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Conservation and genetic diversity of Irish willows



Key external stakeholders:

Biomass growers, Willow breeding companies, Farm foresters, COFORD, DAFM

Practical implications for stakeholders:

- The genetic diversity and potential for breeding of native Irish willows was investigated.
- We found a wealth of genetic variation within Irish willow (*Salix*) populations which can be exploited and utilised by, selection, propagation, testing and breeding varieties with desirable traits such as disease and / or pest resistance.
- The gene markers discovered will facilitate breeding and selection.
- The genetic variation in commercial willow cultivars is restricted and may be improved by introducing relevant genes from the wider gene pool of native Irish willows, *S. viminalis* and *S. caprea*.

Main results:

- All material of *Salix viminalis* and > 80% of *S. caprea* was readily propagated vegetatively. The ploidy level in 68 wild accessions of *S. viminalis* were diploid (2x), and 14 traditional selections were (2x), one triploid (3x) and 11 tetraploid (4x).
- Less genetic variation was detected in *S. viminalis* than in *S. caprea*.
- High levels of genetic variation were observed in populations analysed.
- A few identical clones were discovered among both species.
- Specific markers and haplotypes separated *S. caprea* and *S. viminalis*, apart.
- 50% of genetic markers could be transferred from *Populus* to *Salix*.
- Linkage disequilibrium (LD) index showed a lack of genetic bottlenecks.
- Gene flow is strong within Ireland for *S. caprea* and *S. viminalis*.

Opportunity / Benefit:

There is wealth of genetic variation within *Salix* that is as yet largely untapped for breeding and markers analysed justify a candidate gene approach in association mapping.

The SNP markers developed for genes associated with lignocellulose metabolism and disease resistance can be part of a toolkit for future pre-breeding and commercial breeding of willow varieties.

Collaborating Institutions: Trinity College Dublin, DAFM, National Botanic Gardens, Irish willow growers

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1. Project background:

Salix caprea and *S. viminalis* are important species in Ireland, especially the latter, since it forms the basis for the commercial clones, currently used for the production of renewable biomass. Woody biomass is expected to be a major contributor to achieving Ireland's renewable energy targets. The Renewable Energy Directive (2009/28/EC) has set a target for 16% of final energy from renewables for 2020. Willow material was collected in the wild (*S. viminalis* and *S. caprea*) and also from private collections, including traditional varieties used in basket making. This material was propagated at Teagasc Kinsealy to establish a conservation collection and also as source material for molecular characterisation and for making herbarium specimens.

Willow is well adapted to Irish conditions since it is native here and has the advantages of ease of propagation, fast growth in short rotation coppice (SRC) cycles and minimal fertilizer inputs. Although commercial varieties are high yielding their full potential is limited by leaf diseases, mainly rust, *Melampsora* spp. and also by willow beetle in some years. The local / regional gene pools of Irish germplasm may have evolved natural resistance to common willow diseases and have adapted to local climatic conditions, and so may have potential for breeding, if the genetic diversity of these gene pools are high.

Current willow breeding programmes aim to produce high-yielding clones with, high calorific content, disease and pest resistance and with suitability for mechanical planting / harvesting. The main species used is *Salix viminalis* and its hybrid clones. This species is favoured for SRC because it grows fast, coppices well and maintains a good growth form. Other species are also used such as *S. dasyclados*, *S. schwerinii* or *S. caprea*. The two main characters of interest for breeding are the lignin and cellulose content (for calorific content) and the resistance to the foliar rust disease. However, very little is known about the genetic diversity of willow in Ireland and nothing is known about gene diversity within important coding regions of the genome.

The overall objective of the genetic studies was to investigate the diversity of natural Irish populations and clones of *S. viminalis* and *S. caprea* through the study of ploidy, propagation capacity and molecular genetic markers. Natural levels of variation in genes related to disease resistance and wood formation were also assessed.

2. Questions addressed by the project:

- What is the ploidy level and capacity for vegetative propagation of native collections of *S. viminalis* and *S. caprea*?
- What is the level of diversity in ligno-cellulosic genes and in genes related to rust resistance in natural Irish willow populations?
- Is there a geographic structure in the natural populations across Ireland?
- Are there any differences between *S. caprea* and *S. viminalis* in terms of diversity and population genetic structure?
- What is the extent of gene flow and sexual reproduction in the two species

3. The experimental studies:

We obtained 126 individual accessions of willows from wild and cultivated private sources in Ireland. The material was mainly *S. viminalis*, some *S. caprea* and some were of hybrid origin.

A total of 274 *S. caprea* individuals from 21 semi-natural woodlands and 116 *S. viminalis* individuals were sampled from Ireland as well as several other *Salix* spp. Neutral markers (nuclear and chloroplast microsatellites: SSRs) and also sequences from several gene fragments involved in wood formation or disease resistance were used. As *Salix* has limited genomic resources available online, the resources already available in *Populus*, the sister genus to *Salix*, were transferred to *Salix* to assess levels of variation within the genes of interest.

Eight gene fragments involved in wood formation and nine gene fragments putatively involved in rust resistance were analysed on 183 Irish *S. caprea* individuals and 101 Irish *S. viminalis* individuals. Candidate genes were retrieved from publicly available databases, and Sanger sequencing was then performed. Analyses of the obtained sequences were performed using several population genetic approaches (linkage disequilibrium, fixation index, analysis of molecular variance: AMOVA, multivariate analyses, structure analyses). Eight chloroplast and 6 nuclear microsatellite markers were also genotyped on 183 Irish *S. caprea* individuals and 116 Irish *S. viminalis* individuals.

4. Main results:

All accessions of *S. viminalis* from 24 counties were readily vegetatively propagated. For *S. caprea*, 10 genotypes rooted out of 12 tested (rooting range of 10- 58%). Propagated material was used to establish a field conservation collection and sources of material for growth tests, genetic analysis and for herbarium specimens. The ploidy level in 68 wild accessions of *S. viminalis* was determined as diploid (2x), whereas among 24 basket making clones, 14 were diploid (2x), one was triploid (3x) and 11 were tetraploid (4x). For the wood formation genes, we found a close sequence similarity to Poplar and a high level of gene conservation among species. All sequences varied considerably among *Salix spp.* mainly as single nucleotide polymorphisms (SNPs). An average of 24 mutations was found for *S. caprea* and 18 mutations for *S. viminalis* per 1000 base pairs in the predicted coding regions. Average nucleotide diversity for *S. caprea* was nearly two-fold greater than that observed for *S. viminalis*.

Analyses of Linkage disequilibrium indicated that for each species there was no evidence of genetic population bottlenecks. Linkage disequilibrium was found to decline rapidly with distance in all 8 gene segments for both species. There was no strong subdivision of populations or genetic structuring from the different analyses, particularly for *S. caprea*. An upstream selective pressure was observed for the genes involved in the lignin biosynthesis pathway.

In the genes for putative rust resistance, only 3/9 regions sequenced, gave suitable reads. By comparing the rust resistance gene sequences with lignocellulose genes, more variation was found in the former and average nucleotide diversity was also slightly higher, for both species. Between 25 and 176 SNPs / kbp / gene region were discovered in lignocellulose genes and further analyses to infer their specific functions in *Salix* are desirable.

Nuclear (n) and chloroplast (cp) markers (SSRs) were used to analyse Irish sources of *S. caprea* and *S. viminalis*. All *S. caprea* individuals studied displayed a high level of SSR allelic variation and a high number of haplotypes (79 cpSSR vs. 286 nuclear SSR haplotypes) was found within and among populations. However, a few individuals were found to have the same haplotypes, suggesting clonal samples or in the case of *S. caprea*, introgressed hybrids, since this species is not readily propagated vegetatively. AMOVA results for the chloroplast SSRs study showed that most of the variation was within populations. Population structure and differentiation analyses, as well as isolation by distance tests and UPGMA trees were in accordance and showed low levels of geographical structuring of variation.

In *Salix viminalis* we discovered 31 cp DNA alleles in 8 microsatellite regions. Combining all these cp markers, a total of 79 different haplotypes was discovered and among them 48% were unique to a single individual. Six nuclear markers with 3-16 alleles per locus could differentiate among 165 unique multilocus genotypes.

S. viminalis was found to be less variable than *S. caprea* in the SSR analyses and 37 individuals were found to share the same haplotype with at least one other individual, possibly showing that they are clonal. Relatively high values of observed heterozygosity and gene diversity were detected for *S. viminalis* individuals. Gene flow via pollen dissemination was found to be seven times higher than via seed dissemination and a low level of geographical structuring of variation were shown using different methods. The data are consistent with the outbreeding nature of *Salix* and that gene flow (both pollen and seed mediated) is expected to be strong within Ireland for *S. caprea* and *S. viminalis*.

3. Opportunity/Benefit:

This is the first study that shows there is wealth of genetic variation within native Irish populations of willow species, for both *S. caprea* and *S. viminalis*. These native genetic resources have potential to be exploited and utilised for developing clonal material and pre-breeding stocks with important characteristics such as tolerance to rust diseases and other pests which are common in Ireland. The gene markers that were identified can be used in association mapping to facilitate breeding methodologies which use a candidate gene approach. The number of markers that can be transferred successfully from *Populus* to *Salix* justifies this approach for marker application and development in *Salix*.

4. Dissemination:

Main publications:

Perdereau, A., Douglas, G., Hodkinson, T. & Kelleher, C. 2013. High levels of variation in *Salix* lignocellulose genes revealed using poplar genomic resources. *Biotechnology for Biofuels*, 6, 114

-128. <http://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/1754-6834-6-114>

Perdereau, A. C., Kelleher, C. T., Douglas, G. C. & Hodkinson, T. R. 2014. High levels of gene flow and genetic diversity in Irish populations of *Salix caprea* L. inferred from chloroplast and nuclear SSR markers. *BMC Plant Biology*,

14: doi:10.1186/s12870-014-0202-xMS ID: 12870_2014_202

Perdereau, A. 2014. Investigating genetic variation in species and populations of *Salix* L. with particular emphasis on wood formation and disease resistance genes. Ph.D., University of Dublin, Trinity College.
Douglas G. C. 2012 Final report on Conservation and genetic characterisation of Irish willows for Department of Agriculture Food and Marine for the project 'Conservation of Genetic Resources'

5. Compiled by: Aude Perdereau and Gerry C. Douglas
