**PROJECT PROGRESS REPORT TO THE**
**INTERNATIONAL TROPICAL TIMBER ORGANISATION (ITTO)**
**Submitted by Double Helix Tracking Technologies Pte Ltd**

# Pilot Implementation of a DNA traceability system for *Prunus africana* in Prunus Allocation Units in Cameroon and Democratic Republic of Congo (DRC).

| | |
|---|---|
| **PERIOD OF REPORT** | 01 May 2014 to 30 November 2014 |
| **PROJECT NUMBER** | PP-A/39-162A |
| **EXECUTING/IMPLEMENTING AGENCY** | Ministry of Environment, Nature Conservation and Tourism (MECNT), Democratic Republic of Congo.<br>Ministry of Forestry and Wildlife (MINFOF), Cameroon |
| **COLLABORATING AGENCIES** | Double Helix Tracking Technologies, Singapore<br>Syndicates of Industries in charge of Harvesting, Processing and Exportation of Special Products (SIHPESP) in Cameroon and Democratic Republic of Congo. |
| **DURATION** | 18 months |
| **START DATE** | March 2014 |
| **PROJECT BUDGET** | US$ 360 675 |
| **Project coordinator** | Double Helix Tracking Technologies Pte Ltd<br>3 Science Park Drive<br>#02-12/25 The Franklin<br>Singapore 118223 |
| **DoubleHelix staff** | Darren Thomas<br>Germain YENE YENE |

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## 1. DESCRIPTION OF WORK IMPLEMENTED DURING THE PERIOD

This project seeks to demonstrate that, using DNA techniques, *Prunus Africana* bark can be traced back to specific trees from controlled Prunus Allocation Units (PAUs). The proposed DNA traceability system will enable random verification of traceability documentation to detect bark substitution from uncontrolled areas and associated document fraud to a high level of confidence, allowing for timely corrective actions to be implemented.

The main outputs are: (1) development of genetic markers for *P. africana* suitable for DNA fingerprinting of bark, (2) Capacity building and training of local teams in DNA sample collection and storage, (3) Implementation of DNA verification in two controlled PAUs in Cameroon and DRC from pre-harvest to the factory and then to the exit points.

Principle activities and key results since project launch in March 2014 are as follows:

- Successful trial DNA extraction from *P. africana* bark samples. Initial tests against the bark samples have shown that sufficient DNA can be extracted from the bark, even down to finely chipped bark, on a routine basis. See **Appendix A** for DNA extraction report.

- Organisation and conduct of two stakeholder workshops. One in Kribi, Cameroon and the other in Kinshasa, DRC. The workshop objectives were to explain the goals of the project, work through questions raised by the participants and conduct training. Germain YENE YENE and Darren THOMAS from DoubleHelix, together with Dr. Henri BOUDA from the Thünen Institut attended to conduct training on DNA sample collection, handling and storage. In Cameroon we were able to combine the workshop and training for both *P. elata* and *Prunus africana* projects. Agendas, attendance lists and photographs from the workshops are presented in **Appendix B**.

- Subsequent to the workshops and training, field missions were organised to collect DNA samples of *P. africana* that will form the genetic reference data set. The goal of collecting samples from 20 individuals from 10 different populations across Cameroon and DRC was achieved. This means that we have the sampling stock to develop both a tree to stump traceability system but also a system to discriminate between different populations (harvesting regions) in the future although this is outside the immediate scope of this project. DNA sample records are presented in **Appendix C**, together with photographs from the fieldwork.

- Meetings have been conducted with industry in Cameroon and DRC to learn about the *P. africana* supply chain and processing systems. Supply chain processes have been mapped and we are in the process of designing the sampling plan to collect samples from multiple stages of harvest, transportation and production as part of the system implementation phase.

## 2. EXECUTION OF THE WORKPLAN

### *2.1 Workplan review*

Table 1 below indicates the project implementation in comparison with the detailed workplan provided to ITTO in May 2014. The approved workplan schedule is marked in grey, with the actual implementation indicated by the hatched red cells.

**Table 1: Activity schedule in comparison with workplan**

| OUTPUTS / ACTIVITIES | RESPONSIBLE PARTY | Month | | | | | | | | | | | | | | | | | | STATUS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | |
| **Output 1.1** | | | | | | | | | | | | | | | | | | | | |
| 1.1.1 Trial DNA extraction | DoubleHelix | | | | | | | | | | | | | | | | | | | Completed |
| 1.1.2 Population sampling | MECNT, ANAFOR, DoubleHelix | | | | | | | | | | | | | | | | | | | Completed |
| 1.1.3 DNA marker development | DoubleHelix | | | | | | | | | | | | | | | | | | | In progress |
| **Output 1.2** | | | | | | | | | | | | | | | | | | | | |
| 1.2.1 Workshops and training | MECNT, ANAFOR, DoubleHelix | | | | | | | | | | | | | | | | | | | First workshop complete (Cameroon and DRC) |
| **Output 1.3** | | | | | | | | | | | | | | | | | | | | |
| 1.3.1 Verification system development | DoubleHelix | | | | | | | | | | | | | | | | | | | In progress |
| 1.3.2 Field implementation | MECNT, ANAFOR, DoubleHelix | | | | | | | | | | | | | | | | | | | Not started |
| 1.3.3 Ongoing DNA verification | DoubleHelix | | | | | | | | | | | | | | | | | | | Not started |
| 1.3.4 Statistical analysis | DoubleHelix | | | | | | | | | | | | | | | | | | | Not started |

### *2.2 Progress in implementation of the activities*

**Table 2: Progress of activities conducted during report period**

| Activity | Percentage executed | Originally planned completion date | Estimated completion date |
|---|---|---|---|
| 1.1.1 Trial DNA extraction | 100 | May 2014 | Complete |
| 1.1.2 Population sampling | 100 | September 2014 | Complete |
| 1.1.3 Genetic marker development | 80 | January 2015 | February 2015 |
| 1.2.1 Opening workshop | 100 | June 2014 | Complete |
| 1.3.1 System development | 75 | September 2014 | January 2015 |
| 1.3.2 Field implementation | 25 | June 2015 | July 2015 |

## 2.3 Inputs applied

To date, DoubleHelix has received USD 100,000 in funds that have been applied towards:

- Project management - field coordination for workshop and sampling; system design; project administration.
- Purchase of sampling consumables (silica gel and hole punches);
- Laboratory costs (manpower and consumables) for trial DNA extraction and marker development;
- DoubleHelix expenses (flights and accommodation relating to workshop attendance for DoubleHelix and Thünen Institut staff);

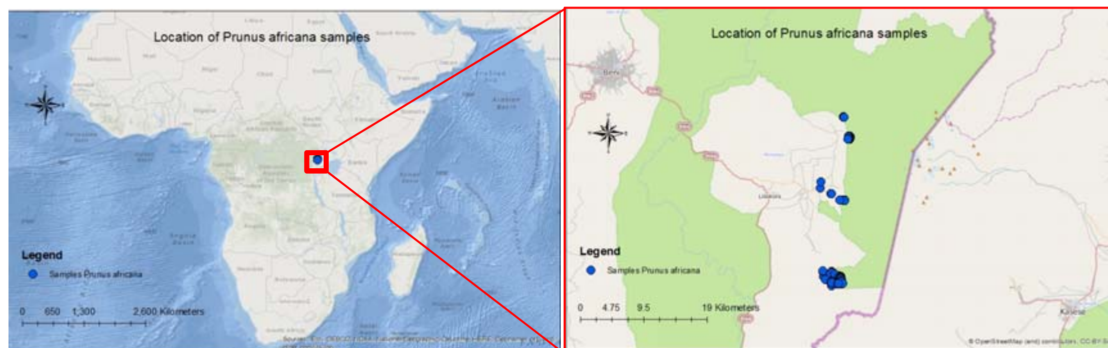
## 2.4 Outputs achievement

***Output 1.1 – Development of genetic markers***

A preliminary test to verify that DNA can be extracted from *P. africana* bark was successfully completed. The results of this DNA extraction test is presented in **Appendix A**.

Following the workshop and training, teams were sent out to collect wood samples from multiple populations in Cameroon and DRC. A map of DNA sample collection areas in DRC is presented in **Figure 1** below. Samples have been delivered to the laboratory in Germany and genetic markers suitable for DNA fingerprinting for *P. africana* are now in progress of being developed. Locations of samples taken in Cameroon are not yet available (yet to be verified) and will be sent in January.

**Figure 1 – Locations of *P. africana* populations in DRC where DNA samples have been collected.**




***Output 1.2 – Capacity building and training of local teams***

The first set of workshops was carried out in Kribi, Cameroon and Kinshasa, DRC. These were well attended by members of the Executing Agencies, industry and education. Agendas, attendance lists and photographs from the workshops are presented in **Appendix B**.

***Output 1.3 – Implementation of DNA verification system***
Meetings have been held with SIHPESP and industry participants in Cameroon and DRC to discuss the design of a routine sampling strategy from bark during harvest, storage and transportation.

## *3. CRITICAL ANALYSIS OF PROJECT PROGRESS*

Project delays are attributable to difficulties working in the local environment.

We experienced difficulties in relatively straight-forward tasks, such as sourcing and delivery of sampling consumables such as silica gel and hole punches which delayed the start of sampling activities by two weeks. Unexpectedly, it was impossible to find willing local suppliers of silica gel in Cameroon. In the end, a supplier in South Africa was found, leading to higher than expected costs of delivery to Cameroon and DRC.

Minor delays were experienced during the collection of reference DNA samples from target tree populations due to wet weather (wood samples should not be collected during wet weather since moisture in samples can lead to mould and sample contamination). Otherwise, implementation of the project has been reasonably straight-forward.

During the project workshops, it was raised by the Executive Agencies the desire to create capacity for conducting at least a portion of DNA extraction and analysis locally. As a result a proposal for the development of laboratory capacity in Cameroon has been developed and submitted for evaluation in September 2014.

During the opening workshops, we were able to research and understand the supply chain mechanism of *P. Africana* in Cameroon and DRC. It is expected that the envisaged system of bark-to-tree matching will work when we are able to make procedural changes to harvesting, bundling and storage processes in collaboration with local community workers and industry. This approach is likely to work with PAUs in the Mount Cameroon area.

In DRC however, bark harvesting and collection is carried out by a large and diverse number of groups and coordination between these groups and conformance to a specific procedure is expected to be impossible. We can foresee that applying a one-on-one matching system in DRC will not be possible. In this case, we recommend that a population analysis approach would be more appropriate and will recommend this type of approach in the final project report.

## *4. CONCLUSIONS*

Achievement of project objectives has been delayed but is still expected to be completed within the approved schedule.

Better understanding of the *P. africana* harvesting and processing systems in Cameroon and DRC means we expect to be able to successfully implement the system using the envisaged bark-to-tree methodology, but recommend using a different population methodology in DRC that will allow assignment to populations (geographic areas) rather than to individual trees. This would better fit the objectives of the ITTO-CITES project whilst requiring less disruption to established harvesting processes.

Whilst we have not so far experienced authorization delays for *P. africana* samples, it is recommended that we implement a process together with ITTO and the Executive Agencies to fast-track requests for export authorization of wood samples and avoid future delays in the sending of samples to Germany.

As per the Special Service Agreement (E) E14/14, we request the disbursement of the second set of funds of USD 60,000.

***Responsible for the Report***

Name: Darren Thomas

Position: Executive Director

Date: 24 December 2014

## *Appendix A – Trial DNA extraction report*

**First analysis of 5 *Prunus africana* samples using SSR markers from *Prunus persica***

***Background***

The Thünen-Institute of Forest Genetics tested the DNA extraction from cambium and bark of *Prunus africana* and the application of known SSR markers of *Prunus persica* for genotyping in *Prunus africana.*

***Methodology***

*Sample preparation and DNA isolation*

The Thünen-Institute of Forest Genetics received 5 samples of *Prunus africana* bark (PrAf1 to 5, Fig. 1).

Fig. 1: *Prunus africana* samples (after sample preparation)

All surfaces of the samples were incubated under UV light, each side for 2 hours. To minimize contaminations with foreign DNA (e.g. fungi) parts of the outer layer of the samples, surrounding the area of sample preparation, were removed, using a sharp scalpel. All together 20 different samples were prepared for DNA isolation (4 different samples were taken from every piece of bark). For cambium preparation (samples labeled with "k") the inner layer of the bark samples was scraped off and $^1/_3$ to $^1/_2$ of a 2ml reaction tube was filled with the resulting shavings. For bark preparation (samples labeled with "a", "b" or "c") the outer and radial layer of the bark samples was scraped off and $^1/_3$ to $^1/_2$ volume of a 2ml reaction tube was filled with the resulting shavings. Two steal balls (diameter 5 mm) were added to each prepared sample, and all samples were frozen in liquid nitrogen and grinded in a Retsch mill (Retsch GmbH, Haan, Germany) 3 times for 100s using a frequency of 17,5 Hz.

The DNA of the samples was isolated according to the custom developed wood DNA isolation protocol using 900µl of extraction buffer, 45µl DTT and 18µl proteinase K. The incubation in extraction buffer was performed overnight at 55°C.

The prepared DNA was solved in 20 µl of water and stored at -20°C. For the determination of DNA concentration and quality all samples were measured using the Nanodrop spectrophotometer (Thermo/ Fisher Scientific, Schwerte, Germany). The results of the spectrophotometric analysis are given in table 1.

| Sample ID | ng/ul | A260 | A280 | 260/280 | 260/230 |
|---|---|---|---|---|---|
| PrAf1a | 6,3 | 0,126 | 0,188 | 0,67 | 0,17 |
| PrAf1b | 8,44 | 0,169 | 0,225 | 0,75 | 0,17 |
| PrAf1c | 3,82 | 0,076 | 0,097 | 0,79 | 0,15 |
| PrAf1K | -170,08 | -3,402 | -0,598 | 5,69 | 1,21 |
| PrAf1K | -11,18 | -0,224 | 0,044 | -5,09 | 80,64 |
| PrAf2a | 19,06 | 0,381 | 0,574 | 0,66 | 0,13 |
| PrAf2b | 20,61 | 0,412 | 0,594 | 0,69 | 0,12 |
| PrAf2c | 3,67 | 0,073 | 0,091 | 0,81 | 0,25 |
| PrAf2K | 61,31 | 1,226 | 1,054 | 1,16 | 0,33 |
| PrAf2K | 2929,62 | 58,592 | 13,899 | 4,22 | 1,46 |
| PrAf3a | 225,27 | 4,505 | 6,36 | 0,71 | 0,41 |
| PrAf3b | 42,72 | 0,854 | 1,216 | 0,7 | 0,18 |
| PrAf3b | 44,07 | 0,881 | 1,279 | 0,69 | 0,18 |
| PrAf3c | 63,23 | 1,265 | 2,269 | 0,56 | 0,14 |
| PrAf3K | 115,12 | 2,302 | 0,913 | 2,52 | 0,61 |
| PrAf4a | 3,88 | 0,078 | 0,118 | 0,66 | 0,19 |
| PrAf4b | 4,27 | 0,085 | 0,078 | 1,1 | 0,22 |
| PrAf4c | 22,18 | 0,444 | 0,971 | 0,46 | 0,1 |
| PrAf4K | 6,84 | 0,137 | 0,24 | 0,57 | 0,15 |
| PrAf5a | 56,33 | 1,127 | 0,968 | 1,16 | 0,36 |
| PrAf5b | 39,6 | 0,792 | 0,645 | 1,23 | 0,51 |
| PrAf5c | 89,53 | 1,791 | 1,687 | 1,06 | 0,37 |
| PrAf5K | 112,99 | 2,26 | 1,099 | 2,06 | 1 |
| H2O | 0,77 | 0,015 | 0,001 | 16,14 | 1,1 |

Tab. 1.: Nanodrop analysis of the *Prunus africana* samples. Listed samples with identical sample IDs are due to repeated measurements of the same sample.

After Nanodrop analysis the samples were purified using the UltraClean 15 DNA Purification Kit (MoBio, Carlsbad, USA, sold by Dianova GmbH, Hamburg, Germany).

*SSR marker analysis*

Isolated DNA of all 5 original samples was used for SSR PCR testing. 5 SSR markers (Pr. UDP98_411; Pr. UDP98_412; Pr. UDP96-001; Pr. UDP96_018; Pr. UDP98-022) developed for *Prunus* persica (Testolin *et al.* 2000) were used for PCR reactions, under standard PCR conditions. Each forward primer carried a fluorescent tag. Thermal cycling was performed with denaturation and annealing steps of 30s and 45 s of elongation time. Cycle number was 33. PCR reactions were run on an ABI 3730 DNA capillary sequencer.

*Results*

Quantifiable DNA was successfully extracted from all 5 *Prunus africana* samples. Fig. 2 shows an example of a PCR-test of 2 *Prunus persica* SSR-markers with the genomic DNA from *Prunus africana*. Both primers amplified well and different alleles could be identified. This indicates that DNA extraction from *Prunus africana* cambium and bark samples is possible and the application of markers developed for other *Prunus* species is suitable for *Prunus africana*.

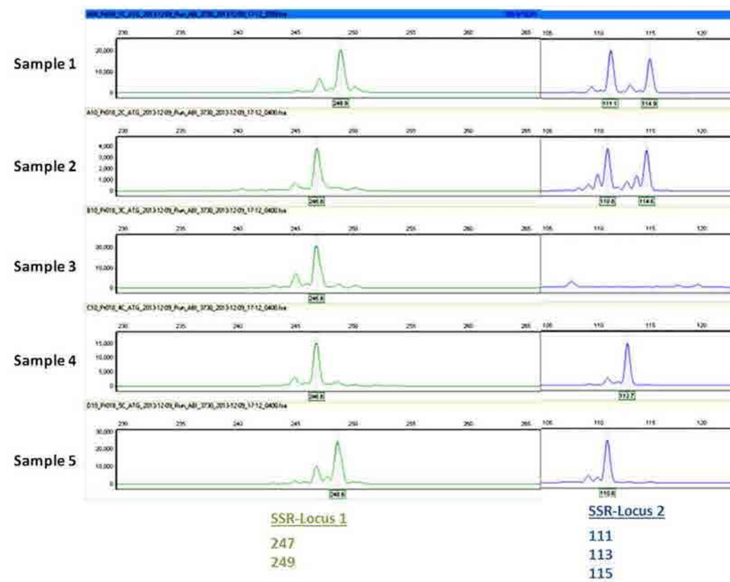



Fig. 2: Example of a PCR-test of 2 *Prunus persica* SSR-markers with genomic DNA from *Prunus africana*

*Literature*

Testolin, R., Marrazzo, T., Cipriani, G., Quarta, R., Verde, I., Dettori, M. T., Pancaldi M., Sansavini, S. (2000). Microsatellite DNA in peach (Prunus persica L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. *Genome*, *43*(3), 512-520.

*Appendix B – Workshop agenda, attendance and photographs*

## Agenda

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# DNA Chain-of-Custody workshop agenda

**DRAFT**

**Day 1**

| | |
|---|---|
| 08:30 | **Participants registration** |
| 09:00 | **Opening remarks** - Government, ITTO representatives |
| 09:30 | **Photo/Picture with all the participants** - Facilitator |
| 10:00 | **Introduction and update on the ITTO-CITES programme** - Jean Lagarde-Betti (ITTO) |
| 10:30 | **Introduction to the project for *pericopsis elata* and *prunus africana***<br>Darren Thomas, Germain Yene Yene (DoubleHelix) |
| 11:00 | Coffee break |
| 11:30 | **Overview of using DNA markers to control origin and Chain-of-Custody**<br>Henri Bouda (Thünen Institut), Darren Thomas |
| 12:00 | **Practical results of a project using DNA for wood tracking (realized in Cameroon)**<br>Henri Bouda (Thünen Institut), |
| 12:30 | Discussion - Facilitator |
| 13:00 | Lunch |
| 14:00 | **Introduction and desk-based training on DNA sampling** - Henri Bouda (Thünen Institut), video<br>a. Tree anatomy<br>b. Tools<br>c. Sampling procedure<br>d. Sample recording and storage<br>e. Logisitics |
| 15:00 | Coffee break |
| 15:30 | **Formulation of sampling plan and schedule for *p.elata* and *prunus africana* + discussion**<br>Jean Lagarde Betti, all. |
| 16:30 | END OF DAY 1 |

It's in our nature

## Day 2 – Field visit

| | |
|---|---|
| 08:30 | **Participants grouping at the hotel** |
| 09:00 | **Departure to the forest reserve of Bidou** |
| 09:30 | **Arrival at the forest reserve site** |
| 10:00 | **Training in the forest reserve**<br>a. Sampling practice for all participants - led by Henri Bouda, Germain Yene Yene<br>b. Adjustment of sampling procedures for *p.elata* and *prunus africana* - Darren Thomas |
| 12:00 | Return travel |
| 12:30 | Debriefing (if necessary) |
| 13:00 | Lunch |
| 14:00 | END OF DAY 2 |

It's in our nature

**Participant list DRC**

A L'ATELIER SUR LA « TRACABILITE D'ADN D'ECORCES DE PRUNUS AFRICANA » EN RDC

**LISTE DE PRESENCE**

*Vendredi, le 06 juin 2014*

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| | | | | |
|---|---|---|---|---|
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| 30. | Katshua Mathieu | DCN | | [illegible] |
| 31. | KISITA LUFUMA | DCN IRET | | [illegible] |
| 32. | EBongo Jean | DGF | | [illegible] |
| 33. | | | | |
| 34. | | | | |
| 35. | | | | |

**Workshop photos**

*__Appendix C – Sample of DNA collection records and photographs__*

Sampling records will be provided separately. They are currently held in Germany and a copy will be obtained in January.

## Sampling in DRC