylla* primer set) could result from higher species divergence.

Table 3. Results of the cross-species amplification tests in the African *Prioria* species. *x* indicates successful amplification.

Species	PriB07	PriB19	PriB23	PriB10	PriB22	PriB04	PriB13	PriB15	PriB02	PriB14	PriB12	PriB03	PriB11	PriB18	PriB08	PriB20	16 SSRs
Prioria balsamifera	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	16
Prioria oxyphylla						X		X				X	X		X	X	6
Prioria buchholzii						X		X	X	X	X		X		X	X	8
Prioria msoo						X		X							X		3
Prioria gilbertii						X							X		X	X	4
Prioria mannii												X			X		2
Prioria joveri																	0
Overall	1	1	1	1	1	5	1	4	2	2	2	3	4	1	6	4	
	PriO03	**PriO23**	**PriO18**	**PriO04**	**PriO13**	**PriO19**	**PriO10**	**PriO22**	**PriO16**	**PriO24**	**PriO01**	**PriO07**	**PriO15**	**PriO06**	**PriO20**		**15 SSRs**
Prioria balsamifera					X												1
Prioria oxyphylla	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		15
Prioria buchholzii	X	X	X		X	X	X	X	X	X	X	X	X	X			13
Prioria msoo		X			X	X			X		X			X			6
Prioria gilbertii	X	X			X	X					X						5
Prioria mannii						X							X	X			3
Prioria joveri						X								X			2
Overall	3	4	2	1	5	6	2	2	3	2	4	2	3	5	1		

4. Conclusions

The developed sets of microsatellite primers for *Prioria balsamifera* and *Prioria oxyphylla*, containing 16 and 15 polymorphic loci respectively, appear to be useful for estimating genetic diversity at population level. The novel markers can be used to study gene flow and spatial genetic structure in *Prioria* species, which is needed to prevent genetic erosion and to set up proper conservation guidelines. Additionally, the microsatellite markers can be valuable for timber tracking through DNA fingerprinting.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/11/1037/s1, Figure S1: Map indicating the sampling locations in the Democratic Republic of Congo: Luki Biosphere Reserve and Yangambi Biosphere Reserve.

Author Contributions: S.V.A., O.J.H. and S.B.J. conceived and designed the experiments, S.V.A. performed the experiments, analysed the data and wrote the manuscript. O.J.H., H.B., B.A.I. and S.B.J. revised the manuscript.

Acknowledgments: We are grateful to Jérémy Migliore, Esra Kaymak (ULB-EBE), Pieter Asselman and Wim Baert (MeiseBG) for their assistance in the laboratory. This study is part of the HERBAXYLAREDD and AFRIFORD projects (BR/143/A3/HERBAXYLAREDD, BR/132/A1/AFRIFORD), funded by the Belgian Belspo-BRAIN program axis 4. This study has also received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement N° 765000, and the Fund for Scientific Research F.R.S.-FNRS (grant J.0292.17F).

Conflicts of Interest: The authors declare no conflict of interest.

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