Population genetics and phylogeny of the plant pathogenic protozoan Spongospora subterranea f. sp. subterranea

Dr Calum Wilson
University of Tasmania

Project Number: PT08032

PT08032

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the potato industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the potato industry.

ISBN 0 7341 2869 X

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

HAL

Horticulture Australia

FINAL REPORT

PT08032: POPULATION GENETICS AND PHYLOGENY OF THE PLANT PATHOGENIC PROTOZOAN SPONGOSPORA SUBTERRANEA F. SP. SUBTERRANEA

REPORT PREPARED BY: **REBECCA DOROTHEA GAU, DOCTORAL STUDENT**

RESEARCH PROVIDER: **INSTITUTE OF INTEGRATIVE BIOLOGY, SWISS FEDERAL INSTITUTE OF TECHNOLOGY, ZURICH, SWITZERLAND; INSTITUTE FOR PLANT AND FOOD RESEARCH LIMITED, LINCOLN, CANTERBURY, NEW ZEALAND**

PROJECT COMPLETION DATE: **19 DECEMBER 2011**

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

HAL
Know-how for Horticulture™

ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

ministry of science + innovation
TE PŪNAHA HIRINGA WHAKAEA

Horticulture™ New Zealand

BRITISH POTATO COUNCIL

PRINCIPAL INVESTIGATOR:

Rebecca Dorothea Gau, Doctoral student
Plant Pathology, Integrative Biology Zurich (IBZ), ETH Zurich, LFW – A27
Universitaetstrasse 2
8092 Zurich – Switzerland
Phone: +41 (0) 44 632 38 64
Fax: +41 (0) 44 632 15 72
Email: Rebecca.Gau@usys.ethz.ch
Home Page: http://www.path.ethz.ch/

PURPOSE:

This project's purpose is the first thorough population genetic analysis of an economically important, plant pathogenic plasmodiophorid. *Spongospora subterranea* f.sp. *subterranea* (*Sss*) is an obligate biotrophic parasite, which causes powdery scab of potato. Up to date, there is no completely effective control method for the disease and breeding of resistant host plants plays a major role in managing powdery scab. For resistance screening during the breeding process, knowledge of the present pathogen strains and their genetic variability is necessary. Before the beginning of this project, very little was known about the genetic variability of *Sss*. Goals of the project were:

1) Determine genetic variability and structure of (*Sss*) and expansion of basic biological and plant pathological knowledge to provide a basis for effective and sustainable management of powdery scab of potato.
2) Obtain insights into the genetic variability, development and optimization of genetic markers, and application of these markers to *Sss* populations obtained from close and widely separate geographical locations from all continents.
3) Establish different scales of genetic relationship between different populations.
4) Fundamental knowledge of the population biology of *Sss* as a basis for understanding its evolutionary potential, which is crucial for risk assessment and managing strategies.

REPORT DATE:

19 December 2011

FUNDING:

This project has been funded by HAL using the potato industry levy and matched funds from the Australian Government. In-kind support was provided by: Swiss Federal Office for Agriculture (FOAG), British Potato Council, Swiss Federal Institute of Technology, Zürich (ETH-Z), New Zealand Ministry of Science and Innovation, and Horticulture New Zealand.

DISCLAIMER:

CONTENTS

Contents 1
Media summary 2
Technical summary 3
Introduction 4
Materials and Methods 6
Samples and DNA extraction 6
Microsatellite library construction 8
Microsatellite amplification and Genotyping 8
Microsatellite data collection 9
Cloning and resequencing of the msat246 locus 10
Sequence analyses of the actin gene and the ITS region 10
Results 11
Objective 1 11
Marker creation optimization and application 11
Sequencing of actin and ITS 11
Objective 2 12
Evolutionary units 12
Global phylogenetic network 12
Geographic races 13
Historic and recent gene flow 13
Objective 3 13
Genetic variation/Clonal diversity 13
Objective 4 14
Mode of Reproduction 14
Discussion 15
Technology transfer 16
Recommendations 17
Acknowledgements 18
Bibliography 19

MEDIA SUMMARY

Powdery scab of potato is an economically important disease, which causes significant losses in all sectors of potato production and processing. It occurs in temperate potato producing regions, but also in hot and dry climates where irrigation is applied.

Infected tubers are not marketable and soils contaminated with the disease stay infectious for many years due to a great number of resting spores produced by the pathogen *Spongospora subterranea* f.sp. *subterranea*, causing powdery scab.

There is currently no completely effective chemical control method for the pathogen and the most promising approach is the breeding of powdery scab resistant host plants.

Breeders usually screen new lines of a crop against the known strains of a pathogen to select for the most durable resistance. In case of *Spongospora*, breeders have been screening new potato lines for resistance without knowledge of the genetic diversity or the number of present strains, because only little is known. However, this knowledge and the role of sexual reproduction in the life cycle of the pathogen play a crucial role in the durability of resistances.

This project aimed at the determination of genetic variability and population structure of the pathogen to expand basic biological and plant pathological knowledge, which provides the basis for developing effective and sustainable powdery scab management, using disease resistance and/or environment manipulation strategies.

The results indicate that there are significant differences between the levels of genetic diversity of the pathogen on the global scale. Some regions show clonality and this means good news for breeders. Resistant varieties being commercialised in one country with low genetic diversity can be sold to and grown in other countries with the same situation as well.

Thus, quarantine measures might have to be taken in those countries to keep the genetic diversity low and to avoid exchange of genetic material. Allowing for exchange might result in an even more aggressive form of the pathogen, which is already difficult to control.

TECHNICAL SUMMARY

The genetic diversity and population structure of *Spongospora subterranea* f.sp. *subterranea*, the cause of powdery scab of potato was described on a global scale.

Highly variable, polymorphic and neutral genetic markers (microsatellites) have been developed specifically for the pathogen to assess the genetic and clonal diversity and to estimate the amount and direction of gene flow.

The internal transcribes spacer (ITS) and the actin gene of the pathogen were sequenced to support the findings from the marker analysis. They have been used for phylogenetic studies and haplotype analyses.

The global genetic and clonal diversity shows significant differences between countries spanning from regions where clonality was found to those with high diversity.

Quarantine measures might have to be taken to prevent spreading of more diverse populations and increasing of global genetic diversity which could lead to more virulent pathogen strains.

INTRODUCTION

Powdery scab of potato is an economically important disease caused by the soil-borne obligate biotrophic parasite *Spongospora subterranea* f. sp. *subterranea* (*Cercozoa*, *Plasmodiophoridae, Sss*). The disease occurs in many of the temperate potato producing and processing regions worldwide, but also in hot and dry climates where irrigation is applied. Long-distance dispersal does not occur in the life-cycle of *Sss* because resting spores are formed in the soil on tubers and roots (Figure 1a-c) and the biflagellate zoospores (Figure 1d) can swim only short distances in moist soil to reach a host and cause infection (Merz & Falloon 2009).Thus, the most likely way of short and long distance dispersal of the pathogen is trade of *Spongospora*- infected seed potatoes (Merz & Falloon 2009).

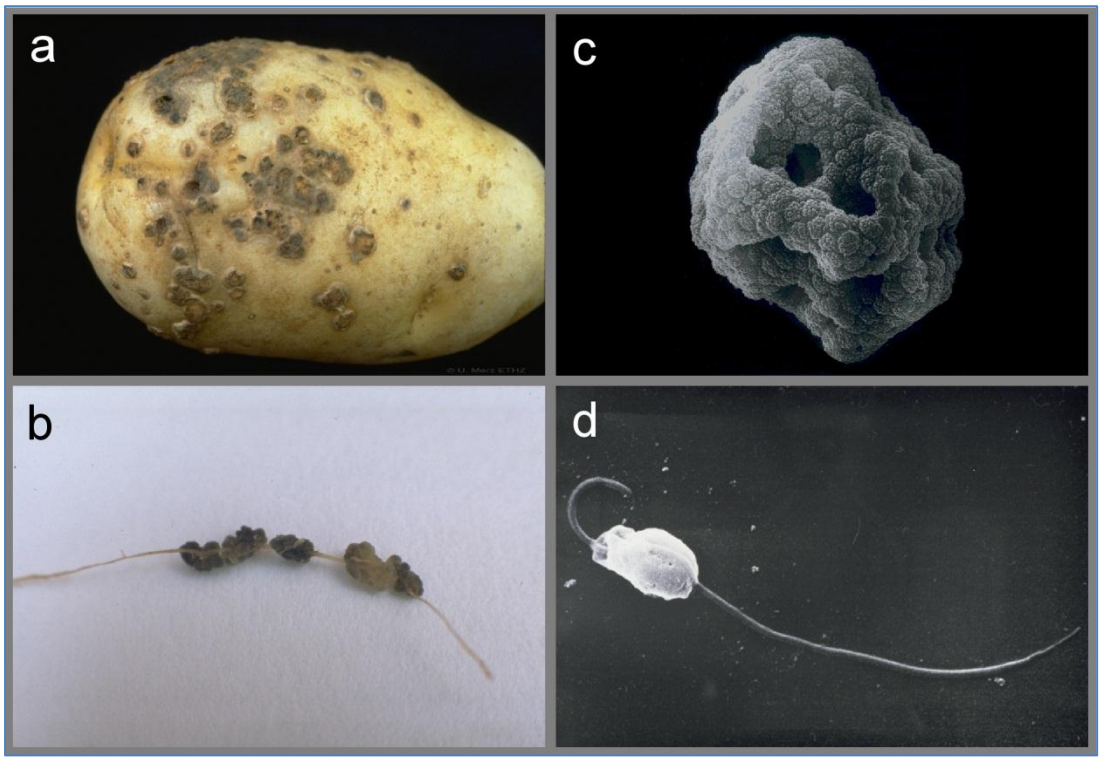

Figure 1: (a) Potato tuber with powdery scab lesions, (b) potato root with galls, (c) *sporosorus* consisting of resting spores, (d) biflagellated zoospore. Pictures taken by Ueli Merz.

Management of powdery scab is difficult. Contaminated soils stay infectious for many years due to the formation of a great number of resting spores. These are thick walled and therefore highly resistant to environmental stresses. Routinely treatment of seed potato tubers with pesticides containing mercury was effectively performed until the 1980s to prevent infection of potato crops (Merz 2008). However, for human health and environmental reasons, mercury has been banned and these treatments are not carried out any more.

Most promising seems the approach of breeding resistant potato cultivars, to control the disease (Merz & Falloon 2009). Until now, plant breeders screen new cultivars and lines for susceptibility to powdery scab without knowledge of genetic variability in *Sss*, but screening is most effective, if the total amount of genetic variation and all strains of a pathogen are known.

The population genetic structure of *Sss* was addressed by a very limited number of studies. These focused mainly on the analysis of ITS-variation and applications for detection and diagnostics (Bulman & Marshall 1998; Qu & Christ 2004). As a marker, ITS is rather conserved. Additionally, all previous

studies incorporated only a low number of samples and provided little insight into the present genetic population structure of *Sss*.

Highly polymorphic markers, which are distributed evenly and unlinked throughout the genome, can provide more information on the population genetic setup of a species. Their design and application was the substance of this project.

The four objectives of the 3 year project:

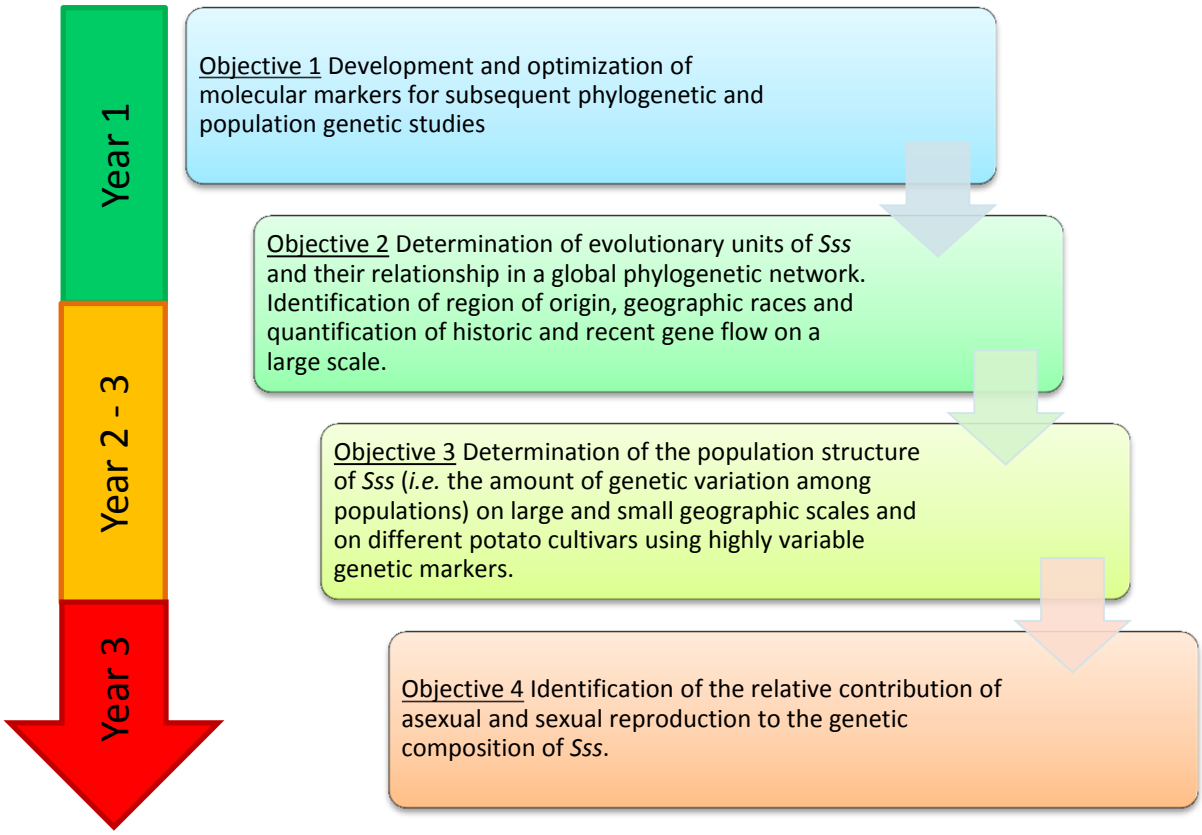

Figure 2: Objective of the project and workflow within the three year project.

MATERIALS AND METHODS

All primer sequences and PCR protocols used for this study are about to be published.

SAMPLES AND DNA EXTRACTION

An extensive global collection of populations of *Sss* has been obtained following a hierarchic sampling scheme (McDonald 1997). Locations, sample sizes and collectors are listed in Table 1.

Table 1: Sample collection for this project. "*" indicate, that the sample was sequenced only and no microsatellite data is available.

Origin	Number of sites and year	Number of samples in the collection	Potato subspecies, cultivar	Tissue
Switzerland				
Wallestalden, Langnau	1 site, 2005, 2007 and 2009	35	Tuberosum, various cultivars	Lesion scrapings
Solothurn, Biezwil	1 site, 2004	34	Tuberosum, Agria	Lesion scrapings
Luzern, Kägiswil	1 site, 2004	21	Tuberosum, Agria	Lesion scrapings
Zürich	1 site, 2005	1	Tuberosum, unknown	Galls
Various	8 sites, 1998, 2000 and 2003-2005	27	Tuberosum, various cultivars	Lesion scrapings
Germany				
Nordrhein-Westfalen, Meinersen	1 site, 2009	22	Tuberosum, various cultivars	Lesion scrapings
Netherlands				
Netherlands, Drenthe, North-East Netherlands	2 sites, 2000 and 2009	4	Tuberosum, unknown	Lesion scrapings
Norway				
Norway, Farmen, Kvelde	1 site, 2008	25	Tuberosum, Redstar	Lesion scrapings
Norway, Rustad, Romeldal	1 site, 2008	25	Tuberosum, Beate	Lesion scrapings
Iceland				
Iceland, South Iceland, Thykkvibær	1 site, 2009	22	Tuberosum, Red Icelandic	Lesion scrapings
Scotland				
Scotland, Dundee	3 sites, 1999 and 2004	3	Tuberosum, Estima and Nadine	Lesion scrapings

Sweden				
Sweden, Uppsala	2 sites, 1994 and 2004	2	Tuberosum, Kultivator	Lesion scrapings
Malta				
Malta	3 sites, 2006	8	Tuberosum, various cultivars	Lesion scrapings
South Africa				
South Africa, Kwazulu-Natal & Sandvelt	1 site, 2009	25	Tuberosum, Mondial	Lesion scrapings
South Africa, Sandvelt, Western Cape	1 site, 2009	32	Tuberosum, Mondial	Lesion scrapings
Pakistan				
Pakistan, Sharan, Kaghan Valley	2 sites, 2009 and 1994	15 + 1	Tuberosum, Barma, Diamond, Cardinal	Lesion scrapings
Sri Lanka				
Sri Lanka	1 site, 2010	4	Tuberosum, various cultivars	Galls
South Korea				
South Korea, Hoenggye	3 sites, 2009 and 2005	50 + 1	Tuberosum, Superior	Lesion scrapings
Japan				
Japan, Hokkaido, Kyogoku-Town	2009 and 1999	25 + 3	Tuberosum, Irish cobbler	Lesion scrapings
Australia				
Australia, Ballarat	3 sites, 2006	57	Tuberosum, unknown	Lesion scrapings
New Zealand				
New Zealand, Canterbury, Lincoln	1 site, 2008 and 1997	113	Tuberosum, Agria and Desiree	Lesion scrapings
United States of America				
USA, Pennsylvania, Potter County	2 sites, 2009 and 2004	25 + 1	Tuberosum, various cultivars	Lesion scrapings
Colombia				
Colombia, Villapinzón	1 site, 2008	18	andigena/phureja, unknown	unknown
Colombia, Nariño	1 site, 2008	15	andigena/phureja, unknown	unknown
Colombia	1 site, 2010	25	andigena/phureja, unknown	Galls

Venezuela				
Venezuela, Mérida State, El Llano	1 site, 2010	24	Andigena, variety testing	Lesion scrapings
Venezuela, Mérida State, Los Muros de Tadeo	1 site, 2010	25	Andigena, Unica	Lesion scrapings
Venezuela, Mérida State, La Toma	1 site, 2010	22	Andigena, Unica	Lesion scrapings
Venezuela	7 sites, 2011	80*	Tuberosum, Granola	Lesion scrapings and galls
Peru				
Peru	2 sites, 1996 and 1999	2*	Andigena/Phureja, Mariva	Lesion scrapings
Ecuador				
Ecuador	1 site, 2000	1*	unknown	unknown

Genomic DNA was extracted from dried lesion scrapings of potato tubers or potato root galls. The extraction followed either the cetrimonium bromide (CTAB) method (Winnepenninckx *et al.* 1993) or was carried out using the QIAgen DNeasy Plant mini kit. As an obligate biotrophic pathogen, *Sss* cannot be grown in pure cultures on artificial media. Thus, the presence of *Sss* DNA in the samples was confirmed using a *Sss*-specific PCR which amplifies a fragment of the ITS region (Bulman & Marshall 1998) prior to further studies. Samples, which did not produce amplicons during PCR were excluded from further experiments.

MICROSATELLITE LIBRARY CONSTRUCTION

An enriched microsatellite library was established using a purified Swiss *Sss* sample (Glenn & Schable 2005). From this library, microsatellite primers specific for *Sss* were developed. In a nutshell: *Sss* DNA was digested with *Rsa*I and *Xmn*I to obtain blunt ended fragments of approximately 500 bp of length. An enriched microsatellite library of these fragments was produced, employing magnetic beads (MyOne T1 Streptavidin Dynabeads, Invitrogen) and biotinylated oligonucleotides. The enriched fragments were ligated and cloned using the TA Cloning® Kit (Invitrogen). Transformants underwent blue-white selection and were sequenced subsequently using an ABI 3730 xl sequencer (Applied Biosystems). Sequences were screened for the presence of microsatellites and a BLAST (Altschul *et al.* 1990) analysis was performed with all obtained sequences to identify non-specific *Sss* fragments (e.g. potato or soil organisms). Using PRIMER3 (Rozen & Skaletsky 2000), primer pairs were designed for all remaining sequences.

MICROSATELLITE AMPLIFICATION AND GENOTYPING

A global collection of 705 Sss DNAs (Table 1) was genotyped using six polymorphic microsatellite loci. Prior to genotyping, separate PCRs were carried out for each of the loci using fluorescent-labeled primers. Separation of amplicons including the internal GeneScan LIZ600 size standard (Applied Biosystems) was performed either on an ABI 3730 xl or on an ABI 3130 sequencer (Applied Biosystems). The dye set was DS-33 and the filter set was G5 as recommended by Applied Biosystems. Processing of data and size calling of alleles was performed with the GENEMAPPER software (Applied Biosystems).

MICROSATELLITE DATA COLLECTION

Using GENOTYPE (Meirmans & Van Tienderen 2004), the allele frequencies were calculated. Clone correction and multilocus genotype collapsing of the microsatellite data set was performed with GENODIVE (Meirmans & Van Tienderen 2004). Several measures of clonal diversity were estimated based on the infinite allele model (IAM):

- Number of genotypes per population (num)
- Site specific genotypes (ssg)
- Nei's corrected diversity (div) index with 1000 permutations with correction for sample sizes
- And the clonal fraction (cf), which describes the proportion of individuals in the sample set originating from asexual reproduction.

Between regions, differences of clonal diversity were calculated based on a bootstrap approach with subsampling to account for different population sizes. Furthermore, population genetic parameters were computed, such as allelic diversity, heterozygosity and inbreeding coefficients to estimate deviation from Hardy-Weinberg equilibrium using G_{IS}. Recent migration rates between regions were estimated with BAYESASS 1.3. (Wilson & Rannala 2003) on default settings and 20.000.000 iterations of which the first 1.000.000 were burn in. The calculation was repeated five times to ensure consistency of results.

In many cases, two microsatellite alleles were observed for the majority of the samples, which lead to the assumption that *Sss* is a dikaryotic organism and all analyses were performed accordingly. The Bayesian-based software STRUCTURE (Pritchard *et al.* 2000) was used to detect population subdivisions in the data set. However, first explorative analyses indicated low or absent subdivision between geographically close sampling locations. Thus, individual sample sites were pooled into seven geographic regions for all subsequent genetic analyses (Table 2).

Table 2: Separation of the total microsatellite data set into geographical regions

Region	Countries	No. of isolates samples (N)
Europe	Switzerland, Germany, Netherlands, Norway, Sweden, Scotland,	219
Malta	Malta	8
Africa	South Africa	57
Asia	South Korea, Japan, Pakistan, Sri Lanka	98
Australasia	Australia, New Zealand	170
North America	United States of America	26
South America	Colombia, Venezuela	129

Using the "admixture model" implemented in STRUCTURE, final analyses were performed and iterated five times with a burn in length of 100.000 and 500.000 repeats for a range of $K = 1$ to $K = 10$ assumed populations. Evaluation of the minimum number of K was performed using the ΔK procedure (Evanno *et al.* 2005) and results were visualized with DISTRUCT (Rosenberg 2004).

CLONING AND RESEQUENCING OF THE MSAT246 LOCUS

During PCR, the marker msat246 produced two fragments, which became obvious during genotyping. To validate this finding and to ensure the correctness of the genotyping results, the loci were amplified in a PCR using template DNA from different samples which were previously genotyped and yielded different results, e.g. double homozygous, double heterozygous and single homozygous for each of the loci. Resulting fragments were cloned using the TA cloning kit (Invitrogen) and subsequently sequenced in an ABI 3730 xl sequencer (Applied Biosystems).

SEQUENCE ANALYSES OF THE ACTIN GENE AND THE ITS REGION

A sample subset of 308 *Sss* DNAs, representing all regions with at least four samples each, was chosen for sequencing and analysis of the ITS region (393 bp) and the partial actin gene (615 bp, no introns). The standard PCR protocol decribed by Bulman & Marshall (1998) was used to amplify ITS. To amplify the partial actin gene, a nested PCR using an “outer” and an “inner” pair of primers had to be applied. On an ABI 3730 xl sequencer (Applied Biosystems) the products were sequenced and resulting data was edited using SEQUENCHER (Gene Codes Corporation). Against Genebank, BLAST analyses (Altschul *et al.* 1990) were done to verify that the sequences stem from *Sss*. Sequence alignments were created using ClustalW (Higgins *et al.* 1996).

Summary statistics were calculated in DNASP (Librado & Rozas 2009). To visualize haplotype relationships and haplotype frequencies across regions, parsimony-based TCS haplotype networks were constructed using TCS (Clement *et al.* 2000) and confirmed with the haploNet function of R (http://www.r-project.org/).

A maximum likelihood (ML) tree on the concatenated data set was constructed using MEGA5 (Tamura *et al.* 2011) and the implemented Kimura-2-parameter model with 1000 bootstrap replications. Genbank sequences of *Plasmodiophora brassicae*, (AB526843.1 and AM411664.1) *Spongospora subterranea* f. sp. *nasturtii* (AF310907.1) and *Polymyxa graminis* (FN393971.1) were added as outgroups.

The data was screened for the presence of recombination using the single break point analysis of Datamonkey, the webserver of the HyPhy package (Delport *et al.* 2010; Pond & Frost 2005; Pond *et al.* 2005).

RESULTS

OBJECTIVE 1

MARKER CREATION OPTIMIZATION AND APPLICATION

The absence of a sequenced genome of *Sss* in Genbank is limiting and restricts the possibilities of molecular approaches. However, microsatellite markers can be designed without knowledge of the genome, but development of specific primers for obligate biotrophic parasites like *Sss* is challenging, because there is no way of isolating pure DNA of the pathogen only. The tissue of host and pathogen are tightly connected, thus each DNA sample contains a mixture of host and pathogen DNA and often also of other soil organisms.

After establishment of an enriched microsatellite library, 87 microsatellite primer pairs were designed and ordered. All markers which did not amplify any fragments, amplified fragments from potato DNA, or were not polymorphic were excluded. Out of 19 promising primer pairs, five pairs remained after these optimization steps. These five microsatellite markers were applied to 705 individual DNA samples, which originated from worldwide field populations. The markers amplified six loci prior to fragment analysis. They were highly polymorphic and yielded reliable results during genotyping and allele size calling. Nevertheless, the number of polymorphic markers obtained in the project is not sufficient for a thorough microsatellite based population genetic analysis.

SEQUENCING OF ACTIN AND ITS

There are only few sequences of plasmodiophorids present in GenBank. In the case of *Sss*, three sequences are available: Polyubiquitin, actin and a partial internal transcribed spacer (ITS) sequence. Polyubiquitin and actin are housekeeping genes, which are in general highly conserved regions in the genome of all organisms. They are essential for basic physiological functions of cells and alterations in the nucleotide sequence of these genes can be lethal in case of non-silent mutations. Sequencing of housekeeping genes connected with phylogenetics is a commonly used technique to distinguish between species and genetically distant populations of a species. In contrast, the ITS region is very variable between individuals and can be used to characterize the genetic variability within and among populations.

To consolidate the results obtained after fragment analysis and genotyping, the actin and the ITS region in the genome of *Sss* were sequenced. Specific primers were applied to a representative sample subset across all populations in the total sample collection. The *Sss* sequencing data was compared to outgroups, *i.e.* actin and ITS sequence data from other plasmodiophorids (accession numbers are given above). The microsatellite and the sequencing data was analyzed and compared using different models and approaches.

During sequence editing and analysis, heterozygote sequences have been detected. These were excluded for the time being, as consensus sequences and haplotypes could not be resolved without further cloning and sequencing, which might be the subject of future studies. The final sequence data set consisted of 308 sequences each for ITS and actin.

OBJECTIVE 2

EVOLUTIONARY UNITS

EVOLUTIONARY UNITS IN THE MICROSATELLITE DATA AND POPULATION STRUCTURE

The first step with the complete microsatellite data set was the definition of true populations using STRUCTURE, which detects subtle structures and clusters in genotyping data. Very few clusters, *i.e.* true populations, were detected in the total data set.

For all subsequent studies, the microsatellite data set was subdivided into geographic regions instead of field populations (Table 2). These regions were compared to each other in all further calculations. Malta was treated as a separate region, because it revealed an exceptional status during clustering with STRUCTURE.

EVOLUTIONARY UNITS IN THE SEQUENCING DATA

All 308 sequences of ITS and actin and the out-groups from Genbank (accession numbers given in the materials and methods section) were edited and trimmed to the same lengths with BIOEDIT. Output was saved as concatenated fasta file and as phylip file.

The DNAsp software was used to determine the sample distribution in the haplotypes of ITS and actin in the data set of only *Sss* sequences excluding the out-groups. Bulman & Marshall (1998) and Qu & Christ (2004) published two haplotypes of ITS. In the present study, more haplotypes were found. For actin, which is a coding sequence, few different proteins (on amino acid sequences), but many different haplotypes were found (on nucleotide sequences).

The numbers of haplotypes are generally very low, indicating that the genetic variation is very low. A haplotype contains only sequences which are completely identical, *i.e.* clonal. The number of clones varied in the different global regions from extremely clonal to moderate diversity.

GLOBAL PHYLOGENETIC NETWORK

Using DNASP, the individual data sets were haplotype collapsed. Multiple sequence alignments were calculated for all detected haplotypes with ClustalW and maximum likelihood (ML) trees were constructed in MEGA5 based on the Kimura-2-parameter model, using 1000 bootstraps and including the gaps in the sequences.

In the haplotype ML trees, out-groups were always resolved as separate branches. In case of ITS, the outgroup sequences were obtained from other plasmodiophorids, including *Spongospora subterranea* f.sp. *nasturtii*, *Plasmodiophora brassicae*, and *Polymyxa graminis*. In case of actin, the outgroup sequence was obtained from *Plasmodiophora brassicae*.

Two main groups of haplotypes are present in the ML tree of the ITS haplotypes (Fig. 5). Few haplotypes were similar to Qu & Christ's (2004) ITS Type II, which contain a characteristic deletion. All other Haplotypes have a complete sequence and are similar to Qu & Christ's (2004) ITS Type I. These types are the haplotypes which have already been described for *Sss* in the literature and must not be mixed up with ITS1 and ITS2, which are the terms in the genetics literature for the two internal transcribed spacers which are located in the genome between the ribosomal genes.

Actin has more haplotypes than ITS on nucleotide level. Most of them originate from silent mutations because the nucleotide sequences were translated *in silico* into amino acid sequences and there were only few proteins for actin of *Sss* detected. Only one haplotype was distributed evenly across the world, all others were restricted to few regions. Differences in results could be seen between root gall and lesion tissue samples of the pathogen.

GEOGRAPHIC RACES

Combining the results of the STRUCTURE analysis of the microsatellites and the analyses on the sequence data set, few main geographic groups can be found. Some are very clonal and occur around the globe, but differ from other groups, which are diverse.

HISTORIC AND RECENT GENE FLOW

The program BAYESASS+ was applied to the microsatellite data set to calculate recent migration rates based on a Bayesian and Markov Chain Monte Carlo (MCMC) approach. The term “recent” refers in this context to the last two generations. All calculations were iterated five times and showed great consistency. The results indicate that currently only one region has migrants moving to other regions.

Based on the sequence data, several approaches were made to estimate ancient/long-term gene flow, *e.g.* using the programs IM and MIGRATE. However, both programs did not converge and yielded inconsistent results when settings were changed (results not shown).

OBJECTIVE 3

GENETIC VARIATION/CLONAL DIVERSITY

The MAC based program GENODIVE collapsed the whole microsatellite data with regional subdivision to all the single clones, also referred to as multilocus genotypes (MLGs) and gave out several measures for clonal and genetic diversity. All these calculations were performed with corrections for the different numbers of samples in the populations.

One region was extremely diverse, in contrast to other regions. Some regions are very clonal, most likely due to asexual reproduction.

Additionally, the software DNASP was used to calculate haplotype diversities for ITS and actin, based on the sequence data. A haplotype is the total amount of individual sequences which are exactly matching. Differences in sequences of two individuals mean that they do not belong to the same haplotype. Haplotypes were separately calculated for ITS and actin and the diversity was found to be low in the majority of the collections.

OBJECTIVE 4

MODE OF REPRODUCTION

The role of asexual and sexual reproduction could not be completely resolved. It is not definitely clear, that *Sss* is a functional dikaryotic organism, but there were many indications during sequencing and genotyping, that *Sss* is dikaryotic. Thus, all calculations were performed based on this assumption and no inconsistencies have been detected.

During sequencing many heterozygous sequences in a specific region were detected. These samples had to be excluded from the data set, because their haplotype could not be resolved. In future studies, these samples will be used to amplify the ITS and actin loci in PCRs and the resulting products will be cloned and sequenced, to resolve the haplotypes of the excluded samples.

Heterozygosity has also been detected for the microsatellites. Many samples, showed double peaks during genotyping, which means that two alleles are present for one marker which correspond to the two chromosomes, each containing one of the two detected alleles.

Using DATAMONKEY, recombination has been detected based on sequence data for a small number of cases only. The presence of recombination strongly suggests that there is sexual reproduction and mating in *Sss*.

DISCUSSION

The results of all objectives allow for a hypothesis: Presumably, *Sss* was introduced to most regions as a stowaway on infected potato roots/tubers or in contaminated soil.

The results imply that the members of the diverse group represent only a snapshot of the great genetic diversity in their region and that the clonal groups of *Sss* still show a founder effect. Founder effect means in this case, that a subpopulation representing only a fraction of the total genetic diversity of *Sss* was brought to those regions on few potato plants.

The historic gene flow could not be calculated, because the data was not diverse enough. This calculation would have helped to determine, if the hypothesis is true.

However, the recent gene flow calculated with BAYESASS answers questions of great importance to powdery scab management. It is obvious, that *Sss* is reaching many regions outgoing from Europe nowadays.

Unintentionally, *Sss* could be a stowaway on shipped seed potato tubers which do not show symptoms of the disease and reach other regions. Having the global diversity in mind this could be a threat for regions with low variability. Greater variability could lead to more aggressive and virulent strains of *Sss*, which makes the management of powdery scab more difficult.

Probably, there is only one mating type of *Sss* in the global populations preventing sexual reproduction. Quarantine measures might have to be taken, if future studies prove these hypotheses to be true.

TECHNOLOGY TRANSFER

Summaries of this research have been presented at two science conferences:

1. Gau RD, McDonald BA, Merz U, Brunner PC, Falloon RE (2010). Global population genetics and phylogeny of the plant pathogenic protozoan *Spongospora subterranea* f. sp. *subterranea*. *Proceedings, 6th Australasian Soilborne Diseases Symposium* (Ed. GR Stirling): 48.
2. Gau RD, McDonald BA, Merz U, Brunner PC, Falloon RE (2011). Global population genetics and phylogeny of the plant pathogenic protozoan *Spongospora subterranea* f. sp. *subterranea.* Oral presentation at the 3rd European Powdery Scab Workshop, 11-13 July, Boldern, Switzerland. The abstract will be available online soon on the Spongospora Homepage: http://www.spongospora.ethz.ch/Zurich_2011/.

The article “Evolution and genetics of the Powdery Scab pathogen-on a global scale” by Mignonne Rawson has summarized this project in the issue June/July 2010 in “Potatoes Australia”, pages 24-25.

A research paper which will be submitted to an appropriate refereed journal is in preparation.

This project’s outcomes will be presented at the “Zürich Mycology Symposium 2012”, which will take place on January 20, 2012 at the Institute of Microbiology, ETH Zürich, Switzerland.

RECOMMENDATIONS

Quarantine measures should be taken to avoid further introductions of *Sss* from the diverse region to the clonal regions. Introduction of new pathogen strains might lead to greater diversity, more recombination and to more virulent pathotypes causing increased disease.

Future studies of the population genetics of *Sss* should address the genetic variation in the diverse regions, for a better understanding of the evolutionary history of *Sss* and to make predictions on how it might evolve. Additionally, a mating type study could help to understand the present distribution of genetic diversity in *Sss*.

The information in this study may help breeders to develop a targeted way of screening new lines of potatoes for resistance against powdery scab, which seems to be easier due to the low genetic diversity of the pathogen in some countries. The low genetic diversity also holds for Australian populations, which are very clonal. Another feature, the Australian *Sss* populations have in common with other populations, is that they do not show indications for the presence of sexual reproduction. This will keep the level of genetic diversity low due to the lack of recombination. As long as no further *Sss* introductions from diverse regions will occur, screening with a low number of strains is sufficient to achieve durable resistance. Furthermore, resistant potato varieties developed in one country with low diversity of *Sss*, can be grown in other low diversity countries, where all strains are clonal and share the same population genetic features, without the risk of disease outbreak.

ACKNOWLEDGEMENTS

This work was supported by grants from Horticulture Australia Ltd (APRP1, University of Tasmania), the Swiss Federal Institute of Technology (ETH-Z), the United Kingdom Potato Council, the New Zealand Ministry of Science and Innovation, Horticulture New Zealand, and the Swiss Federal Office of Agriculture (FOAG).

Data analyzed in this project was generated in the Genetic Diversity Centre of the Swiss Federal Institute of Technology, Zurich, Switzerland, and at the New Zealand Institute for Plant and Food Research Limited, Lincoln, Canterbury, New Zealand.

With the appreciated cooperation of many scientists around the globe, a large number of field collections of *Sss* have been obtained. We thank the following researchers for their contribution: George Babu (Agricultural Research Station Sita Elya, Nuwara Elya, Sri Lanka), Kirsten Bundgaard (Graminor, Ridabu, Norway), Celsa Garcia (Universidad de Colombia, Bogota, Colombia), Hans-Reinhard Hofferbert (Boehm Nordkartoffel Agrarproduktion OHG, Hohenbrünzow, Germany), Shamim Iftikhar (National Agricultural Research Centre, Islamabad, Pakistan), Kim Jeom-Soon (Highland Agricultural Research Center, Pyeongchang, South Korea), Takato Nakayama (National Agriculture Research Center for Hokkaido Region,Sapporo, Japan), Angelo Porta Puglia (Plant Health Directorate, Lija, Malta), Xinshun Qu (Pennsylvania State University, University Park, USA), Dorian Rodriguez (Universidad Centroccidental Lisandro Alvarado Postgrado de Fitopatología, Barquismeto, Venezuela), Maria Sandgren (Swedish University of Agricultural Sciences, Uppsala, Sweden), Halldór Sverrisson (Agricultural University of Iceland, Reykjavik, Iceland), Stuart Wale (SAC, Aberdeen, Scotland), Tonya Wiechel, (DPI, Knoxfield, Australia), and Jessica Wright (University of Pretoria, Pretoria, South Africa).

BIBLIOGRAPHY

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) BASIC LOCAL ALIGNMENT SEARCH TOOL. *Journal of Molecular Biology* **215**, 403-410.

Bulman SR, Marshall JW (1998) Detection of Spongospora subterranea in potato tuber lesions using the polymerase chain reaction (PCR). *Plant Pathology* **47**, 759-766.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Delport W, Poon AFY, Frost SDW, Pond SLK (2010) Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* **26**, 2455-2457.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. *Molecular Evolution: Producing the Biochemical Data, Part B* **395**, 202-222.

Higgins DG, Thompson JD, Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. *Computer Methods for Macromolecular Sequence Analysis* **266**, 383-402.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452.

McDonald BA (1997) The population genetics of fungi: Tools and techniques. *Phytopathology* **87**, 448-453.

Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* **4**, 792-794.

Merz U (2008) Powdery scab of potato - Occurrence, life cycle and epidemiology. *American Journal of Potato Research* **85**, 241-246.

Merz U, Falloon RE (2009) Review: Powdery Scab of Potato - Increased Knowledge of Pathogen Biology and Disease Epidemiology for Effective Disease Management. *Potato Research* **52**, 17-37.

Pond SLK, Frost SDW (2005) Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* **21**, 2531-2533.

Pond SLK, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. *Bioinformatics* **21**, 676-679.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Qu XS, Christ BJ (2004) Genetic variation and phylogeny of Spongospora subterranea f.sp subterranea based on ribosomal DNA sequence analysis. *American Journal of Potato Research* **81**, 385-394.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes* **4**, 137-138.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* (eds. Krawetz S, Misener S), pp. 365-386. Humana Press.

Tamura K, Peterson D, Peterson N*, et al.* (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**, 2731-2739.

Team RDC (2011) *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* **163**, 1177-1191.

Winnepenninckx B, Backeljau T, Dewachter R (1993) EXTRACTION OF HIGH-MOLECULAR-WEIGHT DNA FROM MOLLUSKS. *Trends in Genetics* **9**, 407-407.