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OF WILD FAUNA AND FLORA

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Final report of CITES-financed pilot project: Search for DNA markers to discriminate cultivated from wild gaharu (agarwood)

This document is a report prepared by Marcel C.M. Eurlings and Barbara Gravendeel, of the NHN-Leiden University, under contract with the CITES Secretariat and submitted by the Secretariat.

Introduction

All species of *Aquilaria, Gyrinops* and *Gonystylus* were placed on the Appendix II list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2005 (CITES 2005) to improve control of commercial gaharu trade in all participating countries. Control of international trade by traditional detection methods such as light microscopy fail to distinguish between legally produced agarwood and illegally harvested agarwood (Heuveling van Beek and Phillips 1999, Barden et al. 2000). To improve control of international gaharu trade an alternative identification tool is urgently needed.

Goals

The pilot study carried out in 2005 aimed to: (1) genotype provenances of *Aquilaria crassna* and *Aquilaria malaccensis* from different plantations in Southeast Asia; (2) screen for DNA markers to discriminate cultivated from wild agarwood; (3) develop an easy applicable molecular tool for taxonomic identification of agarwood wood chips.

Remark on the specific goals of MOU S-253. "It must be stated that the proposed study can only be performed when sufficient *A. crassna* and *A. malaccensis* samples from plantations and samples from the wild of other species of these two genera throughout Southeast Asia (i.e. India, Laos, Malaysia and Vietnam) are provided by our international contacts. If no wood samples with branches bearing leaves and flowers or fruits are provided, the proposed study will not be possible".

Plant material obtained

Multiple accessions of *A. crassna* from six different plantations in South East Asia were obtained. However, four of these plantations only contained trees that were recently collected from the wild, and as such do not conform to the definition of a plantation. Several wild-collected accessions from Papua New Guinea were identified as *Gyrinops ledermanii*. We did not succeed in accessing any fresh *A. malaccensis* samples.

Results

The following results were obtained:

1. The consensus chloroplast simple sequence repeats *trn*R-*atp*A (ccSSR4), *trn*R-*Rrn*5 (ccSSR21), *rrn*5-*trn*R (ccSSR17), *ndh*D-*psa*C (ccSSR20), *trn*K (ccSSR1) (Chung et al. 2003) and the plastid DNA region *rps*16 (Nishizawa and Watano 2000) were sequenced for different species of *Aquilaria* (*A. crassna*, *A. malaccensis*, *A. rugosa*) and *Gyrinops ledermanii*. Some of the plant samples used were already present in the collection of NHN-Leiden University and some were collected at plantations in Southeast Asia. The regions tested did not show any interspecific variation with the exception of the *trn*R-*atp*A region, which showed minor differences (a single nucleotide repeat), and *rps*16 which showed some differences between *A. crassna* and *A. rugosa*, the only species which were tested for this region.

   Consensus chloroplast simple sequence repeats of the regions *trn*R-*atp*A (ccSSR4), *rrn*5-*trn*R (ccSSR17) and *rps*16 that were analyzed for different provenances of *A. crassna* showed no variation between *A. crassna* accessions collected at the TRP plantation in Vietnam (An Giang province) and the Kao Yai plantation in Thailand. Nuclear ITS sequences which previously showed clear interspecific variation between *A. crassna*, *A. rugosa* and *Gyrinops ledermanii* (Kiet et al. 2005) failed to reveal sequence divergence at the intraspecific level.

   As the plastid and nuclear regions sequenced provided insufficient variation for the genotyping of provenances of *A. crassna* and could not be used to distinguish plant samples obtained from different plantations, it was decided to develop microsatellites from genomic DNA (Gardner et al. 1999). Of the ten fragments containing seven or more dinucleotide repeats, four were tested for variability (Table 1; 2). Three out of four loci proved to be polymorphic at the intraspecific level (Table 3). More plant samples need to be collected and tested to certify the unique nature of the polymorphisms found.

2. No fresh field collected material of *A. crassna* was available for this study. Microsatellite analyses as carried out on cultivated material (Table 3) could therefore not be repeated on wild material. The samples collected from plantations three to six (Table 3) are treated as cultivated whereas in reality these trees were only recently collected from the wild. The only samples freshly collected from the wild for this study came from Papua New Guinea. These samples were identified at NHN-Leiden University as *Gyrinops ledermanii*. Cultivated samples of this species were not available. The lack of cultivated plant samples and material freshly collected from the wild belonging to the same species made it impossible to develop a screening method to distinguish cultivated from wild collected agarwood. In addition it is likely that most of the plantations are not yet genetically definable, because trees found in the wild are added to the plantation. The high variability found in the microsatellite markers screened in this study, however, suggests that it is possible to genetically define a plantation. Sufficient fresh plant material needs to be collected first, though, to investigate this further.

3. PCR amplification of different regions from several agarwood and other wood samples was achieved using methods described previously (Eurlings and Gravendeel 2005). In several cases, success rates of PCR amplification reactions could be increased by the use of Restorase (Sigma). PCR amplifications were usually successful from chloroplast regions; however some nuclear microsatellites were amplified from wood as well. More wood samples need to be tested in order to determine the potential of microsatellite markers for agarwood identification purposes.

Conclusions

This second pilot study shows that the development of a molecular based identification tool for agarwood is possible. The results obtained show that (1) chloroplast regions show sufficient interspecific variation for identification of plant samples to species level; (2) nuclear microsatellite markers can be used to identify the unique genotypes of plants grown at different plantations; (3) PCR amplification of microsatellite markers from wood is possible. Given further screening of these markers and the availability of sufficient plant samples, cultivated and wild agarwood could be discriminated in the future using DNA techniques. For the control of illegal trade, however, the still very limited DNA sequence database of *Aquilaria* and *Gyrinops* needs to be enlarged first.

Further research

To determine the uniqueness of genotypes from different plantations, more chloroplast regions need to be screened and more microsatellites need to be developed and tested. An increased number of correctly identified plant samples which are derived from both the wild and plantations should be provided in order to ascertain the accuracy of the results obtained so far. In addition, more molecular markers need to be screened in order to get a good overview of the genetic variation of agarwood producing species.

Marcel Eurlings & Barbara Gravendeel
NHN-Leiden University
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Table 1: Characteristics of *Aquilaria crassna* nuclear microsatellites found (repeat ≥ 7 bp)

Repeat Name	Repeat Motif	Insert Size (bp)	Result
3pa17	(CA)15	415	f.i.r.*
10pa17	(CA)12	309	Table 2
14pa17	(CA)7	362	Table 2
16pa17	(CA)19	266	Table 2
59pa17	(CA)7	384	f.i.r.
71pa17	(CA)15	228	f.i.r.
86pa17	(CA)7	464	f.i.r.
6pa18	(CA)8	394	Table 2
12pa18	(CA)7	259	f.i.r.
52pa18	(CA)8	292	f.i.r.

*further investigation required

Table 2: Characteristics of several *Aquilaria crassna* nuclear microsatellites analysed

Locus Name	Primer Name	Sequence 5'-3' sense/anti-sense	Repeat Motif	Sizes (bps)	Number of alleles
10pa17	10PA17F	CACACACTGTTATGGTCTACAGCTT	$(CA)_{12}$	148-156	5
	10PA17R-HEX	TTCGCCATCTCATAATATTCTAATGTA			
14pa17	14PA17F	CGCATATAGAAGCAATCAAAGAGC	$(CA)_7$	134	1
	14PA17R	ATTTGGAATTTTACACCCATTGGA			
16pa17	16pa17-f2	AGTGAACAACTTGACTAGGCTTG	$(CA)_{19}$	133-155	7
	16pa17-5FAM	GCTGAACACAACAAGATATCACC			
6pa18	6PA18F	TGAGGCGTGAGTGAGATATTGATT	$(CA)_8$	180-210	6
	6PA18R-FAM	CCTTCCTCTCTTCTTACCTCACCA			

Table 3: Microsatellite alleles of plant samples analyzed

Species	Plant.	Voucher	10pa17	16pa17	6pa18
A. crassna	1	Eurlings 1	156/152	155	202/194
A. crassna	1	Eurlings 2	156/152	155	196
A. crassna	1	Eurlings 3	156/152	155	196
A. crassna	1	Eurlings 4	156/152	155	196
A. crassna	1	Eurlings 5	156/152	155	202/196
A. crassna	1	Eurlings 6	156	155/145	196
A. crassna	1	Eurlings 7	156	155/153	202/194
A. crassna	1	Eurlings 8	156/152	155	194
A. crassna	2	Eurlings 9	152	155/153	196
A. crassna	1	Eurlings 10	156	155	210/186
A. crassna	1	Eurlings 11	156	155	202
A. crassna	1	Eurlings 12	156/152	155	202
A. crassna	3	HvB 2	154	151	202/198
A. crassna	3	HvB 3	154	151	202/198
A. crassna	4	HvB 4	154	151/145	194
A. crassna	4	HvB 5	154	145/143	202/198
A. crassna	5	HvB 6	154/152	155/143	202/198
A. crassna	5	HvB 8	150/148	155/143	202/198
A. crassna	6	HvB 9		147/133	198
A. crassna	6	HvB 10		147/133	198
A. crassna	6	HvB 11		145	196
A. crassna	?	HvB 12		145/143	198/194
A. rugosa	?	HvB 1		--	180
A. sinensis	?W	Kiet 1943	154	145/143	202/194
A. *sinensis*	?W	Kiet 1944	154/152	145/143	194

Plant. = Plantation numbers: 1. TRP, An Giang, Mt. Giai, Vietnam; 2. TRP, An Giang, Mt. Cam, Vietnam; 3. Mr. Somporn, Trat, Thailand; 4. Nakhon Ratchasima Khao Yai, Thailand; 5. Ms. Chanjira, Trat, Thailand; 6. Konray distr. Mr. Sy, Kontum, Vietnam. ?W = possibly collected from the wild. Voucher specimens are deposited at NHN-Leiden University (L).

References

Barden, A., N. Awang Anak, T. Mulliken and M. Song. 2000. Heart of the matter: Agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. TRAFFIC report. (Barden et al. 2000)

Chung S., Decker-Walters D.S. and Staub J.E. 2003. Genetic relationships within the Cucurbitaceae as assessed by consensus shloroplast simple sequence repeats (ccSSR) marker and sequence analyses. Can. J. Bot. 81: 814-832.

CITES. 1994. The 9th CITES conference of the parties. Inclusion of species in Appendix II. Fort Lauderdale, Florida, United States.

CITES. 2005. Fifteenth meeting of the Plants Committee. Amendments to Appendix II of CITES. Geneva, Switzerland, 17–21 May 2005 unpublished.

Eurlings, M.C.M. and B. Gravendeel. 2005. Identification of gaharu (*Aquilaria* and *Gyrinops*) dry wood using trnL-trnF polymorphisms. Pl. Syst. Evol. 254: 1–12.

Heuveling van Beek H., Phillips D. 1999. Agarwood: Trade and CITES implementation in Southeast Asia. Unpublished report prepared for TRAFFIC Southeast Asia, Malaysia.

Gardner M.G., Cooper S.J.B., Bull C.M. and Grant W.N. 1999. Isolation of Microsatellite Loci from a Social Lizard *Egernia stokesii*, Usin a modified Enrichment Procedure J. Hered. 1999: 90(2) brief communications 301-304.

Kiet l. C., Keßler P.J.A. and Eurlings M. 2005. A new species of *Aquilaria* (Thymelaeaceae) from Vietnam. Blumea 50: 135-141.

Nishizawa T. and Watano Y. 2000. Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. J. Phytogeogr. 48: 63-66.

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