Methods in Plant Cytogenetics

a) Genome differentation in *xTriticoleymus*

b) Cytotaxonomy of the genus *Sorbus*

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Líf- og umhverfisvísindadeild

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Abstract

The first part of this study investigates the genome constituents of the ×*Triticoleymus* genotype U-06, which is a backcross hybrid between the amphiploid U line of ×*Triticoleymus* (hexaploid, 2n=42) and the Icelandic *Leymus arenarius* (octoploid, 2n=56), by means of whole genome *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) performed on chromosome preparations done by the enzymatic root-tip squash method. Fifteen chromosomes originating from the *Triticum* genome were found in this U-06 backcross derivative and three of them were found to have loci of the 18S-5.8S-26S ribosomal genes. These loci were situated on the A1, B1 and B2 wheat chromosomes as well as m1 and m1*Leymus* chromosomes, supporting earlier findings that the U line of ×*Triticoleymus* originated by hybridization of a tetraploid *Triticum* species with *Leymus mollis*. In addition, two ribosomal sites from *L. arenarius*, *i.e.* a2 and a3 loci, could be identified. The total number of chromosomes originating in the *Leymus* genomes could not be assessed with certainty due to either insufficient chromosome spreads or that chromosome loss had taken place in the *in vitro* regenerated line. Genome of this hybrid U-06 is expected to contain 15 *Triticum* chromosomes and 34 *Leymus* chromosomes, of which 6 are from the x *Triticoleymus* seed parent and 28 from the *L. arenarius* pollen donor.

The second part of this study investigates the chromosome numbers for a few representatives of the genus *Sorbus*. Chromosome counts were assessed for six species and one sexual hybrid of the genus *Sorbus*. Earlier chromosome counts for the species *S. mougeotii* (2n=68), *S. aucuparia* (2n=34) and *S. cashmiriana* (2n=68) were confirmed. This study assessed *S. hostii* as a tetraploid (2n=68) species and further evidence was provided when its hybrid with *S. aucuparia* (2n=34) was proved to be triploid (2n=51). Two species of uncertain status, *S. sudetica* and *S. erubescens* were investigated and they were found to be polyploid. Chromosome numbers, however, for these two species could not be accurately assessed since the spreads of chromosomes on the microscopic slides were insufficient.

Útdráttur

Fyrri hluti þessa rannsóknarverkefnis rýnir í erfðamengi F1 blendings milli ×*Triticoleymus* og *Leymus arenarius*. Litningar voru einangraðir úr rótarendum plöntunnar, fjöldi þeirra áætlaður og 18S-5.8S-26S gen kortlögð með þáttatengingu flúrljómandi þreifara (FISH). Jafnframt var uppruni litninganna kannaður með þáttatengingu flúrljómandi erfðamengja melgresis og hveitis við litninga F1 blendingsins (GISH). Þáttatenging flúrljómandi erfðamengis hveitis við litninga blendingsins leiddi í ljós að fimmtán litningar eiga uppruna sinn í hveiti. Þrír af þessum litningum báru 18S-5.8S-26S gen og voru þeir staðsettir á A1, B1 og B2 litningi hveitierfðamengisins. Þessar niðurstöður gefa til kynna að ×*Triticoleymus* á uppruna sinn í kynblöndun fjórlitna hveititegund við melgresistegund. Ekki tókst að telja litninga sem eiga uppruna sinn í melgresi sökum ófullnægjandi dreifingar litninga á sýnaglerjum eða vegna taps litnings við vefjaræktun blendingsins.

Seinni hluti þessa verkefnis kannar litningafjölda nokkurra reynitegunda *(Sorbus* spp.). Litningar voru einangraðir úr toppsprotum sex tegunda og eins tegundablendings. Áður uppgefinn litningafjöldi tegundanna alpareynis (*S. mougeotii*, 2n=68), ilmreynis (*S. aucuparia*, 2n=34) og kasmírreynis (*S. cashmiriana*, 2n=68) var staðfestur. Úlfareynir (*S. hostii*) reyndist vera fjórlitna tegund (2n=68) og blendingur hans við ilmreyni þrílitna (2n=51). Ekki var unnt að ákvarða litningafjölda tveggja tegunda, súdetareynis (*S. sudetica*) og bergreynis (*S. erubescens*), sökum ófullnægjandi dreifingar litninga á sýniglerjum. Þessar tvær tegundir reyndust þó vera fjöllitna.

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Abbreviations

CCD	charge-coupled device
DAPI	4',6-diamidino-2-phenylindole
e.g.	*exempli gratia*, for example
EDTA	ethylenediaminetetracetic acid
et al.	*etalii*, and others
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
GISH	Genomic *in situ* hybridization
HB	*HortusBotanicus*, Botanic Garden
NOR	Nucleolar organizing region
rDNA	ribosomal DNA
rpm.	rounds per minute
s.l.	*sensulato*, in the broad sense

1 Genome differentiation in the U06backcross of ×*Triticoleymus*

1.1 Introduction

1.1.1 The genome of wheat and its hybrids with lymegrass

The domestication of wheat, *Triticum* L., started more than 10.000 years ago in the Near East, in an area that spans modern-day Israel, Jordan, Lebanon and western Syria, into southeast Turkey and, along the Tigris and Euphrates rivers, into Iraq and the western flanks of Iran (Salamini*et al.*, 2002; Feuillet *et al*., 2008; Wilcox, 2013). Ploidy levels of cultivated wheat have through the ages ranged from diploid (2n=14) to hexaploid (6n=42). The diploid species *T. monococcum* L. was widely cultivated until the Bronze Age (started approximately 5000 years ago) when its cultivation started to decline and it was replaced by cultivated tetraploid wheat varieties. All tetraploid varieties of domesticated wheat have a common allotetraploid progenitor, *T. turgidum* L. (AABB), of which genomic constituents are believed to originate from a species closely related to *T. urartu*Thumanjan (AA) and yet an unknown species of *Aegilops*(BB). This allotetraploid, having combined genomes of the parental diploid species, proved more vigorous, higher yielding and more broadly adapted to different environmental conditions than its progenitors. The evolution of hexaploid bread wheat (AABBDD) occurred more recently when the cultivated form of *Triticumturgidum* (AABB) crossed with the wild grass *Aegilopstauschii*Coss (DD) (McFadden & Sears, 1946).

In 2013 wheat is one of the world's leading cereal grains being grown on larger area than any other crops worldwide (Food and Agriculture Organization of the United Nations). The adoption for intense farming methods followed by breeding activities and the introduction of modern high-yielding cultivars have led to reduction of genetic diversity within cultivated wheat (Skovmand*et al*., 2002). This raises concerns about our ability to sustain crop yield and quality in the face of environmental and biotic threats. Therefore, high priority for wheat improvement worldwide is to conserve existing diversity and to enrich the cultivated gene pools by incorporating favourable alleles, genes or gene complexes from land races and wild species (Feuillet *et al.*, 2008). Diverse useful traits

have thus been incorporated into the cultivated gene pool by crossing cultivated wheat with its related wild species of *Triticum* spp. and *Aegilops* spp. Wide crosses of wheat with more distant relatives in the tribe Triticeae, both wild and cultivated, have also served same purposes, either by chromosome transfer or by making new hybrid species. For instance, fertile hybrid cultivars of *Triticum* and *Secale* have successfully been bred, so-calledtriticale (×*Triticosecale* Wittmack), and their cultivation for commercial purposes has increased steadily the last decennia (Food and Agriculture Organization of the United Nations). After several decades of breeding, triticale stands out as a crop of high biomass and grain yield potential, which generally surpasses that of common wheat, as well as having outstanding tolerance to abiotic stresses (reviewed in Blum, 2014). Another synthesized wheat hybrid commercially cultivated is tritordeum(×*Tritordeum*Ascherson & Graebener), a new cereal developed in the late seventies from crosses between the wild barley *Hordeum chilense* Roem. & Schult. and durum wheat (reviewed inMartin *et al*., 1999). Tritordeum is proven to have possibilities to be grown under drought environments such as in the Mediteranean region, since its performance is quite close to wheat and triticale, in addition to its qualitative added values that may improve farmers' income per unit land (Villegas *et al*., 2010). Attempts have also been made to hybridize wheat with various lymegrass species,*Leymus* spp., where traits influencing tolerance to high salinity and drought have especially been targeted along with resistance to virus diseases. Other traits such as tolerance to low temperatures, freezing and ice encasement have also been targeted by hybridization with the northern temperate and arctic species *Leymusarenarius* (2n=56) and *L. mollis*(2n=28) (Anamthawat-Jónsson*et al*., 1997; Anamthawat-Jónsson, 1999). Although fertile hybrids have been obtained they have not yet been utilized in breeding for commercial cultivation.

1.1.2 The genome of the amphiploid line U of ×*Triticoleymus*

The genome of the fertile amphiploid line U of ×*Triticoleymus* has proven to be stable allohexaploid (2n=6x=42) and to consist of 15 pairs of an unknown allotetraploid wheat species (AABB) and 6 pairs of lymegrass chromosomes (*Leymusmollis*). Available information on the exact origin of the amphiploid is limited and the extent of backcrossing with wheat while maintaining the line is not known. Nevertheless, experiments have demonstrated a stable replacement of one pair of *Leymus* chromosomes by a pair of *Triticum* chromosomes. Since the line is fully fertile the replacement must have been

between homoeologous chromosomes, as only then can the genetic content be complemented (Anamthawat-Jónsson, 1999).

1.1.3 Cytotaxonomy

Comparative karyotype analysis has proved to be an important tool for taxonomy and for understanding chromosome evolution. Their number, structure and behaviour have shed light upon the relationship among many taxa such as within the genera *Passiflora* (Hansen *et al.*, 2006) and those in the family Agaveceae (McKelvey& Sax, 1933) and the family Fagaceae (Chokchaichamnankit *et al*. 2007), as well as among species in the tribe Triticeae within Poaceae (reviewed by Goncharov, 2011). The basic principle of cytotaxonomy is that closely related species share a more similar karyotype than less related ones. Detailed analysis of the karyomorphology can provide insights into the mechanisms of chromosomal evolution in plants (Stebbins, 1971). Chromosome number is the karyotype feature most commonly used in cytotaxonomicalanalyses, often accompanied with chromosome mapping with the help of molecular probes (*e.g.* FISH and GISH). Karyotype features, however, are often recurrent, meaning that similarity is not all and plesiomorphy or synapomorphy should be supported by robust phylogenetic trees (Guerra, 2008 &2012).

1.1.4 Fluorescent *in situ* hybridization (FISH)

FISH is a cytogenetic method that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. Fluorescence-labelled probes are hybridized to achromosome preparation fixed on microscopic slide and if homologous sequences are present the fluorescent probes are bound and can be visualized by epifluorescent microscopy. Genome *in situ* hybridization (GISH) is when total genomic DNA is labelled and hybridized with a chromosome preparation (Anamthawat-Jónsson *et al.*, 1990). GISH is an informative and versatile method identifying genome origin in hybrids or allopolyploids and for studying chromosomal evolution and chromosome introgression (Schwarzacher&Heslop-Harrison, 2000; Anamthawat-Jónsson, 2001). Repetitive sequences such as the ribosomal genes 5S and 18S-5.8S-26S have also proved as valuable probes when studying karyotype evolution in species where little information on their genomes is available (*e.g.*Anamthawat-Jónsson&Bödvarsdóttir, 2001; Anamthawat-Jónsson&Thórsson, 2003; Ellneskog-Staam*et al*., 2006).

1.1.5 The ribosomal genes 18S-5.8S-26S

The ribosomal genes 18S-5.8S-26S (rDNA) are usually located on the nucleolus organizing regions (NOR) of the chromosomes in repetitive tandem repeats. Not all NORs are transcriptionally active. Species with multiple NORs can vary in the active proportion.Active NORs are identified in cells in metaphase as weakly stained chromatin regions, often referred to as secondary constrictions (McStay, 2006).

Altogether, seven to eight NORs have been reported in hexaploid bread wheat,*Triticumaestivum* (Jiang & Gill, 1994). Four of these are major (active) loci, namely*Nor-A1*, *Nor-B1*,*Nor-B2* and *Nor-D3*, whereas the other four loci, i.e.*Nor-A7*, *Nor-B6*,*Nor-D4* and rarely*Nor-D8*, are minor loci and are often inactive. Of the four major loci, three have been reported in the line U of the amphiploid ×*Triticoleymus*, *Nor-A1*, *Nor-B1* and *Nor-B2* (Anamthawat-Jónsson, 1999), meaning that this amphiploid may not have the D genome. Three NORs have been positioned on three separate chromosomes of *Leymusmollis*(Anamthawat-Jónsson&Bödvarsdóttir, 2001), whereof two (m1 and m2) have been found in line U of ×*Triticoleymus*(Anamthawat-Jónsson, 1999). In *Leymusarenarius*, six rDNA loci have been positioned on six separate chromosome pairs(a1, a2, a3, a4, a5 and a6), where three had strong signals (hence major sites) and had secondary constrictionsvisible (Anamthawat-Jónsson&Bödvarsdóttir, 2001).

1.1.6 Objectives

The objectives of this project were to investigate the genome constituents of the F1 backcross derivative U-06 by means of whole genome *in situ* hybridization and to characterize the chromosomes by attempting localization of the 18S-5.8S-26S ribosomal genes by fluorescent *in situ* hybridization.

1.2 Materials and methods

1.2.1 Plant material

The backcross derivative U-06 (a single clone, maintained by repeated *in vitro* propagation) was used for this study.The backcross was made by KesaraAnamthawat-Jónsson (University of Iceland) by using the amphiploid line U of ×*Triticoleymus*(hexaploid, 2n=42) as a seed parent and *Leymusarenarius*(octoploid, 2n=56) collected in Seltjarnarnes, Iceland, as a pollen parent.

1.2.2 Enzymatic root-tip squash method

Root tips were collected from plants in pots. Healthy root tips in full growth were collected in ice-water and then kept at 4°C for 28 hours to arrest metaphases. The root tips were then transferred to a freshly made fixative of 1 part glacial acetic acid and 3 parts 96% ethanol. The root tips need to be fixed for at least 2 hours and can be stored at -20°C for up to 3 months. The root tips were then submerged in an enzyme buffer at room temperature for 20 minutes, made of 40 ml of stock solution A, 60 ml of stock solution B and 900 ml ddH_2O. To prepare stock solution A, 10.5 g of citric acid monohydrate was dissolved in water and the volume made to 500 ml. Stock solution B was made by dissolving 14.7 g of trisodium citrate in water and the volume made to 500 ml. The roots were moved to a fresh buffer after 10 minutes. A 10 ml of root tip enzyme mixture contained 1 g (ca. 1000 units) of cellulase (Onozuka R10 from Merck #102321), 1.2 ml (667 units) of pectinase (Sigma P-4716, from *Aspergillusniger*) and 8.8 ml enzyme buffer. About 2-3 mm long tips were dissected from the root tips and incubated in an18 µl drop on an acid-cleaned slide. The slide was placed on a Petri dish with a lid on and incubated at 37°C for 40 minutes. The enzyme mixture was then drawn out and replaced with a big drop of enzyme buffer which was changed once. The enzyme buffer was then replaced by a large drop of 45% acetic acid, changed once and left for a few minutes. In a stereo microscope, with 20x magnification, the root tip was then dissected and the meristem, seen as a dense area of cells in the root tip, was teased out of the root tip with a dissecting needle. Tissue debris was removed and a coverslip placed on top of the meristem droplet. The coverslip was then tapped gently with the dissecting needle to disperse the cells evenly. The slide was then squashed by placing it in between two sheets of filter paper and pressed firmly with a thumb. The slide was evaluated in a phase-contrast microscope and if cells in metaphase were seen the slide was freeze dried with the aid of liquid nitrogen and the cover slip was

flicked off the slide with a razor blade. The slides were air dried and marked with a diamond tipped pen on the underside for the edges where the cover slip had been. The slides were stored at 4°C in an air tight storage box until examination.

1.2.3 Genomic *in situ* hybridization (GISH)

High quality air-dried slides were treated with RNase for 1 hour at 37°C to hinder the probe from binding to RNA and thus clearing the backgroundsignal on the slides later on. Subsequently the slides were washed for five minutes in 2x SSC at 37°C, the washing procedure was repeated once. A pre-made stock solution with 10 µg/ml of RNase (in 10 mMTris pH8 and 15 mMNaCl) was diluted in 2x SSC to give a final concentration of 5 µg/ml. Pre-made 20x SSC (3 M NaCl and 0.3 M trisodium citrate ($Na_3C_6H_5O_7$) adjusted for pH7 with 1M HCL) was diluted with ddH_2O to give the 2x SSC. The slides were then fixed for 10 minutes in 4% paraformaldehyde and subsequently washed twice in 2x SSC for 5 minutes. The slides were then dehydrated through an ethanol series of 70%, 90% and 96% strength for 2 minutes each, then air dried.

Pre-labelled probes were used for this study. The whole genomic probes of *Leymusmollis* and *Triticumaestivum* had been labelled directly using standard nick translation method with green-fluorescing FITC/fluorescein (Roche) for *L. mollis* and red-fluorescing SpectrumRed (Vysis) for *T. aestivum*. Probe mixture of 20 µl was made by mixing 11 µl of formamide (90% stringency), 4 µl of 20% dextran sulphate (heated to decrease thickness), 2 µl of 20x SSC, 1 µl of 10% SDS (decreases surface tension) and 1 µl of each labelled probe. The mixture was boiled for 6 minutes and then kept on ice for 5 minutes. The total amount of 20 µl was applied gently to the slide while air bubbles were avoided and broken if seen, 22 mm cover slip was then placed over the probe. The prepared slide with the probe was denatured at 89°C for 10 minutes in an *in situ*thermocycler (MJ Research Inc., model PTC-100), at 89°C for 10 minutes, followed by a stepwise decrease in temperature to 50°C. Hybridization took place in humid chamber (box with 2x SSC) at 37°C overnight.

Cover slips were removed before washing. The slides were washed briefly two times in 2x SSC at 37°C. More stringent wash was provided by washing the slides in 0.1x SSC at 55-60°C for 5 minutes three times. The slides were then put in 37°C warm 2x SSC and let stand at RT. Then they were washed in 4x SSC with the detergent Tween20 added (0.2% of volume) at RT for 5 minutes. The slides were then stained with DAPIby adding about

10 µl to each slide and after one minute the slides wererinsed briefly with ddH_2O and air dried. A drop of antifade, Citifluor AF1, was added, a 22x30 mm cover-slip mounted and air bubbles gently pressed out. The slides were stored in a cold and dark place until examination.

1.2.4 Fluorescent *in situ* hybridization (FISH)

The best slide was chosen for further fluorescent *in situ* hybridization for localizing 18S-5.8S-26S ribosomal genes on the chromosomes. Green fluorescing FITC labelled rDNA probe were used from clone pTa71 (Gerlach & Bedbrook, 1979), a 9 kb fragment from *Triticum*, which contains a part of 18S, the entire 5.8S and 25S coding regions along with non-transcribed intergenic spacers. The slide was refixed in fresh fixative for 10 minutes at room temperature and then washed twice in 96% ethanol for 10 minutes each and then air dried. Probe mixture of 20 µl was made by mixing 10 µl of formamide (87% stringency), 2 µl of ddH_2O, 4 µl of 20% dextran sulphate (boiled to decrease thickness), 2 µl of 20x SSC, 1 µl of 10% SDS (decreases surface tension) and 1 µl of the labelled probe. Subsequently, same procedure as in the genomic *in situ* hybridization was followed through the hybridization.

1.2.5 Examination of slides

When examined, 10 µl of DAPI were added to the slide and a cover slip was added. After at least 1 minute, the slides were examined in an epifluorescent microscope (Nikon Eclipse 800) using UV filter block with 340-380 nm excitation wavelengths for detecting the DAPI stain at 430 nm emission wavelengths (blue), 450-490 nm excitation wavelengths for detecting the FITC fluorescent stain at 520 nm emission wavelengths (green) and 530-560 nm excitation wavelengths for detecting the SpectrumRed fluorescent stain at 580 nm emission wavelengths (red). The slides were scanned for cells in metaphase using 200x magnification and pictures were taken with 1000x magnification via the CCD camera (Nikon DXM 1200F) for chromosome number determination and karyotyping.

1.3 Results

The slide preparation did result in unsatisfactory spreading of the chromosomes and as a result exact chromosome numbers could not be determined for U-06. GISH, simultaneously using *Triticum* and *Leymus* genomes as probes, revealed 15 chromosomes originating from the *Triticum* genome (as expected) and at the most 33 chromosomes from the *Leymus* genome (34 chromosomes expected). Neither a karyotype nor an ideogram was constructed due to insufficient chromosome spreads. The FISH detected sequences homologous to the rDNA probe on three *Triticum* chromosomes and on seven *Leymus* chromosomes. Chromosomal *in situ* mapping of the ribosomal genes is attempted in Figure1. The three loci for rDNA found on *Triticum* chromosomes were identified as *Nor*-A1, *Nor*-B1 and *Nor*-B2 (as in the wheat genome of the amphiploid seed parent). Out of seven loci of rDNA found on *Leymus* chromosomes, four of them were identified (a2, a3, m1 and m2).The *L. mollis* loci (m1 and m2) are as in the *Leymus* genome in the amphiploid seed parent), but only two out of three major rDNA loci from the *L. arenarius* pollen donor were observed. The a1 locus was missing.One cell in metaphase was found where an unambiguous interpretation could be presented (Figure1.a). A second cell in metaphase is presented to support the former, but an unambiguous reading could not be presented due to insufficient chromosome spreading (Figure1.b). However, in this second cell the rDNA major loci *Nor*-B1, *Nor*-B2 and m1 were clearly visible.

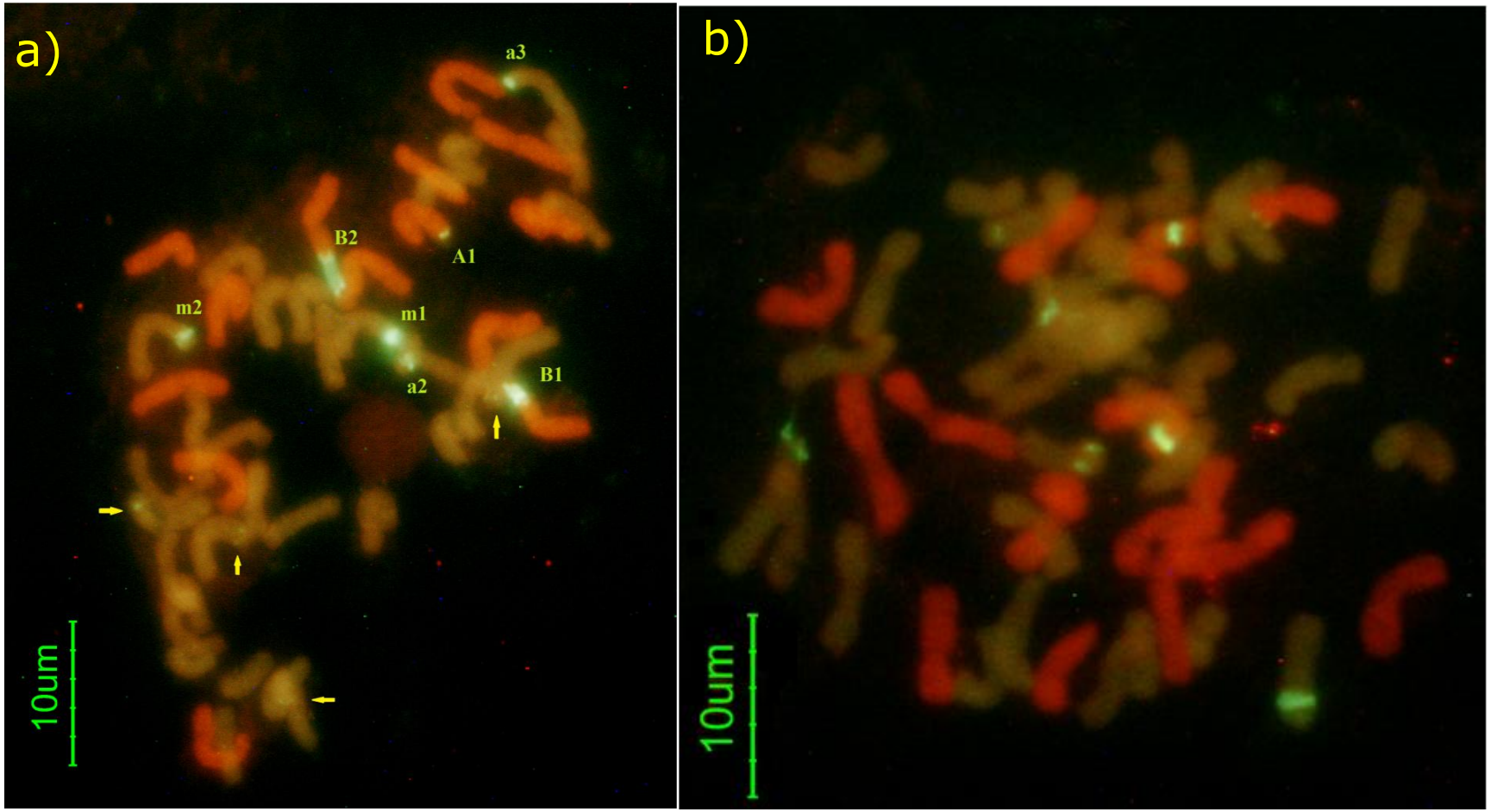

Figure 1: Mitotic chromosomes of the U-06 backcross derivative of ×Triticoleymus after fluorescence in situ hybridization using the whole genomic probes of Leymus mollis (yellow) and Triticum aestivum (red) and a 18S-5.8S-26S rDNA probe. ***a)*** *Green-fluorescing 18S-5.8S-26S loci on Triticum (Nor-A1, Nor-B1 and Nor-B2) and Leymus chromosomes (a2, a3, m1, m2 and arrows).* ***b)*** *Chromosome preparation with insufficient spread for proper reading. Three rDNA loci are seen on three Triticum chromosomes and at least on six Leymus chromosomes.*

1.4 Discussion

1.4.1 Genomic *in situ* hybridization

The results of the genomic *in situ* hybridization support the findings of Anamthawat-Jónsson (1999) that ×*Triticoleymus* behaves as a stable diploid. The F1 backcross U-06 had the expected haploid set of 15 *Triticum* chromosomes of ×*Triticoleymus*. The F1 backcross was expected to have 34 *Leymus* chromosomes as its seed parent (×*Triticoleymus*) should have provided the F1 hybrid its haploid set of 6 *Leymus* chromosomes and the pollen parent (*Leymusarenarius*) the haploid set of 28 *Leymus* chromosomes. This was not detected in the metaphase cells investigated for this study; only 33 *Leymus* chromosomes were counted. The maintenance of the clone by vegetative propagation may have resulted in changes in chromosome number in a similar manner as that occurring with various micropropagation techniques. In studies of bread wheat, deviant chromosome numbers were observed in up to 12% of plantsregenerated by short term somatic embryogenesis (Karp &Maddock, 1984;Henry *et al*., 1996). Similar tendencies have been observed for ×*Triticosecale*regenerants(Armstrong*et al*., 1983) and the phenomenon is known from arange of plant species when micropropagated (*e.g*.Browers& Orton, 1982; McCoy *et al.*, 1982; Armstrong &Phillips, 1988;Al-Zahim*et al*., 1999). The loss of one chromosome could also be explained by chromosome elimination. In crosses between wheat and distantly related species such as maize, pearl millet and sorghum, non-wheat chromosomes are often eliminated from the hybrid during embryogenesis (Barclay, 1975; Laurie & Bennett, 1986; Laurie, 1989). The phenomenon has also been observed in crosses between wheat and *Hordeumbulbosum* L., a wild barley species which is in the same tribe as *Leymus* and *Triticum*; Triticeae (Zenkteler& Straub, 1979). The stable and fertile amphiploids x*Triticoleymus,* including the U line in the present study, maintain only 6 pairs of *Leymus* chromosomes instead of the full set of 7 chromosome pairs per genome (Anamthawat-Jónsson, 1999). The quality of the slide preparations was however not sufficient to exclude the possibility of insufficient spreads of chromosomes. Only one slide was investigated and good chromosome count could only be obtained from one cell.

1.4.2 Chromosomal *in situ* mapping of the ribosomal genes

As expected, the rDNA loci found on *Triticum* chromosomes were the same as Anamthawat-Jónsson (1999) found in line U of ×*Triticoleymus* in her study; *Nor-A1*, *Nor-B1* and *Nor-B2*. The rDNA loci on wheat chromosomes 1BS (*Nor-B1*) and 6BS (*Nor-21*) appeared to be actively expressed as the signals were extended across the secondary constrictions whereas a secondary constriction was not observed for the rDNA locus on wheat chromosome 1AS (*Nor-A1*) and its signal was quite weak. Eight rDNA loci were detected on the *Leymus* chromosomes, presumably six from the pollen parent and two from the seed parent. Signals were significantly weaker on *Leymus* chromosomes than on *Triticum* chromosomes and small secondary constrictions could only be observed at loci identified as m1 and m2 (Anamthawat-Jónsson&Bödvarsdóttir, 2001). This indicates little activity and general suppression of nucleolar formation on *Leymus* chromosomes in the backcross derivative U-06.

Nucleolar dominance is the term given to the phenomenon where nucleolus formation at one organizer is suppressed in the presence of another NOR. Nucleolar dominance has frequently been observed in intergeneric and interspecific hybrids in plants (*e.g.*Navashin, 1934; Chen &Pikaard, 1997; Chen *et al*., 1998; Tucker *et al.*, 2010; Ge *et al*., 2013). Studies have demonstrated that when placed in a wheat genetic background, the NORs of rye are heavily suppressed (Houchins*et al*., 1997). Similarly, this study indicates that NORs of *Leymus* spp. are at least partially suppressed when placed in wheat background.

1.5 Conclusion

Chromosome counts for the F1 backcross derivative U-06 of ×*Triticoleymus* could not be assessed as either chromosome spreads were insufficient or chromosome loss had occurred due to repeated *in vitro* regeneration. By applying methods of whole genome *in situ* hybridization this study showed that 15 chromosomes of U-06 originated in the *Triticum* genome. The identification of rDNA loci by fluorescence *in situ* hybridization supported earlier findings (Anamthawat-Jónsson, 1999) that the probable ancestor of the U line of ×*Triticoleymus*is a tetraploid species with AABB genome composition.

2 Cytotaxonomy of a few species in the genus *Sorbus s.l.*

2.1 Introduction

2.1.1 The genus *Sorbus*

The genus *Sorbus* L. includes at least 250 species that are widespread in the temperate areas of the Northern Hemisphere (Phipps *et al*., 1990). Considerable difficulties in species classification have been met within the genus and consequently species number varies greatly between authors. The main reason for these discrepancies is the numerous polyploid apomicts in the genus and different opinions on how they should be treated (*e.g.*Aldasoro*et al*., 1998; McAllister, 2005).

Interspecific hybridization and genome multiplication are among the fundamental processes in plant diversification and speciation (reviewed in Rieseberg& Willis, 2007). The formation of allopolyploids offers a clear route to sympatric speciation in plants and the associated breeding system determines their long-term survival. Outbreeding encourages introgression and the formation of new allopolyploid offspring, whereas inbreeding will perpetuate the new hybrids and reinforce the reproductive isolation between them and their parental taxa. Asexual or clonal reproduction offers a secure way to maintain new hybrids (Rieseberg, 1997). Apomixis is a form of asexual reproduction where both fruits and seeds are produced without any fertilisation or sexual recombination of genes. The production of seed without fertilisation results in seedlings genetically identical to the parent plant.

The genus *Sorbus*belongs to the subfamily Maloideae of the Rosaceae and has traditionally been divided into the five subgenera *Sorbus, Aria, Chamaemespilus,Torminaria*and*Cormus*.Morphological and molecular data havedemonstrated that the subgenus *Aria* is more related to the genera *Malus* and *Pyrus* than to any of the other subgenera and that the subgenus*Torminaria*is more related to the genus *Crataegus*than to any of the other subgenera(Robertson *et al*., 1991; Campbell *et al.*, 1995). On the basis of this polyphyly, Robertson *et al.* (1991) divided*Sorbuss.l.*into five

genera, corresponding to the subgenera traditionally acknowledged: *Sorbus*, *Aria*, *Chamaemespilus*, *Torminaria* and *Cormus*.

In the genus *Sorbuss.l.*, hybridization, allopolyploidy, autopolyloidy and apomixis have all contributedto the complex patterns among its taxa (Liljefors, 1953&1955; Chester *et al.*, 2007; Dickinson *et al*., 2007;Robertson *et al.*, 2010; Pellicer*et al*.,2012). The origin of the polyploid apomictic species in the genus is an ongoing question. Liljefors (1955) suggested that the sexual diploids *S. aria, S. aucuparia, S. torminalis* and *S. chamaemespilus*, along with the apomictic autopolyploid taxaof *S.aria* (*e.g. S. rupicola, S. eminens*) form the backbone of this complex in Europe. These suggestions have been supported by later findings (*e.g.*Nelson-Jones *et al.*, 2002; Robertson *et al.*, 2010; Pellicer*et al.*, 2012) and it is believed that these intermediate apomicticspecies have originated by hybridization of *S. aria*(or related species) and one of the other sexual diploid species. One taxon, *S. intermedia*, has been suggested to have threediploid species in its genomic makeup; *S. aucuparia*, *S. aria* and *S. torminalis*(Liljefors,1955; Nelson-Jones*et al*., 2002).Indications have also been found that a few taxa (*e.g. S. hostii*) have components of *S. aucuparia, S. aria* and *S. chamaemespilus* genomes (Rehder, 1947; Krüssmann, 1986; Gömöry&Krajmerová, 2008). The basic chromosome sets of each diploid species have been designated different letters in order to make hypothesis on the origin of hybridogenous species. If, for example, diploid *S. aucuparia* (BB) hybridizes with the tetraploid *S. rupicola* (a tetraploid *S. aria*, AAAA), the resulting apomictic microspecies (*e.g. S. arranensis*) would have the basic chromosome sets AAB and be a triploid. If such a species would produce unreduced gametes and pollinate *S. aucuparia* (BB) it could result in an apomictic microspecies with the basic chromosome set AABB (*e.g.S. hybrida*) (Liljefors, 1955; McAllister, 2005). Repeated backcrossing of apomictic derivatives with parental taxa is responsible of new apomictic taxa leading to ongoing reticulate evolution of the genus *Sorbuss.l.* (Liljefors, 1955; Robertson *et al.*, 2010).

This study employs the broad concept of the genus *Sorbus* as difficulties would be met with nomenclature when dealing with the hybridogenous species.

2.1.2 Objectives

The objectives of this study are to investigate chromosome numbers in representatives of the genus *Sorbus*and reflect upon their evolution and taxonomy. Special emphasis is puton species believed to have originated as hybrids between *S. aria*, *S. aucuparia* and *S. chamaemespilus.*

2.2 Materials and methods

2.2.1 Plant material

The species investigated in this study are listed in Table 1. Shoot-tips were collected in the Reykjavík Botanic Garden and in a neighbouring park. In addition to the species listed in Table 1, *Sorbuschamaemespilus*was collected but the species was later excluded as no cells in metaphase were found.

Table 1: List of species investigated, the origins of accessions, their ploidy numbers and theoretical genomic formulas (Liljefors, 1955; Löve & Löve 1956, 1975; Warburg & Karpati, 1968; McAllister 2005). All species were collected in Reykjavík Botanic Garden except for Sorbushostii, which was collected in a neighbouring park. In the genomic formulas, A represents basic set of chromosomes from S. aria, B represents basic set of chromosomes from S. aucuparia and C represents basic set of chromosomes from S. chamaemespilus.One individual of each species was investigated.

Species	Origin and accession number	Apomictic	Ploidy and theoretical genomic formulas
Sorbus sudetica Bluff, Nees &Schauer	Via HBChampexLac(CHE05199207)	Unknown	Tetraploid,AACC
*Sorbusmougeotii*Soy.-Will. &Godr.	Via HB Chambesy/Geneve (CHE02199008)	Yes	Tetraploid, AAAB
*Sorbuserubescens*Kern. ex Dippel	From Haute-Savoie in France, via HB Champex Lac (CHE05198904)	Yes	Unknown
*Sorbushostii*Heynh.	Park ornamental	Facultative	Unknown
Sorbus aucuparia L.	From Vatnsfjörður in the Westfjords of Iceland(ISL06200712)	No	Diploid, BB
*Sorbuscashmiriana*Hedl.	Via HB Nat Dublin (IRL01197702)	Yes	Tetraploid
Sorbushostii × *aucuparia*	Spontaneous hybrid in a plant nursery (ISL26198602)	Unknown	Unknown

2.2.2 Chromosome isolation by the protoplast dropping method

For chromosome isolation, the method of Anamthawat-Jónsson (2003) for protoplast dropping was followed. Shoot tips in active growth were collected (the 28th of May and 4th of June) and placed in ice-water for 25 h to arrest metaphases. Subsequently, the shoot tips were transferred to a freshly made fixative of 1 part glacial acetic acid and 3 parts 96% ethanol. The tips were then submerged in the fixative for at least 2 h, during which time the fixative was changed once. The fixed shoot tips were stored at -20°C until further preparation. The fixed shoot tips were then rinsed in ddH_2O. Scales and outer leaves of the shoot tip were trimmed away gently, trying to leave the innermost part (2-4 mm) containing the meristem. The excised tips were kept in ddH_2O for 30 minutes, during which time, water was changed once. Two to three tips of each sample were placed in 100 µl of enzyme mixture and incubated at room temperature overnight. The enzyme mixture had been prepared beforehand by adding 800 units or 0.8 g, of cellulase (Onozuka R10 from Merck #102321) and 300 units, or 0.3 g of pectinase (Sigma P-4716, from *Aspergillusniger*) to 10 ml of enzyme buffer containing 75 mMKCl and 7.5 mM EDTA with pH 4. The leaf tissue was broken with a pipette tip while still in the enzyme mixture. The suspension was then filtered through a nylon mesh into a 1.5 ml microtube. For hypotonic treatment, to ensure good swelling of the cells and increase spreading of the chromosomes, 1.5 ml of cold 74 mM of KCl was added to the tubes. The tubes were gently invertedseveral times and let stand at room temperature for 15 minutes. The protoplast suspension was then centrifuged at 7000 rpm for 5 minutes and the supernatant was discarded. 1 ml of fresh and cold fixative was added to the precipitate and it was resuspended very gently and let stand at room temperature for at least 5 minutes. The centrifugation step was repeated three times more, always using the same pipette tip to resuspend the pellet of cells gently. After the last centrifugation, fresh and cold fixative was added to the approximate total volume of 50 µl. If the protoplast suspension was cloudy, more fixative was used. A drop of the protoplast suspension was dropped onto an ice-cold slide from a distance of about 20 cm. Just before, or immediately when the drop had dried, the slides were briefly dehydrated in 95% ethanol and air dried on a rack. The slides were stored at 4°C in an air tight storage box until examination.

2.2.3 Examination of slides

When examined, 10 µl of DAPI were added to the slide and a cover slip was placed over. After at least 1 minute, the slides were examined in an epifluorescent microscope using UV filter block with 340-380 nm excitation wavelengths for detecting the DAPI stain at 430-450 nm emission wavelengths (blue). The slides were scanned for cells in metaphase using 20x magnification and pictures were taken with 100x magnification with a CCD camera for chromosome number determination.

2.3 Results

Chromosomes could be countedunambiguously in at least one cell for *Sorbusmougeotii*,*S. hostii*, *S. cashmiriana* and *S. aucuparia* (Figure2). *Sorbusmougeotii*, *S. hostii* and *S. cashmiriana* had the tetraploid chromosome number 2n=68, whereas *S. aucuparia* had the diploid chromosome number 2n=34. Chromosome spreads were not sufficient to facilitate unambiguouschromosome counts for*S. sudetica*, *S. erubescens* and *S. hostii* × *aucuparia*. Approximate numbers, however, could be estimated and their ploidywas assessed (Figure2).Chromosome counts for *S. sudetica* indicated polyploidy as the numbers were more than the diploid number of 34.Chromosome counts for the putative F1 hybrid *S. hostii*× *aucuparia* were at least close to the triploid chromosome number 2n=51 and the counts for *S. erubescens*were close to the tetraploid chromosome number 2n=68.Two supernumerary genomic components, or B chromosomes, were detected in*S. hostii*.

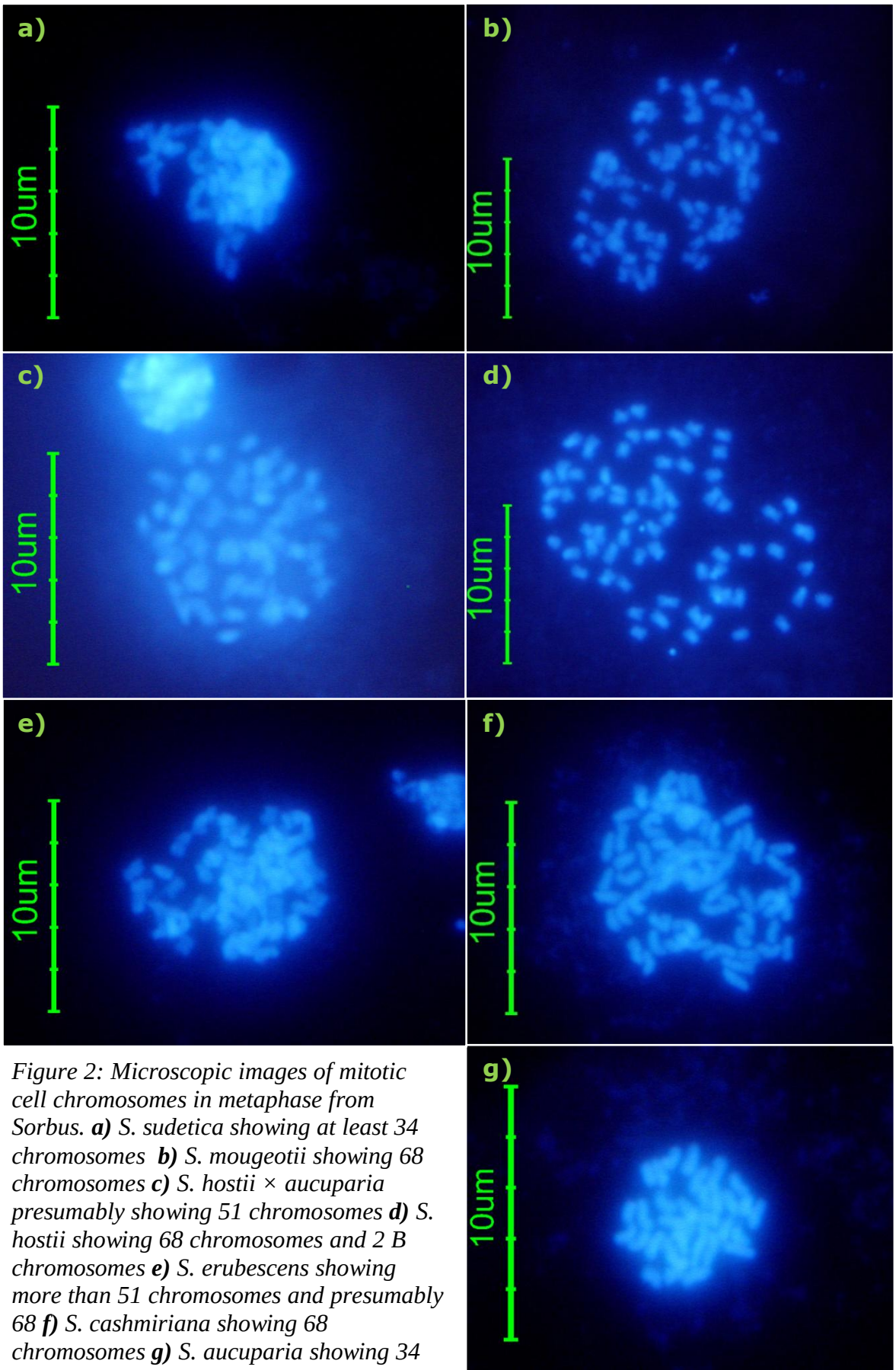

Figure 2: Microscopic images of mitotic cell chromosomes in metaphase from Sorbus. ***a)*** *S. sudetica showing at least 34 chromosomes* ***b)*** *S. mougeotii showing 68 chromosomes* ***c)*** *S. hostii × aucuparia presumably showing 51 chromosomes* ***d)*** *S. hostii showing 68 chromosomes and 2 B chromosomes* ***e)*** *S. erubescens showing more than 51 chromosomes and presumably 68* ***f)*** *S. cashmiriana showing 68 chromosomes* ***g)*** *S. aucuparia showing 34 chromosomes.*

2.4 Discussion

2.4.1 *Sorbuschamaemespilus* and *S. sudetica*

Sorbuschamaemespilus is native to the mountains in central Europe. It can be found in the Alps, the Pyrenees, the Jura Mountains, the Vosges Mountains, the Mountains of the Balkan Peninsula, and the Apennine Mountains (Meyer &Schuwerk, 2000). Chromosome counts have indicated that the species is diploid, but triploids and tetraploids have also been reported (Liljefors, 1955; Pogan*et al*., 1985). Liljefors (1955) argued from morphology and cytological data that autotriploidy could be found in *S. chamaemespilus*.*Sorbuschamaemespilus* was originally included in this study but no cells in metaphase could be found for chromosome counting.

Some degree of uncertainty surrounds the definition of *Sorbussudetica*. *Flora Europaea* (Warburg &Karpati, 1968) and many others (*e.g.*Meyer &Schuwerk, 2000; Nelson-Jones *et al*., 2002) state it to be allotetraploid with its genome components from *S. chamaemespilus* and *S. aria*. In *Flora Europaea*(Warburg &Karpati, 1968), the morphological description, however, does not describe such a putative hybrid. Minor deviants from *S. chamaemespilus*are described and no other hybrids of *S. chamaemespilus* are listed. *Sorbussudetica* is said to be found in the Sudety and Carpathian Mountains, where *S. chamaemespilus*is not known to be found (Meyer &Schuwerk, 2000). In fact, comparatively low genetic diversity among individuals of a species stated as *S. chamaemespilus* was found in the Carpathian Mountains, indicating apomictic reproduction and thus hybridogenous origin or autopolyploidy(Gömöry&Krajmerová, 2008). Some authors cite Liljefors (1955) (*e.g.* Nelson-Jones *et al*., 2002) for the allopolyploidy of *S. sudetica*, although his comprehensive work does not discuss *S. sudetica* or any hybridogenous species with *S. chamaemespilus* as parental species.The present study indicates that the accession of *S. sudetica* from the Reykjavík Botanic Garden is polyploid and although it is not the scope of this study to investigate upon the genomic makeup of the plant it is rather difficult to imagine that the genome of *S. aria* constitutes half of its genome. As the plant of *S. sudetica* is morphologically very similar to *S. chamaemespilus* (except for somewhat larger leaves and more tomentose underside of leaves) it should be investigated further if it is a polyploid form of *S. chamaemespilus* and if indeed a correct name is applied to it.

2.4.2 *Sorbusmougeotii*

Sorbusmougeotii is native to the Western Alps, Jura Mountains and the Pyrenees. This study indicated that it is a tetraploid species and thus confirms other researchers' findings (Liljefors, 1955; Pellicer*et al*., 2012).It has been suggested that its genome components are of *S. aria* and *S. aucuparia* (AAAB) (Liljefors, 1955; Nelson-Jones*et al*., 2002).

2.4.3 *Sorbushostii*and S. erubescens

Sorbushostii has been in cultivation since at least 1820 (Rehder, 1947) and in Iceland at least since 1886 (Schierbeck, 1886). Information on the species is scant but in horticultural guidebooks and manuals it is stated to be a hybridogenous species found native in the Western Alps and the Jura Mountains, co-occurring with the putative parental species, *S. chamaemespilus* and *S. mougeotii*(Rehder, 1947; Krüssmann, 1986).Since this study found *S. hostii* to be a tetraploid species, either unreduced gametes of *S. chamaemespilus* must have been involved, or reticulate events of hybridization must have taken place, probably involving unreduced gametes of unknown triploid species. In cultivation, *S. hostii* has been suggested to be facultative apomictic as half of the seedlings prove to be runts with weak growth (variation in leaf form can be observed) whereas the other half is like the mother species (Ólafur S. Njálsson, personal communication, November 26, 2013).Since *S. hostii* is partly sexual, hybrids with *S. aucuparia*are occasionally identified and one of those was investigated in this study. Although chromosome spread was not good enough for exact counting the F1 hybrid was inferred to be triploid (2n=51). The triploidy of the hybrid supports the findings of the tetraploidy in *S. hostii*, as triploidywould be expected when a diploid species is crossed with a tetraploid species.

In addition to the 68 chromosomes detected in *Sorbushostii*, two smaller B chromosomes were observed in all cells investigated. B chromosomes are supernumerary and dispensible components of the genome in numerous species of plants, fungi and animals. Assessment of their frequencies among angiosperms has shown that B chromosomes exist in more than 150 species of flowering plants distributed over a wide range of families (Stebbins, 1971). Later reviews reported approximately 4% of investigated species had B chromosomes reported in their genomes (Levin *et al*., 2005) andsome estimates have been as high as 10-15% (Jones, 1995). Their distribution among the angiosperm families is quite heterogeneous. In most families, however, there have been few or no reports of species with B chromosomes (Jones, 1995; Levin *et al*., 2005). InRosaceaeB chromosomes have

been reported for *Rosa* spp., *Prunuslaurocerasus* and *Maluspumila* (Meurman, 1929; Chaudary & Mehra, 1975; Lata, 1982).

B chromosomes do not pair with any of the standardA chromosomes at meiosis, have irregular modes of inheritance and vary in copy number among members of the same species (Jones, 1995& 2012). For the most part, they lack coding sequences, but may have a physiological impact on their carriers by affecting gene regulation (Houben*et al.*, 2014). Several possible scenarios have been put forward for their origin but the most accepted is that they are derived from the complementing A chromosomes. A model has been put forward that suggests stepwise evolution of B chromosomes after segmental genome duplication followed by the capture of additional A-derived and organellar sequences and amplification of B-specific repeats. This model predicts that B chromosomes occur primarily in taxa with elevated levels of chromosomal rearrangements and phylogenetic groups with unstable chromosome numbers (Martis*et al*., 2012).Although no conclusions can be drawn from the presence of B chromosomes in the sampled individual of *Sorbus hostii,* the suggested hybridization events leading to the formation of the species probably caused a plethora of genome rearrangements and provided the platform for B chromosomes to emerge. Furthermore, and most interestingly, B chromosomes may have a significant role in the diploidization process in the newly formed alloploids and hybrid species (Jones, 2012).

Information on *Sorbuserubescens*is even more scant than for*S. hostii* andits status as a species can be questioned.Richen (1898) states it as a synonym for *S. sudetica,* being of hybridogenous origin having *S. aria* and *S. chamaemespilus* as parents.Morphologically it is more intermediate between *S. aria* and *S. chamaemespilus* than the plant of*S. sudetica*included in this study. Being a small tree in stature, it has gained some popularity in cultivation and has proved to be apomictic when propagated by seed (Ólafur S. Njálsson, personal communication, November 26, 2013). This study could not assess the chromosome number for the accession with any certainty but most likely it is a tetraploid.

2.4.4 *Sorbusaucuparia*

Sorbusaucuparia is a variable sexual diploid (Löve&Löve 1956&1975) and occurs naturally in Iceland, Madeira and North Africa across Europe, Central and northern Asia to Kamchatka (McAllister, 2005).The plant investigated in this study came thus from one of the westernmost populations of this species in the Westfjords of Iceland. This study confirms that *S. aucuparia* is a diploid species.

2.4.5 *Sorbuscashmiriana*

Sorbuscashmiriana is an apomictic tetraploid microspecies from Kashmir and has been known to cultivation since the early 1930s (McAllister, 2005). This study confirms that this species is tetraploid, with 68 chromosomes. Several other microspecies share characters with *S. cashmiriana* not found elsewhere in the genus, such as large flowers and large, very soft fruits with large and fleshy calyx lobes. These species have all been found to be tetraploid and no related diploid species have yet been found (McAllister, 2005).

2.5 Conclusion

Chromosome counts were assessed for six species and one sexual hybrid of the genus *Sorbus*. Earlier chromosome counts for the species *S. mougeotii* (2n=68), *S. aucuparia* (2n=34) and *S. cashmiriana* (2n=68) were confirmed. Although the systematic status of the species *S. hostii* is uncertain, it is well known to cultivation in Europe and it appears to be maintained clonally through apomictic reproduction. This study assessed *S.hostii* as a tetraploid (2n=68) species and further evidence was provided when its hybrid with *S. aucuparia* (2n=34) was investigated. The hybrid appeared to be triploid, as would be expected when a tetraploid species were crossed with a diploid species. Two supernumerary elements, or B chromosomes, were found among the chromosomes of *S. hostii*. In Rosaceae, B chromosomes have previously been found in cultivars of roses, apples and Cherry Laurel but no references were found for their presence in a species of the genus *Sorbuss.l.* Two species of uncertain status, *S. sudetica* and *S. erubescens* were investigated and they proved to be polyploid. Chromosome numbers, however, for these two species could not be accurately assessed since the spreads of chromosomes on the microscopic slides were insufficient.

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