FA1203: Sustainable management of *Ambrosia artemisiifolia* in Europe (SMARTER)

Short Term Scientific Mission Report

Intra-populational genetic diversity and adaptability of European populations of *Ambrosia artemisiifolia*

STSM details

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Applicant details

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Summary of the STSM

The aim of this STSM was to investigate the link between the intra-populational genetic diversity and the adaptability of European populations of *Ambrosia artemisiifolia*, *Amaranthus retroflexus* and *Chenopodium album*. All these three species are facultative short-day plants and are known to be thermophilic. The two last weeds have invaded a large number of European countries and have successfully established in different biotic and abiotic environmental conditions, even in the north of Europe under cold and long-day conditions (e.g. Scandinavia). However, while *C. album* succeeded to adapt to field conditions located in the North of Europe, *A. retroflexus* did not and is recognized only as ruderal plant along road sides with negligible impact on agriculture.
During my stay at Uppsala I supported Romain Scalone in the detection of the genetic diversity present in field-adapted and non-adapted populations of *Chenopodium album* and *Amaranthus retroflexus* by Amplified Fragment Length Polymorphism (AFLP) and Microsatellite (SSR) technique. The results obtained should be compared with equivalent data coming from populations of *Ambrosia artemisiifolia.* These determinations and comparisons should permit to investigate the invasion potential of *A. artemisiifola* in northern European countries and its possible future impact on northern European agriculture.

Purpose of the STSM

1) Determination of the most efficient way to extract high quality DNA from genetically diverse species like *Ambrosia artemisiifolia*

2) Disclosure of the optimal relations and concentrations of the various ingredients/chemicals in order to provide an optimal milieu for the DNA of the 3 species to be ligated, restricted and amplified
3) Determination of the optimal conditions for AFLP analyses, including testing several primer- and polymerase-combinations in order to get most significant results
4) Determination of the optimal conditions for Microsatellite analyses, including testing several primer- and polymerase-combinations
5) Disclosure of the most effective PCR-protocol for the 3 species, especially what the different primers and polymerases are concerned
6) Preparation of a big sampling with fluorescence-primers, evaluation and interpretation of the results gained in this sampling

Additional impact for me: Exchange of lab know-how and learning new lab techniques which can help to detect the genetic relations of the Austrian populations of *A. artemisiifolia*

Description of the work carried out during the STSM
Description of the main activities and results obtained

Week 1

In the first week of my stay the main focus of Romains and my work lied on the extraction of DNA from the 3 plant species. Therefore, we prepared a small test sampling, containing:

3 populations á 5 individuals of *Ambrosia artemisiifolia*
3 populations á 5 individuals of *Amaranthus retroflexus*
3 populations á 5 individuals of *Chenopodium album*
Total: 45 individuals

QIAGEN-KIT

The “DNeasy Plant Mini Kit” (Qiagen-Kit) provides silica-membrane-based-nucleic acid purification from tissues, which are lysed enzymatically and thereby ideally removes all contaminants and inhibitors. In order to gain high-quality DNA, I cutted the leaf tissue of samples in small pieces and shredded them in the Retsch Mixer Mill (MM400) for 2 minutes on each side at a vibrational frequency of 25 Hz. After shredding, samples were mixed following the protocol of Qiagen: The disrupted samples (< 100 mg wet weight) were mixed with 400µl Buffer and 4µl RNase and incubated for 10 min. at 65°C. After that 130 µl buffer was added and the samples were incubated for 5 min. on ice.
Then the samples were analyzed by the Qiagen-Cube. All materials needed for sample purification were placed onto the instrument worktable prior the run. The sample block contained the pipet tips and the sample material that was lysed and transferred to the filter plate during the run. Disposable troughs contained all necessary buffers and reagents for sample binding, washing and elution. Liquid-handling tasks were performed by a 8-channel pipetting head. After washing, the

pipetting head moved the filter plate to the elution compartment where purified DNA was eluted into elution microtubes. The included strip caps closed the elution microtubes automatically for sample storage.

BRUTUS BUFFER

Since it is recommended to use very fresh plant material for Qiagen-Kit, we also tried to extract DNA with a chemical mixture, called Brutus-Buffer and consisting of following ingredients:

Steps for Brutus Buffer (100 ml)
60 ml of MilliQ-H_2O
Add 7,455 g KCl and mix with a magnetic mixer
Add 10 ml of 1M Tris-HCl (pH 9.5)
Add 2 ml of 0.5M EDTA (pH 8)
Fill the mixture with MilliQ-H_2O up to 100 ml

After preparation of the buffer, the cut and chrushed samples were treated as follows:

Sample preparation with Brutus Buffer
1cm² of leaf disrupted leaf tissue is filled in 2ml-tubes
Add 1ml of Brutus Buffer
Put the tubes in a hot plate at 95°C for 5 minutes
Put the tubes on ice for 5 minutes
Vortex the tubes and freeze them at -20°C for storage

When all samples (Qiagen-Kit & Brutus) were ready for subsequent analyses, the yield of the DNA extraction were measured by using the NanoDrop ND-1000 Spectrophotometer, which uses an absorbance based nucleic acid quantification method. Therefore, 1µl of the sample solution is pipetted onto the end of a fiber optic cable. A second fiber optic cable is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. Thereby, the instrument is controlled by PC based software, and the data is logged in an archive file on the PC.

RESULTS

Since the leaves of the 3 species were harvested too late, the quality of the leaf samples was quite low. Therefore, the DNA extraction with DNA-Kit did not show any applicable results. It was quite obvious that only from leaf samples which still had green parts and did not have reached senescence at harvesting DNA could be extracted. Nevertheless, also for these samples concentration of DNA was too low to proceed. In contrast, the Brutus Buffer worked out very well, delivering high concentrations of DNA. But: As the leave tissues were soaked in the buffer solution, the samples were contaminated with other compounds which lead to biased results with the NanoDrop. To improve the purity and the quality of the DNA of these samples the Brutus-DNA was diltued with MilliQ-H_2O (ultrapure water).

After measuring again with NanoDrop the samples showed a better quality and where then diluted a third time to get standardized samples, each having 50ng DNA/µl.

Week 2

With these standardized samples AFLP procedure was prosecuted as follows:

DIGESTION/LIGATION/RESTRICTION

Samples were standardized with 250 ng DNA = 5 µl of diltued DNA

Ingredients	Concentration	Total amount for 1 sample
NaCl	0.5M	2 µl
BSA	10 mg/µl	0.1 µl
Mse1 (10U/µl)	5 U/µl	0.5 µl
EcoR1 (10U/µl)	5 U/µl	0.5 µl
Mse1 Adapter (10pM/µl)	50 pm	5 µl*
EcoR1 Adapter (10pM/µl)	5 pm	0,5 µl*
T4 DNA Ligase (5 wU/µl)		0.1 µl
T4 DNA Ligase Buffer (10x)		2 µl
MilliQ-H_2O		4.3 µl
	Σ	**15 µl**
+ DNA (250 ng)		5 µl
Total amount/sample		**20 µl**

* amount is for both adapters together; before mixing the adapters the must be heated up in bain-marie for 5 min at 95°C and cooled down slowly until room temperature

For restriction and ligation the samples were put on a heating plate and stored there for 5 hours at 37°C.

PRE-AMPLIFICATION

Ingredients	Total amount for 1 sample
Primer Mse1 (100pM/µl)	0.4 µl
Primer EcoR1 (100pM/µl)	0.4 µl
dNTPs (10mM)	0.6 µl
Taq Polymerase (5wU/µl)	0.2 µl
Taq Polymerase Buffer (10x)	4.5 µl*
MilliQ-H_2O	8.9 µl
	15 µl
+ DNA (10 x diluted)	5 µl
Total amount/sample	**20 µl**

* 1.6 µl of MgCl2 were added to the Taq polymerase buffer

Pre-Amplification Protocol		
Step	**Temp**	**Time**
Initial denaturation	72°C	2 min
Denaturation	94°C	30 sec
Annealing	56°C	1 min
Extension	72°C	1 im
Final Extension	60°C	30 min

→ **30 cycles**

AMPLIFICATION

Ingredients	Total amount for 1 sample
Primer Mse1 (100pM/µl)	0.04 µl
Primer EcoR1 (100pM/µl)	0.04 µl
dNTPs (10mM)	0.5 µl
Taq Polymerase (5wU/µl)	0.08 µl
Taq Polymerase Buffer (10x)	2.5 µl
MilliQ-H_2O	4.34 µl
	7,5 µl
+ DNA	2.5 µl
Total amount/sample	**10 µl**

Amplification Protocol		
Step	**Temp**	**Time**
Initial denaturation	94°C	2 min
Denaturation	94°C	10 sec
Annealing	65°C	30 sec
Extension	72°C	2 min
Denaturation	94°C	30 sec
Annealing	56°C	30 sec
Extension	72°C	1 min
Final Extension	60°C	30 min

→ **12 cycles, decreasing annealing by 1°C each cycle**

→ **32 cycles**

RESULTS

After preparing the samples, the amplification product of the last PCR was migrated on gel-electrophoreses system, but unfortunately did not show any results.
So we were testing different ingredient combinations but didn't receive any results for the AFLPs, too.
After discussion with Matthias Kropf from the University of Natural Resources and Life Science Vienna, who is an expert in AFLP technique, we tested the DNA quality again and finally came to the conclusion that the DNA was not pure enough to run AFLP which requires the best quality of DNA possible. Even though the DNA extracted with the Brutus Buffer showed a high concentration, the ratios for quality and purity did not improve that much after dilution with ultrapure water. Therefore, Romain Scalone instructed me to go on with testing the Microsatellites for *Ambrosia artemisiifolia*, *Amaranthus retroflexus* and *Chenopodium album* because due to the results of various studies SSR technique does not require such high quality DNA as AFLP.

Week 3

For the Microsatellites analyzes for the various species we tested two different polymerases in the mixture below:

- Thermo Scientific Phire Hot Start II DNA Polymerase
- Thermo Scientific DreamTaq DNA Polymerase

PHIRE HOT START II	
Ingredients	**Amount for 1 sample**
Primer (forward)	1 µl
Primer (reverse)	1 µl
Taq Polymerase Phire	0.4 µl
Total for each Primer combination	**2.4 µl**
Taq Polymerase Phire Buffer	4 µl
dNTPs (10mM)	0.4 µl
MilliQ-H_2O	12.2 µl
Mastermix	**16.6 µl**
+ DNA (50 ng)	1 µl
Total amount/sample	**20 µl**

DREAMTAQ	
Ingredients	**Amount for 1 sample**
Primer (forward)	1 µl
Primer (reverse)	1 µl
Taq Polymerase Phire	0.1 µl
Total for each Primer combination	**2.1 µl**
Taq Polymerase Phire Buffer	2 µl
dNTPs (10mM)	0.4 µl
MilliQ-H_2O	14.5 µl
Mastermix	**16.9 µl**
+ DNA (50 ng)	1 µl
Total amount/sample	**20 µl**

PRIMERS USED

PRIMERS *Ambrosia artemisiifolia*			Optimum Annealing Temperatures [°C]*	
Name	**Foward primer sequence (5' → 3')**	**Reverse primer sequence (5' → 3')**	**PhireTaq**	**DreamTaq**
AMBEL 10	CGT CAA TGG ACG ATG AAG AA	CCA CGT CTT CAA GAA TAA CAA AA	62.2	47.2
AMBEL 17	GAA CAT CGA TTA TGA AGA TGC AG	GAT TAA GGT TGT CAA TAA GGA TTG G	65.7	47.8
AMBEL 26	TCA AGA AAT TGA TTT AGA ACC AAG G	GGA GAA CTT GCG CTC GTA TT	63.4	47.3
AMBEL 47	CAA TCA CCA TCG TCA CAT CC	GGA GCC GGT CAT CGT TTT AT	64.4	49.8
AMBEL 67	ACA AAG CCA CTT TTG ATG CC	CCT TCA GAT GTT TGG CCT TC	63.9	50.4
AMBEL 71	GAC TTT CGC TTC CCA AAC AC	CAA ATG TCA TGG GGA GAA GG	63.9	49.7
AMBEL 73	GAC TCA TGC ATA TGG AAC ACG	CCA AAT GGT CTA CCT CCT GC	63.4	49.9
AMBEL 86	TCT GCC TTC TTT GAG GAT CTT T	AAA ATA CCT GCC TAT CAT GGT TGA	66.4	50.1
AMBEL 91	AAA CAT CTT TCG ATT CAA GCT CA	TGG TTT GGA TAT TGA TAG AAC AGC	66.1	48.7

PRIMERS *Amaranthus retroflexus*			Optimum Annealing Temperatures [°C]*	
Name	**Foward primer sequence (5' → 3')**	**Reverse primer sequence (5' → 3')**	**PhireTaq**	**DreamTaq**
AMARE 99	AAA TTG ACA ATG CGC AGC	TTC CTCACC AAA ATT GCC	61.6	46.8
AMARE 105	GTG ATG GTC GTG GTG GAG	GAT TCC CTC ATC TTC GCC	62.7	49.9
AMARE 129	TTC ACG TGG GAA GGA GG	AAA ATT AAT GGG CCT CGC	61.8	47.1
AMARE 132	AAC TTT TGC CTC CTG CAA	TCA AAT GCT GAT CCC AGG	61.5	48.4
AMARE 136	TCA GCA AAA CAT GAT CAA CAA	GTT GCT GCA TTG GTG GTT	62.2	46.2
AMARE 137	CGA AGA TCA TGG GTT TGC	TTG AGA ATA AGG CGT TGA CA	61.3	47.7

PRIMERS *Chenopodium album*			Optimum Annealing Temperatures [°C]*	
Name	**Foward primer sequence (5' → 3')**	**Reverse primer sequence (5' → 3')**	**PhireTaq**	**DreamTaq**
CHENA 28	TGC TCA CCC TAG CAT TTA TAC ACT	ATG AGA CGG AGG GAG CAC TA	62.9	51.7
CHENA 30	TCA TTG GTT AGA TGG TGG AAT G	CCC TCT AGT GCA TAG GAG TTT CTG	66.8	48.5
CHENA 33	CAG GGC AGT CCA CCT CTC TA	ACC TTC TAG TCC TAT GTT CTT GTA TGG	62.8	51.7
CHENA 37	CCG TTC TTC CAG ACC AAT TC	TCA TGA GCC ACT TCA TAC ACG	63.8	50.1
CHENA 46	GCA GGT AAA TCA ACC CTT GC	TGC ATG ATA AAC TAA GCA GAC GA	63.7	50.2
CHENA 48	ACA ATA CAT ACA TAA CCC AAT ATT CAA	TGG AAA TGT CAC TAT GAT TGG A	63.5	45.7

* The optimum temperatures were calculated with the support of Thermo Scientific who supplied the Taq Polymerasen and provide a calculation tool on their homepage: https://www.thermofisher.com/se/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

Due to large differences between the PCR protocols used in the various studies and the instruction of Thermo Scientifc concerning temperature and time intervals, we tested all these different protocols in order to get the best result possible.

SUMMARY OF THE PROTOCOLS

PCR protocol for Phire Taq [Thermo scientific]

Step	Temp	Time	
Initial denaturation	98°C	30 sec	
Denaturation	98°C	5 sec	35 cycles
Annealing	62°C	5 sec	
Extension	72°C	15 sec	
Final Extension	72°C	1 min	

PCR protocol for Dream Taq [Thermo scientific]

Step	Temp	Time	
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	40 cycles
Annealing	48°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	10 min	

PCR protocol due to Lee et al. (2008) for *Amaranthus hypochondriacus*

Step	Temp	Time	
Initial denaturation	94°C	3 min	
Denaturation	94°C	30 sec	50 cycles
Annealing	55°C*	45 sec	
Extension	72°C	45 sec	
Final Extension	72°C	20 min	

* Primer AMARE 99: Annealing Temperatur 50°C following the protocol of Lee et al. (2008)

Touchdown PCR protocol due to Maughan et al. (2004) for *Chenopodium quinoa*

Step	Temp	Time	
Initial denaturation	94°C	1 min	
Denaturation	94°C	30 sec	5 cycles, decresing annealing by 1°C each cycle
Annealing	55°C	30 sec	
Extension	72°C	1 min	
Denaturation	94°C	30 sec	10 cycles
Annealing	50°C	30 sec	
Extension	72°C	1 min	
Denaturation	94°C	30 sec	5 cycles, decresing annealing by 1°C each cycle
Annealing	50°C	30 sec	
Extension	72°C	1 min	
Denaturation	94°C	30 sec	10 cycles
Annealing	45°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	

RESULTS

After amplification the PCR-products of *A. retroflexus* and *C. album* were analyzed using gel electrophoreses. For *C. album* the gel electrophoreses did not show any results when evaluating the PCR-products under UV-light. For *A. retroflexus* there were only very slight bar marks visible. After testing several gel intensities and loading-dye/PCR-product-combinations which always delivered the same results I decided to go for a test run with purified DNA gained from Qiagen-Kit from fresh leave material of all 3 species. I wanted to check if there is something wrong with the gel-electrophoreses apparatus since it hasn't been used a quite long time, according to the lab technicians. After this run, a failure of the gel-electrophoreses apparatus could be excluded, since the run with the purified DNA showed very good results under UV-light.

Our conclusion was, that the DNA quality gained with the Brutus buffer was not sufficient to get clear PCR results visualized by subsequent gel electrophoreses. Thus, all analyzes were again performed using the MultiNA Microchip Electrophoresis System which is very sensitive to DNA-fragments and can therefore be used for low quality DNA, too.

Week 4

While *A. retroflexus* showed good PCR-results when analyzing with MultiNA Microchip Electrophoreses the touchdown PCR for *C. album* and *A. artemisiifolia* were unfortunately without any result. Thus, we did several SSR-analyses in order to find the optimal protocol as well as the optimal Primer- and Polymerase conditions for all 3 species.

SUMMARY OF THE TEST-RUNS

***Amaranthus retrofelxus & Chenopodium album*:**
6 primers, 5 individuals => 30 samples each per run
Ambrosia artemisiifolia:
9 primers, 5 individuals => 45 samples

AMARANTHUS RETROFLEXUS

Run	Polymerase	Protocol	Amount of DNA	Primer/Polymerase Amount
1	Phire	Lee et al. (2008)	50 ng	Thermo Scientific
2	Dream	Lee et al. (2008)	50 ng	Thermo Scientific
3	Phire	Thermo Scientific	50 ng	Thermo Scientific
4	Dream	Thermo Scientific	50 ng	Thermo Scientific

After these 4 runs the results showed clearly that the protocol used by Lee et al. (2008) delivered better results than the protocol suggested by Thermo Scientific. Thus, the following runs were performed following this protocol. Furthermore, it was noticeable that the samples treated with Phire Hot Star II performed better than those with the DreamTaq.

Run	Polymerase	Protocol	Amount of DNA	Primer/Polymerase Amount
5	Phire	Lee et al. (2008)	25 ng	Thermo Scientific
6	Dream	Lee et al. (2008)	25 ng	Thermo Scientific
7	Phire	Lee et al. (2008)	50 ng	Increased by 1/3
8	Dream	Lee et al. (2008)	50 ng	Increased by 1/3

After examination of the results of these various runs, the best results for *Amaranthus retroflexus* was shown under the following conditions:

Amount of DNA: **50 ng**
Polymerase: **Phire Hot StarII**
Protocol: **Lee et al. (2008)**
Primer/Polymerase Amount: **Increased by 1/3**

AMBROSIA ARTEMISIIFOLIA & CHENOPODIUM ALBUM

After several similar tests with *A. artemisiifolia* and *C. album* trying different primer-polymerase amounts, different polymerases and different protocols we still did not receive any significant results, independent of the primer used and neither on gel electrophoreses nor on MultiNA Microchip Electrophoreses.

Week 5 and 6

After discussing these results with Matthias Kropf and Gerhard Karrer, Romain Scalone and me decided to go on with *A. retroflexus,* and so, we started with the preparation of the big sampling for at least *A. retroflexus*. In total, the sampling comprised 13 populations each consisting of 20 individuals (= 260 samples). The leave tissues were – as in the pre-sampling – cut and shredded with Retsch Mixer Mill (MM400) for 2 minutes on each side at a vibrational frequency of 25 Hz and then mixed with Brutus Buffer as indicated above.
After measuring the DNA concentration with NanoDrop ND-1000 Spectrophotometer the samples were diluted to get a standard concentration of 50 ng/µl DNA.
To check if the quality of the samples is at least the same of that in the pre-test several PCRs were performed and the result were checked on MultiNA Microchip Electrophoreses.
After receiving constantly satisfying results Romain Scalone instructed me to prepare a test-run with 10 individuals for the Uppsala Genome Center, which was responsible for the analyzes of the big sampling. Therefore, I was provided with fluorescent forward primers (Tag Copenhagen), whereas the reverse primers stayed the same as in the test run. Since the increased primer-polymerase amount turned out to deliver the best results we went for this. According to the protocol of Lee et al. (2008) the relation between fluorescent forward primer and non-fluorescent reverse primer was 1:4. Phire Hot Star II was used as polymerase and PCR was executed using the protocol of Lee et al. (2008).

PHIRE HOT STAR II	
Ingredients	**Amount for 1 sample**
Primer (forward) fluorescent	0,375 µl
Primer (reverse)	1.5 µl
Taq Polymerase Phire	0.6 µl
Total for each Primer combination	**2.475 µl**
Taq Polymerase Phire Buffer	6 µl
dNTPs (10mM)	0.6 µl
MilliQ-H_2O	9.925 µl
Mastermix	**19 µl**
+ DNA (50 ng)	1 µl
Total amount/sample	**20 µl**

Before delivering the samples to Uppsala Genome Center the PCR products were diluted in the relation 1:10, 1:20 and 1:40 according to the instruction of Uppsala Genome center to see which dilution suites best for these samples.

RESULTS

After receiving the results from Uppsala Genome Center for the fluorescently marked Primers we had to confess that all PCRs did not show feasible results. Approximately 50 % of the samples didn't show anything, and the results of the remaining were very bad, not allowing further analyses.
Therefore, the protocol was changed again. In contrast to the protocol of Lee et al. (2008) we went for equal concentrations of forward (fluorescent) primer and reverse primer. Additionally, the primer-polymerase ratio was again lowered by 1/3.

PHIRE HOT STAR II	
Ingredients	**Amount for 1 sample**
Primer (forward) fluorescent	1 µl
Primer (reverse)	1 µl
Taq Polymerase Phire	0.4 µl
Total for each Primer combination	**2.4 µl**
Taq Polymerase Phire Buffer	4 µl
dNTPs (10mM)	0.4 µl
MilliQ-H_2O	12.2 µl
Mastermix	**19 µl**
+ DNA (50 ng)	1 µl
Total amount/sample	**20 µl**

After amplification the PCR products were diluted 1:40 and sent to Uppsala Genome Center again. The results obtained from this test run were very promising – at least some of the primers used work very well for *A. retroflexus* so that the big sampling could be prepared by Romain Scalone after the end of my stay at SLU.

Further use of data within SMARTER and foreseen publications/articles resulting from the STSM

This STSM contributes to the goals of the WG2, especially in terms of predicting the future establishment potentials of *A. artemisiifolia* as well as in terms of understanding the causes and mechanisms of ragweed invasion on a genetic basis. Assuming that there is mechanistic similarity between the genetic variation of a successfully established weed (*A. retroflexus*) and the upcoming *A. artemisiifolia* land users/managers may be able to adopt preventative measures to avoid the establishment of *A. artemisiifolia* in their arable fields.
This collaboration is the result of discussion between R. Scalone, M. Kropf and G. Karrer during the second SMARTER Genetic Task force Meeting, which took place in Tulln in April 2015. Based on this collaboration a paper can be expected to be submitted to Molecular Ecology.

Confirmation by the host institution of the effective execution of the STSM

Copy of the e-mail sent to Dr. Maurizio Vurro, the Training Coordinator of the COST Action FA1203, is to find attached.

Vienna, 30th November 2015

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