ANNEX 6

Report on the ring test results

Ring testing of molecular markers for genetic traceability of *Entandrophragma cylindricum*, *Milicia excelsa* and *Triplochiton scleroxylon*

Céline Blanc-Jolivet 02.09.2015

Aims of the ring test:

Genetic fingerprinting protocols have been recently developed to control declarations of species and region of origin of timber. In well-equipped laboratories, large sets of SNPs markers have screened to build genetic maps of reference for the species *Entandrophragma cylindricum*, *Milicia excelsa* and *Triplochiton scleroxylon*. Application of genetic fingerprinting methods in the producers' countries is a very important achievement to improve detection of false declarations in timber before exportation.

Three African reference laboratories for genetic fingerprinting have been supported by the project to get ready to conduct DNA testing on timber (KEFRI in Nairobi/Kenya; IRET in Libreville/Gabon; FORIG in Kumasi/Ghana). Because cost and time-effective methods are needed to conduct such analysis, sets of very informative molecular markers have been identified. This ring test aims at ensuring the quality of the DNA extraction of fresh and timber material, as well as the successful amplification, with low occurrence of DNA contamination, of the newly developed genetic markers in the African reference laboratories. Another partner Institution (NERC) will also take part to this ring test organized by the Thünen Institute.

Design:

Four molecular markers were identified for each species. Each laboratory will receive both materials to extract (dried leaves and timber) and already extracted DNA from the same individuals. The goal of the test is to amplify all genetic markers and to estimate the fragment size on an agarose or polyacrylamide gel. All selected loci can be used for timber tracking and genotyping can be conducted through sequencing, PCR-RFLP or fragment analysis, depending on the locus.

The Thünen Institute prepared DNA extraction kits, primers and samples for each participating laboratory.

Evaluation of the results:

Each participant will have to report amplification success and fragment size for each sample and locus. **Results have to be communicated to Céline Blanc-Jolivet until 30.06.2015**.

Instructions

Each participant will receive a package containing: DNA extractions kits, four fresh samples from each species, four dried DNA samples from each species, and four primer pairs per species (total: 24 samples and 12 primer pairs). **Other reagents (PCR buffers, agarose, acrylamide, DNA ladder, tubes, tips…) have to be provided by the participants themselves** as formerly explained by email. A budget was attributed to the participants for this purpose (see project description).

The testing might be conducted as follows:

- **Preparation of the samples (timber and leaf)**

Put some of material from the samples SA_1, SA_2, SA_3, SA_4 (Sapelli), IR_1, IR_2, IR_3, IR_4 (Iroko) AY_1, AY_2, AY_3 and AY_4 (Ayous) in tubes and extract the DNA according to the provided protocol with the innuPREP Plant DNA Kit.

- **Preparation of the concentrated DNA stock solution for the provided dried DNA samples**

Dissolve the provided dried DNA samples (1,5 mL Tubes) with 15µL (SA_7; IR_5; AY_7) or 25µL (SA_5, SA_6 and SA_8; IR_ 6, IR_7, IR_8; AY_5, AY_6, AY_8) with sterile water.

- **Dilution of DNA to 10 ng/µL**

Dilute provided dried DNA samples to 10ng/µL for PCR (see table below) as well as samples newly extracted with the InnuPREP Kit (estimate the DNA concentration with the standard protocol applied in your laboratory).

Sample	concentration	comments
SA_5	245 ng/µL	Dilute to 10 ng/ µL
SA_6	966,1 ng/µL	Dilute to 10 ng/ µL
SA_7	-	Dilute 1:10
SA_8	-	Dilute 1:10
IR_5	-	Dilute 1:10
IR_6	433,9 ng/µL	Dilute to 10 ng/ µL
IR_7	-	Dilute 1:10
IR_8	1685,6 ng/µL	Dilute to 10 ng/ µL
AY_5	-	Dilute 1:10
AY_6	270,7 ng/µL	Dilute to 10 ng/ µL
AY_7	-	Dilute 1:10
AY_8	111,9 ng/µL	Dilute to 10 ng/ µL

- **Preparation of the primers**

Use the information on the attached list (Oligonukleotid Synthese Report) to find the appropriate amount of water to add in each tube. The provided primers are marked in color on the list. After adding the water, let the pellet resuspend at room temperature for one or two hours before diluting to 10 µM.

- **Preparation of the PCR**

We provided the optimal PCR protocols used in Thünen, please note that some adjustments have been done for timber samples. Because the reagents might be different in other laboratories, optimization of the protocol might be necessary.

Samples SA_1 to SA_8 (Sapelli) might be tested with primers pairs SA_m20477 F+R, SA_2101 F+R, SA_0387 F+R, and SA_rbcL_1.1a F+ SA_rbcL_2.1a R.
Samples IR_1 to IR_8 (Iroko) might be tested with primers pairs IR_0342 F+R, IR_0536 F+R, IR_3246 F+R and IR_rbcl_2.1a F + IR_ rbcL_3.3a R.
Samples AY_1 to AY_8 (Ayous) might be tested with primers pairs AY_4681 F+R, AY_5909 F+R, AY_1559 F+R, AY_rbcL_3.3a F +AY_rbcL_4.1a R.

- **Visualization of the results**

Amplified DNA should be visualized on an agarose and/or acrylamide gel. Repeat the PCR if necessary. Please fill the following table according to your results and send to Céline Blanc-Jolivet celine.blanc-jolivet@ti.bund.de **until 30.06.2015**.

Results

- **Description of the samples provided**

Blind ID	Real ID	Sample description
SA_1	CIV_12_ENTC_01	Timber
SA_2	Coffee	Leaf- Negative control
SA_3	DRC_3_ENTC_01	Leaf
SA_4	CB_06_ENTC_18	Leaf
SA_5	DRC_3_ENTC_01	DNA from leaf
SA_6	CB_06_ENTC_18	DNA from leaf
SA_7	CIV_12_ENTC_01	DNA from timber
SA_8	H_2O	Negative control
IR_1	Coffee	Leaf- Negative control
IR_2	CB_21_IROE_11	Timber
IR_3	GH_4_IROE_8	Leaf
IR_4	DRC_30_IROE_9	Leaf
IR_5	CB_21_IROE_11	DNA from timber
IR_6	DRC_30_IROE_9	DNA from leaf
IR_7	H_2O	Negative control
IR_8	GH_4_IROE_8	DNA from leaf
AY_1	CIV_03_TRI_11	Timber
AY_2	GH_3_TRI_4	Leaf
AY_3	DRC_02_TRI_05	Leaf
AY_4	Coffee	Leaf- Negative control
AY_5	H_2O	Negative control
AY_6	GH_3_TRI_4	DNA from leaf
AY_7	CIV_03_TRI_11	DNA from timber
AY_8	DRC_02_TRI_05	DNA from leaf

- **Results provided by the participants**

One African laboratory (KEFRI, Nairobi, Kenya) and NERC could successfully amplify DNA and address fragment sizes. Results are reported below. The two other laboratories (IRET, Libreville, Gabon; FORIG, Kumasi, Ghana) did not manage to amplify DNA due to very long delays for chemicals delivery and conservation problems which damaged the products.

Sapelli samples NERC

	SA_m20477 F+R			SA_2101 F+R			SA_0387 F+R			SA_rbcL_1.1a F+ SA_rbcL_2.1a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
SA_1	Y	150	165	N	-	80	Y	350	93	Y	100	100
SA_2	N	-	-	N	-	-	N	-	-	Y	100	100
SA_3	N	-	174	N	-	80	N	-	93	Y	100	100
SA_4	Y	150	157	Y	90/(400)	80	Y	100/350	93	Y	100	100
SA_5	Y	160	174	Y	50/90	80	Y*	50	93	Y	100	100
SA_6	Y	150	157	Y	90/(400)	80	Y	100/350	93	Y	100	100
SA_7	Y	160	165	Y	50/90	80	Y	100/350	93	Y	100	100
SA_8	N	-	-	Y*	50	-	Y*	50	-	N	-	-

* the fragment observed at 50 bp was probably a “primer cloud”, so no amplification for this sample

Sapelli samples KEFRI

	SA_m20477 F+R			SA_2101 F+R			SA_0387 F+R			SA_rbcL_1.1a F+ SA_rbcL_2.1a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
SA_1	N	-	165	Y	75/100	80	Y	125	93	Y	275	100
SA_2	N	-	-	Y	75/100	-	Y	275	-	Y	125/225	100
SA_3	Y	50/180	174	Y	100	80	Y	125	93	Y	125/225	100
SA_4	Y	50/150	157	Y	100	80	Y	125/375	93	Y	125	100
SA_5	Y	180	174	Y	100	80	N	-	93	Y	125	100
SA_6	Y	150	157	Y	100	80	Y	75/125	93	Y	125	100
SA_7	Y	150	165	Y	50/100	80	Y	125/375	93	N	-	100
SA_8	N	-	-	N	-	-	N	-	-	Y	175	-

Iroko samples NERC

	IR_0342 F+R			IR_0536 F+R			IR_3246 F+R			IR_rbcl_2.1a F + IR_ rbcL_3.3a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
IR_1	N	-	-	N	-	-	N	-	-	N	-	190
IR_2	Y	140	140	N	-	104	Y	(180)/500	151	Y	190	190
IR_3	Y	140	140	Y	100	104	Y	180	151	Y	190	190
IR_4	Y	140	140	Y	120	104	Y	180	151	Y	190	190
IR_5	Y	140/300/ (500)	140	Y	180/400	104	Y	(180)/500	151	Y	190	190
IR_6	Y	140	140	Y	120	104	Y	180	151	Y	190	190
IR_7	N	-	-	N	-	-	N	-	-	N	-	-
IR_8	Y	140	140	Y	100	104	Y	180	151	Y	190	190

Iroko samples KEFRI

	IR_0342 F+R			IR_0536 F+R			IR_3246 F+R			IR_rbcl_2.1a F + IR_ rbcL_3.3a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
IR_1	N	-	-	Y	125/275	-	Y	150	-	Y	200	190
IR_2	Y	175	140	Y	175/150	104	Y	200/500/700	151	Y	200	190
IR_3	Y	175	140	Y	100/400	104	Y	200	151	Y	200	190
IR_4	Y	175	140	Y	125	104	Y	200	151	N	-	190
IR_5	N	-	140	Y	75/150/430	104	Y	200/500/700	151	Y	200	190
IR_6	Y	175	140	Y	125	104	Y	200	151	Y	200	190
IR_7	N	-	-	Y	75/150	-	N	-	-	N	-	-
IR_8	Y	175	140	Y	75/130	104			151	Y	200	190

Ayous samples NERC

	AY_4681 F+R			AY_5909 F+R			AY_1559 F+R			AY_rbcL_3.3a F +AY_rbcL_4.1a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
AY_1	N	-	86	N	-	81	N	-	92	N	-	250
AY_2	N	-	86	N	-	81	N	-	92	N	-	250
AY_3	Y	700	86	N	-	81	N	-	92	N	-	250
AY_4	N	-	-	N	-	-	N	-	-	Y	250	250
AY_5	Y	700	-	N	-	-	N	-	-	N	-	-
AY_6	Y	700	86	Y	90	81	Y	100	92	Y	250	250
AY_7	Y	700	86	Y	90/(200)/(400)	81	Y	100	92	Y	250	250
AY_8	N	-	86	Y	90	81	Y	100	92	Y	250	250

Ayous samples KEFRI

	AY_4681 F+R			AY_5909 F+R			AY_1559 F+R			AY_rbcL_3.3a F +AY_rbcL_4.1a R		
	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments	PCR success (Y/N)	Fragment size	Comments
AY_1	Y	125/175	86	Y	100	81	Y	125	92	Y	240	250
AY_2	Y	125	86	N	-	81	Y	125	92	Y	240	250
AY_3	Y	125	86	Y	100	81	Y	125	92	Y	240	250
AY_4	N	-	-	N	-	-	Y	325/650	-	Y	240	250
AY_5	N	-	-	N	-	-	N	-	-	N	-	-
AY_6	N	-	86	N	-	81	N	-	92	Y	240	250
AY_7	N	-	86	N	-	81	Y	125	92	N	-	250
AY_8	Y	125/175	86	Y	100	81	Y	125	92	Y	240	250

- **Interpretation pf the results**

Each participant could successfully amplify DNA from timber, either extracted by Thünen or extracted by themselves, and for most tested markers. This demonstrates the utility of the InnuPREP Kit for the DNA extraction from timber.

The presence of multiple bands reported by both participants at a few loci reveals the lack of optimization of PCR conditions. All loci were tested by Thünen on the same samples and provided good results with the PCR conditions provided to the participants.

Most discrepancies among the results provided by NERC and the expected fragment sizes result from amplification failure (especially in Ayous extracted by NERC) and PCR optimization problems. Signal at around 50 bp on the agarose gel was misinterpreted as a positive amplification, while it might only be due primer clumps ("primer cloud"). Only one contamination seemed to have occurred (AY_5 at AY_4681 F+R), but the fragment reported is too long, which is not critical for the use of this PCR product for timber tracking.

As for KEFRI, PCR optimization, but maybe also fragment size estimation problems occurred. The participant laboratory mentioned that they did not go through an optimization step, therefore we do not see this as critical. More importantly, seven cases of contamination were observed, with at least two cases corresponding to the expected fragment size in other samples (SA_2 at SA_2101 F+R; IR_1 at IR_3246 F+R), indicating a contamination from the same species. Therefore, more caution should be taken during PCR preparation and during dilution of PCR product aliquots. These results highlight the need of further discussion between Thünen and KEFRI to tackle this problem.