Development, Characterization, and Cross-Amplification of Microsatellite Markers in the Understudied African Genus Anthonotha (Fabaceae)

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Primer Note

Development, characterization, and cross-amplification of microsatellite markers in the understudied African genus *Anthonotha* (Fabaceae)[1]

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- *Premise of the study: Anthonotha macrophylla* (Fabaceae) is a common tree species throughout the Guineo-Congolian forest that is sometimes confounded with other congeneric species; it is expected to be an interesting phylogeographical model to infer the history of the African dense forests. We developed 18 microsatellite markers from this species and tested their transferability in 15 congeneric species.
- *Methods and Results:* A genomic library was obtained using the Illumina platform, and 18 polymorphic microsatellite loci were developed. The polymorphic microsatellites displayed two to 24 alleles (average: 11.9 alleles per locus, expected heterozygosity range: 0.18–0.91, mean: 0.64) in three populations of *A. macrophylla* from Benin, Liberia, and Cameroon. Cross-amplification in one to nine individuals of 15 congeneric *Anthonotha* species (*A. acuminata*, *A. brieyi*, *A. cladantha*, *A. crassifolia*, *A. ferruginea*, *A. fragrans*, *A. gilletii*, *A. lamprophylla*, *A. mouandzae*, *A. noldeae*, *A. pellegrinii*, *A. pynaertii*, *A. stipulacea*, *A. wijmacampensis*, and *A. xanderi*) showed successful amplification in six to 17 loci, making most of these markers useful at the generic level.
- *Conclusions:* This set of markers will be useful to study species delimitation and the genetic structure of *Anthonotha* species, and thus to better understand the history of tropical African rainforests.

Key words: *Anthonotha macrophylla*; Fabaceae; microsatellites; next-generation sequencing; rainforest history.

Anthonotha P. Beauv. (Fabaceae) is an African native genus belonging to the monophyletic tribe Detarieae. *Anthonotha* species are found in evergreen to deciduous tropical African forests. Breteler (2010) recognizes 17 species almost completely confined to the Guineo-Congolian region, but species distinction is not always easy without flowers. Among these 17 species, *A. macrophylla* P. Beauv. is the most common and frequently collected species of the genus. It is a shrub or tree that usually grows 4–20 m tall and is one of the forest tree species found in the Holocene Climate Optimum forest relics in the Dahomey Gap. Its wide and nearly continuous distribution from Guinea to the Democratic Republic of the Congo (west and central African rainforest) should be useful to study the impact of past climate change on tropical African forest from genetic diversity pattern and phylogeographic and demographic inferences. To date, no microsatellite resources have been developed for *Anthonotha* species.

In this paper, we isolated and characterized a set of 18 polymorphic microsatellite markers for *Anthonotha*. These markers will complement the ones developed for *Terminalia superba* Engl. & Diels (Demenou et al., 2015) to study the history of fragmentation of the tropical African rainforest in the Dahomey Gap. We also attempted cross-amplification in 15 congeneric *Anthonotha* species.

METHODS AND RESULTS

***Microsatellite development*—**Total genomic DNA of *A. macrophylla* was extracted (ca. 5 µg) from 30 mg of silica gel–dried leaf collected from a sample coded OH3840 (2.30018°N, 25.02499°E; Appendix 1) from the Democratic Republic of the Congo using a cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2005). The extracted DNA was used to prepare a DNA genomic library without enrichment, following the protocol of Mariac et al. (2014), and sequenced using the Illumina (San Diego, California, USA) MiSeq platform (sequencing performed at CIRAD, Montpellier, France) as described in Demenou et al. (2015), which generated 28,902 150-bp-long paired-end reads. After assembling the paired reads with PANDAseq (Masella et al., 2012), the identification of simple sequence repeats (SSRs) and design of primers were performed with the bioinformatics pipeline QDD (Meglécz et al., 2014) following three steps: (1) selection of sequences containing SSRs, (2) elimination of redundant sequences, and (3) primer design. We detected 1109 loci (≥7 repeats) between 3246 reads containing microsatellite motifs. From these, we selected 48 primer pairs representing the longest dinucleotide repeats with PCR product length ≥100 bp and flanking region length of at least 15 bp from the microsatellite. Finally, using an M13-like protocol of Micheneau et al. (2011), we attached one of the four possible linkers (Q1–Q4) to the 5′ end of the forward primer of each locus to label PCR products with the distinct fluorochromes FAM, NED, VIC, and PET.

Amplification for each pair of designed primers was evaluated in three individuals of *A. macrophylla* from Benin (EE271; 6.96013°N, 2.67641°E), Cameroon (BS102; 5.10500°N, 11.40056°E), and Côte d'Ivoire (GK1034; 6.42321°N, 7.48098°W) (Appendix 1). PCR reactions (13 µL) were performed using 1 µL

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of DNA (ca. 50 ng/μL), 1.5 μL PCR buffer (10×), 0.6 μL $MgCl_2$ (25 mM), 0.45 μL dNTP (10 mM each), 0.3 μL of each primer (0.25 μM), 0.08 μL TopTaq DNA polymerase (5 U/μL; QIAGEN, Venlo, The Netherlands), and 8.77 μL H_2O using the following conditions: an initial step at 94°C for 4 min; followed by 30 cycles of 30 s at 94°C, 45 s at a primer annealing temperature of 55°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. PCR products were visualized on a 1% agarose gel and stained with SYBR Safe (Invitrogen, Merelbeke, Belgium).

All but two of the 48 primer pairs amplified consistently. Polymorphism was assessed on the same three previously amplified individuals (Appendix 1). For this step, PCR amplification was performed for each of 46 loci with fluorescent labeling in a total volume of 15 μL, combining: 0.3 μL of the reverse (0.2 μM) and 0.1 μL of the forward (0.07 μM) microsatellite primers with a Q1–Q4 universal sequence at the 5′ end, 0.3 μL of Q1–Q4 labeled primer (0.2 μM each), 3 μL of Type-it Microsatellite PCR Kit (QIAGEN), H_2O, and 1.5 μL of DNA. Cycling conditions were as described above with 30 cycles and primer annealing temperature of 55°C. A mix of 1 μL of each PCR product with 12 μL of Hi-Di Formamide (Life Technologies, Carlsbad, California, USA) and 0.3 μL of MapMarker 500 labeled with DY-632 (Eurogentec, Seraing, Belgium) was run on an ABI3730 Capillary Sequencer (Applied Biosystems, Lennik, The Netherlands). Electropherograms were analyzed with GeneMapper version 3.7 (Applied Biosystems). Twenty-eight loci were discarded because of lack of amplification, genotyping difficulties, or unreadable electropherograms. The remaining 18 selected polymorphic loci were combined into three multiplexed reactions (Table 1) using Multiplex Manager 1.0 software (Holleley and Geerts, 2009).

***Microsatellite marker data analysis*—**We evaluated the quality of these 18 microsatellite markers in three populations of *A. macrophylla* from southern Benin (N = 19), southern Liberia (N = 35), and eastern Cameroon (N = 28) (Appendix 1). Multiplex PCR reactions were carried out as described above to check polymorphism except that we added 3 μL of 5× Q-solution and readjusted the quantity of H_2O for a total volume of 15 μL. Multiplex PCR programs consisted of 94°C (5 min); followed by 22 cycles of 95°C (30 s), 56°C (90 s), and 72°C (1 min); followed by 10 cycles of 94°C (30 s), 53°C (90 s), and 72°C (1 min); and a final extension of 10 min at 72°C.

We computed the parameters of allele size range, observed number of alleles (A) per locus, observed (H_o) and expected (H_e) heterozygosities, inbreeding coefficient (F), and null allele frequencies (r) with INEst 1.0 (Chybicki and Burczyk, 2009) for each locus and population. We also tested deviation from Hardy–Weinberg equilibrium (HWE) for each locus with SPAGeDi (Hardy and Vekemans, 2002).

The number of alleles per locus ranged from two to 24 (average of 11.9 alleles per locus; Table 2). H_o and H_e ranged from 0 to 0.74 (average: 0.38) and from 0.05 to 0.89 (average: 0.48) for the Benin population, from 0 to 0.86 (average: 0.41) and from 0 to 0.93 (average: 0.58) for the Liberia population, and from 0 to 0.75 (average: 0.43) and from 0.04 to 0.89 (average: 0.63) for the Cameroon population (Table 2), repectively. Significant deviation from HWE (Table 2) was observed for four loci (AntM-ssr08, AntM-ssr09, AntM-ssr27, and AntM-ssr06) in the Benin population, for seven loci (AntM-ssr26, AntM-ssr42, AntM-ssr09, AntM-ssr04, AntM-ssr33,

Table 1. Characteristics of 18 polymorphic microsatellite loci for *Anthonotha macrophylla*.

Locus[a]	Primer sequences (5′–3′)[b]	Fluorescent label	Repeat motif	Allele size range (bp)	GenBank accession no.
Multiplex 1					
AntM-ssr22	F: TAGGAGTGCAGCAAGCATTATGTGCTAAGAAGAGCCTTAGCTT R: TCGATCAGGTCGTAACGAGG	Q2-NED	$(AG)_9$	149–160	KX865149
AntM-ssr26	F: CACTGCTTAGAGCGATGCGCCATAAAGAAGATGAGGACAA R: AGGCAGAGCGTGATATCGTC	Q3-VIC	$(GA)_8$	176–182	KX865151
AntM-ssr08	F: TGTAAAACGACGGCCAGTGTGCAAAGGATAGCAGCGTG R: TGCTCATTTCAGAGATGGTGTT	Q1-6-FAM	$(CT)_8$	179–201	KX865144
AntM-ssr42	F: CTAGTTATTGCTCAGCGGTTTGCAGGCAAACATGAGC R: AAACAGAGTTGTCCTTCTCCG	Q4-PET	$(TC)_7$	166–203	KX865157
AntM-ssr15	F: TAGGAGTGCAGCAAGCATGAGACTCAAAGTCCCTACGAAA R: AGATATGGAAGCCATGGACG	Q2-NED	$(TC)_9$	211–235	KX865146
AntM-ssr09	F: TGTAAAACGACGGCCAGTAAGGAAGGATGAGAGGGAAA R: GCTTAGGCATCAAATACGGG	Q1-6-FAM	$(CT)_{25}$	238–331	KX865145
Multiplex 2					
AntM-ssr36	F: CACTGCTTAGAGCGATGCAAAGGCAGAAACACAATGGC R: CGCTTTCATCATTCACTCAGA	Q3-VIC	$(GA)_{11}$	117–135	KX865154
AntM-ssr27	F: CACTGCTTAGAGCGATGCAAGGGAAATCGTAAAGCTCG R: TCTTTAGGAGATGGGCTAGTGG	Q3-VIC	$(TC)_7$	166–192	KX865152
AntM-ssr41	F: CTAGTTATTGCTCAGCGGTGGGTAGTAATCCGCAAGAAGG R: CTCTGCGCTAGAGGCTAGGA	Q4-PET	$(GA)_7$	176–194	KX865156
AntM-ssr24	F: TAGGAGTGCAGCAAGCATTTTACCAACCCAGAAAGCAA R: TGAGAAATGGAAGTCCACCA	Q2-NED	$(GA)_8$	177–222	KX865150
AntM-ssr39	F: CTAGTTATTGCTCAGCGGTTCCAACAGCTTCCTACTAACTAGC R: CCTTGTGATACACAGCCTGC	Q4-PET	$(GA)_{14}$	201–227	KX865155
AntM-ssr04	F: TGTAAAACGACGGCCAGTGAGGAAACGAGCTCTCCATC R: CTCTTGCGTCCTGATCTTCC	Q1-6-FAM	$(GA)_7$	222–230	KX865142
AntM-ssr02	F: TGTAAAACGACGGCCAGTTACTCAGAGGTGAGCTAAGCCG R: AATCCAGCTACTCCTGCTCC	Q1-6-FAM	$(AG)_{10}$	349–387	KX865141
Multiplex 3					
AntM-ssr33	F: CACTGCTTAGAGCGATGCTGGAAGTCCTCTGGCAGATT R: TGAATGGAACCATGGGTATGT	Q3-VIC	$(GA)_{12}$	146–166	KX865153
AntM-ssr21	F: TAGGAGTGCAGCAAGCATTATGGGTGCAGATTCCAGTG R: CACTCTCGCAAGATTTGCTT	Q2-NED	$(TC)_7$	158–160	KX865148
AntM-ssr43	F: CTAGTTATTGCTCAGCGGTTAAAGTACCAGCACGCAGCA R: TGAACCGGCAAAGATTGTGT	Q4-PET	$(CT)_8$	170–216	KX865158
AntM-ssr16	F: TAGGAGTGCAGCAAGCATATGCAGGTTCCCAAGGTATG R: TCCCTTAGCCATCGATCTCA	Q2-NED	$(GA)_9$	307–363	KX865147
AntM-ssr06	F: TGTAAAACGACGGCCAGTGATTCTGACTGACCAATGGGA R: AACCTGTTTACTCGAGTTGGG	Q1-6-FAM	$(CT)_8$	345–377	KX865143

[a]Optimal annealing temperature was 55°C and 53°C for Phase 1 and 2.
[b]The linkers (Q1, Q2, Q3, Q4) attached to the forward primers are underlined.

TABLE 2. Genetic properties of the 18 polymorphic microsatellite loci for three populations of *Anthonotha macrophylla*.[a]

		Benin (Pobè, $N = 19$)				Liberia (Nimba, $N = 35$)					Cameroon (Southeast, $N = 28$)				
Locus	A_T	A	H_o	H_e	F^b	r	A	H_o	H_e	F^b	r	A	H_o	H_e	F^b	r
Multiplex 1																
AntM-ssr22	6	4	0.26	0.33	0.21	0.08 ± 0.08	3	0.31	0.34	0.09	0.03 ± 0.03	5	0.50	0.58	0.14	0.06 ± 0.06
AntM-ssr26	6	3	0.21	0.29	0.27	0.00 ± 0.00	4	0.29	0.63	0.55**	0.26 ± 0.06	4	0.07	0.30	0.75***	0.32 ± 0.08
AntM-ssr08	17	4	0.11	0.20	0.48*	0.12 ± 0.12	8	0.37	0.42	0.12	0.00 ± 0.00	13	0.54	0.73	0.27**	0.07 ± 0.05
AntM-ssr42	20	9	0.63	0.86	0.27	0.10 ± 0.08	19	0.40	0.93	0.57***	0.33 ± 0.07	9	0.46	0.85	0.46***	0.28 ± 0.07
AntM-ssr15	12	4	0.32	0.29	–0.10	0.00 ± 0.00	4	0.31	0.28	–0.11	0.00 ± 0.00	9	0.61	0.65	0.06	0.01 ± 0.03
AntM-ssr09	24	9	0.53	0.74	0.29*	0.11 ± 0.07	10	0.26	0.79	0.67***	0.33 ± 0.12	16	0.68	0.82	0.17	0.01 ± 0.04
Multiplex 2																
AntM-ssr36	11	6	0.53	0.54	0.03	0.00 ± 0.00	9	0.66	0.67	0.01	0.00 ± 0.00	10	0.64	0.79	0.19	0.06 ± 0.04
AntM-ssr27	7	2	0.00	0.47	1.00***	—	6	0.29	0.35	0.19	0.08 ± 0.06	4	0.15	0.31	0.53**	0.35 ± 0.27
AntM-ssr41	7	2	0.05	0.05	0.00	0.00 ± 0.00	3	0.26	0.23	–0.11	0.00 ± 0.00	4	0.61	0.45	–0.35	0.00 ± 0.00
AntM-ssr24	13	4	0.68	0.61	–0.12	0.00 ± 0.00	11	0.57	0.84	0.32	0.17 ± 0.06	6	0.63	0.74	0.15	0.06 ± 0.07
AntM-ssr39	13	7	0.53	0.49	–0.08	0.00 ± 0.00	6	0.46	0.50	0.08	0.01 ± 0.04	9	0.75	0.84	0.19*	0.08 ± 0.05
AntM-ssr04	4	3	0.21	0.29	0.27	0.00 ± 0.00	3	0.14	0.37	0.61**	0.25 ± 0.08	2	0.04	0.04	0.00	0.00 ± 0.00
AntM-ssr02	17	8	0.74	0.89	0.17	0.04 ± 0.06	11	0.74	0.83	0.11	0.05 ± 0.04	13	0.68	0.89	0.23**	0.11 ± 0.05
Multiplex 3																
AntM-ssr33	10	5	0.58	0.54	–0.08	0.00 ± 0.00	6	0.31	0.60	0.47**	0.22 ± 0.12	7	0.57	0.70	0.18	0.10 ± 0.07
AntM-ssr21	2	2	0.26	0.31	0.15	0.04 ± 0.06	1	0.00	0.00	1.00***	—	2	0.25	0.31	0.14	0.06 ± 0.10
AntM-ssr43	18	3	0.11	0.10	–0.01	0.00 ± 0.00	13	0.86	0.89	0.04	0.00 ± 0.00	7	0.43	0.70	0.37***	0.21 ± 0.07
AntM-ssr16	13	8	0.63	0.76	0.17	0.03 ± 0.04	8	0.80	0.82	0.02	0.02 ± 0.02	6	0.69	0.75	0.32**	0.13 ± 0.12
AntM-ssr06	14	8	0.47	0.78	0.39**	0.17 ± 0.12	11	0.26	0.86	0.70***	0.43 ± 0.06	4	0.00	0.53	1.00***	—

Note: A = number of alleles; A_T = total numbers of alleles observed among all three populations; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; r = null allele frequency.

[a] Voucher and locality information are provided in Appendix 1.

[b] Significant deviation from Hardy–Weinberg equilibrium: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 3. Results of cross-amplification (allele size ranges) of microsatellite loci isolated from *Anthonotha macrophylla* tested in 15 other *Anthonotha* taxa.[a]

Locus	*A. acuminata* (*N* = 8)	*A. brieyi* (*N* = 8)	*A. cladantha* (*N* = 2)	*A. crassifolia* (*N* = 9)	*A. ferruginea* (*N* = 3)	*A. fragrans* (*N* = 7)	*A. gilletii* (*N* = 1)	*A. lamprophylla* (*N* = 4)	*A. mouandzae* (*N* = 3)	*A. noldeae* (*N* = 3)	*A. pellegrinii* (*N* = 1)	*A. pynaertii* (*N* = 4)	*A. stipulacea* (*N* = 9)	*A. wijmacampensis* (*N* = 1)	*A. xanderi* (*N* = 3)
Multiplex 1															
AntM-ssr22	149–153	149–152	151	153–158	151–153	151	152	149–154	155–162	151–152	—	151–153	151–155	151	149–151
AntM-ssr26	—	—	189	—	180	—	—	—	184	—	—	178–189	—	—	—
AntM-ssr08	177–178	181–195	188–202	179–183	179–190	179–183	200–204	184–216	183–186	175–189	184–191	177–188	171–195	184–193	177–199
AntM-ssr42	—	172	—	164–193	171	171–193	—	—	—	—	—	174–185	172–193	—	166
AntM-ssr15	213–222	213–220	220–228	211–233	213–215	213–224	213	215–233	213–215	215–220	220–224	213–218	215–220	215–222	222–228
AntM-ssr09	250	—	250	—	250	248–252	—	250	250	—	—	248–262	250	—	250
Multiplex 2															
AntM-ssr36	117–125	115–117	117–123	123–135	121–127	121	140–144	123–146	127–144	131–135	123–125	117–129	123–138	115	125–131
AntM-ssr27	172	172–194	180	170–174	—	—	—	172	212	172–186	—	172	—	—	182–220
AntM-ssr41	176	176	176	176–183	176	176	176	176	176	176	176	176	176	176	176
AntM-ssr24	185–187	212–214	—	183–199	185	183–209	189	—	—	187–191	—	183–216	—	—	—
AntM-ssr39	199–221	205–219	207–215	203–217	203–229	203–221	211	205–213	207	203–215	—	205–221	205–215	207	203–205
AntM-ssr04	217–228	226–228	228	222–228	228	228	230	228	228–230	228–230	—	228	228	228	226–230
AntM-ssr02	359	351–359	357–392	348–371	351–355	346–371	—	351–363	359–361	—	—	355–382	351–355	—	353–359
Multiplex 3															
AntM-ssr33	146–154	151–157	147–149	144–146	146–150	146–158	151–152	150–151	151	147–159	173–196	143–152	150–168	152	146–152
AntM-ssr21	158–172	160–190	146–158	158–164	158–160	158–160	162	158–168	160	158–174	178–194	158–162	158–174	160–188	160–182
AntM-ssr43	164	160–164	164	172–200	164–184	177–204	164	164	164	164	—	164–194	160–177	164	164
AntM-ssr16	—	—	—	—	311	309–325	—	—	—	—	—	—	—	—	—
AntM-ssr06	—	—	353–355	—	355	357	—	—	—	—	—	355	355–361	—	—
Private allelic richness (avg. over loci)	0.10	0.09	0.17	0.11	0.03	0.05	0.18	0.13	0.03	0.17	0.28	0.03	0.06	0.13	0.08

Note: *N* = number of individuals used.

[a]Voucher and locality information are provided in Appendix 1.

AntM-ssr21, and AntM-ssr06) in the Liberia population, and for nine loci (AntM-ssr26, AntM-ssr08, AntM-ssr42, AntM-ssr27, AntM-ssr39, AntM-ssr02, AntM-ssr43, AntM-ssr16, and AntM-ssr06) in the Cameroon population due to the presence of null alleles. After accounting for the effect of null alleles, INEst inferred no inbreeding across populations ($F = 0.00 \pm 0.00$), indicating an outcrossing mating system. The sequences of the developed microsatellite loci have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Bioproject ID PRJNA352928).

***Cross-amplification in 15 congeneric* Anthonotha *species*—**The selected loci were then tested in one to nine individuals of 15 other *Anthonotha* species (Table 3) using the PCR conditions described above to check their transferability. Among the 18 loci, six to 17 (mean: 13) successfully amplified depending on the species and displayed one to nine alleles per locus (results not shown). The allelic size varies among species for a given locus, but a few alleles are shared by up to 10 species (e.g., alleles 158 and 160 for locus AntM-ssr21, alleles 205 and 207 for locus AntM-ssr39, alleles 213 and 215 for AntM-ssr15). Private allelic richness (average over loci) computed with HP-rare 1.1 (Kalinowski, 2005) for each species indicated that *A. pellegrinii* Aubrév. shows the highest value (0.28), followed by *A. gilletii* (De Wild.) J. Léonard (0.18), *A. cladantha* (Harms) J. Léonard (0.17), and *A. noldeae* (Rossberg) Exell & Hillc. (0.17); therefore, these species are likely the most divergent with *A. macrophylla*. According to the data of this study, no allele of a given locus is shared by all species.

CONCLUSIONS

In this study, 18 polymorphic microsatellite markers were developed for *A. macrophylla*. This set of microsatellite markers showed its tranferability in most of 15 congeneric species. These microsatellite markers and those published on *T. superba* will be useful for investigating phylogeographic patterns, dispersal patterns, and demographic history of *Anthonotha* species to provide a better understanding of the fragmentation history of tropical African rainforests in the Dahomey Gap. With them, one can start to disseminate, for example, paleovegetative information for this region.

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Appendix 1. Voucher and collection locality information of the samples used in this study.

Species	*n*	Voucher no.[a]	Collection locality	Latitude	Longitude
Anthonotha macrophylla P. Beauv.	1	OH3840[b]	DRC	2.30018	25.02499
	1	BS102[c]	Cameroon	5.10500	11.40056
	1	GK1034[c]	Côte d'Ivoire	6.42321	−7.48098
	19	BoD306, BoD314, BoD315, BoD317, BoD318, BoD322, BoD323, BoD324, EE0271[c], EE0272, EE0273, EE0274, EE0275, MH476, MH2278, MH2281, MH2282, MH2283, MH2284	Pobè–Etchéde, Benin	6.9	2.6
	35	Bod1724, Bod1726, Bod1745, Bod1767, Bod1783, Bod1798, Bod1896, Bod1800, Bod1809, Bod1813, Bod1849, Bod1857, Bod1858, Bod1916, Bod1933, Bod1946, Bod1968, Bod1996, Bod2019, Bod2039, Bod2049, Bod2076, Bod2083, Bod2089, Bod2097, Bod2100, Bod2105, Bod2113, Bod2115, Bod2117, Bod1577, Bod1579, Bod1581, Bod1596, Bod1607	Nimba, Liberia	7.4	−8.6
	28	BS0077, BS0078, JFG0411, JFG0500, LD0044, LD0045, LD0046, LD0116, LD0125, LD0153, LD0179, LD0184, LD0231, MH1290, MH1360, MH1407, MH1444, MH1848, MH1849, OH1009, OH1020, OH1055, OH1061, RP0013, RP0015, RP0192, SVO0077, SVO0155	Southeastern Cameroon	3	13
Anthonotha acuminata (De Wild.) J. Léonard[d]	8	WAG0355893[e]	DRC	−2.91733	28.49783
		WAG0360982[e]	DRC	−1.20000	28.21667
		WAG0161175[e]	DRC	−3.50000	28.43333
		WAG0380740[e]	DRC	−0.86667	18.13333
		WAG0161180[e]	Cameroon	2.81667	11.13333
		WAG0160988[e]	Cameroon	2.38333	11.28333
		TOD1242	Cameroon	4.82475	9.70107
		WAG0161181[e]	Gabon	1.58333	11.58333
Anthonotha brieyi (De Wild.) J. Léonard[d]	8	WAG0161089[e]	Cameroon	2.81667	10.63333
		WAG0235096[e]	Gabon	−0.89667	13.84667
		WAG0128248[e]	Gabon	0.58333	10.43333
		WAG0161169[e]	Gabon	−2.60000	10.58333
		WAG0251111[e]	Gabon	−0.70250	12.97783
		WAG0123291[e]	Gabon	0.90000	10.51667
		GiD0318, GiD1602	Gabon	−1.79100	10.17100
Anthonotha cladantha (Harms) J. Léonard[d]	2	WAG0161261[e]	Cameroon	3.30000	14.00000
		BS0064	Cameroon	2.22760	13.95950
Anthonotha crassifolia (Baill.) J. Léonard [d]	9	WAG0157486[e]	Benin	6.88333	2.63333
		WAG0161136[e]	Côte d'Ivoire	5.04926	−7.04879
		WAG0250767[e]	Gabon	0.41667	11.91667
		WAG0250767[e]	Gabon	−0.78400	13.78550
		WHA0052	Ghana	5.58833	−2.43976
		WAG0012833[e]	Guinea Bissau	12.38333	−13.78333
		WAG0060575[e]	Guinea Conakry	10.41667	−9.30000
		WAG0323828[e]	Liberia	5.65683	−8.17467
		WAG0060577[e]	Sierra Leone	9.85000	−11.31667
Anthonotha ferruginea (Harms) J. Léonard[d]	3	WAG0161150[e]	Gabon	−1.36333	10.61333
		WAG0122594[e]	Gabon	−2.21533	9.66750
		GiD2141	Gabon	−0.76000	10.54250
Anthonotha fragrans (Baker f.) Exell & Hillc.[d]	7	WAG0204481[e]	Cameroon	5.01667	8.80000
		WAG0235123[e]	Gabon	−0.85667	13.26167
		WAG0152975[e]	Côte d'Ivoire	5.74500	−4.12500
		Bod1667	Liberia	7.47875	−8.64761
		Bod1866, Bod1908, Bod2184	Liberia	7.55824	−8.63344
Anthonotha gilletii (De Wild.) J. Léonard[d]	1	WAG0161147[e]	DRC	−4.06667	15.56667
Anthonotha lamprophylla (Harms) J. Léonard[d]	4	WAG0021831[e]	Cameroon	2.80000	10.01667
		PM5206	Cameroon	5.06200	8.85400
		WAG0103899[e]	Gabon	0.47350	10.25717
		WAG0127962[e]	Gabon	0.50000	10.36667
Anthonotha mouandzae Breteler[d]	3	WAG0161237[e]	Gabon	−2.33333	10.41667
		WAG0028668[e]	Gabon	−2.25583	9.70806
		WAG0161177[e]	Gabon	−2.55000	10.53333
Anthonotha noldeae (Rossberg) Exell & Hillc.[d]	3	WAG0161248[e]	Gabon	6.56667	10.68333
		WAG0161263[e]	Cameroon	4.08333	9.10000
		WAG0161090[e]	Burundi	−2.70000	29.25000
Anthonotha pellegrinii Aubrév.[d]	1	WAG0161250[e]	Gabon	0.75000	9.83333

Appendix 1. Continued.

Species	*n*	Voucher no.[a]	Collection locality	Latitude	Longitude
Anthonotha pynaertii (De Wild.) Exell & Hillc.[d]	4	WAG0250507[e]	DRC	−7.21667	17.96667
		WAG0281070[e]	Gabon	−0.80833	13.86833
		WAG0323827[e]	Liberia	5.64733	−8.18133
		WAG0409718[e]	Liberia	5.30617	−8.75117
Anthonotha stipulacea J. Léonard[d]	9	WAG0394765[e]	Gabon	0.58819	9.33542
		WAG0416743[e]	Gabon	−2.01253	10.48131
		WAG0122464[e]	Gabon	0.81667	10.23333
		WAG0061693[e]	Gabon	−0.58833	10.46833
		WAG0161258[e]	Gabon	−1.93333	9.83333
		GiD0264	Gabon	−1.73000	10.19900
		GiD0283	Gabon	−1.73300	10.20800
		GiD0396	Gabon	−1.42059	10.30705
		GiD1849	Gabon	−0.83155	10.46154
Anthonotha wijmacampensis Breteler[d]	1	WAG0161128[e]	Cameroon	3.00000	11.35000
Anthonotha xanderi Breteler[d]	3	WAG0161217[e]	Cameroon	2.65000	9.90000
		WAG0237446[e]	Cameroon	4.35200	10.42450
		WAG0204351[e]	Cameroon	4.98333	8.83333

Note: DRC = Democratic Republic of the Congo; *n* = number of individuals.

[a] Vouchers are deposited at the Herbarium of the Université Libre de Bruxelles (BRLU), Brussels, Belgium, silica gel collection of Dr. Olivier Hardy.

[b] Individual used to create a DNA genomic library.

[c] Individuals used for the first amplification test and for polymorphism testing.

[d] Individuals used for cross-amplification.

[e] Specimen codes for samples collected from material deposited in the National Herbarium of The Netherlands (WAG), Leiden, The Netherlands.