Norwegian University of Life Sciences
Department of Ecology and
Natural Resource Management

Master Thesis 2015
60 credits

Flower colour variation, pollination biology and population ecology in *Dactylorhiza sambucina*

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Abstract

The dimorph *D. sambucina* grow only yellow in Norway, and comes in a range of colour gradients from yellow to red elsewhere in Europe. DNA analysis of leaf tissue could not map population structures at Norwegian locations due to unreproducible AFLP's retrieved from external laboratory. Germination experiments were not successful due to incorrect protocol or laboratory procedures, but seeds survived and developed into seedlings. Colour density of conspecifics seem to be more important than the colour and colour proportions of co-flowering species in regenerating the monochrome Norwegian populations. In Norway *Bombus lapidarius* is the main pollinator and *D. sambucina x D. fuchsii* hybrids are found intermixed in these populations.

Keywords

D. sambucina, AFLP's, pollinator, colour, hybrids, co-flowering, inventory, germination.

Introduction

Plant flowers show a great variation in colours, shapes, sizes and fragrances. These characters have been interpreted as adaptations that attracts specific pollinators (Fægri & van der Pijl, 1980). However, there are increasing evidence that pollinator behaviour might not be directly involved in the maintenance of colour polymorphism (Jones & Reithel, 2001; Morgan & Schoen, 1997; Schemske & Bierzychudek, 2001).

In general orchids depend upon insects or rarely birds for pollination, and offer their visitors different kinds of rewards like oil, floral fragrances and, most frequently, floral nectar (Arditti, 1992; Dressler, 1993; van der Cingel, 1995; van der Pijl & Dodson, 1966). Some taxa do not offer reward to their pollinators, but lure insects by mimicking a food model blooming at the same time (Dafni, 1984, 1986; Johnson, 2000) or by displaying attractive flowers. Generalized food mimics often bloom in dense populations, early in the season, and are polymorphic, for example in corolla colour (Nilsson, 1992).

It has been suggested that the high levels of intraspecific floral diversity observed in some species of orchids may be related to their deceptive system of pollination in which orchids offer no rewards to their pollinators, nor mimic any specific rewarding-producing species (Ackerman & Galarzaperez, 1991; Dafni, 1984; Heinrich, 1975; Nilsson, 1980). The early blooming and the display of polymorphic deceptive flowers may increase their ability to attract naive pollinators. It has been hypothesised that the ability to deceive is increased by

this variability in colour, because it takes pollinators longer to discriminate the morphs, and thus they make more mistakes in avoiding the species (Heinrich, 1975; Petterson & Nilsson, 1983).

Rewardless orchids seems to be pollinated by naive insects in Europe, mainly bumblebee queens (Kropf & Renner, 2005; Nilsson, 1980). Because of its prominent floral morphology and excretion of highly fragrant terpenes bumblebees are tricked a couple of times before they learn (Nordal & Wischmann, 1987). Bumblebees tend to switch between colour morphs when not rewarded with nectar. This behaviour results in overvisiting of rare colour morphs (Smithson & Macnair, 1997).

D. sambucina is a common rewardless European terrestrial orchid with a boreal-alpine distribution (Tutin et al., 1980), and normally this species prefers rich soils (pH<5.0), but may also grow on dry, acidic, and nutrient-poor siliceous soils as long as the meadows are open with a lot of light (Kropf & Renner, 2005). *D. sambucina* is generally diploid with 2n = 40 (exceptionally 2n = 42) chromosomes (Pedersen, 2006). The Norwegian populations represent the outermost northwest area of distribution, and studies here conclude that *D. sambucina* demands moderately nutrient soils, and is definitely not a calcium demanding species (Nordal & Wischmann, 1987). *D. sambucina* is blooming in the middle of May and early June in Norwegian populations (Nordal & Wischmann, 1987). This early in the season the conditions for the plants are wet from snowmelt in the mountains, but still fairly hot and much light where *D. sambucina* grow (Norderhaug, Bakkevik, & Skogen, 1997). *D. sambucina* has a symbiotic relationship with fungus. One of these fungus are *Rhizotonia sp*. (Marchisio, Berta, Fontana, & Mannina, 1985).

Studies of *D. sambucina* reveals that the yellow colour morph attracts more pollinators (a higher male reproduction success - RS) than red and pink morphs, and that the pink colour morph makes less viable seeds (lower female RS) than yellow and red morphs , but fails to show a significant difference in both male and female RS for all colour morphs (Pellegrino, Bellusci, & Musacchio, 2005). Pellegrino's studies shows a post-pollination barrier in *D. sambucina*, but the involvement of pollinators are not sufficient to explain the maintenance of colour polymorphism in this species and the loss of a morph could be entirely due to genetic drift in small populations (Kropf & Renner, 2005; Smithson, Juillet, Macnair, & Gigord, 2007).

(Gigord, Macnair, & Smithson, 2001) found the highest pollinia export, receipt, and fruit set in mixed populations, and that negative frequency-dependent selection might be a mechanism that maintains colour polymorphism in *D. sambucina*. Pellegrino, Caimi, Noce, & Musacchio (2005) found opposing results indicating that the contribution to the total reproductive success deriving from the two colour morphs does not conform with the predictions of negative-dependent selection. Only individual plant height and population density had a positive effect on pollen export, and pollination experiments confirm that *D. sambucina* is non-agamospermous, incapable of automatic selfing, self-compatible, and pollinator-limited (Kropf & Renner, 2005). The mean frequency of yellow-flowered *D. sambucina* across natural populations in Europe is 53% ± 2.6 (n = 174), and the two colour morphs are identical for floral scent (Nilsson, 1980).

Tamm (1972) found that *D. sambucina* individuals grow very old and have some vegetative reproducibility, and is reproducing and maintained just to a small degree by seeds. Drainage conditions may be vital for the species (Mattiasson, 1986), and it also seems that trampling and summer drought favours *D. sambucina* to re-establish and maintain viable populations (Pettersson, 1958).

Reproductive success of food-deceptive orchids may be affected by interactions with co-flowering rewarding species (Pellegrino, Bellusci, & Musacchio, 2008). An important co-flowering nectar provider for *D. sambucina* in West German populations is the purple *Lamium purpureum* (Kropf & Renner, 2005). We do not find many purple flowers this early in the summer in Norwegian populations, and *L. purpureum* is not common in the area where *D. sambucina* grow. Instead there are mostly yellow and blue coloured co-flowering species, and very few purple ones (Norderhaug et al., 1997). This early in the season when *D. sambucina* is blooming (May), they have few co-flowering species at all in their open and dry patches where they grow in Norway (pers. obsv.).

In western part of Germany (Kropf & Renner, 2005) and in Norway there are populations of exclusively monochrome yellow coloured *D. sambucina* (Lid & Lid, 2007). In Norway the red morph is totally lacking and its closest dimorphic populations are found on the east coast of Sweden (Lid & Lid, 2007).

This study aims to find causes for lack of flower colour polymorphism in Norwegian *D. sambucina* populations by studying DNA for the expressed colour and how they interact with

the fitness of the Norwegian yellow colour-morph of this species (Gaskett, 2011). To assess these questions I have tested three hypothesis:

1. The allele coding for red colour is missing or silenced in the Norwegian *D. sambucina* populations.

2. *D. sambucina* seed production depends on co-flowering colourmorphs of other species.

3. Population density variations cause differences in seed production rates in Norwegian *D. sambucina* populations.

Revealing more knowledge of colour morphology through DNA and function of density, co-flowering colourmorphs, fitness, and frequency-dependent reproduction success in this species might give new information in how to maintain and monitor this species for future survival in their natural habitat in Norway.

Materials and Methods

Study and sampling area

In May 2012, four populations of *D. sambucina* were sampled in Telemark, Norway. In May 2013, one population was sampled at Stora Karlsö on the island of Gotland in Sweden and one population was sampled in the Sila Mountains in Italy.

Telemark, Norway.

Protected species. Permit obtained from Direktoratet for Naturforvaltning.

Hjartdal - Bøllås, elevation 550-575 m (>1000 individuals distributed over an area of 400 m^2): **N** 6623677 / **E** 138796 (UTM 33 N).

Seljord - Blika, elevation 550-600 m (>1000 individuals distributed over of 400 m^2): **N** 6623566 / **E** 136923 (UTM 33 N).

Seljord - Brekkegrend, elevation 400-425 m (>1000 individuals distributed over 300 m^2): **N** 6606179 / **E** 153048 (UTM 33 N).

Tokke - Eidsborg, elevation 450-500 m (>1000 individuals distributed over 200 m^2): **N** 6611364 / **E** 104179 (UTM 33 N).

All Norwegian locations were former pasture fields and abandoned for the last two decades, but still the grass is cut and removed to manage and maintain the cultural diversity of plants in the field. All locations are mainly slopes facing south-southwest.

The bedrock at the study sites in Norway consisted of gneisses and quartzite, impermeable hard bedrocks, with strands of amphibolite, rhyolite and feldspar that give a slightly calcareous soil (pH 4.4 - 6.0). The four populations of *D. sambucina* were studied between April and June 2012, and between May and July 2013.

Gotland, Sweden.

Protected species. Permit obtained from Länsstyrelsen Gotlands Län.

Stora Karlsö – Gotland, elevation 30 – 40 m (>1000 individuals distributed over an area of 500 m^2): **N** 6352776 / **E** 679689 (UTM 33 N).

The location were mainly on sheep pasture and a gentle slope slightly facing south – southeast, but otherwise relatively flat terrain.

The bedrock at the Swedish site was mainly calcareous consisting of fossil coral reef.

Sila Grande, Calabria, Italy.

Species categorized as least concern (LC) on the 2011-2014 IUCN Red List in Europe and thus not protected in Italy other than in certain protected areas.
Centro sci di fondo "Carlo Magno"– Silvana Mansio – Consenza, elevation 1450 – 1470 m (>1000 individuals distributed over an area of 500 m^2): **N** 4349283 / **E** 632511 (UTM 33 N).

The location was on pastureland and on a gentle slope slightly facing north but in otherwise relatively flat terrain.

The bedrock in Sila Grande, Italy consisted mainly of limestone.

All samples were randomly chosen for each population at the sites described. Sites and populations were chosen to secure a large number of individuals in each population to retrieve random samples.

Sampling and DNA test

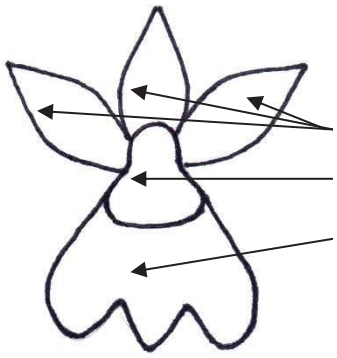
To measure the inter- and intra-genetic variation and phylogenetic relationships, a total of 210 samples consisting of about one cm^2 tissue from young leaves were collected in Norway, 80 samples in Sweden, and 60 samples in Italy, and put into plastic bags with silica gel for drying (Hedren, Fay, & Chase, 2001). First 10 preliminary samples were sent for AFLP analysis in September 2012 and later 200 samples including five repeats were sent in September 2013, all samples sent to Ecogenics in Zurich-Schlieren, Switzerland. This lab also did DNA extraction.

Genomic DNA was digested with the restriction enzymes EcoRI and MseI and ligated with restriction-site specific adaptors to allow subsequent PCR amplification of a subset of fragments with selective primers, binding partially to the adaptor and partially within the restriction fragment. In a second round of PCR amplification with more selective primer combinations, EcoRI site-specific primers labelled with a fluorophore were used. Generated PCR fragments were separated by capillary electrophoresis (CE) on an Applied Biosystems 3730 DNA Analyser.

Colour modelling

To find the proportions of the different colour morphs in each population at the sites in Sweden and Italy, a flower colour-scoring model was constructed, and 60 individual plants from Stora Karlsö and 51 individual plants from Silvana Mansio were scored for their colour gradients. The model were constructed to group the different colourmorphs into separate colour classes by combining code letters for each colour grouping part of the flower.

Flower model of *D. sambucina*.



Positions:

1. Tepals
2. Column and upper labellum
3. Lower labellum

Colour codes

y = yellow, n = pink, r = red, l = purple

Figure 1. Flower model of *D. sambucina.*

Pollinator studies

The observation of potential pollinators of *D. sambucina* was done throughout all fieldwork at all sites, and documented by using a Panasonic Lumix DMC-TZ30 pocket camera. Video recording by the use of two GoPro Hero 3 cameras, one of model white and one of model silver, on tripods were done in June 2013 for documenting all species in the field plots, and possibly detecting actions of potential pollinators in Norway only.

Field inventory, species frequencies and reproduction

To investigate if frequencies of *D. sambucina* or co-flowering species of certain colours influenced the reproduction of the orchids a field experiment was performed. Three Norwegian populations in Telemark were studied in June 2013 for this purpose, and revisited in July the same year to register and collect seed capsules. These studies were performed at Bøllås in the municipality of Hjartdal, at Blika in Svartdal and at Todalen in Brekkegrend, the latter two located in the municipality of Seljord. The experimental design included in total 90 one by one meter square plots randomly chosen in each population, 30 at each of the locations. Photos were taken of all plots for control of the inventories. The inventories of the three locations were listed in an excel sheet. Frequencies of *D. sambucina* and co-flowering species were also listed in excel sheets for each location.

Seed sampling and germination tests

Background Information

Orchid seeds germinate in nature under suitable conditions with the assistance of specific fungi (Rasmussen, Andersen, & Johansen, 1990). The natural symbiotic fungi serve at least two functions. 1) Support necessary nutrients to the embryo at germination stage 2) Kills off any other fungi or organisms that will harm the seeds germinating.

Germinating orchid seeds *in vitro* in the lab can be done for certain orchid species by mimicking natural conditions by making an agar with all ideal nutrients in the correct amounts. The difficulty using the symbiotic fungi in the lab can be substituted by sterile

conditions. Under such conditions, most orchid seeds will germinate successfully without its natural symbiotic fungi.

The Orchimax agar is a nutrient rich media used for several orchid genera, and is suitable for most Dactylorhiza species. Orchimax contains all macro- and micro-nutrients in ideal amounts as well as activated charcoal to stabilize pH and the necessary nitrogen source needed. Without any additives the ready blended agar will have the ideal pH of 5.8 -6.2. Seeds are applied on top of the agar and the semi-solid agar makes support for the roots of the germinating embryos.

Seed sampling were done in Telemark, Norway in July 2013 at the same plots and populations as investigated for colour studies in June to investigate reproduction of *D. sambucina*. Numbers of seed capsules for each individual for each plot at the three locations in Telemark were noted in excel sheets. Seed sampling were also done at Stora Karlsö, Sweden in July and seeds from Sila Grande, Italy was provided by Guiseppe Pellegrino at the Unical University of Rende, Calabria, in Italy and sent by mail the same week as sampled. Germination tests were done ex situ at the INA ecology lab at UMB ÅS, Norway following an *in vitro* protocol I made for making agar out of an Orchimax Orchid maintenance medium (Duchefa catalogue 2010 – 2012). The germination protocol for photoperiods follows (Rasmussen H., 1990).

Protocol to make 1000mL of Orchimax agar (makes about 50 Orchimax agar plates):

1. Weigh out the following into a 1L Erlenmeyer flask: 5.5g Plant agar, 27.8g Orchimax from Duchefa, and (dH2O) to 1000mL.

> Note: If your laboratory has pre-mixed Orchimax agar powder, use the suggested amount instead of the other dry ingredients above.

2. Swirl to mix, the contents do not have to be completely in solution, but any powder left on the sides of the flask will caramelize on the glass during autoclaving.

3. Cover the top of the flask with aluminium foil and label with autoclave tape.

4. Autoclave the liquid for 20 minutes or according to your autoclave's specifications.

5. After removing the solution from the autoclave, allow the agar solution to cool to 55°C.

> Note: This can be done by placing the flask in a 55°C oven or water bath, as this will hold the temperature, and it can be left unattended for some time.

6. When pouring plates, keep your bench area sterile by working near a flame or Bunsen burner.

7. Pour ~20mL of Orchimax agar per 10cm polystyrene Petri dish.

> Note: Pour slowly from the flask into the centre of the petri dish. When the agar has spread to cover about 2/3 of the dish stop pouring and the agar should spread to cover the entire plate. You may need to tilt the plate slightly to get the agar to spread out completely. If you pour in too much, the plate will be fine, but it will reduce the number of plates you can make per batch.

> Note: If bubbles are introduced during the pouring, these can be removed by quickly passing the flame of an inverted Bunsen burner over the surface of the plate. Be careful, if you leave the flame too long, it will melt the petri dish.

9. Place the lids on the plates and allow them to cool for 30-60 minutes (until solidified) then invert the plates. Let sit for several more hours or overnight.

10. Label the bottom of plates with antibiotic and date and store in plastic bags or sealed with para-film at 4°C.

For sowing, and germination of *D. sambucina* in germination chamber, I used a protocol based on germination of *D. majalis* by (Rasmussen H., 1990).

Protocol to treat seeds of *D. sambucina* and how to sow them in Orchimax agar

Before sowing, the seeds need a treatment to sterilize and disturb the surface enough to germinate.

1. Crack the seed capsule open using a tweezer into a small strainer (a tea strainer will do) over a small glass beaker (50 ml).
2. Sterilize the seeds by pouring 5 ml NaOCl 10 % into the beaker, cover with a lid and shake the beaker to rinse the seeds.
3. Filter the seeds on a water resistant filter in a glass funnel into a sterilized beaker.
4. Filter once again using ddH_2O to rinse the seeds for NaOCl.
5. Rub the filter gently on to the agar surface of the petri dish.
6. Cover the petri dish with a lid and wrap with plastic foil (para-film).
7. Keep the petri dishes in a germinating chamber at room temperature using the following protocol for germinating *D. majalis* seeds. The seeds should germinate within 2 months.

Perform prickling and sow into perlite/seramis.

Protocol to germinate *D. sambucina* seeds in a germinating chamber

1. Put all petri dishes on racks and place the racks into slots in the germinating chamber.
2. Start the incubation by programing the chambers software to 6 days of constant darkness at optimum temperature of 20°C with following interruption of two consecutive photoperiods before day 8 and not later.
3. Start 14 days of 16h photoperiods on day 7 using intensity 4 (corresponds to ca. 11 Wm^{-2}) at 24°C, ending on day 21.

The seeds were considered to have germinated if the testa was ruptured and/or if rhizoids had developed (Rasmussen H., 1990).

Results

DNA test

The preliminary test results based on ten tissue samples (Table 2) collected at the three locations in Norway in June 2012 were done at the Ecogenics laboratory in Switzerland. Further analyses of these results revealed that some primer combinations (Table 1) produced too little information of interest and were discarded before the main lot of 200 samples were analysed in 2013 at the same laboratory in Switzerland.

Table 1. Primer combinations showing number of bands found for each combination.

	E32	E33	E35	E36	E37	E38
M47	5	10	6	8	65	11
M48	5	79	12	7	37	30
M49	4	62	19	15	67	33
M50	failure	48	6	32	62	23

Table 2. Names of preliminary genomic samples identifying origin. Red samples are *D. fuchsii*, the rest are yellow *D. sambucina* samples.

Name	Short name
BRP-1A BLIKA RED	Ds_01
BRP-2B BLIKA RED	Ds_02
B-1 BLIKA	Ds_03
B-2 BLIKA	Ds_04
D1 BERGE	Ds_05
D2 BERGE	Ds_06
A-1 BOLLAS	Ds_07
A-2 BOLLAS	Ds_08
C-1 TODALEN	Ds_09
C-2 TODALEN	Ds_10

Eight *D. sambucina* (two from each location) and two *D. fuchsii* (mixed into a large *D. sambucina* population) were analysed with 24 AFLP primer combinations for finding the

most informative AFLP's (Tables 1 – 2). These preliminary data were analysed with GenAlEx (Tutorial A. see appendix) generating a PCoA of 640 AFLP's (Figure 2), SplitsTree4 (Tutorial B. see appendix) constructing a Neighbour-Join out of 179 AFLP's (Figure 3), and Structure (not shown).

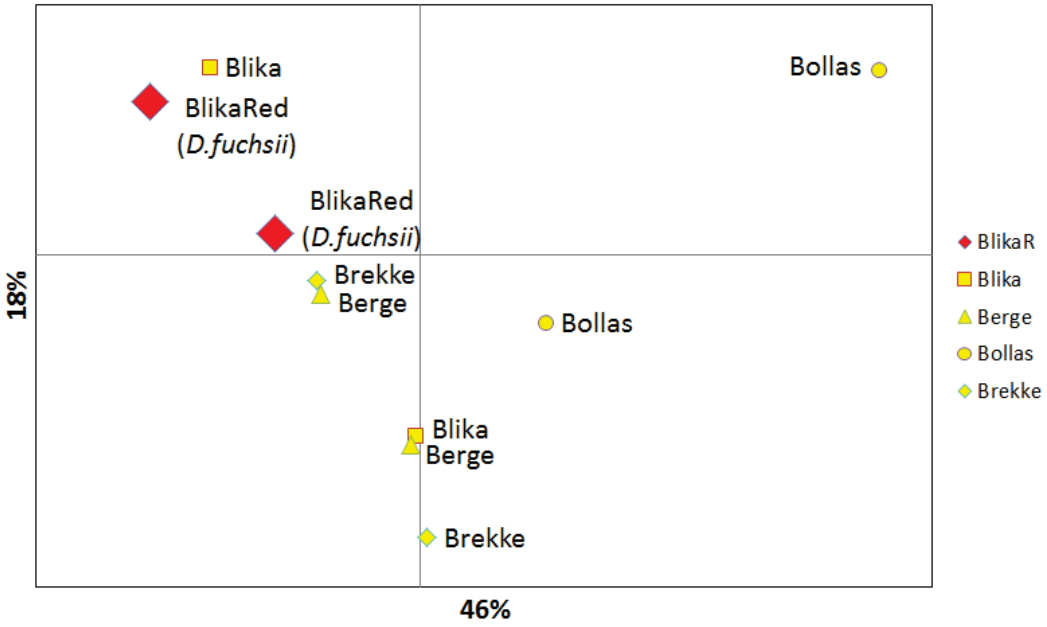


Figure 2. PCoA with 10 plants and 640 AFLP's.

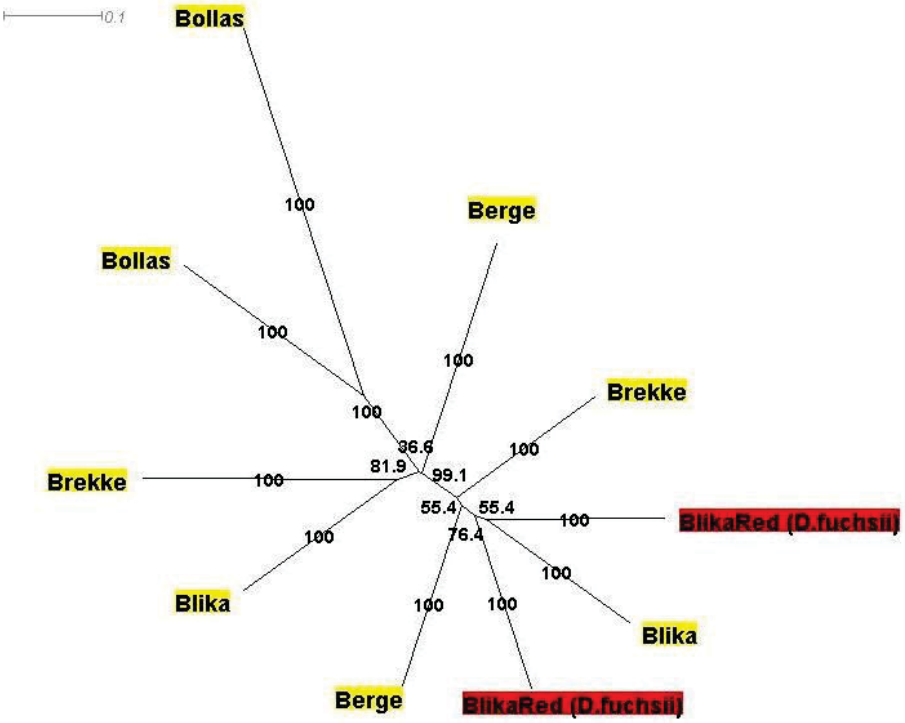


Figure 3. Neighbour-Join with 10 plants and 179 AFLP's.

The preliminary results does not show a clear differentiation of *D. fuchsii* from *D. sambucina* as one of the yellow orchids from Blika interfere the expected results (Figure 2 - 3).

Table 3. Tissue samples and IDs from seven populations in Norway, Sweden and Italy.

25.06.13 LAB INA ÅS: Dactylorhiza sambucina. Labeling of samples forDNA-analyses at Ecogenics.																				
BØLLÅS			BLIKA			BREKKE			BERGE			KARLSØ-Y			KARLSØY-R			KARLSØ-I		
A3	=	001	B3	=	018	C3	=	035	D3	=	052	M1	=	069	L1	=	086	N1	=	103
A4	=	002	B4	=	019	C4	=	036	D4	=	053	M2	=	070	L2	=	087	N2	=	104
A5	=	003	B5	=	020	C5	=	037	D5	=	054	M3	=	071	L3	=	088	N3	=	105
A6	=	004	B6	=	021	C6	=	038	D6	=	055	M4	=	072	L4	=	089	N4	=	106
A7	=	005	B7	=	022	C7	=	039	D7	=	056	M5	=	073	L5	=	090	N5	=	107
A8	=	006	B8	=	023	C8	=	040	D8	=	057	M6	=	074	L6	=	091	N6	=	108
A9	=	007	B9	=	024	C9	=	041	D9	=	058	M7	=	075	L7	=	092	N7	=	109
A10	=	008	B10	=	025	C10	=	042	D10	=	059	M8	=	076	L8	=	093	N8	=	110
A11	=	009	B11	=	026	C11	=	043	D11	=	060	M9	=	077	L9	=	094	N9	=	111
A12	=	010	B12	=	027	C12	=	044	D12	=	061	M10	=	078	L10	=	095	N10	=	112
A13	=	011	B13	=	028	C13	=	045	D13	=	062	M11	=	079	L11	=	096	N11	=	113
A14	=	012	B14	=	029	C14	=	046	D14	=	063	M12	=	080	L12	=	097	N12	=	114
A15	=	013	B15	=	030	C15	=	047	D15	=	064	M13	=	081	L13	=	098	N13	=	115
A16	=	014	B16	=	031	C16	=	048	D16	=	065	M14	=	082	L14	=	099	N14	=	116
A17	=	015	B17	=	032	C17	=	049	D17	=	066	M15	=	083	L15	=	100	N15	=	117
A18	=	016	B18	=	033	C18	=	050	D18	=	067	M16	=	084	L16	=	101	N16	=	118
A19	=	017	B19	=	034	C19	=	051	D19	=	068	M17	=	085	L17	=	102	N17	=	119
									CONTROLS			CONTROLS			CONTROLS			CONTROLS		
SILA-Y			SILA-R			SILA-I			RED FUCHSII			RED FUCHSII			SILA MASCUL			REPEATS		
G1	=	120	I-1	=	137	H1	=	154	BRP1A	=	171	BER1	=	174	K1	=	182	069	=	196
G2	=	121	I-2	=	138	H2	=	155	BRP2B	=	172	BER2	=	175	K2	=	183	113	=	197
G3	=	122	I-3	=	139	H3	=	156	BRP3B	=	173	BER3	=	176	K3	=	184	167	=	198
G4	=	123	I-4	=	140	H4	=	157				UPP1		177	K4	=	185	075	=	199
G5	=	124	I-5	=	141	H5	=	158				UPP2		178	K5	=	186	013	=	200
G6	=	125	I-6	=	142	H6	=	159				UPP3		179	K6	=	187			
G7	=	126	I-7	=	143	H7	=	160				UPP4		180	K7	=	188			
G8	=	127	I-8	=	144	H8	=	161				UPP5		181	K8	=	189			
G9	=	128	I-9	=	145	H9	=	162							K9	=	190			
G10	=	129	I-10	=	146	H10	=	163	B=BLIKA			BE=BERUM			K10	=	191			
G11	=	130	I-11	=	147	H11	=	164				UPP=UPPLAND			K11	=	192			
G12	=	131	I-12	=	148	H12	=	165							K12	=	193			
G13	=	132	I-13	=	149	H13	=	166							K13	=	194			
G14	=	133	I-14	=	150	H14	=	167							K14	=	195			
G15	=	134	I-15	=	151	H15	=	168												
G16	=	135	I-16	=	152	H16	=	169												

These preliminary data was supposed to be extended by including seven populations each represented by 40 lines and typical unrelated *D. fuchsii* controls (Table 3). These laboratory results failed to be reproduced at the Ecogenics laboratory in Switzerland in 2013 (Table 7).

Table 4. Enzymes and primer names with sequences used for AFLP tests of the 200 tissue samples from all sites in Norway, Sweden and Italy in June 2013.

Name	Primer sequence 5'-3' (selective bases)	Fluorescence label	Restriction site specificity	Purpose
EcoRI_A	GACTGCGTACCAATTCA	-	*EcoRI*	Used for 1st round of PCR
MseI_C	GATGAGTCCTGAGTAAC	-	*MseI*	
E32	GACTGCGTACCAATTCAAC	FAM	*EcoRI*	Used for 2nd round of PCR together with various *MseI*-specific primers
E33	GACTGCGTACCAATTCAAG	ATTO550	*EcoRI*	
E35	GACTGCGTACCAATTCACA	ATTO565	*EcoRI*	
E36	GACTGCGTACCAATTCACC	FAM	*EcoRI*	
E37	GACTGCGTACCAATTCACG	ATTO550	*EcoRI*	
E38	GACTGCGTACCAATTCACT	ATTO565	*EcoRI*	
M47	GATGAGTCCTGAGTAACAA	-	*MseI*	Used for 2nd round of PCR together with various *EcoRI*-specific primers
M48	GATGAGTCCTGAGTAACAC	-	*MseI*	
M49	GATGAGTCCTGAGTAACAG	-	*MseI*	
M50	GATGAGTCCTGAGTAACAT	-	*MseI*	

Table 5. The primer combinations used for the DNA analyses of the 200 tissue samples from all sites and locations in June 2013, showing potential interesting information.

E32_M48_blue	1 out of 5 bands
E33_M48_yellow	1 out of 79 bands
E33_M49_yellow	1 out of 62 bands
E33_M50_yellow	1 out of 46 bands
E36_M49_blue	2 out of 15 bands
E36_M50_blue	1 out of 32 bands
E37_M50_yellow	2 out of 62 bands
E38_M49_red	1 out of 33 bands

To find the primer combinations that worked for *D. sambucina*, the results of the analyses were scored for significant differences in band signals between the red *D. fuchsii* and the yellow *D. sambucina* morphs. Out of the 24 primer combinations (Table 1) in the preliminary analyses in 2012, eight primer combinations (Tables 4 - 5) were found useful with some distinguishable information, and these were used for analysing the main lot of 200 samples sent to the Ecogenics laboratory in 2013, the rest failed to elicit any information of interest.

Further analyses of the results from the main 200 DNA-samples from the external laboratory in 2013 showed that six out of the eight primer combinations produced some potential interesting information (Table 6).

Table 6. Bands per primer set expressing genetic information out of 200 samples.

	BAND DISTANCE			
PRIMERSET	3 bps	2bps	1bps	0bps (All included)
E33 M49	6	10	64	350
E33 M50	1	5	37	502
E33 M48	1	7	58	492
E37 M49	3	15	56	419
E37 M50	0	4	48	421
E37 M47	3	4	21	562

Using the chosen primers (Table 6), all labelled tissue were extracted and analysed at the Eurogenics laboratory in Switzerland. The analysis this laboratory returned generated a lot of information, but produced inconsistent data when comparing control repeats with their identical originals (Table 7).

Table 7. Ten control repeats out of 200 samples sent for DNA analysis at Eurogenics.

Control repeats	G	N	AR	AX	BS	CL	CX	ES	HP
196 = 69									
Run1_Ds069.fsa	0$0	1$5354	1$10346	0$0	1$1006	0$0	0$0	0$0	1$4935
Run1_Ds196.fsa	1$1851	1$1135	1$4950	0$0	1$2439	0$0	1$6058	1$2009	1$1098
197 = 113									
Run1_Ds113.fsa	1$14574	0$0	1$29674	0$0	0$0	0$0	0$0	0$0	0$0
Run1_Ds197.fsa	0$0	0$0	1$6734	0$0	1$1318	0$0	0$0	1$3774	0$0
198 = 167									
Run1_Ds167.fsa	0$0	0$0	1$2743	0$0	0$0	1$4694	1$6581	0$0	0$0
Run1_Ds198.fsa	1$2237	0$0	1$6886	0$0	0$0	1$1350	0$0	0$0	0$0
199 = 075									
Run1_Ds075.fsa	1$4236	0$0	1$11190	1$5475	0$0	0$0	1$16042	1$10326	0$0
Run1_Ds199.fsa	1$2165	1$4854	0$0	1$1692	1$2660	1$1337	1$4981	0$0	0$0
200 = 013									
Run1_Ds013.fsa	0$0	0$0	1$22432	0$0	0$0	0$0	1$5319	1$1426	0$0
Run1_Ds200.fsa	0$0	0$0	1$4128	0$0	0$0	0$0	1$8907	1$2173	0$0

Colour studies

Table 8. Colour scoring of flower *D. sambucina* colour at Stora Karlsö in Sweden.

									Color range:		
									y=yellow		
									n=pink		
									r=red		
ID:	Photo no:	Colour code:									

		Yellow	Yellowish pink		Pink intermediates				Pinkish red		Red
		yyy	nyy	ryy	nyn	nnn	nyr	nnr	ryr	rnr	rrr
1	1080453							nnr			
2	1080459	yyy									
3	1080464	yyy									
4	1080470						nyr				
5	1080475										rrr
6	1080481							nnr			
7	1080485	yyy									
8	1080488	yyy									
9	1080507	yyy									
10	1080512									rnr	
11	1080519	yyy									
12	1080525	yyy									
13	1080533	yyy									
14	1080541									rnr	
15	1080547								ryr		
16	1080557								ryr		
17	1080569	yyy									
18	1080576	yyy									
19	1080586									rnr	
20	1080591	yyy									
21	1080596									rnr	
22	1080623				nyn						
23	1080634				nyn						
24	1080639				nyn						
25	1080650				nyn						
26	1080657				nyn						
27	1080672				nyn						
28	1080678		nyy								
29	1080686								ryr		
30	1080695								ryr		

31	1080704				nyn						
32	1080714				nyn						
33	1080721								ryr		
34	1080730									rnr	
35	1080741						nyr				
36	1080749					nnn					
37	1080760				nyn						
38	1080774				nyn						
39	1080779				nyn						
40	1080788						nyr				
41	1080802						nyr				
42	1080808								ryr		
43	1080818									rnr	
44	1080825									rnr	
45	1080830								ryr		
46	1080838								ryr		
47	1080847								ryr		
48	1080855									rnr	
49	1080862									rnr	
50	1080865								ryr		
51	1080873										rrr
52	1080880	yyy									
53	1080889	yyy									
54	1080896	yyy									
55	1080904	yyy									
56	1080913	yyy									
57	1080921	yyy									
58	1080928	yyy									
59	1080940	yyy									
60	1080949	yyy									

Table 9. Summary colour sampling at Stora Karlsö, Sweden.

Yellow	Yellowish pink		Pink intermediates				Pinkish red		Red	Totals
yyy	nyy	ryy	nyn	nnn	nyr	nnr	ryr	rnr	rrr	All colours
20	1	0	11	1	4	2	10	9	2	60
33,30 %	1,67 %	-	18,30 %	1,67 %	6,67 %	3,30 %	16,67 %	15 %	3,30 %	100 %
33,30 %	1,67 %		30 %				31,67 %		3,30 %	100 %
35 %			30 %				35 %			100 %

Table 10. Colour scoring of flower *D. sambucina* colour at Sila Grande, Italy.

Color range:
y=yellow n=pink r=red l=purple

ID:	Photo no:	Colour code:												
		Yellow	Yellowish pink		Pink intermediates				Pinkish red		Red	Purple		
		yyy	nyy	ryy	nyn	nnn	nyr	nnr	ryr	rnr	rrr	lyl	lyn	lyr
1	1060768											lyl		
2	1060781				nyn									
3	1060795	yyy												
4	1060796											lyl		
5	1060800													lyr
6	1060801				nyn									
7	1060811											lyl		
8	1060815											lyl		
9	1060818	yyy												
10	1060827											lyl		
11	1060834												lyn	
12	1040977					nnn								
13	1040981					nnn								
14	1040991						nyr							
15	1040993	yyy												
16	1050003							nnr						
17	1050060				nyn									
18	1050063				nyn									
19	1050064a	yyy												
20	1050064b					nnn								
21	1050067	yyy												
22	1050068						nyr							
23	1050069				nyn									
24	1050070					nnn								
25	1050071a				nyn									
26	1050071b				nyn									
27	1050072					nnn								
28	1050073				nyn									
29	1050075	yyy												
30	1050076									rnr				
31	1050077				nyn									
32	1050084				nyn									
33	1050088	yyy												
34	1050095						nyr							
35	1050103	yyy												
36	1050107	yyy												
37	1050113	yyy												
38	1050115						nyr							

39	1050120		nyn			
40	1050129		nyn			
41	1050132		nyn			
42	1050137			nnn		
43	1050140		nyn			
44	1050149			nnn		
45	1050152					rnr
46	1050155	yyy				
47	1050157					rnr
48	1050159		nyn			
49	1050176		nyn			
50	1050177	yyy				
51	1050182	yyy				

Table 11. Summary colour sampling at Sila Grande, Italy.

Yellow	Yellowish pink		Pink intermediates				Pinkish red		Red	Purple			Totals
yyy	nyy	ryy	nyn	nnn	nyr	nnr	ryr	rnr	rrr	lyl	lyn	lyr	All colours
13	0	0	16	7	4	1	0	3	0	5	1	1	51
25,49	-	-	31,37	13,73	7,84	1,96	-	5,88	-	9,80	1,96	1,96	100,00
25,49 %			54,90 %				5,88 %			13,73 %			100 %
13			28				3						44
29,55 %			63,64 %				6,82 %						100 %

Scoring the flowers in Sweden and Italy by using the colour scoring model grouped the colours into five main colour classes (Tables 13 – 15, see appendix) where the three main colour classes yellow, pink and red showed more evenly distributed colour frequencies at Stora Karlsö in Sweden than did in Italy (Tables 8 – 11). At Sila Grande in Italy, colours were biased towards the pink colour morph, with few red colour morphs and showing a different purple colour morph that was not found in Sweden. Photos below the tables show the colour range.

Pollinator studies

The use of video by hero cameras on tripods did not produce any results. No potential pollinators were detected this way. I managed to take some photographs of one of the naïve bumblebees, *B. lapidarius* with three pollinia from *D. sambucina* attached to its clypeus, in action in the field at Blika, Telemark, Norway (Figures 16 - 17).

Field inventory, species frequencies and reproduction

During fieldwork in May, June, and July 2013 all locations in Telemark, Norway were examined for all plant species present at the time and registered. Photos of all plots taken during field examination were studied a second time during winter 2014 to verify that all present species were registered. The registered species were listed as a table of inventory (Table 12).

Table 12. Inventory Telemark 2013.

Latin		Bøllås	Blika	Brekke	
Equisetum arvense	ssp. arvense				
Botrychium lunaria		1	1		
Pteridium aquilinum	ssp. Latiusculum				1
Woodsia ilvensis		1			
Dryopteris filix-mas					1
Phegopteris connectilis					1
Polypodium vulgare					1
Pinus sylvestris		1	1		1
Picea abies		1	1		1
Juniperus communis		1	1		1
Salix caprea		1			1
Populus tremula			1		1
Betula pubescens		1	1		1
Alnus incana					1
Ulmus glabra		1	1		1
Rumex acetosa		1	1		1
Bistorta vivipra		1	1		
Viscaria vulgaris		1	1		
Silene dioica		1	1		
Anemone nemorosa		1	1		1
Ranunculus acris		1	1		1
Cardamine pratensis	ssp. pratensis	1			
Sorbus aucuparia		1	1		1

Noccaea caerulescens	1	1	
Hylotelephium maximum		1	1
Filipendula ulmaria	1	1	1
Geum rivale	1		
Potentilla crantzii	1	1	
Potentilla erecta	1	1	1
Fragaria vesca	1		1
Rubus saxatilis	1	1	1
Rubus idaeus		1	1
Rosa mollis			1
Rosa dumalis	1	1	1
Alchemilla alpina	1	1	
Alchemilla monticola	1	1	1
Trifolium repens	1	1	1
Trifolium pratense	1	1	1
Triolium medium	1	1	1
Lotus corniculatus	1	1	1
Vicia cracca		1	1
Viccia sepium	1	1	1
Lathyrus linifolius	1	1	1
Geranium sylvaticum	1	1	1
Polygala vulgaris	1	1	1
Acer platanoides		1	1
Hyperikum maculatum	1	1	1
Viola riviniana	1	1	1
Viola palustris	1		
Viola tricolor	1	1	1
Anthriscus sylvestris	1	1	1
Pimpinella	1	1	1
Calluna vulgaris	1		1
Vaccinium myrtillus	1		1
Galium boreale		1	1
Fraxinus excelsior		1	1
Myosotis sylvatica	1		
Ajuga pyramidalis			1
Veronica chamaedrys	1	1	1
Veronica officinalis	1	1	1
Veronica arvensis	1		
Melampyrum sylvaticum		1	1
Rhinanthus angustifolius	1		
Rhinanthus minor	1	1	1
Plantago lanceolata	1	1	1
Valeriana sambucifolia			1
Knautia arvensis	1	1	
Antennaria dioica	1	1	1
Achillea millefolium	1	1	1
Leucanthemum vulgare		1	1
Cirsium heterophyllum		1	1

Centaurea jacea			1	1
Hypochaeris maculata		1	1	1
Leontodon autumnalis	var. taraxaci	1	1	1
Taraxacum ruderalia		1	1	1
Hieracium pilosella		1		1
Hieracium vulgatum			1	1
Dactylorhiza sambucina		1	1	1
Dactylorhiza fuchsii			1	1
Platanthera bifolia	ssp. bifolia			1
Luzula pilosa		1		
Luzula multiflora		1	1	1
Carex bigelowii	ssp. rigida	1		
Carex nigra	var. nigra			
Carex pallescens		1	1	1
Anthoxanthum odoratum		1	1	1
Phleum pratense		1		
Phleum alpinum		1		
Agrostis capillaris			1	1
Avenella flexuosa			1	1
Dactylis glomerata		1	1	1
Festuca rubra	ssp. rubra			1
Nardus stricta		1	1	1
Elytrigia repens			1	1
Draba sp.			1	
Polytrichum juniperinum		1		
Rhytidadelphus squarrosus			1	1
Climacium dendroides			1	
		67	69	74

Number of co-flowering species in each of the 30 plots was registered for the three locations in Telemark; Bøllås, Blika and Brekke (Tables 13 - 15, see appendix), and grouped into five colour-classes during one week in June 2013. Number of *D. sambucina* seed capsules were registered for the identical plots later in the season during one week in the middle of July 2013 (Tables 16-18, see appendix).

None of the specific colour-classes in this study had any significant impact on the seed-production of *D. sambucina.* Only when the numbers of yellow colour were merged together with the numbers of *D. sambucina* a positive coherency occurred, indicating that the density of *D. sambucina* rather than colour of co-flowering species have a positive impact on seed-production of these orchids (Table 19).

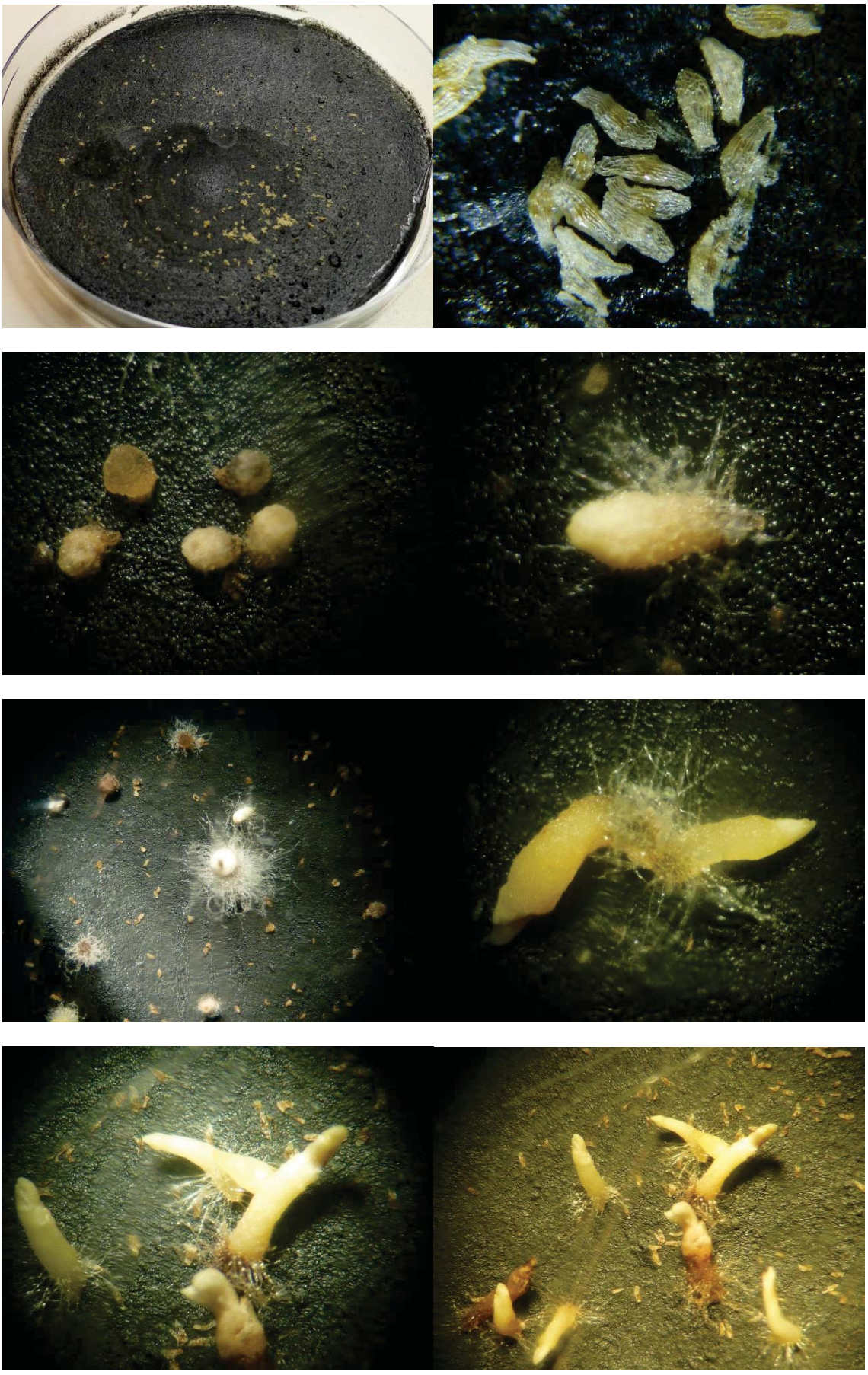
Germination experiments *in vitro* were a challenge to perform, and I did not succeed in germinating any of the *D. sambucina* seeds sown under sterile condition in our laboratory following the described protocol, at least not during the eight week timeframe I had available for a master thesis study. The minute seeds did not show any change even when I rechecked the petri dishes one month later.

Table 19. Frequencies of ripe *D. sambucina* seed capsules and co-flowering species per colour.

	Bøllås	Blika	Brekke
Total number of *D. sambucina* capsules	5249	8435	1960
Ripe seed capsules of *D. sambucina*	969	1219	185
Ripe seed capsules in % of total capsules	18 %	14 %	9 %
Total number of *D. sambucina* inflorescences in % of all flowers			
Light yellow	7 %	9 %	7 %
Total number of co-flowering species in % of all flowers			
Yellow	12 %	5 %	5 %
Green	75 %	80 %	65 %
Blue	3 %	3 %	20 %
Red	2 %	3 %	2 %
White	1 %	1 %	1 %
Total number of all yellow flowers (*D. sambucina* and co-flowering species)			
All yellow	19 %	14 %	12 %

However, results had occurred as I rechecked the petri dishes in April 2015 before storing all my work and submitting my thesis to my supervisors. I managed to make a photo series through my stereo magnifier to show the process of germination of the *D. sambucina* seeds. The photos show seeds where the testa is rupturing (Figures 1-2) and rhizoids are developing at the suspensor end (Figures 3-4). The embryos then imbibes and increases in circumference as forming protocorms (Figure 5-6), growing apical meristem at the chalazal end, and later forms into seedlings (Figure 7-8).

During the preliminary fieldwork in May 2012, I found an intermixed *D. fuchsii* at Blika in Telemark, Norway (Figures 12 – 13). The photos show a variant with wider floral lobes, wider leaves, and a thicker stem than the common variant that occur in the woods later in June.



Figures 4-11: D. sambucina seeds grown *in vitro* developing into protocorms and seedlings.

Figure 12. *D. sambucina* and one of the intermixed *D. fuchsii* at Blika**.**

Figure 13. Details from one of the intermixed *D. fuchsii* at Blika.

Figure 14. Orchis mascula at Silvana Mansio, showing a bright purple colour.

Figure 15. Details from one of the intermixed *D. Sambucina* at Silvana Mansio.

Figure 16. *B. lapidarius* with three yellow pollinia in the field at Blika.

Figure 17. Three pollinia attached to the clypeus of *B. lapidarius.*

Discussion

DNA test

The obtained PCoA and Neighbour-Join (Tab. 1 and 2) from the preliminary analyses are not as expected when assuming a significant interspecies distinction between *D. sambucina* and *D. fuchsii*. We excluded technical mistakes during the sampling and the DNA analyses and assumed that the used *D. fuchsii* lines represent *D. fuchsii x D. sambucina* hybrids (Fig 12 - 13).

Taking a second look at the sampled *D. fuchsii* might confirm the hybrid nature, because these lines have thicker stems and wider leaves (Fig. 13), but that might also be an environmental effect due to the atypical growing conditions for *D. fuchsii*. This hybrid was also found at Bøllås in May 2009, and this is four weeks earlier than expected for *D. fuchsii* in its normal habitat.

The DNA results of the second and main tissue analyses from the laboratory in Switzerland came back in November 2013, and results were further analysed with the software described above for the preliminary analyses. The results of this work was at first very confusing, as it did not make much sense. Analyses of the repeat controls revealed that the DNA analyses were not reproducible at the laboratory in Switzerland by comparing the repeats with their originals (Table 7). These comparisons showed that the expected identical pairs were far from identical and it was not much point in further work as we must assume the rest of the data we received would be just as inconsistent.

The cause for these errors could of course be that the samples were not labelled correctly when sampled in the field, or the repeats were mixed by packing at our laboratory before transport to the external analysis laboratory. However, this work were done following detailed procedures in the field, brought straight to our laboratory. They were further stored in labelled containers, in a locked cooler at our university at NMBU - Ås, until they were controlled and packed under supervision by my supervisor. Therefore, we do not find it likely to have made errors here, as the controls were randomly chosen, labelled and packed in cooperation, rather than solely by the student alone. If this is the case, then the error must have occurred at the Eurogenics laboratory by mixing samples there, or their AFLP protocol and procedures were not effective enough to be reproducible as our repeats does clearly show (Table 7).

One problem could have been that the data received from Eurogenics contained too much information due to not being selective enough of fragments, and not reducing the complexity of the banding pattern resulting in far too many bands in the PCR reactions, or in the CE separation of fragments. On the other hand, there would be a problem finding meaningful patterns if the further discarding of weak and blueprinting signalling bands were not done carefully enough by the student in analysing the received data. But then again such cleaning of data after PCR and CE will be extremely difficult if not impossible if the information generated are too complex. Anyway, it is not possible to make any conclusion in the study of mine, as the repeat controls point to the received data not being reproducible in the first place.

Learning this, I would rather have done the DNA extraction and the PCR in our laboratory at the university at Ås, Norway to gain control of all processes in my study.

The frame of this study and the use of AFLP's, and not e.g. microsatellites, limits and prevents the possibility to find causative alleles for the expressed colours in *D. sambucina.* (Hyp.1). As the DNA analyses also failed, it is not possible to reveal much more about the intra- and inter-specific relationship of *D. sambucina* and its relatives in this study.

However, if this study had produced some sound results from the DNA analyses, it might have been possible to calculate and show the relationship between the different populations by using analysing tools like GenAlEx, SplitsTree and Structure, and to tell more about how the individuals of the different populations group together and how close or distant these populations are related. I would expect the PCoA and the Neighbour-Join to show the *D. fuchsii* individuals clearly separated from the yellow *D. sambucina* individuals, and that the populations of the *D. sambucina* individuals from the three Norwegian locations are separating from one another. Perhaps I could even see that the closest populations, Blika and Bøllås occur less separated then the third population at Brekke that is more distant to the south. Especially it would have been interesting to see how the intermixed *D. fuchsii* individual fits in a Neighbour-Join using SplitsTree, or compare how the different *D. sambucina* populations from Norway, Sweden and Italy, and their genetic structure would look like using Structure. I would expect the Swedish and Italian population would differ more from the Norwegian population than the Norwegian populations differ from one another in Norway. Studies of other species in the *Dactylorhiza* family has shown this possible by several different scientists (De Hert et al., 2011; Hedren et al., 2001; Paun et al., 2011; Sonstebo, Borgstrom, & Heun, 2007).

Colour studies

The idea of *D. sambucina* being of dimorph character may be stringent interpreted. Looking closer at the colour morphs during sampling, I found intermediate coloured individuals filling most of the colour range between yellow and red at the locations of Stora Karlsö and Sila Grande (tables 8 – 11). In addition, I found individuals at Sila Grande with a purple colour character. This is not what was expected when looking for a dimorph orchid species described as yellow and red.

Interesting notation at Stora Karlsö.

The distribution of the three main colour classes yellow, pink and red at Stora Karlsö were divided fairly equally between the classes: yellow 35%, pink 30% and red 35% (Tab.9). Collecting more data might show that the colour frequencies does not differ that much between the locations. My dataset of 50 samples from two locations only is probably to minute to satisfy a representative selection of the total population.

Interesting notation at Silvana Mansio.

The distribution of the three main colour classes yellow, pink and red at Sila Grande were biased towards the pink intermediates, with very few of the red colour: yellow 29,55%, pink 63,64% and red 6,82% (Tab.11). The purple coloured flowers here is suspected to be *O. mascula x D. sambucina* hybrids as the purple colour resembles the colour of the co-flowering *O. mascula* (Fig.14) at the same location. One of the flowers on one of the purple *D. sambucina* was bicolored purple and yellow and could be a strange local mutation (Fig. 15). The selection of samples were done randomly and should produce a representative picture of the frequencies of colours, but the number of samples might be too small to generate a significant result. I think that more studies would have to be done with a larger number of samples and locations to make a more solid conclusion.

Pollinator of *D. sambucina* found at Blika

During the preliminary studies at Blika in 2012, one of the main pollinators, *B. lapidarius*, was registered in the field. This is an interesting notation, as it has been demonstrated in Sweden that *B. lapidarius* is frequently visiting *D. sambucina*, and acts as one of at least two vital contributors to pollinating the *D. sambucina* populations there (Nilsson, 1980). As far as I know, the pollinator has not been registered in Norway before for this orchid species. It has been assumed the same pollinator I Norway as well and now this fact is confirmed for Norway too (Fig. 16 and 17). The use of video by hero cameras on tripods was no success. During all fieldwork in Norway in June 2013, where this method was used, the weather was chilly with a slight movement of wind that probably did not attract much insect activity at all.

Field inventory, species frequencies and reproduction

The inventory is a picture of the different plant species found in the field at the actual time when the field experiments were done, and does not reflect the total inventory throughout the whole year. However, my species lists are similar to those obtained by Ann Norderhaug in 1987 – 1995 (Norderhaug et al., 1997) at the same location. Local farmers at Bøllås and Blika has maintained the fields by sheep grazing and cutting grass only when reproduction of *D. sambucina* is over from July 15th each year. This has favoured *D. sambucina* in these fields and these population numbers seems to have risen in these fields and in the area below.

The results of the field experiments does not show any coherency between *D. sambucina* reproduction and co-flowering species colour for any colour but yellow, and even this relationship is weak (Hyp. 2). There are not many red coloured co-flowering species in the Norwegian *D. sambucina* populations and it would be interesting to look closer to see if the lack of red co-flowering species have any influence on reproduction of the red *D. sambucina* morph in dimorph populations by making an experiment adding red co-flowering species.

In addition, the results of the field experiments does not show any coherency between density of *D. sambucina* and the number of ripe seed capsules (Hyp. 3). The density of *D. sambucina* does not seem to have any effect on its reproduction and indicates that other ecological factors

may come into play. This means that to find more answers I will need more field studies trying out other factors influencing reproduction of *D. sambucina.*

Seed sampling and germination tests

All seeds were sown on agar in petri dishes and in sterile conditions at our laboratory at Ås, but none of the dishes showed any germination after 21 days in growing chambers. The Italian seeds were sent by mail in closed plastic tubes without preliminary drying. They arrived in a miserable condition; the capsules were warm, wet and contained damaging fungi. Unfortunately, they were probably damaged already at arrival to Norway. The petri dishes showed contamination of both bacteria and fungi in more than 50% of the petri dishes and most of the growing media was dried out when removed from the growing chamber.

The petri dishes was re checked six weeks later and there had not been any change since the petri dishes was removed from thee germination chamber. The lack of germination might have several causes; one might be using a protocol used for *D. majalis*, which did not fit *D. sambucina* without further adjustments. The temperature and the light cycles might have been set wrongly, or the seeds used were infertile due to incompetent storage and preparation. There is also a possibility that the seeds needed more time hibernating, or the seeds did not receive the correct preparation before sowing. Finally working in sterile conditions is always difficult and demands laboratory training. Perhaps in the end I just lacked enough such experience and patience to succeed.

Nevertheless, while clearing my office before delivering my thesis in April 2015 I found the box with all petri dishes that failed germinating in August and October 2013. Now, when I rechecked them a second time, I surprisingly found living protocorms and seedlings in several dishes (Fig. 8-12). I think this points to a lack of hibernating of the seeds, as they had been stored in total darkness and in a chilly room on the first floor at my home in Telemark.

Conclusion

It was not possible conclude on the DNA studies due to lacking results. To get results I would need working data. It would have been great to do the DNA extraction and DNA analysis in the local laboratory instead of sending the lot to an external laboratory to gain more control of this part of the process to avoid errors. To do so I will have to do a new experiment once again.

The colour studies indicates that colour of co-flowering species may not be significant to reproduction of *D. sambucina*, but the inflorescence size and colour densities of conspecifics may matter. I would like to study the relationship between *Dactylorhiza* orchids and their co-flowering species closer to understand what kind of effect they might endure upon orchids in general.

As there were not registered any pollinia bearing insects other than *B. lapidarius* at any of the populations studied in Norway, this could indicate *B. lapidarius* to be the main pollinator of *D. sambucina* in Norway too. There was little insect activity in the fields during my fieldwork period in Telemark so more field studies would be needed to collect sufficient data on pollinator behaviour and possibly find other pollinators of *D. sambucina* too.

The germination experiment was not a success, but I learnt much about working under sterile conditions in the laboratory, and that using correct protocols is essential, especially when working with orchids that has proven difficult to germinate *in vitro*. What more learnt was that being patient is crucial to science and scientists finding several of the petri dishes in activity germinating one and a half year later.

Finding that seeds had survived and germinated was inspiring at the end of this study, unfortunately just a bit too late for me to be able to analyse germination percentages before submitting my thesis, but I might carry on these studies some time later. It would have been interesting to compare the seeds germination capacity in the different populations in Norway and Sweden.

I need to thank my supervisors Manfred Heun and Mikael Ohlson for patience and professional guidance along my master thesis work. Without their support and interest, my efforts would just be half the fun.

Appendix

Table 13 - Co-flowering species Bøllås Telemark - Excel doc.

Table 14 - Co-flowering species Blika Telemark - Excel doc.

Table 15 - Co-flowering species Brekke Telemark - Excel doc.

Table 16 - Bøllås seed capsules - Excel doc.

Table 17 - Blika seed capsules - Excel doc.

Table 18 - Brekke seed capsules - Excel doc.

Tutorial A. SplitsTree for dummies - Tutorial for new beginners by D. Mundal - Word doc.

Tutorial B. GenAlEx6.5 for dummies - Tutorial for new beginners by D. Mundal - Word doc.

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Tabel 13. Co-flowering species Bøllås Telemark

Specis name	Colour	Plot number and amount																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30			
Dactylorhiza sambucina	Light yellow	74	30	55	26	14	12	10	19	15	18	5	12	10	15	24	5	3	4	2	2	4	6	7	9	10	4	3	3	1	1			
Ranunculus acris	Yellow	3		4		2		18	6	18	1				7	45	1	2	5	53	87	3	5			1		4	6	12	23			
Taraxacum ruderalia	Yellow	5	2	2	3		2	4												3			4	1							4			
Potentilla crantzii	Yellow					4				7						1		1										1						
Potentilla erecta	Yellow													109		25									4									
Rhinanthus minor	Yellow					3				59						10											18	1	59	1				
Lotus corniculatus	Yellow																												10					
Plantago lanceolata	Green	48	15	5	8	7		20		69	12	2		18	29	42	9	6	8	8		2		4				6	31	38	30			
Anthoxanthum odoratum	Green	35	24	5	43	195		118		393	115	70	181	108	143	40	126	156	159		119	266	93		12		274	165	264	319	173			
Alchemilla monticola	Green	4		1				8		7													23	4							2			
Phleum pratense	Green																			4		3												
Carex pallescens	Green								34																									
Viola riviniana	Blue			2			6		2	37	3	3		13					13							1								
Polygala vulgaris	Blue	3												1			20	1	14						7	1				1			
Veronica chamaedrys	Blue								6	16						2												4						
Viccia sepium	Blue																7																	
Myosotis sylvatica	Light blue							6																										
Viola tricolor	Purple							1																				1	1		5			
Geranium sylvaticum	Purple								4			1							1															
Lathyrus linifolius	Purple														5																			
Viola palustris	Purple																					2												
Rumex acetosa	Red		2		1			2	4						1	5	2	1	1	14	6							8						
Trifolium pratense	Red																4												1					
Luzula multiflora	Brown					2						1		3	1				2															
Noccaea caerulescens	White							1									8		2	1														
Anemone nemorosa	White											16		5	12								1											
Bistorta vivipra	White									1																								
Fragaria vesca	White																									1		1						
Antennaria dioica	White																								9									
Antennaria dioica	Pink																								6									
	BØLLÅS TOTALS																																TOTALS	%
	Light yellow	74	30	55	26	14	12	10	19	15	18	5	12	10	15	24	5	3	4	2	2	4	6	7	9	10	4	3	3	1	1	403	7%
	Yellow	8	2	6	3	9	2	22	6	84	1	0	0	109	7	81	1	3	5	56	87	3	9	1	4	1	18	6	75	13	27	649	12%
	Green	87	39	11	51	202	0	146	34	469	127	72	181	126	172	82	135	162	167	12	119	271	116	8	12	0	274	171	295	357	205	4103	75%
inlcuding light blue	Blue	3	0	2	0	0	6	6	8	53	3	3	0	14	0	2	27	1	27	0	0	0	0	0	7	2	0	4	0	0	1	169	3%
incl. pink, purple, brown	Red	0	2	0	1	2	0	3	8	0	0	2	0	3	7	5	6	1	4	14	6	2	0	0	6	0	0	9	2	0	5	88	2%
	White	0	0	0	0	0	0	1	0	1	0	16	0	5	12	0	8	0	2	1	0	0	1	0	9	1	0	1	0	0	0	58	1%
	Totals	172	73	74	81	227	20	188	75	622	149	98	193	267	213	194	182	170	209	85	214	280	132	16	47	14	296	194	375	371	239	5470	100%	
	Yellow	5%	3%	8%	4%	4%	10%	12%	8%	14%	1%	0%	0%	41%	3%	42%	1%	2%	2%	66%	41%	1%	7%	6%	9%	7%	6%	3%	20%	4%	11%			
	Green	51%	53%	15%	63%	89%	0%	78%	45%	75%	85%	73%	94%	47%	81%	42%	74%	95%	80%	14%	56%	97%	88%	50%	26%	0%	93%	88%	79%	96%	86%			
	Blue	2%	0%	3%	0%	0%	30%	3%	11%	9%	2%	3%	0%	5%	0%	1%	15%	1%	13%	0%	0%	0%	0%	0%	15%	14%	0%	2%	0%	0%	0%			
	Red	0%	3%	0%	1%	1%	0%	2%	11%	0%	0%	2%	0%	1%	3%	3%	3%	1%	2%	16%	3%	1%	0%	0%	13%	0%	0%	5%	1%	0%	2%			
	White	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	16%	0%	2%	6%	0%	4%	0%	1%	1%	0%	0%	1%	0%	19%	7%	0%	1%	0%	0%	0%			
																																Totals		
Total number of D.sambucina capsules		837	275	915	287	197	105	121	342	229	238	56	137	120	232	369	71	45	55	22	24	47	72	139	80	70	44	59	22	16	23	5249		
Ripe seed capsules of D.sambucina		88	37	162	65	35	17	40	116	103	49	7	28	14	16	34	19	11	7	6	0	11	9	39	18	17	9	6	4	0	2	969		
Ripe seed capsules in % of total capsules		11%	13%	18%	23%	18%	16%	33%	34%	45%	21%	13%	20%	12%	7%	9%	27%	24%	13%	27%	0%	23%	13%	28%	23%	24%	20%	10%	18%	0%	9%	18%		

Tabel 14 . Co-flowering species Blika Telemark

Specis name	Colour	Plot number and amount																														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
Dactylorhiza sambucina	Light yellow	6	4	12	13	8	19	28	13	8	8	10	6	3	13	18	14	38	10	63	36	36	18	12	31	12	39	18	28	41	16	
Ranunculus acris	Yellow	3						1				9	2	15				1	5		4		1	29	9	7	5	16	5	6	18	
Taraxacum ruderalia	Yellow									2																	2		2	4	1	
Potentilla crantzii	Yellow																			1	1	1	2				2	10		6		
Potentilla erecta	Yellow							29	1							1	2			2		4	10		14		8	2	42		20	
Rhinanthus minor	Yellow																															
Lotus corniculatus	Yellow			2											1		4			8	6		4				4	6		16	1	
Plantago lanceolata	Green	132	3	49	7	58	50	32	4	29	57	8	87	129	57	28	126	133	97	28	4	3	43	1	35		8	10	10	2		
Anthoxanthum odoratum	Green		87	255	203	215	173	95	135	93	143	225	22	163	144	248	7	23	244	65	117	175	66	162	213	73	54	26	133	99	41	
Alchemilla monticola	Green	17								2	1	14		7	3				20	37	1	10	17	24	30	21	43	80	5	9	49	
Phleum pratense	Green																															
Carex pallescens	Green																			61	17		1				17		2	13		
Viola riviniana	Blue							1			2		10		2	2	11	1														
Polygala vulgaris	Blue												2		1		4	4		18	1	4	10		1	1	6	8	10	32	8	
Veronica chamaedrys	Blue						4		4									9					10	48								
Viccia sepium	Blue																															
Myosotis sylvatica	Light blue																															
Viola tricolor	Purple	2		8																												
Geranium sylvaticum	Purple														2																	
Lathyrus linifolius	Purple																															
Viola palustris	Purple																															
Rumex acetosa	Red									3						1								5				1				
Trifolium pratense	Red					1																										
Luzula multiflora	Brown							12		3						1					1		8				9	1	18			
Noccaea caerulescens	White										2				13			1														
Anemone nemorosa	White																															
Bistorta vivipra	White																						3		2		2		6		32	
Draba sp.	White	1																														
Fragaria vesca	White																															
Anthriscus sylvestris	White																											11				
Antennaria dioica	White																															
Antennaria dioica	Pink				13																											
Viscaria vulgaris	Pink				1																			1								
Dactylorhiza fuchsii	Pink																															
	BLIKA TOTALS																														TOTALS	%
	Light yellow	6	4	12	13	8	19	28	13	8	8	10	6	3	13	18	14	38	10	63	36	36	18	12	31	12	39	18	28	41	16	581	9%
	Yellow	3	0	2	0	0	0	30	1	2	0	9	2	15	1	1	6	1	5	11	11	5	17	29	23	7	21	34	49	32	40	357	5%
	Green	149	90	304	210	273	223	127	139	124	201	247	109	299	204	276	133	156	361	191	139	188	127	187	278	94	122	116	150	123	90	5430	80%
inlcuding light blue	Blue	0	0	0	0	0	4	1	4	0	2	0	12	0	3	2	15	14	0	18	1	4	20	48	1	1	6	8	10	32	8	214	3%
incl. pink, purple, brown	Red	2	0	8	14	1	0	12	0	6	0	0	0	0	2	2	0	0	0	0	1	0	8	6	0	0	9	2	18	0	0	91	1%
	White	1	0	0	0	0	0	0	0	0	2	0	0	0	13	0	0	1	0	0	0	0	3	0	2	0	2	11	6	0	32	73	1%
	Totals	161	94	326	237	282	246	198	157	140	213	266	129	317	236	299	168	210	376	283	188	233	193	282	335	114	199	189	261	228	186	6746	100%
	Yellow	2%	0%	1%	0%	0%	0%	15%	1%	1%	0%	3%	2%	5%	0%	0%	4%	0%	1%	4%	6%	2%	9%	10%	7%	6%	11%	18%	19%	14%	22%		
	Green	93%	96%	93%	89%	97%	91%	64%	89%	89%	94%	93%	84%	94%	86%	92%	79%	74%	96%	67%	74%	81%	66%	66%	83%	82%	61%	61%	57%	54%	48%		
	Blue	0%	0%	0%	0%	0%	2%	1%	3%	0%	1%	0%	9%	0%	1%	1%	9%	7%	0%	6%	1%	2%	10%	17%	0%	1%	3%	4%	4%	14%	4%		
	Red	1%	0%	2%	6%	0%	0%	6%	0%	4%	0%	0%	0%	0%	1%	1%	0%	0%	0%	0%	1%	0%	4%	2%	0%	0%	5%	1%	7%	0%	0%		
	White	1%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	0%	0%	6%	0%	0%	0%	0%	0%	0%	0%	2%	0%	1%	0%	1%	6%	2%	0%	17%		
																															Totals	
Total number of D.sambucina capsules		101	73	129	171	134	227	368	199	123	130	127	80	61	246	279	211	488	145	906	446	675	300	197	469	154	511	247	347	626	265	8435	
Ripe seed capsules of D.sambucina		29	7	21	30	29	36	59	39	21	32	25	32	4	63	51	51	97	11	75	74	56	78	27	29	19	42	20	28	87	47	1219	
Ripe seed capsules in % of total capsules		29%	10%	16%	18%	22%	16%	16%	20%	17%	25%	20%	40%	7%	26%	18%	24%	20%	8%	8%	17%	8%	26%	14%	6%	12%	8%	8%	8%	14%	18%	14%	

Table 15. Co-flowering species Brekke Telemark

Specis name	Colour	Plot number and amount																																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30							
Dactylorhiza sambucina	Light yellow	6	12	13	9	20	10	6	2	5	3	3	2	2	2	1	1	2	2	6	8	8	3	3	2	4	3	8	5	11	19							
Ranunculus acris	Yellow		2			6	2	5	4																4	3				5									
Taraxacum ruderalia	Yellow																																					
Potentilla crantzii	Yellow																																					
Potentilla erecta	Yellow		11					35															2	1			5		15		2							
Rhinanthus minor	Yellow																																					
Lotus corniculatus	Yellow																																					
Melampyrum sylvaticum	Yellow				2								1	45											1													
Anthoxanthum odoratum	Green		3		63			176	10	23	15	102	214	123	29	82	51	5		2	93	42	64	69			115	29	84		35							
Plantago lanceolata	Green		2						1												12	3					2											
Alchemilla monticola	Green			5	7	42																				46												
Phleum pratense	Green																																					
Carex pallescens	Green					1		7	8																													
Elytrigia repens	Green							1																														
Viola riviniana	Blue																												2									
Polygala vulgaris	Blue																																					
Veronica chamaedrys	Blue			5	4	101	5	254	52			2			4										45		3			2								
Viccia sepium	Blue	1				1	1																				1											
Ajuga pyramidalis	Blue													20																								
Myosotis sylvatica	Light blue																																					
Viola tricolor	Purple											4										1																
Geranium sylvaticum	Purple																																					
Lathyrus linifolius	Purple						1		2		2							5			1																	
Viola palustris	Purple																																					
Rumex acetosa	Red						1	2				1													2													
Trifolium pratense	Red																																					
Luzula multiflora	Brown																																					
Noccaea caerulescens	White																																					
Anemone nemorosa	White				7		5		2		1																											
Bistorta vivipra	White																																					
Fragaria vesca	White			1							1			2															1	7								
Platanthera bifolia	White																			1																		
Antennaria dioica	White																																					
Antennaria dioica	Pink																																					
Dactylorhiza fuchsii	Pink																								1													
	BREKKE TOTALS																																				TOTALS	%
	Light yellow	6	12	13	9	20	10	6	2	5	3	3	2	2	2	1	1	2	2	6	8	8	3	3	2	4	3	8	5	11	19		181	7%				
	Yellow	0	11	0	2	0	0	35	0	0	0	0	1	45	0	0	0	0	0	0	0	0	2	1	1	0	5	0	15	0	2		120	5%				
	Green	0	5	5	70	43	0	183	19	23	15	102	214	123	29	82	51	5	0	2	105	45	64	69	0	46	117	29	84	0	35		1565	65%				
inlcuding light blue	Blue	1	0	5	4	102	6	255	52	0	0	2	0	0	4	0	0	0	0	0	0	0	0	0	45	0	4	0	2	2	0		484	20%				
incl. pink, purple, brown	Red	0	0	0	0	0	2	2	2	0	2	5	0	20	0	0	0	5	0	0	1	1	0	0	3	0	0	0	0	0	0		43	2%				
	White	0	0	1	7	0	5	0	2	0	2	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	7	0		28	1%				
	Totals	7	28	24	92	165	23	481	77	28	22	112	217	192	35	83	52	12	2	9	114	54	69	73	51	50	129	37	107	20	56		2421	100%				
	Yellow	0%	39%	0%	2%	0%	0%	7%	0%	0%	0%	0%	0%	23%	0%	0%	0%	0%	0%	0%	0%	0%	3%	1%	2%	0%	4%	0%	14%	0%	4%							
	Green	0%	18%	21%	76%	26%	0%	38%	25%	82%	68%	91%	99%	64%	83%	99%	98%	42%	0%	22%	92%	83%	93%	95%	0%	92%	91%	78%	79%	0%	63%							
	Blue	14%	0%	21%	4%	62%	26%	53%	68%	0%	0%	2%	0%	0%	11%	0%	0%	0%	0%	0%	0%	0%	0%	0%	88%	0%	3%	0%	2%	10%	0%							
	Red	0%	0%	0%	0%	0%	9%	0%	3%	0%	9%	4%	0%	10%	0%	0%	0%	42%	0%	0%	1%	2%	0%	0%	6%	0%	0%	0%	0%	0%	0%							
	White	0%	0%	4%	8%	0%	22%	0%	3%	0%	9%	0%	0%	1%	0%	0%	0%	0%	0%	11%	0%	0%	0%	0%	0%	0%	0%	0%	1%	35%	0%							
																																Totals					
Total number of D.sambucina capsules		41	120	167		209	154	65	26	59	24	29	26	25	23	18	10	19	13	69	55	93	34	33	17	42	27	101	66	143	252		1960					
Ripe seed capsules of D.sambucina		5	21	30		14	7	4	10	7	0	0	0	6	2	2	3	0	0	6	12	13	1	9	0	12	5	0	7	1	8		185					
Ripe seed capsules in % of total capsules		12%	18%	18%		7%	5%	6%	38%	12%	0%	0%	0%	24%	9%	11%	30%	0%	0%	9%	22%	14%	3%	27%	0%	29%	19%	0%	11%	1%	3%		9%	9%				

Tabel 16. Bøllås seed capsules Origin of seeds for germination experiment

Plot	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23		24		25		26		27		28		29		30	
Ind.	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No
1	2	11	1	12	0	14	0	12	0	6	0	11	3	13	6	24	3	13	9	18	2	12	0	10	1	14	0	12	0	14	2	9	5	21	0	13	2	13	0	11	1	8	0	8	4	10	0	14	4	11	1	12	2	16	0	12	0	16	2	23
2	0	10	5	13	5	20	5	16	0	16	2	14	3	11	1	18	1	15	2	10	1	9	2	9	1	11	0	10	0	13	2	5	1	8	1	10	4	9	0	13	1	14	3	13	4	12	0	6	3	10	1	8	3	20	4	10				
3	0	9	2	9	3	17	1	8	0	14	1	12	1	9	2	16	9	13	5	15	3	12	2	13	0	12	1	15	2	16	4	16	5	16	1	15					1	9	4	10	1	12	1	7	1	13	2	10	1	23				
4	1	12	3	25	0	7	2	14	0	17	5	13	7	13	1	18	7	17	1	14	5	14	5	11	1	14	1	17	0	14	0	14			5	17					8	16	1	16	0	11	4	9	5	16	5	14						
5	0	18	0	12	4	18	1	13	3	13	1	14	4	16	12	17	6	16	1	12	4	9	5	12	3	10	5	15	6	13	9	19											0	15	2	18	5	10	4	20								
6	2	13	3	13	1	14	3	11	6	11	1	18	3	10	10	19	16	20	0	15			1	12	0	11	5	21	1	12	2	8											1	10	1	12	2	8										
7	5	9	5	17	2	16	1	18	1	7	1	8	4	12	3	16	7	15	1	8			0	13	1	6	1	11	0	14															6	14	2	10										
8	4	8	0	13	3	13	1	7	3	12	6	15	5	14	4	15	17	20	0	11			0	10	0	11	0	16	3	12															8	15	1	6										
9	0	12	0	13	1	17	1	9	0	15			5	11	6	20	5	18	0	14			2	8	2	10	0	19	0	17															3	22	3	10										
10	2	18	0	8	0	15	2	8	1	17			5	12	4	17	4	14	3	18			5	11	2	9	0	16	3	19															10	13												
11	0	12	2	11	9	20	2	12	7	20					1	18	1	7	6	14			3	10	3	12	1	13	3	14																												
12	1	7	1	10	1	9	2	8	6	21					6	15	11	16	2	13			3	18			1	15	0	15																												
13	2	12	2	18	8	28	5	17	2	15					7	12	8	12	0	8							0	25	0	5																												
14	1	19	3	15	3	24	3	18	6	13					10	20	5	13	2	12							1	15	2	27																												
15	0	8	0	14	1	14	1	15							12	20	3	20	5	18							0	12	3	23																												
16	0	11	2	15	0	15	4	16							5	19			0	13									1	19																												
17	0	22	1	12	4	10	5	21							10	18			4	13									2	24																												
18	3	16	0	12	1	10	5	12							2	19			8	12									6	17																												
19	2	14	6	9	1	9	7	9							14	21													1	14																												
20	4	14	0	10	8	16	4	15																					1	14																												
21	0	12	1	14	9	21	4	17																					0	10																												
22	1	11			1	15	6	11																					0	8																												
23	0	10			8	22																							0	17																												
24	0	11			2	15																							0	18																												
25	0	6			2	12																																																					
26	0	8			0	10																																																				
27	2	11			8	20																																																				
28	0	12			6	20																																																			
29	3	12			3	14																																																			
30	1	10			4	16																																																			
31	0	12			5	23																																																			
32	0	10			3	13																																																			
33	0	9			4	23																																																			
34	0	11			1	11																																																			
35	0	7			3	16																																																			
36	0	8			3	18																																																			
37	3	13			1	19																																																			
38	0	8			1	14																																																			
39	0	13			3	13																																																			
40	4	17			5	18																																																			
41	0	12			1	15																																																			
42	1	17			0	20																																																			
43	0	18			3	22																																																			
44	4	9			2	23																																																			
45	8	14			5	21																																																			
46	3	13			2	17																																																			
47	0	6			0	23																																																			
48	4	14			4	12																																																			
49	4	16			4	18																																																			
50	0	6			0	9																																																			
51	1	15			1	16																																																			
52	2	9			6	18																																																			
53	0	7			0	13																																																			
54	2	12			0	14																																																			
55	2	13			0	19																																																			
56	0	10			7	16																																																			
57	0	19																																																									
58	0	12																																																							
59	2	8																																																						
60	0	13																																																						
61	0	17																																																						
62	0	11																																																						
63	0	13																																																						
64	1	11																																																						
65	1	14																																																						
66	3	19																																																						
67	0	10																																																						
68	3	18																																																						
69	4	15																																																						
Total	88	837	37	275	162	915	65	287	35	197	17	105	40	121	116	342	103	229	49	238	15	56	28	137	14	120	16	232	34	369	19	71	11	45	7	55	6	22	0	24	11	47	9	72	39	139	18	80	17	70	9	44	6	59	4	22	0	16	2	23
Mean	1,28	12	1,76	13	2,89	16	2,95	13,05	2,50	14,07	2,13	13,13	4,00	12,10	6,11	18,00	6,87	15,27	2,72	13,22	3,00	11,20	2,33	11,42	1,27	10,91	1,07	15,47	1,42	15,38	3,17	11,83	3,67	15,00	1,75	13,75	3,00	11,00	0,00	12,00	2,75	11,75	1,50	12,00	3,90	13,90	2,00	8,89	3,40	14,00	2,25	11,00	2,00	19,67	2,00	11,00	0,00	16,00	2,00	23,00
Mean %	11%		13%		18%		23%		18%		16%		33%		34%		45%		21%		27%		20%		12%		7%		9%		27%		24%		13%		27%		0%		23%		13%		28%		23%		24%		20%		10%		18%		0%		9%	

Snitt 19%

Ripe: Capsules with seeds per individual/florescense
No: Total capsules per individual/florescense with and without seeds

Tabel. 17 Blika seed capsules Origin of seeds for germination experiment

Plot	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23		24		25		26		27		28		29		30	
Ind.	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No
1	1	16	1	18	0	11	1	11	4	19	0	10	2	15	11	23	2	18	0	16	2	10	8	12	2	27	7	15	0	22	7	18	0	8	0	11	0	10	0	9	1	10	0	10	2	12	0	19	1	15	0	11	1	15	1	10	1	10	0	15
2	4	16	0	17	1	8	4	13	3	15	1	10	2	14	1	6	6	18	3	16	6	21	3	11	0	20	7	20	7	17	4	16	0	8	3	15	0	13	1	12	0	11	1	12	0	12	0	13	0	16	2	10	1	6	0	15	2	12	4	17
3	5	23	0	17	1	10	5	13	3	15	6	15	3	16	1	11	5	19	4	20	6	17	9	13	2	14	7	16	7	18	3	14	0	11	2	17	0	19	1	10	1	18	2	18	2	20	0	16	0	11	3	10	2	12	0	10	6	17	2	13
4	8	15	6	21	4	12	4	14	6	20	1	13	0	13	2	16	3	19	10	16	5	15	1	16			9	15	0	15	0	15	4	16	1	12	0	9	0	8	0	16	13	30	5	15	0	9	1	8	6	19	2	15	0	14	6	29	3	14
5	9	18			1	6	3	15	3	18	1	16	2	13	0	14	0	18	6	16	2	18	4	14			1	14	0	14	2	12	3	12	0	18	0	14	0	10	3	24	8	15	2	24	0	14	2	11	0	11	3	18	0	17	2	10	3	20
6	2	13			2	8	0	15	5	16	1	13	3	10	0	21	0	14	4	16	0	15	7	14			7	17	3	14	2	10	1	12	0	12	0	12	0	9	0	13	1	13	4	15	0	8	2	13	0	12	0	12	2	11	1	13	4	20
7					2	13	0	10	2	15	3	14	5	14	0	16	5	17	2	14	2	9					2	19	1	16	5	17	3	13	0	15	0	6	0	14	0	23	3	16	0	11	0	14	1	11	0	8	0	14	0	11	2	23	4	12
8					2	16	1	11	3	16	1	9	5	15	6	20			3	16	0	14					3	11	5	12	3	14	4	16	0	13	0	10	0	10	0	15	8	22	4	21	0	13	0	11	3	14	2	15	0	12	2	10	1	14
9					4	12	0	14			3	20	1	21	1	15					2	8					9	24	9	18	3	17	5	15	1	16	0	15	0	12	1	16	10	25	2	14	0	15	2	14	2	7	1	15	0	11	8	28	4	20
10					2	13	4	19			4	25	6	13	2	13											0	13	4	22	1	16	6	13	4	16	0	11	0	9	0	13	6	16	2	17	0	15	2	10	0	7	2	8	0	8	0	8	4	14
11					0	11	1	13			3	17	3	18	1	12											2	15	0	12	0	18	2	10				3	23	0	12	0	18	2	13	3	18	0	11	2	20	0	15	1	21	0	14	1	15	2	23
12					2	9	2	10			3	14	1	15	4	17											2	14	0	12	5	14	4	12				0	18	0	11	0	12	6	14	1	18	1	12	6	14	0	16	0	19	1	13	4	18	0	16
13							5	13			2	12	0	12	10	15											2	19	4	19	5	11	1	14				0	7	0	11	0	13	2	14			0	18			0	11	0	17	2	14	2	14	3	17
14											2	11	0	10													2	19	3	13	11	19	1	10				3	19	0	17	0	17	3	21			0	16			0	15	1	14	3	11	0	12	1	12
15											2	13	2	10													3	15	0	12			1	20				0	12	2	5	0	12	3	16			0	17			0	12	1	12	0	12	3	14	7	18
16											3	15	4	10															0	9			1	10				0	11	2	8	1	20	4	13			0	7			0	13	0	6	0	10	3	21	5	20
17													1	8															2	15			1	12				0	7	1	14	3	30	4	15			0	18			3	17	1	9	4	12	7	31		
18													0	16															6	19			0	13				0	11	3	6	0	14	2	17			0	7			4	18	2	19	3	18	2	12		
19													0	10																			0	21				1	16	0	10	1	18					0	14			0	12			1	10	4	17		
20													0	13																			3	10				0	22	2	19	0	23					0	15			3	26			1	16	6	30		
21													1	12																			4	16				0	22	5	22	0	12					0	16			0	15			0	12	0	11		
22													0	17																			7	18				0	13	3	16	0	17					1	22			0	14			1	11	0	8		
23													0	11																			3	11				0	15	2	13	0	16					0	18			0	10			0	17	0	18		
24													3	12																			3	11				0	23	3	11	0	25					2	21			1	18			0	11	0	13		
25													10	14																			2	9				0	9	5	14	2	17					2	18			1	12			0	8	0	18		
26													3	13																			3	14				0	7	0	14	6	16					1	13			0	10			1	12	0	15		
27													2	12																			4	16				0	8	3	11	0	25					3	14			0	5			4	11	2	16		
28													0	11																			5	9				0	13	5	11	5	20					4	12			0	12			4	16	0	8		
29																																	0	9				0	14	4	13	2	22					3	15			0	11					4	14		
30																																	1	11				0	15	3	16	1	23					5	12			1	15					7	18		
31																																	3	17				1	14	6	16	4	24					1	13			0	15					3	18		
32																																	1	12				1	22	2	18	4	26					6	24			1	12					0	12		
33																																	1	8				3	12	7	20	9	33									1	10					0	14		
34																																	5	17				0	13	5	11	1	19									3	13					1	10		
35																																	2	14				0	15	8	13	7	26									2	12					1	20		
36																																	3	15				0	16	1	11	4	18									3	15					0	9		
37																																	4	10				1	16													1	21					1	10		
38																																	6	15				0	20													2	12					0	7		
39																																					6	18													0	15					0	12		
40																																					5	20																			3	20		
41																																					2	13																			3	11		
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59																																					2	21																						
60																																					1	10																						
61																																					6	31																						
62																																					4	19																						
63																																					1	12																						
Total	29	101	7	73	21	129	30	171	29	134	36	227	59	368	39	199	21	123	32	130	25	127	32	80	4	61	63	246	51	279	51	211	97	488	11	145	75	906	74	446	56	675	78	300	27	197	29	469	19	154	42	511	20	247	28	347	87	626	47	265
Mean	4,83	17	1,75	18	1,75	11	2,31	13,15	3,63	16,75	2,25	14,19	2,11	13,14	3,00	15,31	3,00	17,57	4,00	16,25	2,78	14,11	5,33	13,33	1,33	20,33	4,20	16,40	2,83	15,50	3,64	15,07	2,55	12,84	1,10	14,50	1,19	14,38	2,06	12,39	1,56	18,75	4,33	16,67	2,25	16,42	0,91	14,66	1,58	12,83	1,08	13,10	1,11	13,72	1,00	12,39	2,12	15,27	2,94	16,56
Mean %	29%		10%		16%		18%		22%		16%		16%		20%		17%		25%		20%		40%		7%		26%		18%		24%		20%		8%		8%		17%		8%		26%		14%		6%		12%		8%		8%		8%		14%		18%		

Snitt 17%

Tabel 18. Brekke seed capsules Origin of seeds for germination experiment

Plot	1		2		3		4 *		5 **		6		7		8 ***		9		10 ***		11 ***		12 ***		13		14		15		16		17 ***		18 ***		19		20		21		22		23		24 ***		25		26		27 ***		28 ***		29		30	
Ind.	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No	Ripe	No
1	0	9	0	13	0	11	-	-	0	10	1	19	2	18	5	13	2	17	0	10	0	15	0	14	3	12	0	12	2	18	3	10	0	9	0	5	0	10	0	6	0	11	0	12	1	6	0	17	3	12	1	13	0	11	0	9	0	8	0	16
2	0	8	3	5	1	13			0	11	0	13	0	11	5	13	2	17	0	14	0	14	0	12	3	13	2	11					0	10	0	8	0	12	0	5	2	12	0	10	4	13			4	15	4	14	0	15	4	16	0	13	0	18
3	1	11	0	10	0	11			1	15	0	21	0	12			1	14																			1	12	1	11	0	10	1	12	4	14			5	15			0	12	0	12	0	15	0	15
4	4	13	0	12	0	10			0	9	0	20	0	12			2	11																			1	20	3	6	0	16									0	10	3	14	0	16	0	13
5			3	18	5	10			0	15	0	19	2	12																							4	15	3	10	0	11									0	15	0	15	0	18	0	17
6			4	10	4	17			0	8	0	12																											3	9	5	10									0	13			0	15	0	15
7			2	10	2	11			0	10	0	18																											2	8	2	15									0	9			0	17	0	11
8			4	8	3	16			0	6	1	22																													4	8									0	16			0	11	0	14
9			2	16	7	18			0	11	5	10																																										0	12	0	15
10			3	18	1	10			0	10																																										1	18	0	8
11					3	14			0	12																																												2	15
12					3	12			1	8																																											0	9
13					1	14			1	17																																											0	10
14									2	14																																											1	10
15									3	20																																											0	14
16									3	18																																											1	13
17									3	15																																											0	16
18																																																					2	9
19																																																						2	14
Total	5	41	21	120	30	167	0	0	14	209	7	154	4	65	10	26	7	59	0	24	0	29	0	26	6	25	2	23	2	18	3	10	0	19	0	13	6	69	12	55	13	93	1	34	9	33	0	17	12	42	5	27	0	101	7	66	1	143	8	252
Mean	1,25	10	2,1	12	2,31	13			0,82	12,29	0,78	17,11	0,80	13,00	5,00	13,00	1,75	14,75	0,00	12,00	0,00	14,50	0,00	13,00	3,00	12,50	1,00	11,50	2,00	18,00	3,00	10,00	0,00	9,50	0,00	6,50	1,20	13,80	1,71	7,86	1,63	11,63	0,33	11,33	3,00	11,00	0,00	17,00	4,00	14,00	2,50	13,50	0,00	12,63	1,40	13,20	0,10	14,30	0,42	13,26
Mean %	12%		18%		18%				7%		5%		6%		38%		12%		0%		0%		0%		24%		9%		11%		30%		0%		0%		9%		22%		14%		3%		27%		0%		29%		19%		0%		11%		1%		3%	

Snitt 11%

*: Utgått p.g.a. slått
**: 10% skadet p.g.a. slått
***: Ingen frøkapsler plukket

Brekke er meir tilgrodd enn Blika og Bøllås

SplitsTree – Learning how to start and use the software program

SplitsTree for dummies – tutorial for new beginners

(like myself)

Tips: To utilize this program you will need three other programs and an extension file:

Microsoft Excel 2010 (works with the 2007 version also but slower)

Notepad (text editor)

R (Statistical software program) http://www.r-project.org/

AFLP.dat (R-extension) http://www.nhm.uio.no/english/research/ncb/aflpdat/

Download and install SplitsTree for free from here: http://www.splitstree.org/

Now you have got the tools you need to get going.

To use Splitstree you need to load your DNA data as a nex file. That means converting your files to ".nex", before importing them into SplitsTree.

You will usually get your DNA data from analyses as an Excel file. That's ok. We can use Excel for several operations to prepare our data for use in Splitstree.

A DNA excel-file may look like this when you receive it from an analyzing lab:

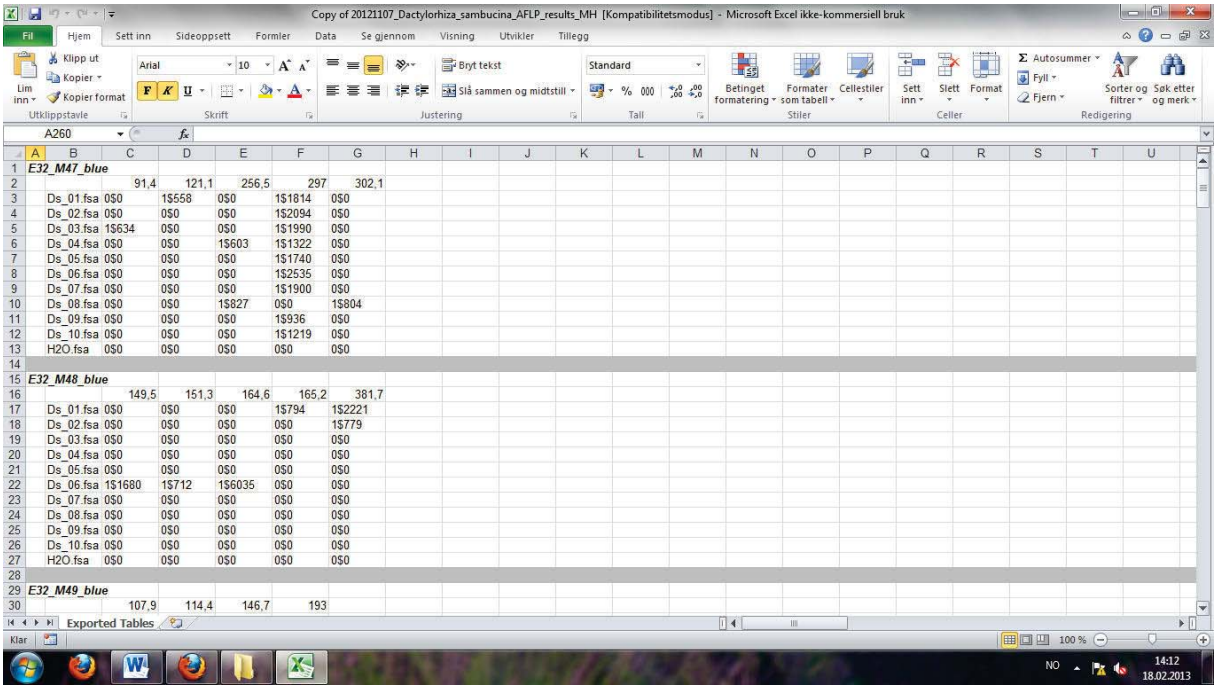


Figure 1: Excel sheet with a data matrix showing DNA bands in columns and samples in rows.

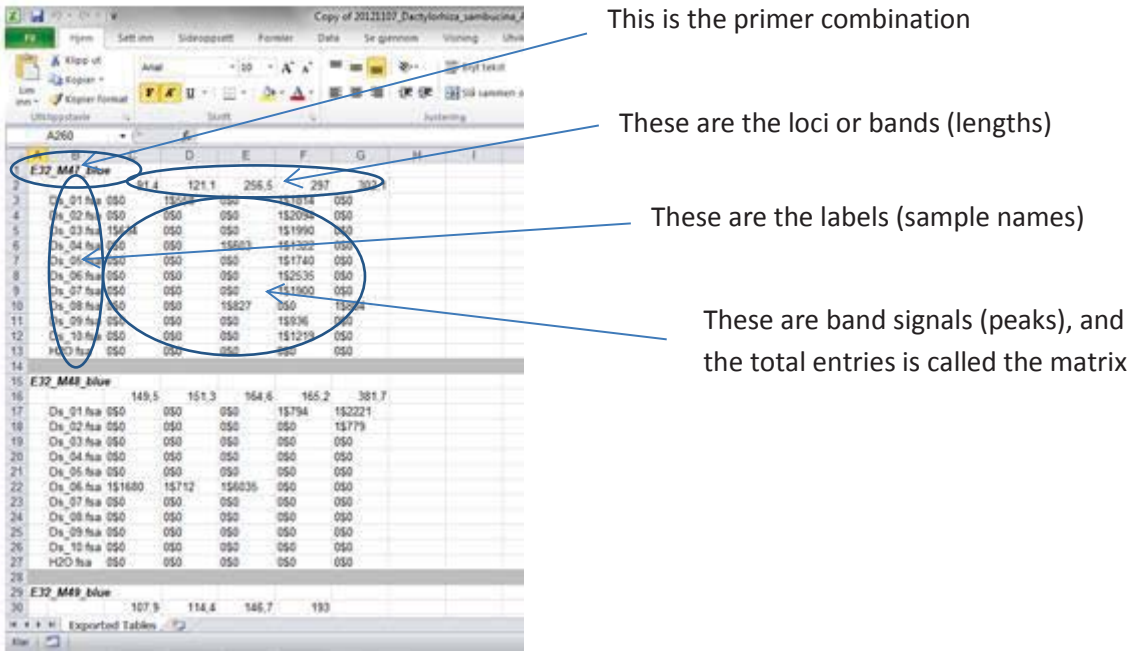


Figure 2: Explaining the excel sheet and the different categories.

Join in and work with me: We start with an easy exercise. (**EX. 1 - Identification**)

The first we have to do is to score the bands. In this DNA set the two first rows are a different species than the other eight rows beneath. These two groups make two species in total. We want to find the bands which have info that separate these two first individuals from the other eight. We do that by finding columns where the two first bands are identical, but different from the other eight. (0,0 vs 1,1,1,1,1,1,1,1 or 1,1 vs 0,0,0,0,0,0,0,0)

Tips: Mark the two first rows green, and the columns that separate the species red:

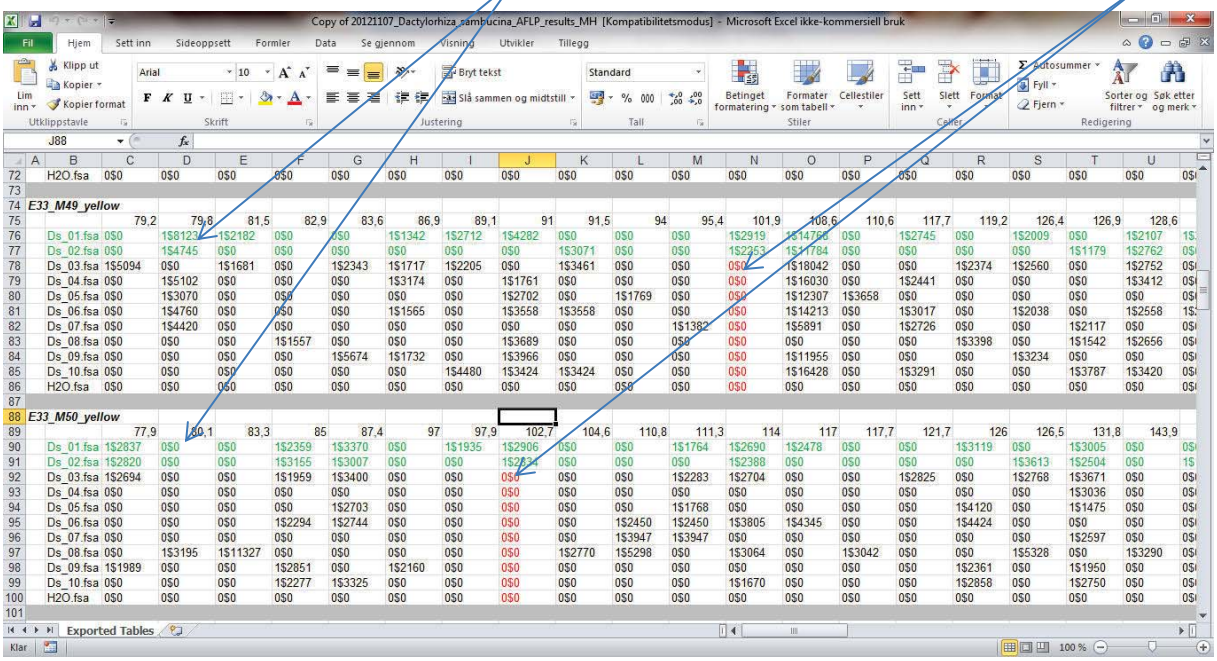


Figure 3: In the figure the columns that separate the two species (row 1 and 2) vs (row 3-10) are marked in red. The other columns do not separate the two species groups. You might find more than one column per primer-set that separate the two species groups.

The primer combinations of interest are then the ones where we are able to find species differentiation. Now you try with your samples! Make a nice file with these colors like in figure 3. Change the sheet tab name at the bottom of your sheet to “ID” and save your work.

(**EX.2 - Saving the primer combinations of interest**)

We now choose to work with the primer combinations of interest only (only those that give info that separate the two species in the matrix – the ones with red colored bands).

Make a new excel sheet (sheet 3) and copy over (mark the sheet in the grey cell between column A and row 1 and copy (Ctrl C)) all data from the first sheet (ID) into sheet 2 (mark cell A1 and paste (Ctrl V)). Then delete the rows you do not longer need (Those primer combinations with a matrix without a red color).

Now score all numbers starting with 0 in the matrix as 0 and all numbers starting with a 1 as 1 like in figure 4.

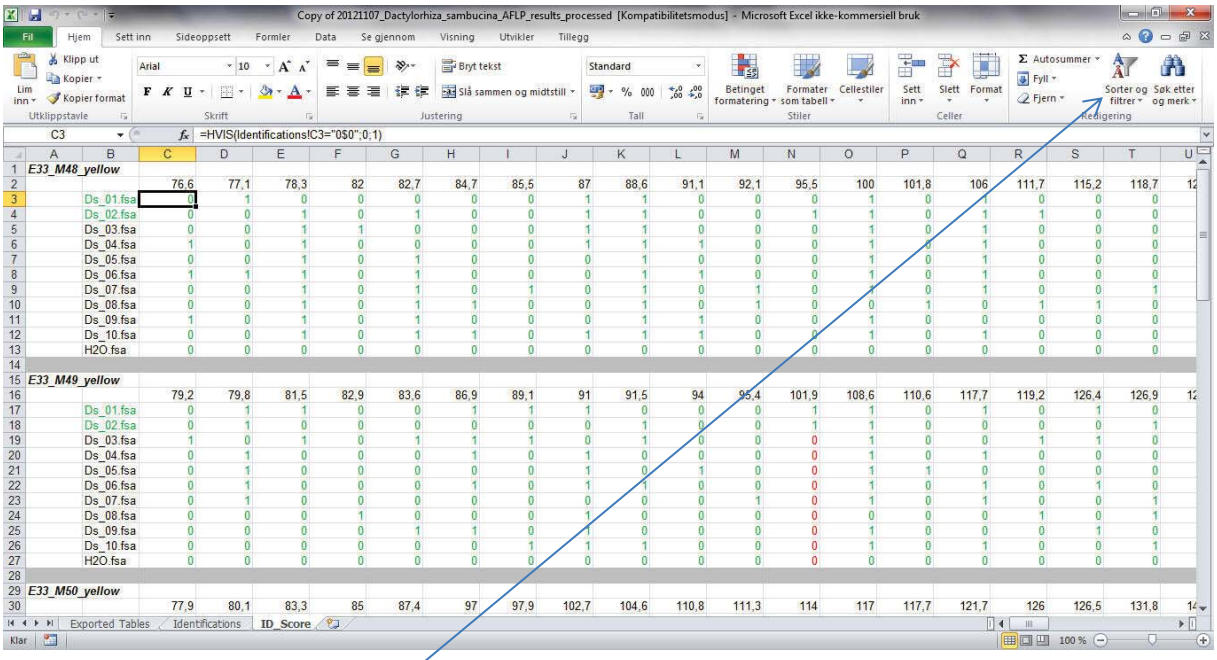


Figure 4: Scoring all numbers starting with 0 as 0, and all numbers starting with 1 as 1.

Tips: use the “Find & substitute” –tab in the upper right corner of the excel window:

Step 1: Find “0*” and substitute with “0”, that will change all numbers starting with 0, and
Step 2: Find “1*” and substitute with 1, that will change the rest of the numbers.

Delete all H2O rows also, these are water only and should all score 0 anyway as they are controls, when checked, we do not need them anymore, and they are not wanted in SplitsTree.

Change the sheet tab name at the bottom of the sheet to “Scores”, and save your work.

(**EX.3 – Make a matrix of all loci**)

This exercise will teach you how to prepare the excel file for converting into a text file.

Make a new sheet and copy all data from sheet 3 (Scores) to the next sheet (sheet 4) and work from here. Note that excel 2007 is constricted to 256 columns, that is column IV to the far right, if you are using this version and exceed 255 loci you need to continue on a next sheet. In the 2010 version of excel there is no such limitation and you may work with several hundred loci in the same sheet.

Now use the first primer combination set and add the other matrix data from the subsequent sets to the first column to the right. Do so for all subsequent data sets in the sheet. Only matrix data is to be copied and pasted, delete the rest when you are finished.

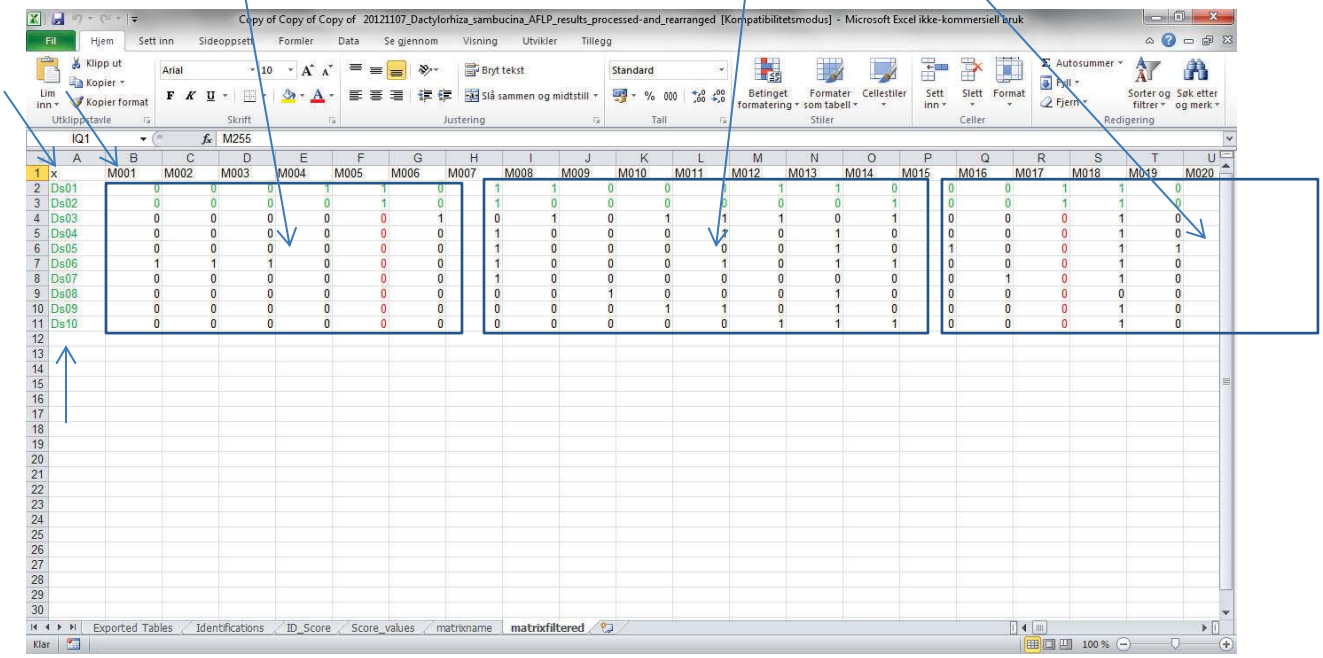


Figure 5: Here is the final arranged matrix where data from all primer combinations of interest are added into one dataset in the right direction (three of these show up in this window). Our dataset has 255 columns as the 2007 version of excel is used here (see fig.6).

Note that the matrix need to fill out cell A-1 with an x, and the subsequent cells in the first row make up the loci numbers with the same amount of integers in each cell in this row. To accommodate this put in a character before 001 like e.g. “M001”, and continue out that row.

Tips: write M001 in cell B-1 and M002 in cell C-1. Mark both these cells and use the nob at the bottom right of these cells to drag the series all the way to the far right by using your mouse.

Note also that you only need to keep the sample-ID once in the first column (fig.5). They also need the same number of integers/characters in all cells in the column, or else Splitstree will not accept the imported data.

Now we are ready to convert files. We will do that in the next exercise.

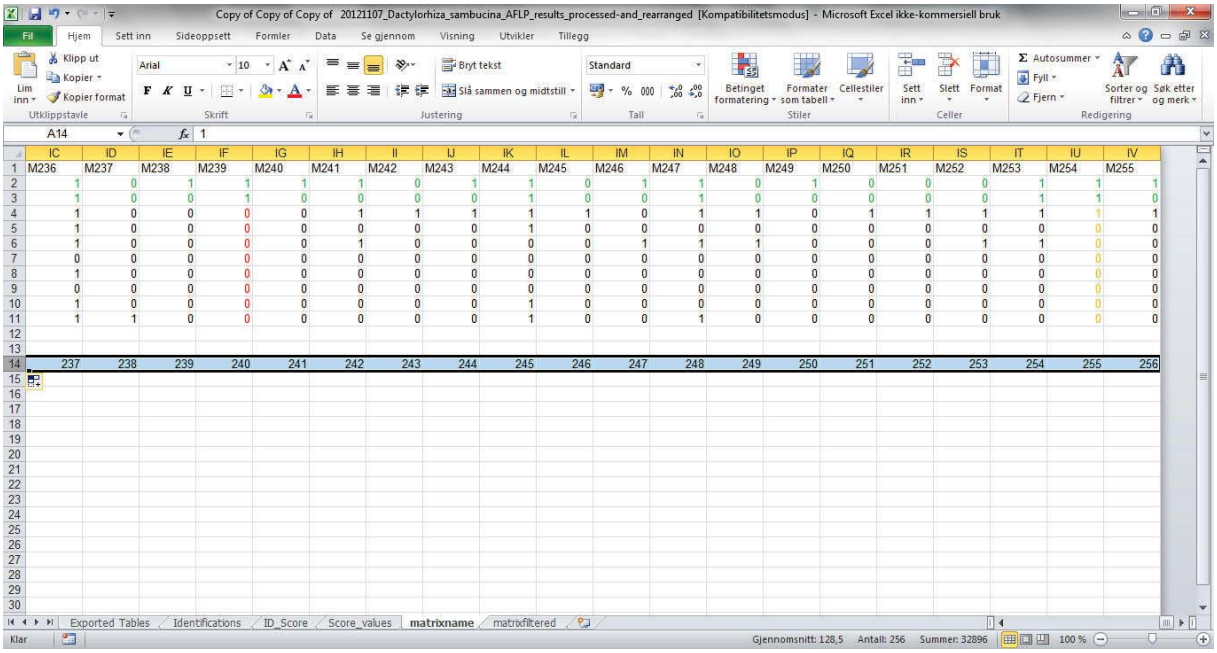


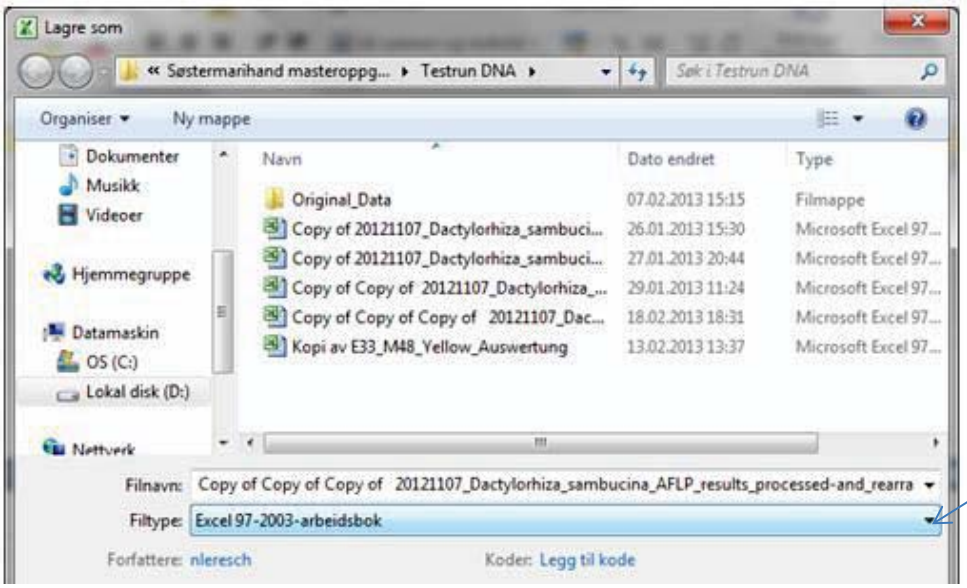
Figure 6: The final arranged matrix has 256 columns and use 1 label column at the far left (see fig.5) with the sample ID’s + 255 columns of data.

(**EX.4 – Converting an excel-file into a text-file**)

This is an easy match for you to do. Go to file → Save as. In the dropdown menu to the right of the file type box at the bottom of your window choose “text (tab-separated)” + folder.

Just follow the 5 steps here: 1.  2. 

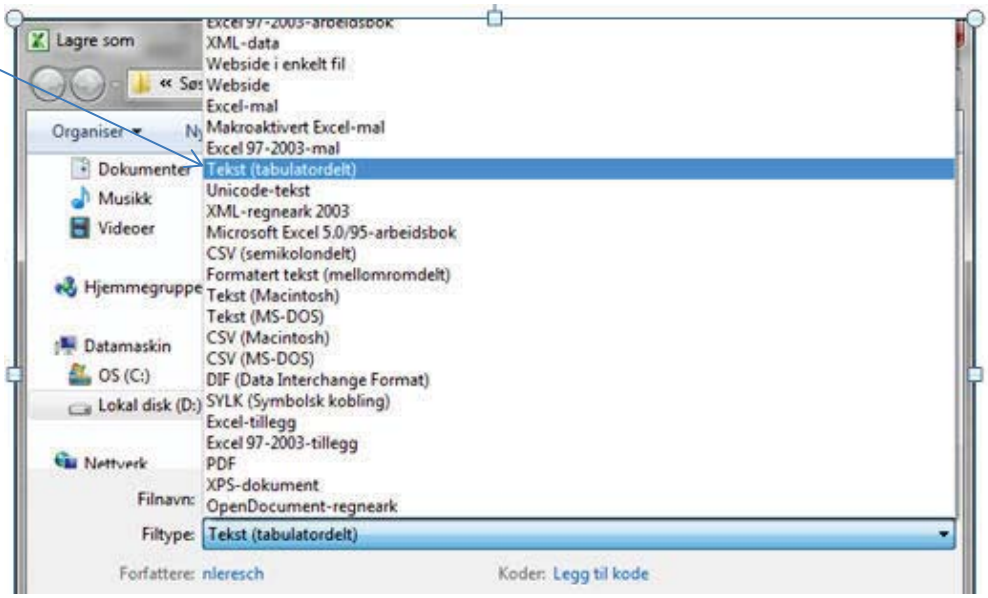
3.



Click

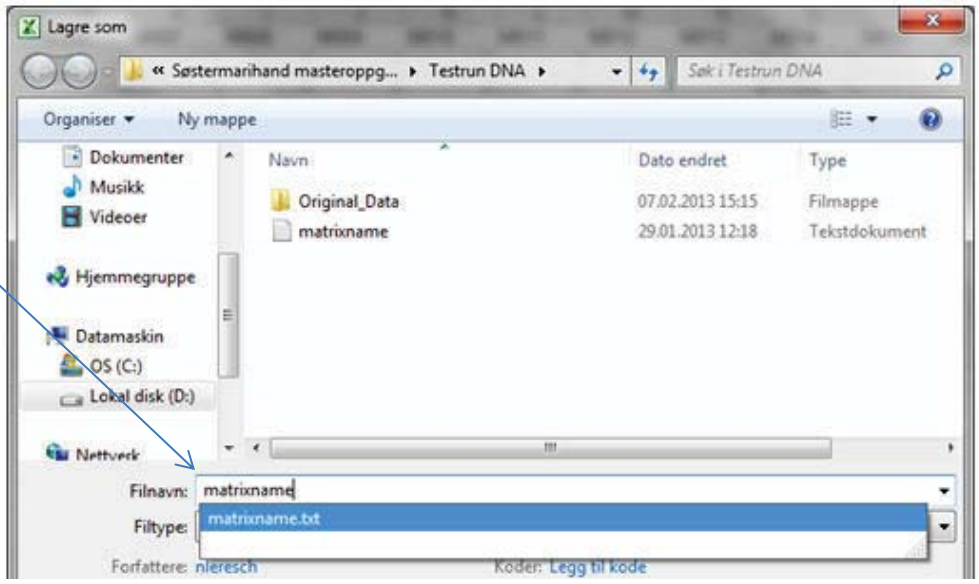
Select

4.



Write

5.



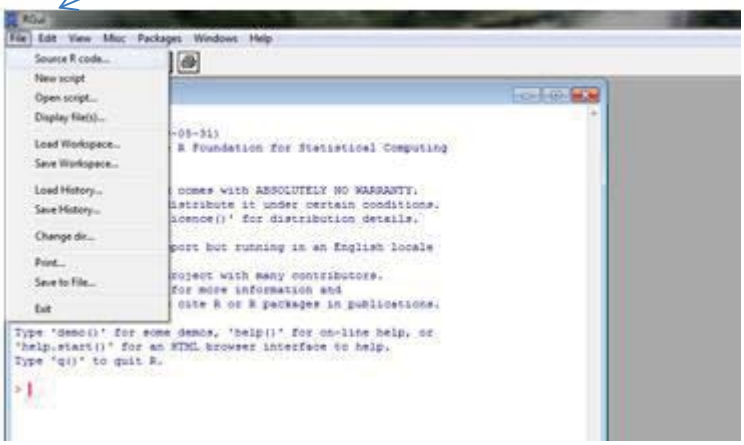
Now you should have a text-file with any name you have given it (eg. “matrixname.txt”), in the folder you chose to put it into (if no change in directory, it ends in the same folder as the original excel file).

To be able to import it into Splitstree, you will need to convert it once more, this time into a nex-file. We will do so in the next exercise.

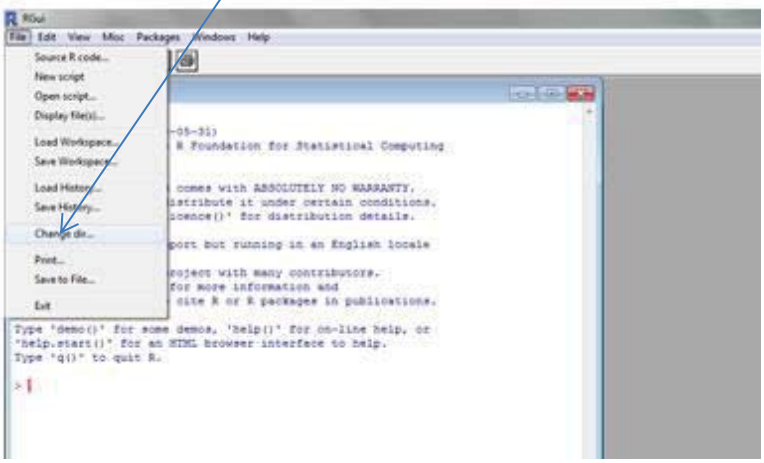
(**EX.5 – Converting a text-file into a next-file**)

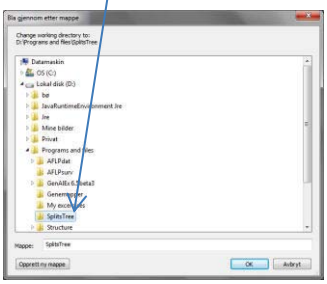
This step requires use of R and the R-extension-file called AFLP.dat.

1. Open R and go to File → Source R code… (Select file to source) to tell R the directory where to save your files. Download your ALFP.dat – file to the same folder and work from there (Select the ALFP.dat – file: File -> Source R code -> …-> ALFP.dat). You can find the AFLP.dat-file (script and documentation) in a zip-file here (unzip files and copy file to target-folder): http://www.nhm.uio.no/english/research/ncb/aflpdat/

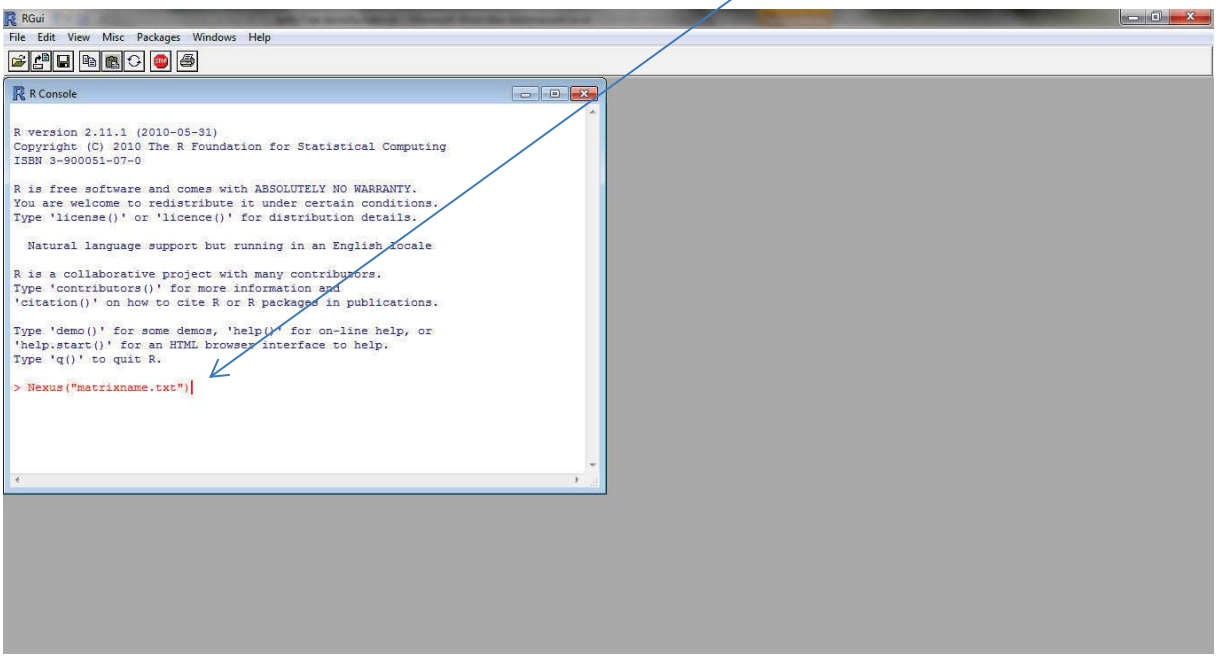


1. Go to File → Change dir… (Change working directory to:) and browse the folder where your ALFP.dat-file is stored (here we choose the same folder to keep it tidy).





2. Write this command in the R Console window: *Nexus("matrixname.txt")* → Enter, to convert your text-file into a nex-file.

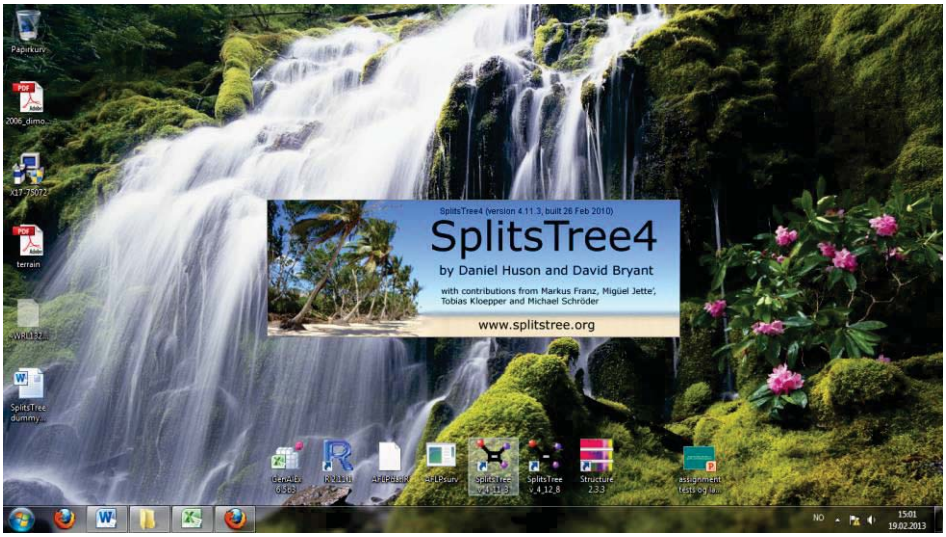


If you now look in the folder where you stored your text-file, you will find a new file also having the same name as your text-file, but with the file extension "nex" (.nex).

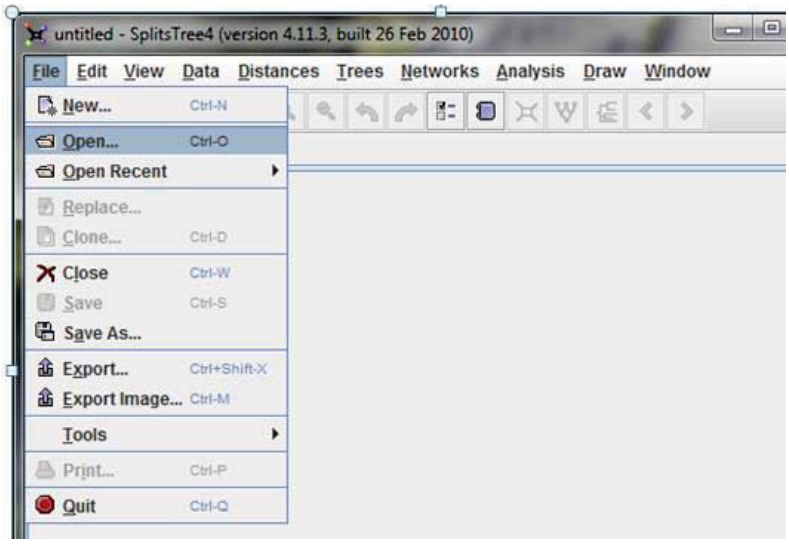
Note that if you do this operation twice you do not get two separate nex-files. The second operation just overwrites the first nex-file. To avoid that you may change the name of your generated nex-file before a subsequent operation.

Now you should have succeeded in making a file that is possible to import into SplitsTree. We will look at the import operation in the next exercise.

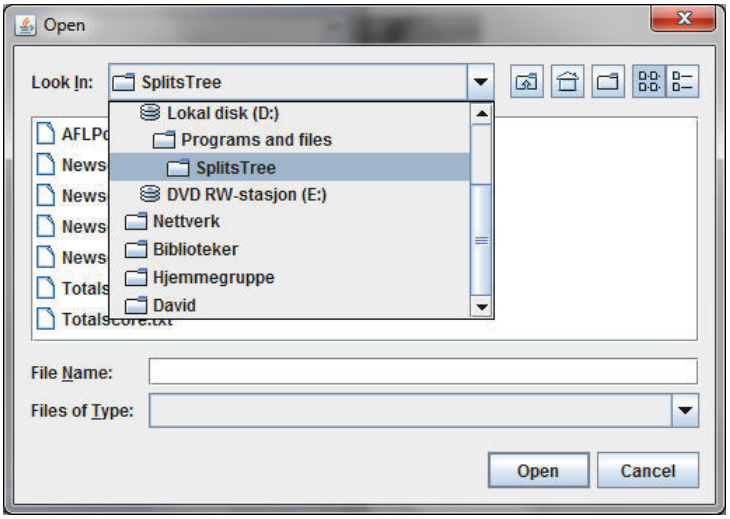
(**EX.6 – Importing a nexus-file (.nex) into SplitsTree**)

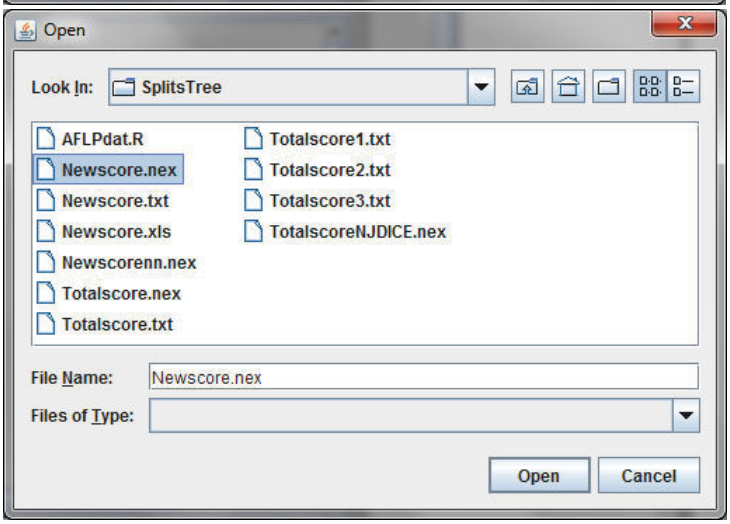


1. Open the SplitsTree software program and go to to File → Open



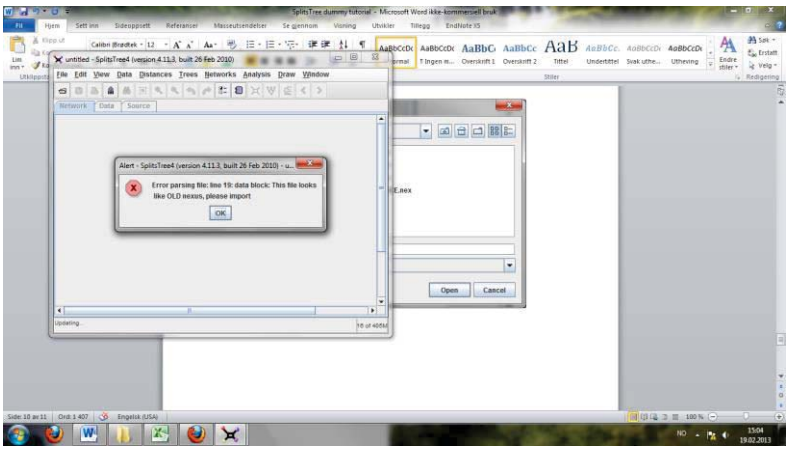
2. Browse the folder where you have stored your nex-file and select it →Open.

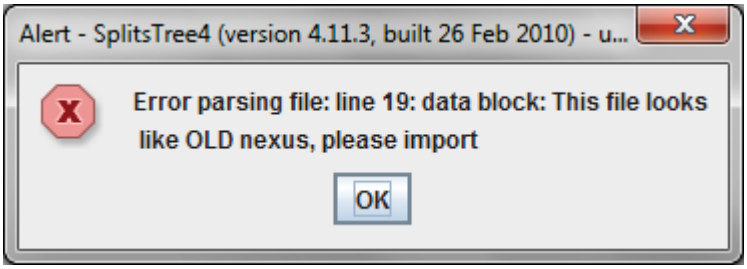




(Here in this case I just use an arbitrary file of my earlier exercises to show you how).

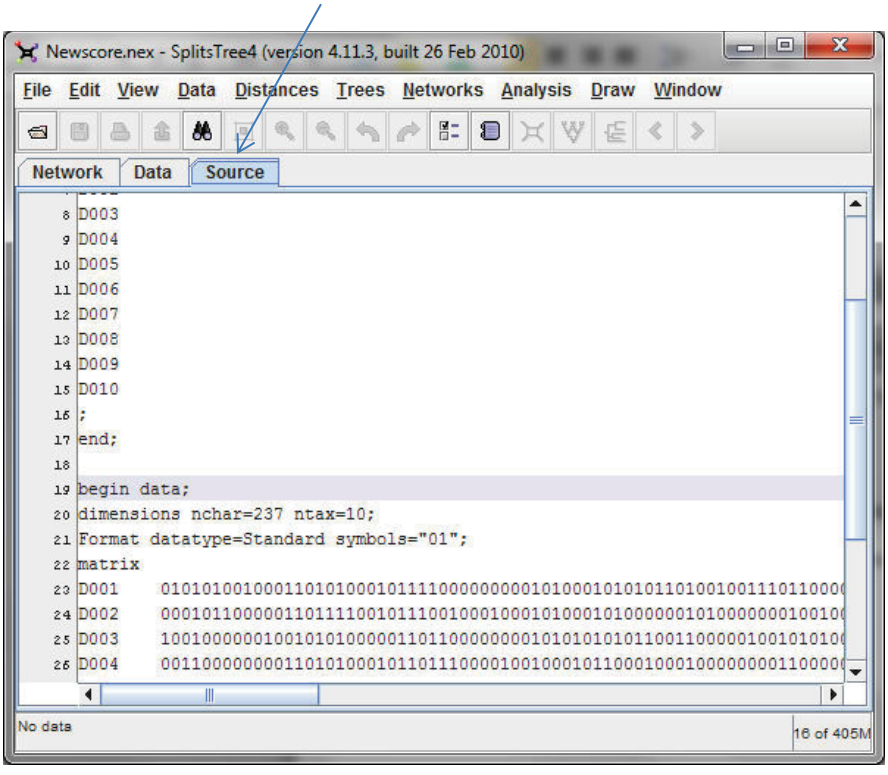
Using the older version you may need to modify the file to make it work. If that is needed SplitsTree will tell you by showing this message:





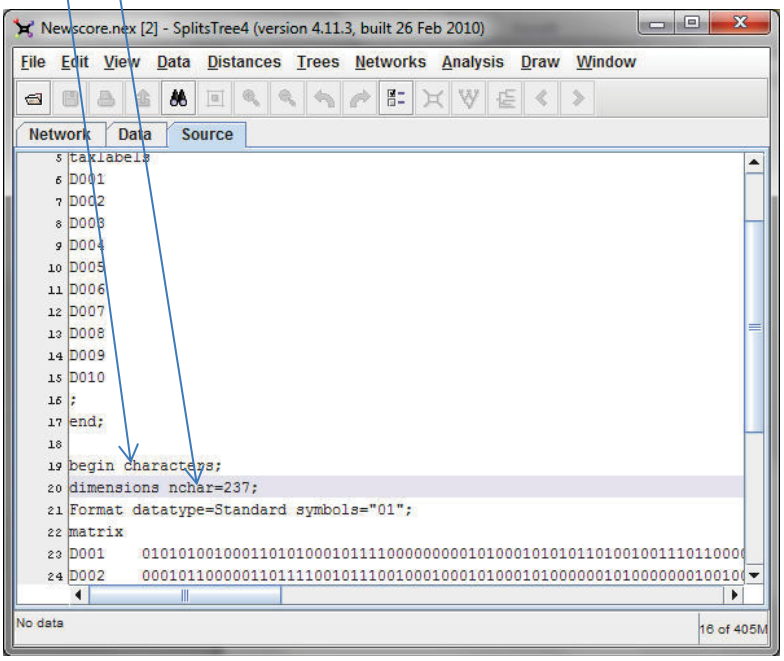
Just press the ok-button and I will show you two simple steps that modify this file so it becomes applicable.

You are brought to the source-tab window to do the modifications:

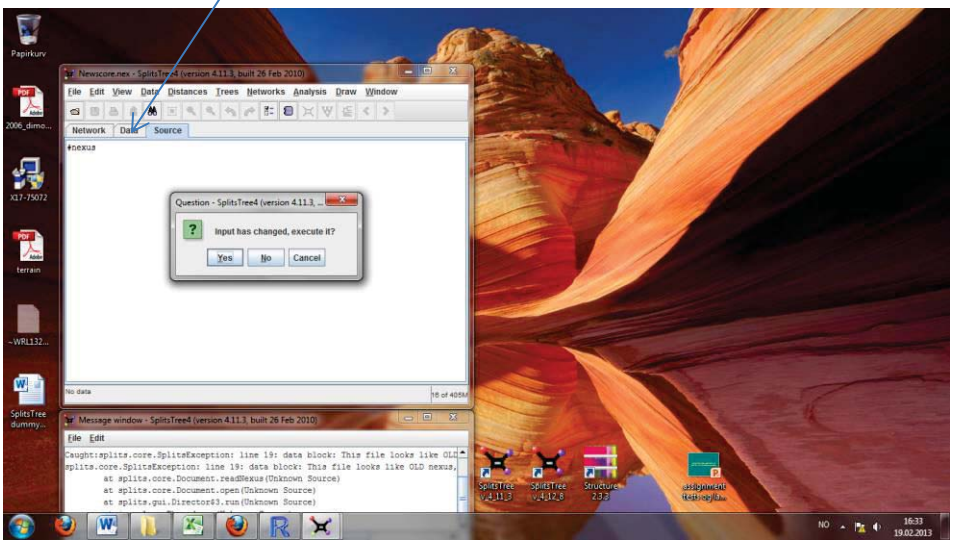


In line 19 put your marker in front of “data”, delete it and substitute with “characters” so it reads “begin characters;”.

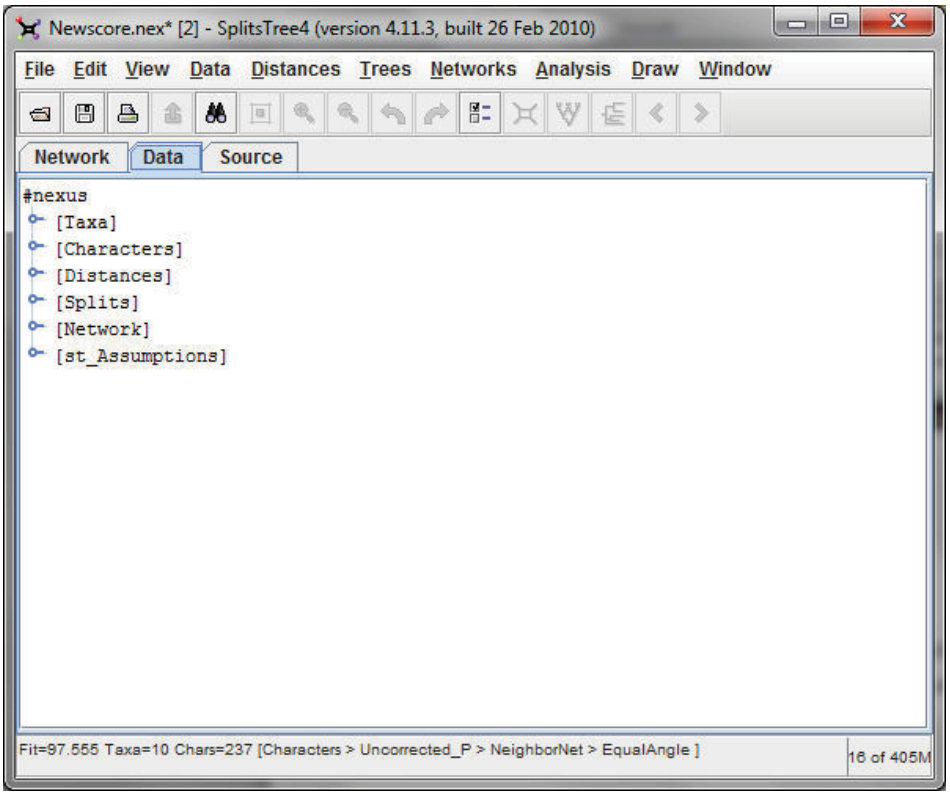
In line 20 you just delete the space +“ntax” so it reads, as in my case, “dimensions nchar=237” (you may have another number of loci in your dataset).



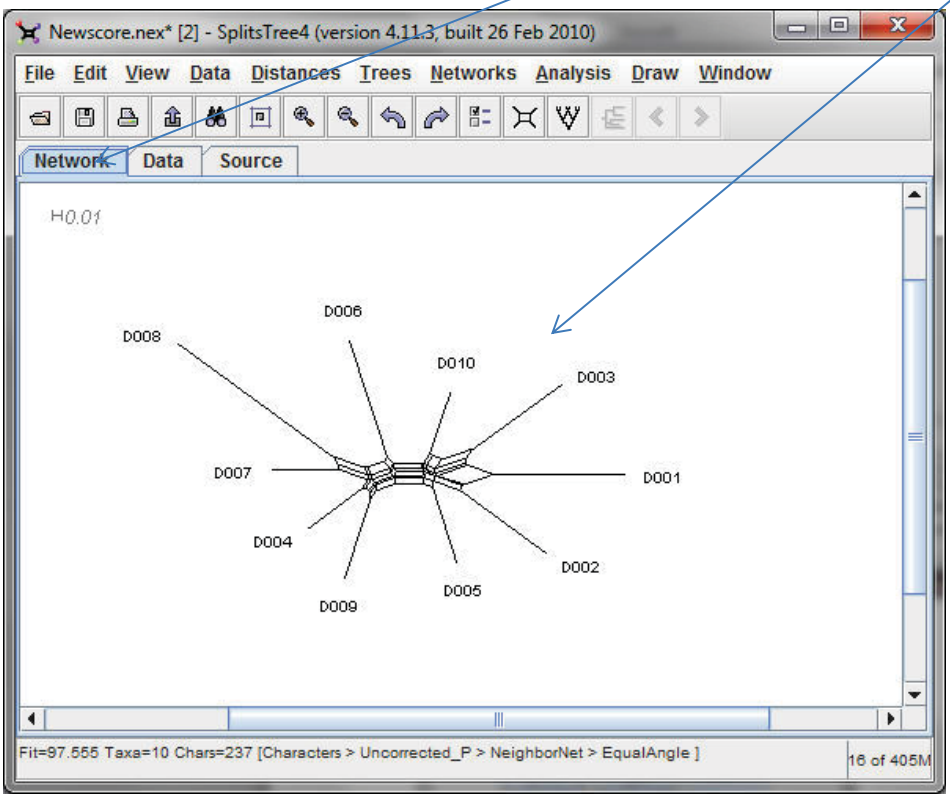
Now click on the Data-tab and SplitsTree automaticly modifies and update your command, the operation itself and generate the right data:



Just click “Yes” in this box and proceed.



The data is now updated so you may go to the network tab and find your NeighbourNet that SplitsTree has generated for you. Volá!



We did it! Congratulations! Now we are up and running in Splitstree. Let us see what we can do with the SplitsTree program, join me in the next exercise to learn more. ☺

(**EX.7 – Learning how SplitsTree works**)

From here you may use the User Manual for SplitsTree4 V4.12.8 which you can download from here: http://ab.inf.uni-tuebingen.de/data/software/splitstree4/download/manual.pdf

This program will let you analyze your dataset, make bootstraps, show genetic distances (eg. DICE or Jaccard) and even make phylogenetic trees.

Shall we do some more exercises, or do you want to proceed on your own from here?

NOTE:

If you, when preparing your excel-file, need to change row-data with column-data (swap places) simply copy all data in your sheet (CTRL-A), open a new sheet (bottom pane +), right-click and paste in selection and remember to tick the option for changing rows and columns.

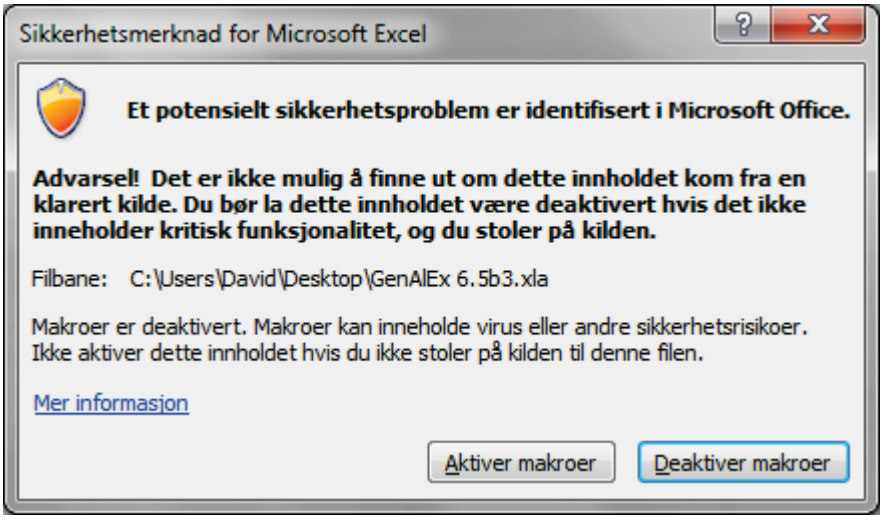
David Mundal, UMB, Ås 19.02.2013.

GenAlEx6.5 for dummies – tutorial for new beginners

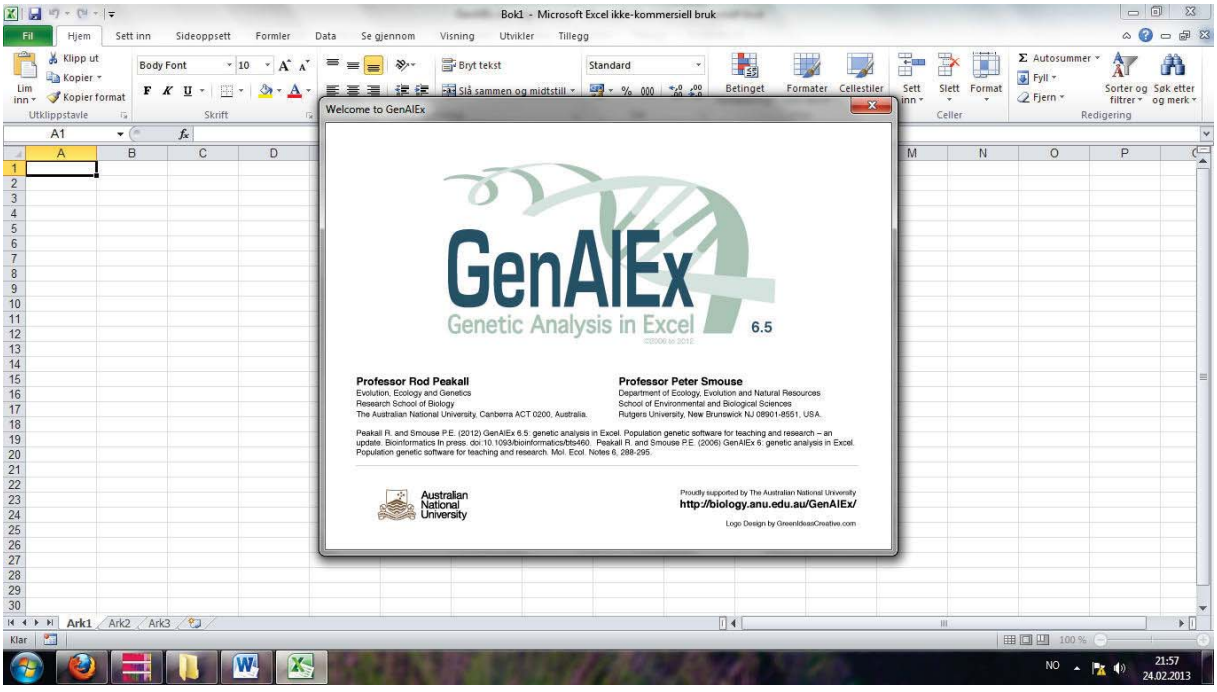
GenAlEx6.5 is an add-in for Excel and works best together with excel 2010. Excel 2007 works also but may run slower. Note that excel 2007 only takes 256 columns of entries while as excel 2010 has no such restrictions.

GenAlEx4.6 can be downloaded from here:
http://biology.anu.edu.au/GenAlEx/Welcome.html

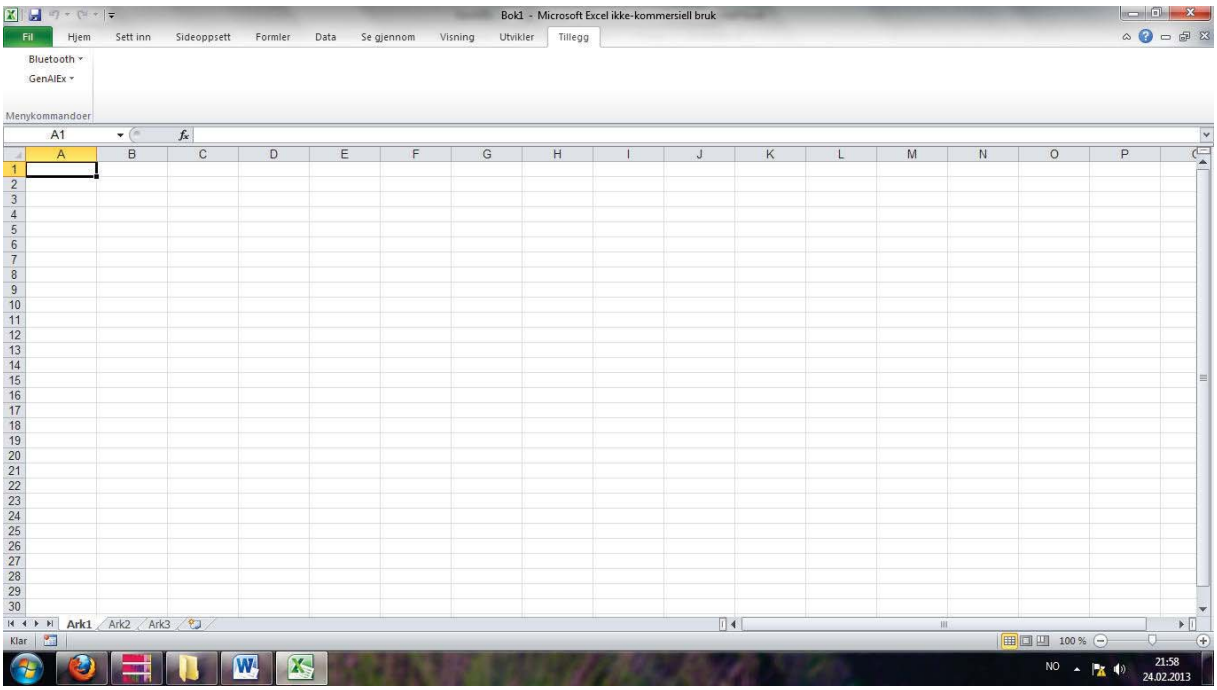
Start excel 2010, and then open GenAlEx4.6. Give your firewall permission to open the file by clicking on the button: (Activate macros).



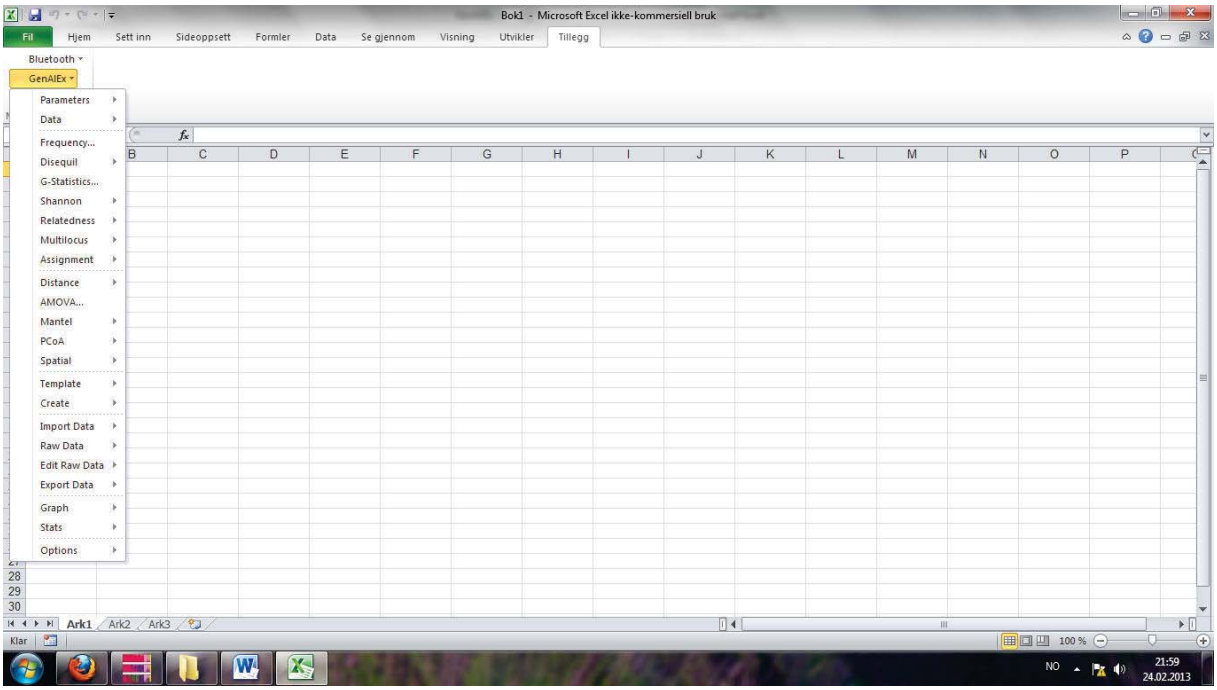
Wait some seconds for GenAlEx to occur in the excel 2010 window, -it shows up as a tab called “AddIns”.



Wait some seconds for GenAlEx to occur in the tab menu of excel2010, -it shows up as a tab called “Add-ins”.



Click on the Add-in dropdown menu at the far left where it says GenAlEx:



Then you access all the analyzing tools.

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