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A pilot study on hybrid speciation in alpine willows (*Salix*)

Eine Pilotstudie zur Speziation von Hybriden an alpinen Weiden (*Salix*)

Background

The discrimination of willows by means of morphological characteristics is often problematic due to their high phenotypic plasticity and great variation in diagnostic morphological characters. Also the molecular characterization using standard systematic primers revealed almost no differences between species (Leskinen and Altström-Rapaport 1999, Azuma et al. 2000, Hamzeh and Dayanandan 2004). For willows (genus *Salix)* sequence information is very scarce in contrast to poplars (genus *Populus*). *Populus* is considered the model system for genetic and genomic studies in forest trees (Taylor 2002) and is the third plant species (after *Arabidopsis* and rice) to have its genome sequenced (Tuskan et al. 2004). Due to the close taxonomic relationship (willows and poplars are both members of the Salicaceae) genetic and genomic information may be transferable between *Populus* and *Salix* (Hanley et al. 2006).

Single nucleotide polymorphisms (SNPs) are widely used markers for hybrid detection and characterization. Instead of the rather expensive and time-consuming sequence analysis for SNP detection high resolution melting curve analysis (HRM) is a promising approach to detect single or short nucleotide polymorphisms (for short review see Mader et al. 2008). HRM measures exactly the decreasing fluorescence of an intercalating dye in the process of dissociation of double stranded DNA due to a heating process between 45°C to 90°C. The double stranded DNA is usually an amplification product with a length of about 100 bp from real-time PCR reactions obtained with specific primers. The shape of the melting curve depends on the GC content, length and sequence of the amplicon. SNPs

and other mutations can be detected immediately after PCR and HRM without sequencing. Another advantage of HRM is that it can discriminate between homozygous and heterozygous genotypes (Mader et al. 2008).

Material and Methods

DNA was extracted from leaves from herbarium specimens (Table 1) using a CTAB extraction method after Pirtillä et al. (2001). DNA content was measured and the concentration was brought to 5 ng/µl DNA. PCR/HRM reactions were carried out in a final volume of 10 µl containing 5 ng DNA, 100 nM of each primer, 3.5 mM $MgCl_2$, 1x Buffer B2 (Solis BioDyne, Tartu, Estonia), 100 µM dNTPs (Solis BioDyne, Tartu, Estonia), 3 µM BEBO (TATAA Biocenter, Bengtsson et al. 2003) , 0.4 Units HotFirePol DNA Polymerase (Solis BioDyne, Tartu, Estonia). PCR with immediately following HRM analysis was performed on a RotorGene 6500 (Corbett Research Ltd., Sydney, Australia) with HRM module using the appropriate tubes (Corbett Research Ltd., Sydney, Australia). The following thermal profile was applied for the real-time PCR reaction: 94°C for 15 min, following 40 cycles of 94°C for 10 s, 57°C – 60°C (depending on the annealing temperature of the primers) for 20 s and 70°C for 20 s. HRM was carried out from 70°C to 90°C at 0.1°C steps with 2 s holds. All HRM reactions were carried out in duplication. Results were analysed using the RotorGene 6000 series software, Version 1.7.65.

Table 1: Herbarium specimens for DNA extraction (Derivations Bayreuth derive from the Botanical Garden of the University of Bayreuth, Germany). Specimens in gray were investigated with all the primer pairs listed in Table 2.

Species	Herbarium	Derivation	Year of Collection	Herbarium Specimen
Salix foetida	Hörandl	France	1992	M3: 4529
Salix foetida	Hörandl	France	1992	M3: 4493
Salix foetida	Hörandl	Austria	1990	M3: 149
Salix foetida	Hörandl	Austria	1990	M3: 120
Salix foetida	Hörandl	Italy	1992	M3: 4393
Salix foetida	Hörandl	Austria	1990	M3: 121
Salix foetida	Hörandl	Austria	1989	M3: 270
Salix foetida	Hörandl	Switzerland	1990	M3: 78
Salix foetida	Hörandl	Switzerland	1990	M3: 8
Salix foetida	Hörandl	Switzerland	1990	M3: 74
Salix foetida	Hörandl	Switzerland	1990	M3:77
Salix foetida	IAB VetMed	Bayreuth	2008	991021
Salix helvetica	Hörandl	Italy	1987	M3: 292
Salix helvetica	Hörandl	Austria	1990	M3: 144
Salix helvetica	Hörandl	Austria	1990	M3: 10
Salix helvetica	Hörandl	Austria	1990	M3: 129
Salix helvetica	Hörandl	Austria	1990	M3: 141
Salix helvetica	Hörandl	Austria	1990	M3: 134
Salix helvetica	Hörandl	Austria	1989	M3: 30
Salix helvetica	Hörandl	Austria	1990	M3: 135
Salix helvetica	Hörandl	Dolomiten	1998	M3: 8
Salix helvetica	Hörandl	Dolomiten	1998	M3: 15
Salix helvetica	Hörandl	Dolomiten	1998	M3: 18

Salix helvetica	Hörandl	Dolomiten	1998	M3: 21
Salix helvetica	IAB VetMed	Bayreuth	2008	991022
Salix helvetica	IAB VetMed	Bayreuth	2008	991023
Salix helvetica	IAB VetMed	Bayreuth	2008	991024
Salix foetida x helvetica	Hörandl	Austria	1990	M3:128
Salix foetida x helvetica	Hörandl	Austria	1990	M3: 137
Salix foetida x helvetica	Hörandl	Austria	1990	M3: 130
Salix foetida x helvetica	Hörandl	Switzerland	1990	M3: 11
Salix foetida x helvetica	Hörandl	Switzerland	1990	M3: 6
Salix foetida x helvetica	IAB VetMed	Bayreuth	2008	991028
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 42
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 56
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 58
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 39
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 48
Salix hegetschweileri	Hörandl	Switzerland	1990	M4: 52
Salix hegetschweileri x myrsinifolia	Hörandl	Switzerland	1990	M4: 25
Salix hegetschweileri x myrsinifolia	Hörandl	Switzerland	1990	M4: 26
Salix hegetschweileri x myrsinifolia	Hörandl	Switzerland	1990	M4: 33
Salix hegetschweileri x myrsinifolia	Hörandl	Switzerland	1990	M4: 38
Salix hegetschweileri x myrsinifolia	Hörandl	Switzerland	1990	M4: 28
Salix myrsinifolia	Hörandl	Austria	1990	M4: 470
Salix myrsinifolia	Hörandl	Austria	1990	M4: 72
Salix myrsinifolia	Hörandl	Austria	1990	M4: 86
Salix myrsinifolia	Hörandl	Austria	1990	M4: 1036
Salix myrsinifolia	Hörandl	Austria	1990	M4: 303
Salix myrsinifolia x hegetschweileri	Hörandl	Switzerland	1990	M4: 5
Salix myrsinifolia x hegetschweileri	Hörandl	Switzerland	1990	M4: 23
Salix myrsinifolia x hegetschweileri	Hörandl	Switzerland	1990	M4: 3
Salix myrsinifolia x hegetschweileri	Hörandl	Switzerland	1990	M4: 22
Salix alba	Hörandl	Austria (Stmk)	1990	M1: 89
Salix alba	Hörandl	Austria (Ktn)	1990	M1: 370
Salix fragilis	Hörandl	Austria (NÖ)	1990	M1: 277
Salix fragilis	Hörandl	Austria (NÖ)	1990	M1: 273
Salix purpurea	Hörandl	Austria (Szbg)	1990	M1: 430
Salix purpurea	Hörandl	Austria (Wien)	1990	M1: 70

Development of HRM primers

At the IAB HRM primer pairs to distinguish different *Salix* species were developed within some other projects. On the basis of polymorphisms in sequence alignments of chloroplast genes of *S. vitellina*, *S. purpurea*, *S. viminalis*, *S. triandra*, *S. pentandra*, *S. babylonica*, *S. fragilis* and *S. alba* eleven primer pairs were developed. Nine primer pairs were designed on the sequence of two nucleus genes (*Salix discolor* leafy/floricaula mRNA, GenBank: AY230817 und *Salix viminalis* wound-induced trypsin inhibitor gene, GenBank: U29092). The 20 primer pairs were investigated with different *Salix* species and 5 primer pairs were chosen, 3 primer pairs from chloroplast genes (Table 2, Nr. 1, 2 and 3) and 2 from nucleus genes (Table 2, Nr. 4 and 5).

The 5 primer pairs were used for HRM with DNA extracts from *S. foetida*, *S. helvetica*, *S. hegetschweileri*, *S. myrsinifolia* and some hybrids (Table 1). Analysing the different HRM curves an enormous polymorphism between different genotypes within each *Salix* species was found. Rarely two genotypes within one species show the same HRM curve. Because *S. hegetschweileri* and *S. mysinifolia* are hexaploid and closely related and *S. foetida* and *S. helvetica* are diploid and not very closely related, it was decided to continue only with *S. foetida*, *S. helvetica* and their hybrids and to develop more primer pairs for HRM.

Because sequence information in *Salix* is scarce *Populus* sequences were used. PCR primers were developed producing approximately 500 bp amplification products using four *Populus* sequences (*P. tremuloides* mitochondrial LPD1 mRNA, GenBank: EF150636; *P. trichocarpa* x *P. deltoides* MT3b mRNA, GenBank: AY594300; *P. tremula* x *P. alba* TEL2 gene, GenBank: EF462992; *P. tremuloides* mitochondrial $gdcT_2$ mRNA, GenBank: EF150634). These primers were used in PCR reactions with DNA from one *S. alba*, *S. fragilis* and *S. purpurea* genotype, and the amplification products were sequenced. Not all the sequences were readable, therefore only alignments from three *Populus* genes (LPD1, MT3b und $gdcT_2$) could have been developed. All three sequence alignments confirm the high degree of relationship between *Populus* and *Salix*. One of the alignments is shown as an example in Figure 1.

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#Populus_EF150634 TCT GGG TGC TGA AGT AAT ACT CAA ACA ACT TGC AGA AGG
#5_S. alba        --- --- --- -TG ... ... ... ... ... ... ... ... ...
#5_S. fragilis    --- --- --- -TG ... ... ... ... ... ... ... ... ...
#5_S.caprea       --- --- --- -TG ... ... ... ... ... ... ... ... ...

#Populus_EF150634 TCC AAA AA TCA GGC TTG TAG GAT TTA CCT CTA CTG GTC
#5_S. alba        C.. ... .. ... ... ... ... ... ... ... ... ... ...
#5_S. fragilis    ... ... .. ... ... ... ... ... ... ... ... .C. ...
#5_S.caprea       ... ... .. ... ... ... ... ... ... ... ... .C. ...

#Populus_EF150634 CAC CTC CCA GAT CCC ACA GTG AGA TTC AGG ATG AGA AAG
#5_S. alba        ... .G. ... ... ... ... ... ... ... ... .C. ... ...
#5_S. fragilis    ... .G. ... ... ... ... ... ... ... ... ... ... ...
#5_S.caprea       ... .G. ... ... ... ... ... ... ... ... ... ... ...

#Populus_EF150634 GGA CTA ACA TTG GAG AAA TTA CAA GTG GAG GAT TTA GCC
#5_S. alba        ... ... C.. .C. ... ... ... ... ... ... ... ... ...
#5_S. fragilis    ... ... C.. .C. ... ... ... ... ... ... ... ... ...
#5_S.caprea       ... ... C.. .C. ... ... ... ... ... ... ... ... ...

#Populus_EF150634 CAT GCC TCA AGA AAA ATA TAG CCA TGG GGT ACG TGA AAT
#5_S. alba        ... ... ... ... ... ... ... ... ... ... ... ... ...
#5_S. fragilis    ... ... ... ... ... ... ... ... ... ... ... ... ...
#5_S.caprea       ... ... ... ... ... ... ... ... ... ... ... ... ...

#Populus_EF150634 CTG GTT CAC ACA AGG CAG GCA CCA AAG CTA AAA TAC TGG
#5_S. alba        ... ... .C. ... ... ... ... ... ... ... ... ... ...
#5_S. fragilis    ... ... .C. ... ... ... ... ... ... .C. ... ... ...
#5_S.caprea       ... ... .T. ... ... ... ... ... ... ... ... ... ...

#Populus_EF150634 TAC GTG GGA AGG CCT ATG ATG GAG TTG TCA CAA AAA AGC
#5_S. alba        ... ... ... ... ... ... ... .G. ... ... ... ... ...
#5_S. fragilis    ... ... ... ... ... ... ... ... ... ... ... ... ...
#5_S.caprea       ... ... ... ... ... ... ... ... ... ... ... ... ...

#Populus_EF150634 CAT TTG TAC CAA CTA AAT ATT ACA AGC CAT CGT AAC TCA
#5_S. alba        ... ... ... ... ... ... ... ... .. ... .T. ... ..-
#5_S. fragilis    ... ... ... ... ... ... ... ... ..T ... .T. ... ...
#5_S.caprea       ... ... ... ... .C. ... ... ... ..T ... .T. ... ...
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Figure 1: Sequence alignment of *P. tremuloides*, *S. alba*, *S. fragilis* and *S. purpurea* for the gene $gdcT_2$ ("-" no sequence available, "." base identical to *Populus*)

Based on the sequence alignment for the MT3b gene a HRM primer pair (Table 2, Nr. 6) was developed which should differentiate between S*. alba* and *S. fragilis*. The results from HRM analysis showed for *S. alba* and *S. fragilis* only slightly differing HRM curves, which can not be explained by the sequence alignment. These results are an indication that the MT3b gene is a multicopy gene. In multicopy genes it is possible that different primer pairs prefer different gene copies, which can be due to mutations in the primer regions. This is resulting in different sequences depending on the used primer. Problems due to the multicopy character could be avoided using DNA sequences from the chloroplast genome or ribosomal DNA which are often used for systematic studies, but in our studies with different Salix species we only could find mean polymorphisms in these DNA regions (results not shown). Because of the similarity between *Populus* and *Salix* we decided to develop further HRM primers directly on the sequence of *Populus* nucleus genes. Ten HRM primer pairs were developed based on different *Populus* nucleus gene sequences. The primer pairs were checked with a few *Salix* genotypes and 5 primer pairs worked well (Table 2, Nr. 7 to 11).

Table 2: Primer pairs for HRM analysis

Nr.	**Gene or number of populus SSR**	**Forward und Reverse Primer**	**Primer Name**
1	tRNA-Ser gene and trnS-psbZ intergenic spacer	TGGGTGTGAGTCGATGAGTCTA ACCCCTTTCCCACTTTATTCG	Salix-trnS-psbz-190F Salix-trnS-psbz-342R
2	psbZ gene for photosystem 2 subunit Z	CTCTCATCTCTTGAACCCAGATACAA AAGTTTGACCCCTCCCTCTCAT	Salix_psbZ-8F Salix_psbZ-146R
3	tRNA-Lys gene intron and matK gene for maturase K, clone A	CCGTAAAAAACTCGTCCAATAATTC TTCGACTTTCTTGTGTTAAAACTTTG	Salix-tRNA-Lys-412F Salix-tRNA-Lys-513R
4	AY230817[1]: *Salix discolor* leafy/floricaula mRNA	AGAAGGCAGGAGCAAGCTACAT TCCTAAGTGCATTAGACGCATCC	Salix-floricaula-815F Salix-floricaula-916R
5	U29092[1]: *Salix viminalis* wound-induced trypsin inhibitor gene	AATCACATTCTCACCGGTTGTAGA AACGAATCCCCGCATTGTT	Salix-wound-482F Salix-wound-632R
6	AY594300[1]: *Populus trichocarpa x Populus deltoides* metallothionein 3b (MT3b) mRNA	GGTTCTGGCTGCACCTGC TCCCAATCCCATATCCCACA	SalixHRM 2-S67-11F SalixHRM 2-S67-91R
7	EF418792[1]: *Populus trichocarpa* actin mRNA	AGACCTTCAATGTGCCTGCAA AGGATGGCATGTGGAAGGG	EF418792-531F EF418792-684R
8	EF418792[1]: *Populus trichocarpa* actin mRNA	TGTGCTCAGTGGTGGTTCCA TGCAAGGATTGACCCTCCAA	EF418792-1048F EF418792-1198R
9	EF396922[1]: *Populus balsamifera* KNOX3 (KNOX3) gene	TCTCTTGCTGCTACACTGTATATGCATT GATCGTTGTCTTGATTCAAACTCTTG	EF396922-212F EF396922-313R
10	EF396897[1]: *Populus balsamifera* teosinte-branched-like protein (TB1) gene	CATTCTTTGCCTTCGTCCCTC CGGCAAGAGTGTTTGTTCCA	EF396897-630F EF396897-730R
11	EU760897[1]: *Populus tomentosa* cinnamyl alcohol dehydrogenase (CAD) mRNA	GGAAAAGGGATTGGCCTCC GCTTGCTACCAGCAACATCGA	EU760897-942F EU760897-1063R
12	*Populus* ORPM SSR 28	GGATCGACTTCCAACCCATA AATTCCCAGATGAAGGCTCA	ORPM_28F ORPM_28R
13	*Populus* ORPM SSR 446	GGGCTGCAGACAAATTAAGG TGGGACATGCTCCATGGTAT	ORPM_446F ORPM_446R
14	*Populus* ORPM SSR 488	CTCCAGCCGCTTCTATCCTT TGTCGTGGGAAAGAACCAGT	ORPM_488F ORPM_488R

[1]Genbank number

For *Populus* also SSR (microstellite) primers are published (http://www.ornl.gov/sci/ipgc/publications.htm), and 3 SSR primer pairs also worked for *Salix* in our investigations (Table 2, Nr. 12 to 14).
The specimans *of S. foetida*, *S. helvetica* and their hybrids (Table 1) were investigated by HRM analysis with all 14 primer pairs. Two genotypes from *S. alba*, *S. fragilis* and *S. purpurea*, respectively were also included in the HRM investigation.

Results

The HRM curves we obtained with the first three primer pairs (based on chloroplast DNA) showed polymorphisms within the investigated *Salix* species. In both species the same or very similar curves were found, so it was not possible to assign typical HRM curves to a specific *Salix* species.
All the other primer pairs, which were developed from DNA sequences from nucleus genes or are SSR markers, showed a much higher degree of polymorphism. Because of the enormous polymorphisms between and within one species it was also not possible to assign typical HRM curves to a specific species.
Figure 2 to 6 shows HRM curves obtained with primer pair 14 (*Populus* ORPM SSR 488).

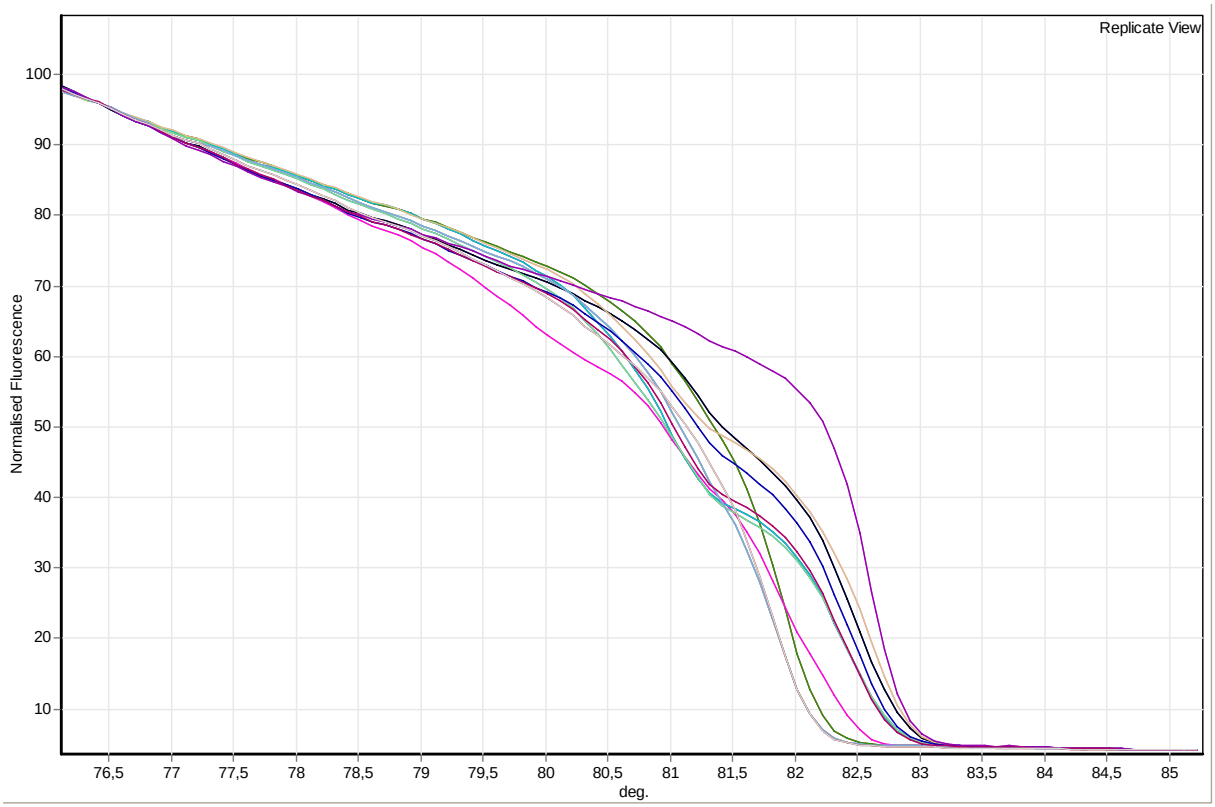

Figure 2: HRM curves of the different *S. foetida* specimens with primer pair 14

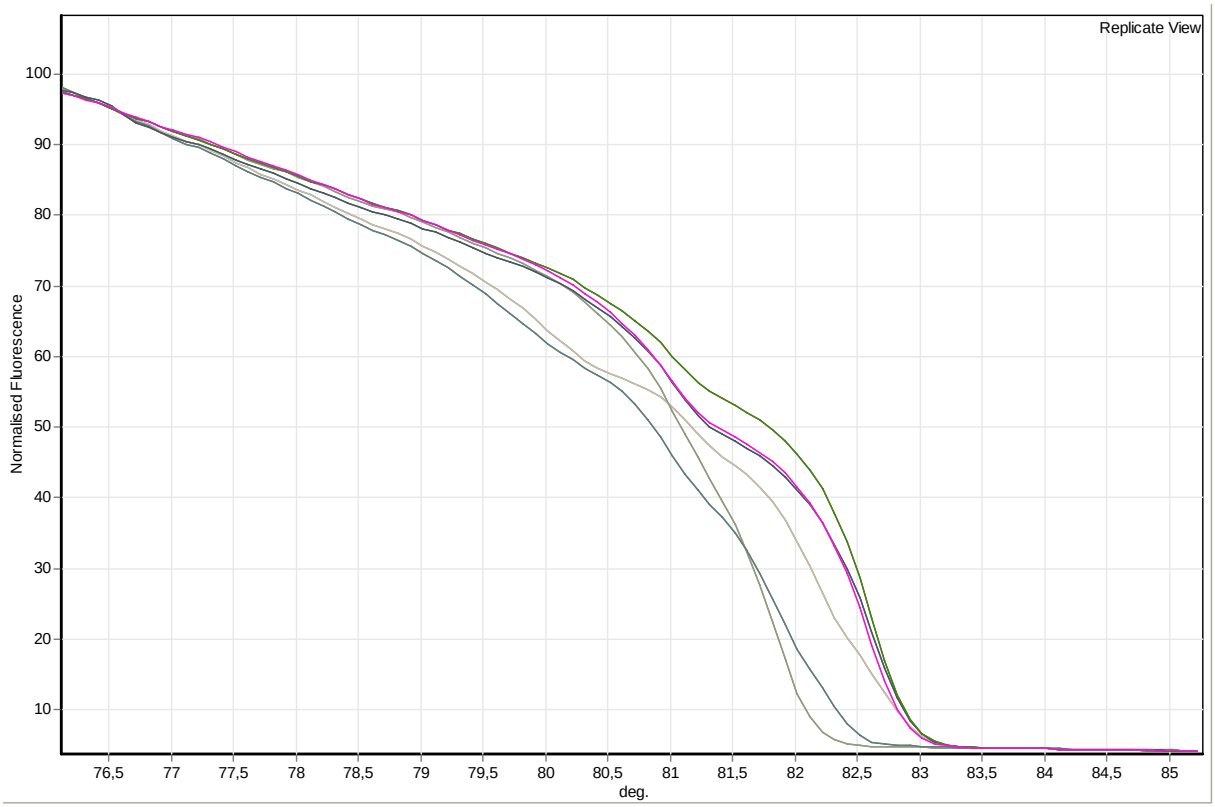

Figure 3: HRM curves of the different *S. foetida x S. helvetica* specimens with primer pair 14

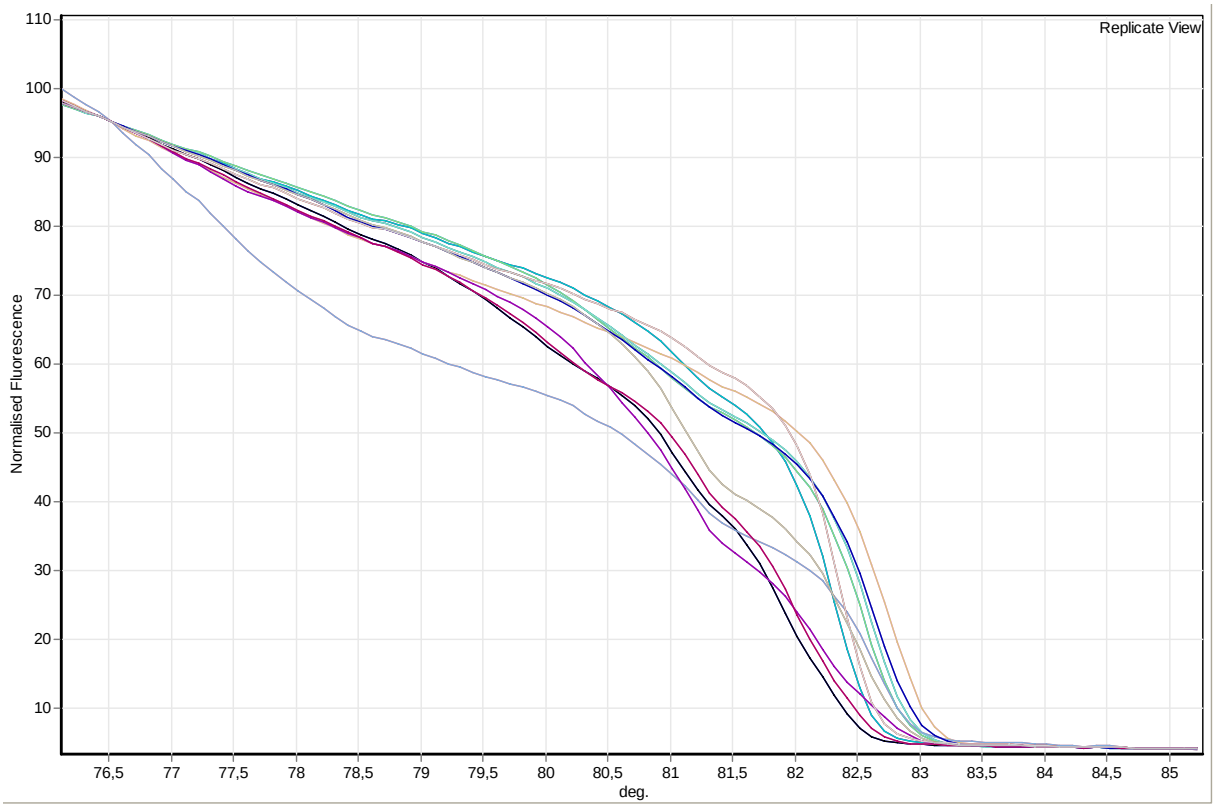

Figure 4: HRM curves of the different *S. helvetica* specimens with primer pair 14

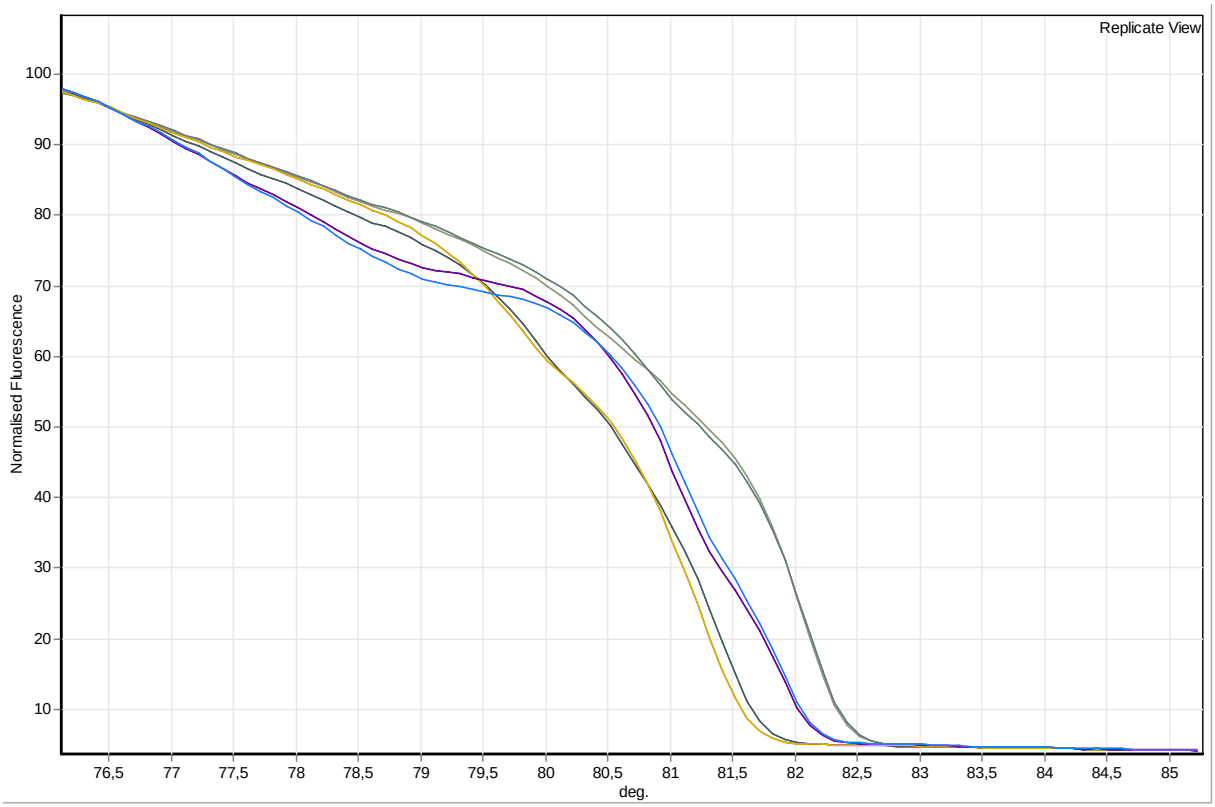

Figure 5: HRM curves of the different *S. alba* (yellow and black*), S. fragilis* (blue and red) and *S. purpurea (*gray*)* specimens with primer pair 14

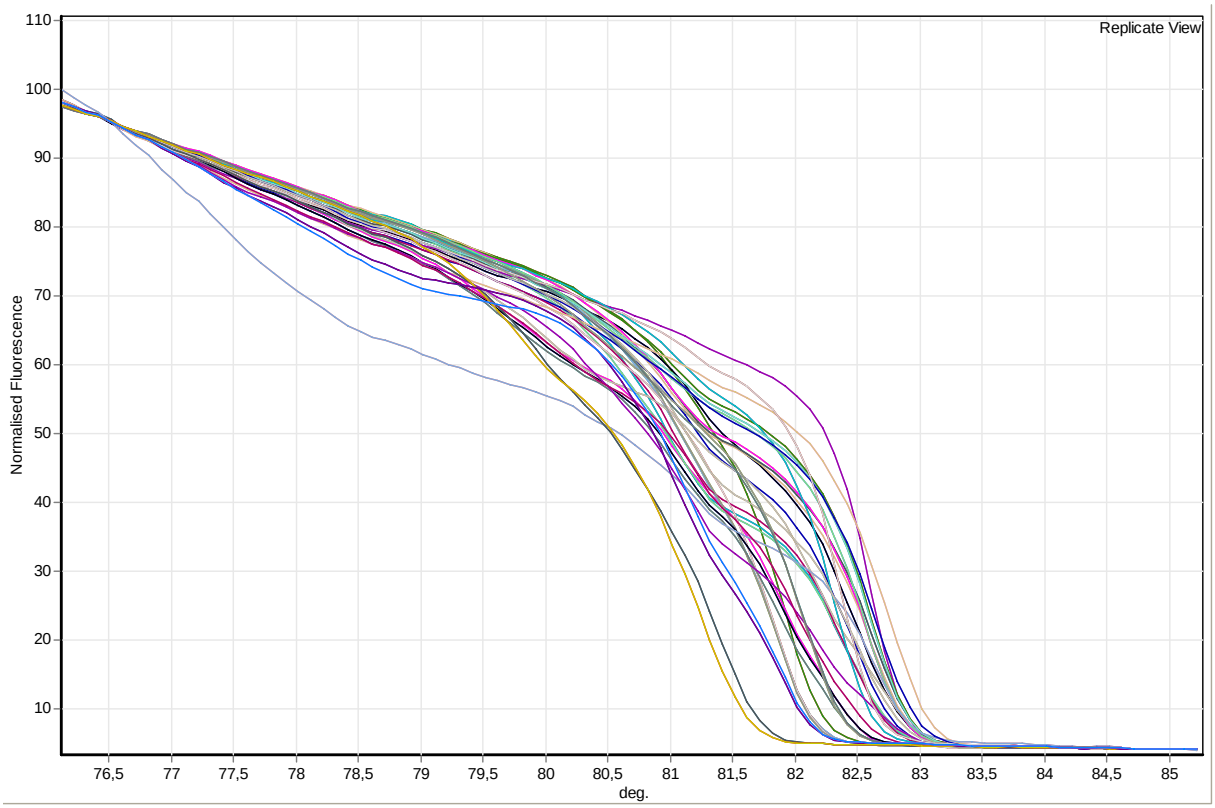

Figure 6: Overall view of all the HRM curves of the *S. foetida*, *S. helvetica*, *S. foetida x S.helvetica*, *S. alba*, *S. fragilis* and *S. purpurea* specimens with primer pair 14. The bottom curves in black and yellow belong to *S. alba*.

Summary

In order to find nucleotide polymorphisms to develop markers for hybrid detection and characterization in *Salix* species primers for HRM analysis were developed. Primer pairs designed on the basis of *Populus* nucleus gene sequences and *Populus* SSR (microsatellite) primers yielded HRM curves showing a great polymorphism between and within the investigated *Salix* species.
It could be demonstrated that it is possible to use the sequence information available from the *Populus* genome for *Salix*, and that HRM analysis is an appropriate tool to detect polymorphisms in *Salix*.
Based on these results strategies will to be developed to use the HRM approach in further studies of hybridisation and speciation in the complex genus *Salix*.

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