DEVELOPMENT OF MULTIPLEX MICROSATELLITE PANELS FOR POPULATION GENETIC STUDIES OF TRITURUS CRISTATUS

Lars Daniel Bottolfs Håland

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Lars Daniel Bottolfs Håland

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Abbreviations

- BSA : Bovine Serum Albumin
- BJO : Bjørsrud
- bp : base pair
- DNA : deoxyribo-nucleic acid
- dNTPs : deoxyribo-nucleoside triphosphate
- EtBr : Ethidium Bromide
- F_{ST}: : Fixation index
- F_{IS} : Inbreed coefficient
- H-W : Hardy-Weinberg
- Viv : Vivestald
- LAH : Lahell
- $MgCl_2$: Magnesium chloride
- PCR : Polymerase Chain Reaction
- SNP : single-nucleotide polymorphism
- SOL : Solheim
- SSR : simple sequence sepeat
- STR : short tandem tepeats
- TAE : Tris-EDTA Acetate buffer
- TE : Tris-EDTA buffer
- UTM : Universal Transverse Mercator
- °C : Degree Celsius

Glossary

- **Allele :** Variant forms of a gene detected as different phenotypes
- **Amplicon :** A piece of DNA or RNA that is the source and/or product of natural or artificial amplification or replication events, such as PCR.
- **Biodiversity :** The variety of plant and animal life in the world or in a particular habitat.
- **Exonucleolytic :** Cleaving a nucleotide chain at a point adjacent to one of its ends.
- **Fixation index (F_{ST}) :** A measure of population differentiation due to genetic structure and is frequently estimated from genetic polymorphism data.

- **Gene Flow :** The alteration of frequencies of alleles of particular genes in a population, resulting from interbreeding with organisms from another population having different frequencies.
- **Genetic drift :** Random changes in the frequency of alleles in a gene pool, usually of small population.
- **Genotype :** The genetic constitution of an individual organism
- **Heterozygote :** An individual having two different alleles of a particular gene or genes.
- **Homozygote :** An individual having two identical alleles of a particular gene or genes.
- **Inbreed coefficient (F_{IS}) :** Is the probability that two alleles at a random chosen locus are identified by descent.
- **Linkage Disequilibrium :** A nonrandom association of alleles at different loci in a population, as is produced when two loci are closely linked and selection operates to keep certain gene combinations together.
- **Locus :** The location of a gene or a significant sequence on a chromosome or on a linkage map.
- **Microsatellite :** Repetitive DNA in certain DNA motifs, ranging in length from 2 to 5 base pairs.
- **Phenotype :** The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.
- **Philopatric :** Tendency of an organism to stay in or return to habitat.
- **Poikilotherm :** Organism that whose internal temperature varies considerably.
- **Universal Transverse Mercator :** Two dimensional coordinate system.

Abstract

A genotype method for nothern crested newt (*Triturus cristatus*) was developed at Inland Norway University of Applied Sciences, Campus Hamar, by using already validated microsatellite markers. The population of amphibians is known to decline in rapid speed. Development of polymorphic microsatellite markers have the potential to estimate genetic variation in northern crested newt populations and investigate the effect of human intrusive, which causes changes to their habitat.

The program MPprimer designed primers from already validated microsatellite markers and the primers were amplified by a PCR reaction followed by agarose gel. Successfully amplified primers were employed to develop a multiplex PCR with the help of the program Multiplex Manager 1.2. Two different multiplexes with seven and four primer sets were developed with the help of Applied Biosystems 3130xl Genetic Analyzer accompanied by GeneMapper (v 5.0).

DNA from 131 individuals were isolated in 4 different populations from the county Buskerud and Hedmark in Norway. Out of the 11 microsatellite markers, 8 were observed to be polymorphic loci and 3 were monomorphic loci with a mean of number of alleles per loci of 5.2. Two of the markers, AJ292517 and KF442197 had high margin of error and no peaks were observed in the genotyping on the population of Bjørsrud. Overall, over the 4 different population genotyped, the microsatellite markers observed peaks 73% of the time. The population study showed genetic similarities between the populations Bjørsrud and Solheim, while Vivestad and Lahell were more isolated.

1 Introduction

This study took place at the Biotechnology Section of the Dept. of Natural Science & technology at Inland Norway University of Applied Sciences. This thesis focuses on molecular genetics. A main focus was to develop microsatellite marker panels for genotyping population of the salamander species *Triturus cristatus*, also known as the northern crested newt or great crested newt.

1.1 Salamanders, *Triturus cristatus*

Newt is a common name for salamanders that live in water. Northern crested newts live mainly in northern and central Europe, north of the Alps. It is absent from Ireland and most of Scandinavia. Their numbers have fallen in the second half of the 20th century and become rare in many countries("Protection of the great crested newt," 2016). They belong to the family Salamandridae, which consist of true salamanders and newts (Species, 2017). It has a moderately slender body, medium sized legs and a narrow tail base. The skin is warty in texture. (see figure 1) Breeding males develop a jagged crest along the body and tail and this is deeply extended at the base of the tail. Females have a length of about 160 mm and weight 6 to 15 g. Males are lighter and are in the range of 140 to 150 mm (Edgar & Bird, 2006).

Figure 1: Male northern crested newt from (Drechsler et al., 2013).

1.1.1 Prevalence in Norway

In Norway, the salamander lives in the eastern region from the Swedish border and around the Oslo fjord to Skien southwest of Oslo stretching up to the middle of Telemark County, and then as far north as Land, Lillehammer in Oppland and Ytre Rendal in Hedmark. There are also populations between Boknafjorden/Haugesund and Bergen and a few in Nordmøre and on the south- and north sides of Trondheim fjord. This is shown in figure 2 (Dervo, Pedersen, & Bærum, 2016). As a specie that has been considered threatened in Norway. With the support of the Berne Convention (list 2) and EU's habitat Directive, northern crested newt are in dire need of stronger protection in Norway. Even though new northern crested newt localities have been discovered, the number of habitats are strongly declining. As of 2008, about 375 localities of habitats with northern crested newt were discovered. The number of localities with habitats that are considered good is estimated to be 250 (Dolmen, 2008). According to the Wildlife Act, the northern crested newt is considered wild in Norway, and therefore fall under the conservation principle. It is forbidden to capture, hunt, kill or hurt protected wild organisms. This also means that a pond with northern crested newt cannot be harmed without consent (Lovdata, 2017).

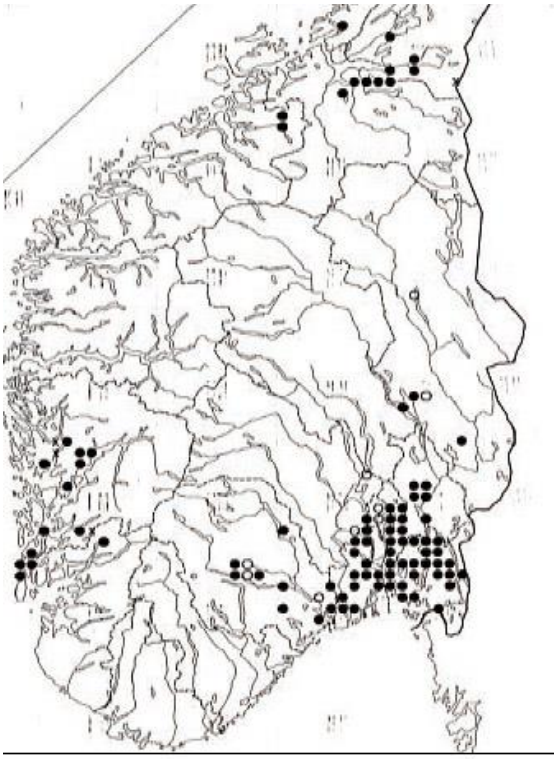

Figure 2: Prevalence of *Triturus cristatus* in Norway as of 1992 (Dolmen, 2008).

1.1.2 Life cycle

The northern crested newt has a complex life cycle where it exchanges between living in the water and on land. In the autumn and winter, it lives in hibernation in soil caves. This happens because the temperature gets so low, and the salamander, as a poikilotherm animal, can't move properly. They are found from 300 to 1300 meters away from the breeding pond. It reduces its metabolism to survive the reduced intake of food. In the spring, it migrates to its breeding pond. Rain signalize that the winter is over, which works as stimulus for waking up after the winter. The standard temperature for this wander is 4 to 5 °C, but lower temperature down to 0 °C has also been observed. Flirtation starts at a temperature on 10 °C, and the female lays 5 to 15 eggs per 24 hours on leaves one by one, from 200 to 300 per season. The larvae are released from the eggs 2 to 3 weeks after. Newly metamorphosed salamanders move quickly to a protective terrestrial environment. Expected lifetimes are 16 to 18 years (Dervo et al., 2016; Skei, Dervo, van der Kooij, & Kraabøl, 2010). For a relative species, *Tritirus dobrogicus*, it was shown that individuals left the pond in approximately the same direction year after year, more often than would be expected by chance from the migration profile for the population as a whole (Jehle & Arntzen, 2000). A study has estimated that juveniles survival was 0.2 and annual adult survival was estimated at about 0.68 based on return rates and do not take emigration into account (Akcakaya et al., 2004). In another study where northern crested newt was colonized to a newly created pond and studied in a six year period had a survival rate for juveniles on 0.22 and adults survival rate of 0.49 (J. Arntzen & Teunis, 1993).

1.1.3 Habitat choice

The northern crested newt is a natural amphibian living very close in contact with water. It prefers small stagnant water bodies: ponds, beaver floodings, temporary natural depression and water bodies situated in quarries. They must contain aquatic vegetation and it prefers clear water, at least partially exposed to sun, and preferably with a forest and grassland within a 50-meter radius. The water bodies should not be more than 500 meters apart, which is the usual distance great crested newts are capable of crossing on dry land ("Protection of the great crested newt," 2016). On leaving the pond, the newts migrate above ground for a few hours, covering relative large distances and takes place in all directions, but with significant preferences for angles towards certain habitat type, where terrestrial habitat at a relative long distance from the pond. (Jehle & Arntzen, 2000). They prefer areas with bushes, hedgerows and trees, and avoid pastures and open areas. Migration in the direction of a habitat type

characterized by trees and underground shelters is favored over migrations in other directions (Jehle & Arntzen, 2000). Key habitat features are low degree of shading for adults and larvae as well as high density of submerged vegetation for larvae. Pond size and depth are not significant (Maletzky, Kyek, & Goldschmid, 2007).

1.1.4 Studies of habitat

In a study from Estonia, 325 water bodies were investigated and northern crested newts were present in 110 (34%) of them and fish were present in 85 of them, of which 16 (19%) also had northern crested newts (Akcakaya et al., 2004). A similar study was conducted in Denmark, where 99 (47%) of the 210 ponds had northern crested newts present and fish was present in 24 ponds, of which two of them (8%) were inhabited by northern crested newts (Akcakaya et al., 2004). Isolation of populations is a very serious threat, which leads to gradual loss of population/genetic diversity. A relatively large population of 100-200 newts had a relatively high risk of extinction if they remained isolated for 50 years or more (Akcakaya et al., 2004).

1.1.5 Threats

One of the main reasons for the decrease of northern crested newts is the lack of small water bodies suitable for breeding. Fish are also a great threat to northern crested newt. They do not eat the adult newts, but feed on their eggs and larvae. Fish also makes the water unclear by eating zooplankton, thus contributing to the proliferation of algae, which reduces the possibilities of the egg to hatch and larvae to feed ("Protection of the great crested newt," 2016). A study done to the age structure suggest that ponds inhabited by fish mainly had young newts (2-3 years old), suggesting that they leave such ponds after their first breeding season. (Miaud, Joly, & Castanet, 1993). Under natural conditions population, persistence and local extinction are influenced by deterministic factors, and stochastic factors play a minor role. However, in cultural landscapes, stochastic events due to human activities have a severe impact on amphibian populations on a longer timescale (Sztatecsny, Jehle, Schmidt, & Arntzen, 2004).

1.1.6Action plan

In Europe the northern crested newt was considered least concern (LC) in the red list as of 2004 and lower risk/conservation dependent (LR/cd) in 1996. In Norway it was considered near threatened in 1999 and the statue was later changed to endangered in 2006 although other concurrent studies discovered more populations than expected and the statue as of Spring 2017 is now near threatened (Edgar & Bird, 2006; naturforvaltning, 2008). Threats to amphibians across Europe have a wide range, most of which are directly related to human

activities. The general lack of long distance mobility makes the crested newt species vulnerable and the rate of population decline contains some of the most rapidly declining amphibian in Europe. In 2006 there was an action plan made by convention on the conservation of European wildlife and natural habitats to stop the rapid decline in population (Edgar & Bird, 2006). In Norway, Directorate of Nature Management made in 2008 an action plan for Northern crested newt. This plan have measures to preserve northern crested newt in existence localities and areas. This includes securing areas from drainage, physical destruction, pollution and against introduction of fish. Areas with more than one pond within reach has priority because of the exchange of animals between ponds, which leads to a greater exchange of genes (naturforvaltning, 2008).

1.2 Genetic markers

Normal cellular operations or interactions with the environment leads to mutations to all organisms, leading to genetic variation (polymorphism). The genetic variation can be within and among individuals, species and higher taxonomic groups. A genetic marker is a DNA-sequence associated with a given phenotype or genotype and can be used to separate individuals, species and higher taxonomic groups on the background of their genetic variation based on mutation over time (Liu & Cordes, 2004). Genetic markers are divided into two types, protein and DNA. Protein markers can be such as allozymes and haemoglobin, while examples of molecular markers are SNPs and microsatellites (Abdul-Muneer, 2014). Molecular markers appear particularly useful for measuring local gene flow and migration, assigning individuals to their most likely population of origin, measuring effective population size through the between-generation comparison of allele frequencies and detecting past demographic bottlenecks through allele frequency distortions.

1.3 Microsatellites

Microsatellites, also known as Short Tandem Repeats (STRs) or Simple Sequence Repeats, (SSR) are short tandemly repeated DNA sequences of 1-6 units of nucleotides up to 100 nucleotides (See figure 3). Most STRs are found in noncoding regions of the genome, while only 8% of STRs are found in coding regions. STRs are classified into different types, where you have mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats, where the most common are the dinucleotide repeats (Fan & Chu, 2007). Trinucleotide repeats were found to be twice as frequent in coding regions in the *Arabidopsis* genome, suggesting selection for certain stretches of amino acids (Hoth et al., 2002). There are 80% GC-rich trinucleotides in exons, whereas AT-rich trinucleotides were disturbed evenly throughout the genomic components;

coding sequences, untranslated regions (UTRs), introns and intergenic spaces. In the non-coding areas, tetranucleotides were predominately situated. Microsatellites have biological functions that can lead to phenotypic changes. These can be variation in intronic SSRs, which can affect gene transcription, mRNA splicing, or export to cytoplasm. Changes in trinucleotide SSRs in UTRs or introns can induce heterochromatin-mediated-like gene silencing. It has been reported that relevant biological processes, such as the regulation of transcription and translation, organization of chromatin, genome size and the cell cycle (Vieira, Santini, Diniz, & Munhoz, 2016).

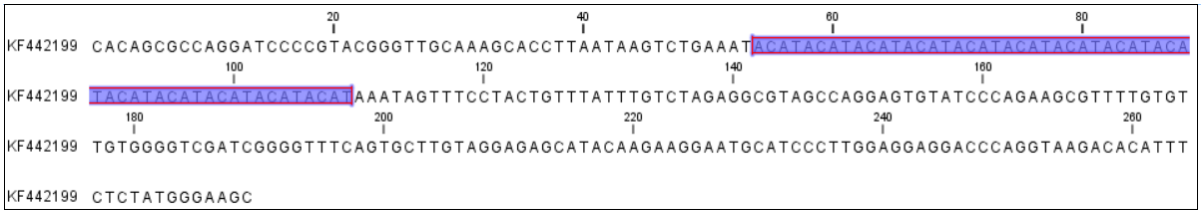

Figure 3: Example of a microsatellite sequence. The microsatellite has a tetranucleotide (ACAT) repeat region with 14 repeats marked in the figure.

1.3.1 Mutation of microsatellites

Microsatellites are evolutionary relevant due to their instability. Microsatellites mutate at the rate of 10^{-2} to 10^{-6} per locus per generation, while unique eukaryotic DNA sequences mutate a rate of approximately 10^{-9} per nucleotide per generation (Ellegren, 2000; Fan & Chu, 2007). Longer loci mutate more often than shorter loci (Ellegren, 2000). The stepwise mutational model (SMM) is model describing a mutation mechanism that result from the addition or deletion of the entire repeat unit or motifs, creating a Gaussian-shaped allele frequency distribution. High mutation rates in SSRs can be because of two mutational mechanisms. The first one involves DNA slippage during DNA replication where the template and nascent strand are mismatched (Selkoe & Toonen, 2006; Vieira et al., 2016). These errors are usually corrected by mismatch DNA repair, which is the most effective and exonucleolytic proofreading, but some mutations occur. Other error are caused by recombination between DNA strands, which means unequal crossing over or over gene conversion can lead to large scale contractions and expansions in the repeat array. There is possibly an association between microsatellites and recombinant hotspots, which means that recombination occur nonrandom in these small clustered regions (Vieira et al., 2016).

1.3.2 Microsatellite loci as DNA-based markers

One type of microsatellite markers is where oligonucleotide primers are complementary to specific SSRs and PCR amplification occurs. Every synthetic oligonucleotides each represents

a specific SSR (Gupta & Varshney, 2000). Microsatellite markers allow the use of small tissue samples which are easily preserved for future use. DNA is easily stored at 96% ethanol and preserved and can still be amplified despite some DNA degradation. Microsatellites are species-specific, so cross-contamination by non-target organisms is much less of a problem compared with techniques that employs universal primers. It has a high information gain because more marker locus can be run with additional loci, so multiple samples can be tested at once (Selkoe & Toonen, 2006). Microsatellites are preferred genetic markers for purposes such as population genetics because of their intrinsic genetic characteristics, which includes high polymorphism, co-dominant, Mendelian inheritance, stability and specificity (SUN et al., 2008). Some disadvantages includes the appearance of shadow or stutter bands, presence of null alleles, which are existing alleles that are not observed using standard assays, homoplasy and too many alleles at certain loci that would demand very high sample size for analysis (Abdul-Muneer, 2014). A new STR locus can be formed by evading the DNA mismatch repair system. Different alleles may exist at the same STR locus, which means that SSR are more informative than other molecular markers, including SNPs (Vieira et al., 2016).

1.3.3 Challenges using microsatellite markers

To amplify the marker regions, specific primer sequences needs to be developed. The primer binds must be identical with start of the sequence that wants to be amplified with few or no mutations. Each primer set rarely works across taxonomic groups, so primers are usually developed anew for each species. Mutation mechanism for microsatellites are complex and still not yet fully understood. In the majority of applications, it is not important to know the exact mutation mechanism for each locus. However, several statistics based on estimates of alleles frequencies rely explicitly on a mutation model. The infinite allele model (IAM), where every mutation event creates a new allele has been the model of choice for population genetics analyses, and continues being used as a default. The phenomenon called "homoplasy" can occur, which is where alleles have the same size, but different lineage. It dampens the visible allelic diversity of populations and may inflate estimates of gene flow when mutation rate is high. There are detectable and undetectable types of homoplasy, where point mutation will leave the size of an allele unchanged, and insertions and deletions in the flanking region might create a new allele with the same size an existing allele. A fraction of genotypes at a fraction of loci have detectable homoplasy appears to be marginal in the majority of cases. When two alleles are identical in sequence, but not identical by descent it is undetectable. This can occur from the random-walk behavior of the stepwise mutation process when there is a "back-mutation" to a previous existing size, or when two unrelated alleles

converge in sequence by changing repeat number in two different places in the sequence. Homoplasy can be problematic for applications in which populations are distantly related. For species with very large population sizes, or for loci with strong allele size, constraints and high-mutation rate (Selkoe & Toonen, 2006).

1.4 Multiplex PCR

Usage of PCR is needed to analyze a microsatellite based genotype. In a multiplex PCR, more than one target sequence can be amplified by including more than one set of primers in the reaction. This will save considerable amount of time and effort within the laboratory without the loss of results. By trial-and-error an optimized reaction can be reached by changing the PCR parameters (Elnifro, Ashshi, Cooper, & Klapper, 2000). Amplifying all the loci individually to see if they amplify individually should be done. For successful multiplex PCR assay, concentration of the primers, concentration of the PCR buffer, balance between magnesium chloride and deoxyribonucleotide concentration, cycling temperatures, and amount of template DNA and DNA polymerase are important. To obtain highly specific amplification products, an optimized combination of annealing temperature and buffer concentration can be reached (Markoulatos, Siafakas, & Moncany, 2002).

1.4.1 Primer design

Genotyping with fluorescent has revolutionized molecular marker-based analysis over the past decade, which has allowed a more rapid data collection compared to earlier methods such as those based on radioactive isotopes/autoradiography and silver staining. Genetic research has in particular benefited greatly from this, and for microsatellites it is the method of choice. The method involves using fluorescently labelled locus-specific primers that ensure PCR amplicons are end-labeled with fluorophores prior to analysis, see Table 1 for universal primers. It is important that the universal primers share limited or no identity with the target genome (Blacket, Robin, Good, Lee, & Miller, 2012).

By using fluorescently labelled universal primers the cost can greatly be reduced by improving PCR efficiency and reducing associated laboratory work for molecular ecological studies that typically use 8-15 microsatellite markers (Blacket et al., 2012).

Table 1: Universal primers and their multiplex PCR fluorophore, Tail sequence and tail length (bp)(Blacket et al., 2012).

Universal Primers	Multiplex PCR fluorophore	Tail sequence (5'-3')	Tail Length (bp)
Tail A	FAM	GCCTCCCTCGCGCCA	15
Tail B	VIC	GCCTTGCCAGCCCGC	15
Tail C	NED	CAGGACCAGGCTACCGTG	18
Tail D	PET	CGGAGAGCCGAGAGGTG	17

1.5 Conservation genetics

Conservation genetics is the application of genetics to preserve species as dynamic entities capable of coping with environmental change. It is motivated by the need to reduce current rates of extinction and to preserve biodiversity, which is the variety of ecosystems, species, populations within species, and genetic diversity within species. (Frankham, Briscoe, & Ballou, 2002)

There are many major genetic issues in conservation genetics(Frankham et al., 2002):

- Inbreeding depression: The deleterious effects of inbreeding on reproduction and survival.
- Outbreeding depression: Deleterious effects on fitness that sometimes occur as a result of outcrossing.
- Loss of genetic diversity and ability to evolve in response to environmental change.
- Fragmentation of populations and reduction in gene flow.
- Genetic drift that overrides natural selection as the main evolutionary process.
- Use of molecular genetic analyses to understand aspects of species biology.

In a study of inbreeding depression in natural situations, it was reviewed 157 valid data sets of wild populations, which included 34 species. In 141 (90 %) cases inbred individuals had poorer attributes than comparable outbreeds (Crnokrak & Roff, 1999). Analyze of large portions of data from marker loci or DNA sequencing data could be used to separate arts groups, populations or individuals. To achieve an effective conservation of endangered species is it important to analyze the genetic variation. The variation within a population is in principal affected by genetic drift, possibility of inbreed and loss of rare alleles. Variation between populations are affected by local customization, selection, gene flow and isolation (Hedrick, 2001).

A general analyze to study population genetics is the Hardy-Weinberg principle, which states that in a large random mating population with no selection, mutation, or migration, the allele frequencies and the genotype frequencies are constant from generation to generation, which is a simple relationship between the allele frequencies and the genotype frequencies (Guo & Thompson, 1992).

The most commonly reported test for loci is conformity to HWE, in which observed genotype frequencies are compared with frequencies expected for an ideal population (random mating, no mutation, no drift, no migration). Heterozygote deficit can occur when a population contains more homozygotes than expected and a heterozygote surplus occur when a population contains less homozygotes than expected. Heterozygote deficit, the more common of the two types, can be due to biological realities of violating the criteria of an ideal population, such as strong inbreeding or selection for or against a certain allele (Selkoe & Toonen, 2006).

Amphibians are good model for investigating the genetics for wild animal population study because they are: widely disturbed in most ecosystems; easy to sample in breeding assemblages; often philopatric to breeding sites, generating high levels of population genetic structure; amendable to controlled crossings in the laboratory and are a major conservation concern. Microsatellite, which are used in this study, are also successfully been used in studies of amphibians effective population size and structure, and in assessing the consequences of hybridization (Beebee, 2005).

1.6 Aim of study

In this master's thesis, the main purpose was to establish a microsatellite-based genotyping method to see the genetic variations of the newt species: *Triturus cristatus*. Samples from different ponds in Norway were collected and different DNA extraction protocols for tissue, eggs and larvae tissue were tested. New microsatellites primers were designed and tested to be specific to *Triturus cristatus*. During this study, 131 different samples of *Triturus cristatus* were analyzed and these results were analyzed using bioinformatics software.

2 Materials and methods

2.1 Collection of samples

The collection of samples follows the procedures given in (J. W. Arntzen, Smithson, & Oldham, 1999) and (naturforvaltning, 2008). In the county of Buskerud, eggs were sampled

from plastic ribbons (1x40 cm) attached to rods that were put into the bottom on 1 m depth, with the ribbon attachment at 40-50 cm depth, and floating towards the surface. In the study ponds in the county of Hedmark, this method was unsuccessful, and samples were collected by collecting larvae by means of a landing net, removing the tail tip of larvae and storing it in 96 % ethanol at -20 °C (see table 2). The samples were stored in ethanol at -20 ˚C. It was collected larvae tails from Solheim (SOL) and Bjørsrud (BJO) and eggs from Lahell (LAH) and Vivelstad (VIV). It was collected in two places in each pond and at two occasions, except in LAH where the water level sank due to tapping for irrigation purposes, leaving the egg sampling ribbons in the air before the second sampling. It was noted that the population of SOL could have samples that are smooth newt.

Table 2: Amount of samples, when and where they were collected.

County	Pond	Samples	Collection Date	UTM
Hedmark	Bjørsrud	30	June	N6730947 E291641
Hedmark	Solheim	30	June	N6730525 E292634
Buskerud	Vivelstad	38	May	N6635831 E232747
Buskerud	Lahell	33	May	N6630212 E236686

The samples were taken from four ponds in the south-east of Norway, more specifically in the counties Buskerud and Hedmark(see figure 4). Picture of the ponds are shown in figure 3 A to 6 A in the appendix.

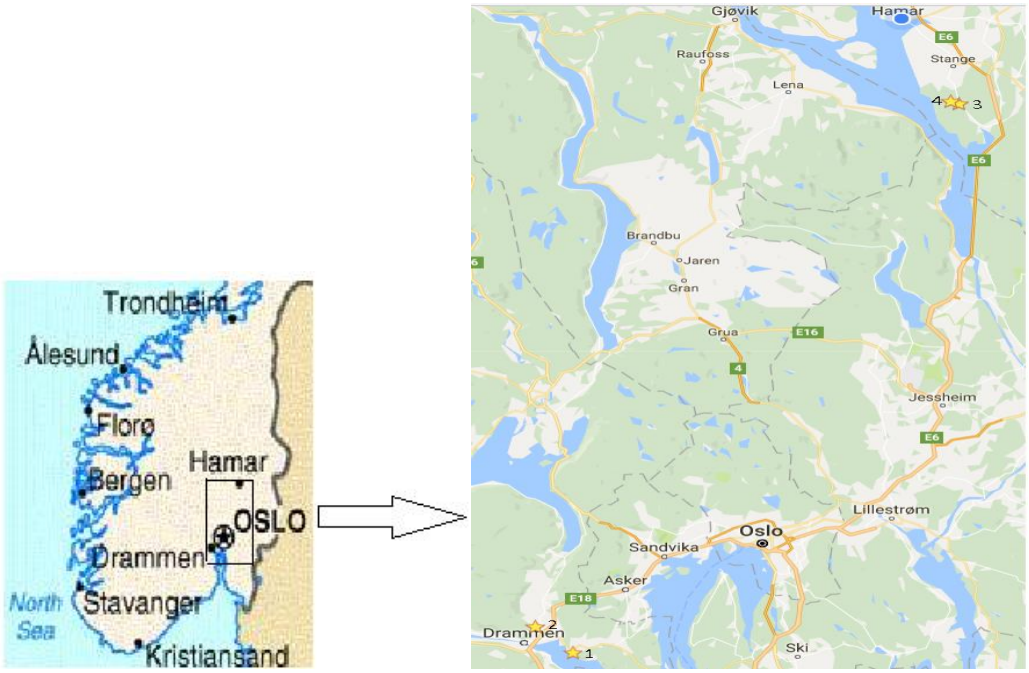

Figure 4: Samples taken from the places marked with star and number: 1: Lahell, 2: Vivelstad 3: Solheim 4: Bjørsrud.

For testing the primers and making the multiplexes a fully grown salamander was used, which was accidentally killed on the road, collected and kept at -20 °C.

2.2 Isolation of Northern crested newt DNA

2.2.2 Tissue

A small part of a tissue was sliced from the salamander and weighted to be in between 20 and 30 mg. This tissue was added to an Eppendorf tube. DNA was then isolated with the use of Bioline's protocol: "Genomic DNA kit: standard protocol; Purifying DNA from cultured cells and human or animal tissue". The only difference was when unsolvable parts were spotted after adding 200 µl lysis buffer G3 and incubated for 70 ˚C, so the sample was centrifuged and the supernatant transferred to a new Eppendorf tube. The isolated DNA was stored at -20 °C. The quality of DNA was investigated using nanodrop.

2.2.3 Egg or larva tissue

The egg samples was directly transferred to an Eppendorf tube and DNA was isolated, while larva was cut to half and transferred to an Eppendorf tube and DNA was isolated. DNA isolation was done using OMEGA bio-tek protocol: "E.Z.N.A. ® Tissue DNA Kit protocol – Tissue", May 2013. The samples were usually observed to weigh less than 5 mg most of the time. Some unsolvable parts were spotted, but it were not enough to change the isolation protocol. The amount and quality of DNA was checked using nanodrop and stored at -20 ˚C.

2.3 Primer design

Microsatellites were collected from various sources from scientific articles. 30 microsatellites of both northern crested newt and smooth newt was collected, and primers were designed using the software MPprimer (Shen et al., 2010) on linux with the following settings:

- Primer length: 17-30, optimal: 22
- Melting temperature (T_M): 59-62 °C, optimal: 60 °C
- G/C content: 30-70 %, optimal: 50 %
- The production size range was different from Lissotriton and Triturus, where Lissotriton was: 60-250 251-400 and the production size range for Triturus was: 75-150 151-250 251-350 351-400.
- Other parameters was set to default.

2.3.1 Testing the primers

Microsatellites were taken from articles (Drechsler et al., 2013; Krupa et al., 2002; Sotiropoulos et al., 2008) and the primers were designed using MPprimer. The microsatellites markers had to be tested before ordering them with labeled fluorescent. Twenty-five primers of both forward and reverse primers were ordered. It was added 0.1x TE buffer to the ordered primers. E.g: if the primer had 21.9 mM it was added 219 µl 0.1x TE buffer.

PCR was done with the total volume of 15 µl with the following components: 2.0 mM $MgCl_2$, 10% 10x B1 buffer (1.5 µl), 0.1 µM forward and reverse primer, 0.1 mM dNTPs, 0.05U Hot Firepol® DNA polymerase and 3.33 ng genomic DNA. There was also a negative control without DNA-template. PCR was done on Veriti 96 well Thermal Cycler (ABI) with the following temperature profile: Initiation activation on 95 °C for 10 minutes, followed by 35 cycles of denaturation on 95 °C for 15 seconds, annealing on 58 °C for 15 seconds and DNA synthesizing on 72 °C for 30 seconds. After the cycles, the final step was a 5 minute DNA synthesizing on 72 °C.

The mix had 5 µl of the sample with 5 µl H_2O and 1.2 µl Loading buffer and analyzed by agarose gel electrophoresis (1.5 % agarose / 1 x TAE) with EtBr with a concentration of 0.5 µg/ml. The electrophoresis had an electric potential of 80 Volts and the duration was 40 minutes. The gel was visualized by UV illumination and pictured with a Kodak image taker (Electrophoresis documentation and analysis system)

The genomic DNA were taken from each of the two salamander sorts, northern crested newt and smooth newt. Runs were also performed as described above, but with the annealing temperature of 52 °C and 54 °C in two different experiments. Two runs with annealing temperature of 58 °C and 52 °C with 2.5 mM $MgCl_2$ and 0.2 µM of forward and reverse primers was also performed.

2.3.2 Testing the primers with labeled fluorescence

Using the program Multiplex Manager (version 1.2), multiplexes for northern crested newt were designed (not shown). Originally 3 multiplexes for northern crested newt using a 4 universal primer system of NED, 6-FAM, VIC and PET, where the markers was added to the forward primers. Loci with overlapping base pair size had different dye.

The primers were tested individually, where the PCR end volume was 15 µl and with the concentrations: 2.0 mM $MgCl_2$, 10% 10x B1 buffer (1.5 µl), 0.1 µM forward, 0.2 µM reverse primer, 0.1 µM universal primer (See table 1), 0.1 mM dNTPs, 0.05U Hot Firepol® DNA polymerase, 0.1 ng/µl BSA 100x and 5 ng genomic DNA. The PCR cycle was the same as before. A run without DNA-template was also done for each primer.

It was transferred 0.5 µl of the PCR product to a mix of 9.5 µl formamide and 0.1 µl 500 liz™ size standard. The mix was heated at 95 °C for 2 minutes in GeneAmp® PCR system 9700 (ABI). The mix was then put in the Applied Biosystems 3130xl Genetic Analyzer with the following settings: GS75-300Liz (-250), G5_36cm_POP7_GS500.

After testing primers single with a specific dye, primers that gave results were used in a multiplex, redesigned with Multiplex manager, where 2 multiplex designs were used.

2.4 Genotyping

A total of 131 samples that included egg, larvae tissue and tissue samples were genotyped on Applied Biosystems 3130xl Genetic analyzer from 4 different populations; LAH, SOL, BJO and VIV. Hot Start PCR samples were run on the Applied Biosystems 3130xl Genetic Analyzer with the adjusted run module Fragment Analysis G5_36cm_POP7_GS500 and the results of this were imported and analyzed by GeneMapper (v5.0). The relative dye concentration was plotted against fragment size. The pre-run voltage was held at 10.0 kV for 500 seconds. The electronic injection procedure was executed during 23 seconds under a voltage of 1.2 kV. An injection solution such as Hi-Di™ Formamide (highly deionized) was supplied to denature the PCR samples and preserve the electrical connection among the polymer in the capillaries and the injection wells. Polymer used was Performance Optimized

Polymer™ 7 (POP-7™ polymer). Electrophoresis temperature was maintained at 60 °C by an oven to keep DNA strands denatured.

2.5 Computer analysis

GENEPOP v.4.10 (Raymond & Rousset, 1995) was used to calculate allele frequency, number of different alleles (Na) and number of effective alleles (Ne) for varied amount of samples (N).

Expected heterozygosity (He), Observer heterozygosity (Ho), Inbreed coefficient (F_{IS}) was calculated by ARLEQUIN software (version 3.5.2.2) (Excoffier & Lischer, 2010).

Pairwise genetic differentiation and AMOVA was calculated by ARLEQUIN software (version 3.5.2.2) (Excoffier & Lischer, 2010).

Linkage equilibrium (LE) were tested (Markov chain length 1 000 000 and 100 000 dememorization steps) with ARLEQUIN software (Excoffier & Lischer, 2010).

STRUCTURE software 2.3.4 (Porras-Hurtado et al., 2013) was used to infer the most likely number of population clusters (K) constituting each sample. Five different runs were performed for each K (1-6). Length of burn in period was set to 20 000 and the number of Monte Carlo Markov chain (MCMC) reps after burnin was set to 20 000. The K with the highest value of ΔK was selected. Harvest software (Earl, 2012) was used to make computations done by STRUCTURE software into a set of readable data and CLUMPP software (Jakobsson & Rosenberg, 2007) was used to calculate a means to 5 of the runs in the STRUCTURE software.

Maximum likelihood estimates of relatedness and relationship were calculated by software ML relate (Kalinowski, Wagner, & Taper, 2006).

Principal coordinates (PCoA) were calculated by GenAlEx 6.5(Peakall & Smouse, 2006).

3 Results

This study entailed the development of microsatellite markers to be used in studies evaluating genetic variation in populations of northern crested newt (*Triturus cristatus*) in Norway. The development of two multiplex reactions were achieved after testing published microsatellite markers (Drechsler et al., 2013; Krupa et al., 2002; Sotiropoulos et al., 2008). Genotyping 131 samples northern crested newt from 4 different populations and one sample of smooth newt was performed.

3.1 Isolation of DNA

Isolation of DNA of tissue, egg and larvae tissue was successful . The quality of the DNA was tested and quantified using Nano drop measurement.

BJO and LAH gave a mean concentration of 54 ng/µl and 25.6 ng/µl, while VIV had a lower concentration of 14.1 ng/µl and SOL had a higher concentration of 268 ng/µl. (See table 3 for mean concentration and appendix; table 1 A for concentration of each individual)

Table 3: Means concentration ng/µl of isolated DNA measured by Nanodrop of 4 different populations.

Population	BJO	SOL	VIV	LAH
Concentration, ng/µl	54.0	268	14.1	25.6

There were also isolated DNA from two different individuals of smooth newt, where the values were registered to 703.9 and 673.4 ng/µl.

3.2 Primer Design

Primers were designed by MPprimer software program (version 1.4) (Shen et al., 2010). Thirty primers were originally found from articles. Twenty-four primers passed the first test, which implied they could not have any chance of making a dimer with another primer in the MPprimer software. Eighteen primers passed the second test, with the goal to show an amplified PCR reaction on a gel electrophoresis. Out of the 18 primers, 10 passed the third test, which was to be able to amplify with a labeled fluorescence and be able to show an amplified PCR reaction on the Applied Biosystems 3130xl Genetic Analyzer in a multiplex reaction. Three primers from smooth newt that was also able to get an amplified PCR reaction

on gel electrophoresis and was then tested with labeled primers on northern crested newt DNA. See tables 4 and 5 for their accession number, dye, primer sequence, primer length and predicted size range. Out of the 18 primers for northern crested newt primers, 14 (77.8 %) were tetra nucleotide and 4 (22.2 %) were dinucleotide.

Table 4: List of northern crested newt microsatellites that includes locus name, what dye they are labeled with, what motive they have, their primer sequence, length of primer sequence and predicted size range (bp).

Locus/GeneBank accession	Motive	Dye	Primer sequence (5'→ 3')	Primer length (F/R)	Predicted size range (bp)
Tcri29 AJ292505 (Discarded)	$(TTTC)_{22}(CA)_{11}$	PET	F: ATGGCTTGCTTTGTTGCTTATTTTT R: CTCTTAGACCTGTTTGTATGAATTTGC	25 27	225-257
Tcri43 AJ292511	$(GAAA)_{30}$	NED	F: ACTCTCCTACAACTATCTCCATCTG R: GGTCGACCACCCTAACTGTTAG	25 22	212-248
Tcri27 AJ292517	$(GAAA)_{27}$	6-FAM	F: AAGAAAACAAGCGAGGTGGAGA R: GTCAGTCACTGAGTCAGGAGAC	22 22	191-240
Tcri35 AJ292490 (Discarded)	$(GAAA)_{32}$ Interrupted	PET	F: GCTTCCACAGACCTAAACAAGC R: AATCAGTGTGCTTTATCTATCTTGCT	22 26	180-224
Tcri36 AJ292491 (Discarded)	$(GAAA)_{36}$ Interrupted	VIC	F: CTCTGGAAACCTGTCCAGCATG R: ACGTTTGGCCAGAGAGAAAGTT	22 22	241-257
Tcri46 AJ292494 (Discarded)	$(TTTC)_{23}$	NED	F: AGAGCTGAAAAACCCTGTTTGG R: AGCCATAGATCCGAGGACAATC	22 22	215-253
Tcri50 KF442195 (Discarded)	$(ACTC)_{18}$	NED	F: CAACAAGTGGCTTTCCCCTTTC R: CGGAACTGCTTCTGTTGTTCAG	22 22	140-231
Tcri52 KF442196	$(ATTG)_{17}$	6-FAM	F: AGTGCACTTACAATTCCCCTGA R: TCAATTGGTTGTAGCAGCCAGA	22 22	118-134
Tcri66 KF442197	$(ATCC)_{18}$	PET	F: TGTACACCACTGGCAAACAAAG R: TGGACCATATTGCACAGTTACCT	22 23	117-149
Tcri70 KF442199 (Discarded)	$(ACAT)_{14}$	6-FAM	F: CGGGTTGCAAAGCACCTTAATA	22 22	134-150

			R: AAACGCTTCTGGGATACACTCC		
Tcri69 KF442202	$(AGAT)_{13}$	PET	F: GTGCAATCGGTATCCAGACAAC R: GAGCTTGATCCTGGCATGAAAT	22 22	134-154
Tcri7 KF442203	$(ACAT)_{11}$	PET	F: AGGACGAGACTCTACGACCTAT R: CTGCTGAAGCGTTACATGTGAA	22 22	115-135
Tcri74 KF442204 (Discarded)	$(AATC)_{13}$	NED	F: ATACTGCCCATTTCGTTTTGCA R: AGTAGTGCGCTCTACAAATGCT	22 22	132-164
Tcri85 KF442205 (Discarded)	$(AATC)_{11}$	PET	F: TTGTTAGACCTCGCATCTGTTG R: GGGTGAGTAGTGCGCTTAAAAA	22 22	91-99
TCM-110 EU760902	$GG(TG)_5GG(TG)_8T(TG)_3CACG(TG)_6$	VIC	F: GCAGGACCAAAACGGTAACATA R: ACGCATACCTTTACCATGCATG	22 22	142-162
TCM-277 EU760904	$(CTT)_2CT(GT)_{21}$	NED	F: AAAGTGAAGTTTGAGCATGGCC R: TTCTCATCTGCTCTATGACCCC	22 22	143-147
TCM-414 EU760906	$(CA)_2CG(CA)_{11}$	NED	F: CACCAATCAACGTATAAGGCGG R: CACGTAGTGGTGCAGAGTGAG	22 21	125-135
TCM-496 EU760908	$(AC)_{14}$	VIC	F: CTGCACCTTTAGTAAACCACTGG R: TGAAATCTAGGTCTCCCCCTCT	23 22	119-131

Table 5: List of smooth newt microsatellites that includes locus name, what dye they are labeled with, what motive they have, their primer sequence, length of primer sequence and predicted size range (bp).

Locus/Gene Bank accession	Motive	Dye	Primer sequence (5'→ 3')	Primer length (F/R)	Predicted size range (bp)
Lm_528 GU574495	$(TATC)_{16}$	VIC	F: CCCTTCAGGCTTGAAGAGAAGA R: ACGTCTTTAGACATGCAGAGGA	22 22	233-278
Lm_521 GU574496 (discarded)	$(GATA)_{19}(GACA)_{11}$	VIC	F: ACGACAGACATACAGAAAGGCA R: GAGGGAGTGGAAAGAAAAAGCC	22 22	207-254
LVG-250 EU568353 (Discarded)	$(AC)_5AA(AC)_{10}AA(AC)_4$	PET	F: TTGGGAGAGCCCTATCTTTGAC R: TGCCAATAGTTTAATCTCTGGCAA	22 24	186-205

3.2.1 Testing of the primer design

PCR reactions were set up for testing the designed primers with genome from northern crested newt to amplify the microsatellite loci. PCR reaction and gel electrophoresis were performed, where a combination of forward and reverse primer of a specific microsatellite loci were tested (see figure 5). Successful amplification was verified by the presence of bands in the gel electrophoresis and comparison with the100 bp ladder size standard. KF442205, KF442196, KF442197, KF442204, KF442202, KF442199, EU760906 and EU760907 showed a value between 100 bp and 200 bp. AJ292517, KF442203, AJ292511, AJ292505, EU760902, KF442195 and EU760908 showed a value between200 bp and 300 bp. KF442195 and KF442199 showed lower amplification than the rest. KF442203 yielded longer amplicons than the expected115-135 bp predicted by MPprimer. Poor intensity of bands were observed on four of the bands: KF442195, KF442199, EU760904 and EU760907. There was also observed 3 lanes with a potential of heterozygosity: KF442202, EU760906 andEU760908, where two bands were present in one lane. PCR products that did not give an amplification in the PCR reaction are not shown. This includes AJ292490, AJ292491, AJ292494, KF442200, KF442201, AJ292487, EU760900 and EU760903.

To give successful amplification for the microsatellite genomic samples a series of runs were performed to establish a final concentration of primers and PCR reaction mix.

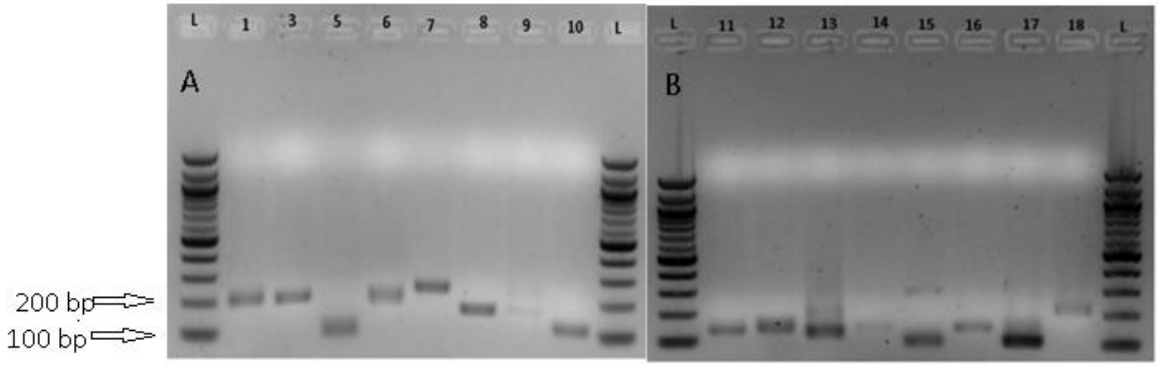

Figure 5: Agarose gel electrophoresis picture of PCR products of isolated genomic DNA with annealing temperature of 58 °C. L: 100 bp ladder, 1: AJ292517, 3: KF442203, 5: KF442205, 6: AJ292511, 7: AJ292505, 8: EU760902, 9: KF442195, 10: KF442196, 11: KF442197, 12: KF442204, 13: KF442202, 14: KF442199, 15: EU760906, 16: EU760904, 17: EU760908 and 18: EU760907.

3.3 Designing Multiplex

A multiplex reaction was developed of the 18 successful amplification markers (primer pairs) by the use of Multiplex Manager (Version 1.2) (Holleley & Geerts, 2009). Primers were ordered with labels made in Multiplex Manager and made by Biomers.net.

Primers were tested by gel electrophoresis after being labeled with specific fluorescence (Figure 6). EU760908 was not tested due to no peaks when it was genotyped on the Applied Biosystem 3130xl Genetic analyzer.

AJ292517, AJ292511, KF442195, KF442204 and KF442204 gave no bands. Bands of the range from 100 to 200 were observed on KF442203, KF442205, AJ292511, EU760902, KF442196, KF442197, KF442202, KF442199, EU760906 and EU760908 with similar intensity of bands. Band of the range 200 and more got observed on AJ292511. The wells on lane 6 and 7 got connected, so it was verified on a retest that it was 7 that gave the results, while 6 gave no results (not shown).

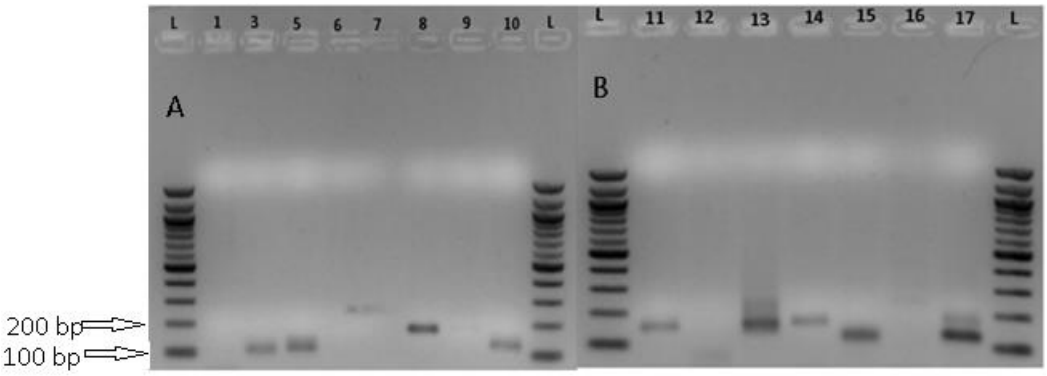

Figure 6: Agarose gel electrophoresis picture of PCR products of isolated genomic DNA with annealing temperature of 58 ºC. L: 100 bp ladder, Panel A: 1: AJ292517, 3: KF442203, 5: KF442205, 6: AJ292511, 7: AJ292505, 8: EU760902, 9: KF442195 and 10: KF442196. Panel B: 11: KF442197, 12: KF442204, 13: KF442202, 14: KF442199, 15: EU760906, 16: EU760904 and 17: EU760908.

After testing the primer sets with a single, specific tailed primers, the primer sets which results were used in a multiplex, redesigned with Multiplex manager. The first runs were done with a concentration of tailed primers of 0.075 nM for all of the 4 tailed primers. The results came back with a high value of VIC which resulted in a parameter “off-scale” this was marked with a yellow danger sign in Gene mapper (5.0). The values were scaled down gradually to finalize a result that had no “off-scale”. The end result of the multiplex testing are given in table 6 with the concentrations of forward and reverse primer. Concentration of the tailed primers is given in table 7.

Table 6: Multiplexes made using multiplex, microsatellites GeneBank accession ID and their concentration in the multiplex.

Multiplex 1		Multiplex 2	
GeneBank accession	Concentration, F / R, nM	GeneBank accession	Concentration, F / R, nM
AJ292511	0.1/0.2	KF442202	0.075/0.15
KF442196	0.075/0.15	KF442203	0.075/0.15
EU760906	0.075/0.15	AJ292517	0.075/0.15
EU760902	0.075/0.15	KF442197	0.075/0.15
EU760908	0.05/0.1		
EU760904	0.075/0.15		
GU574495	0.075/0.15		
KF442205	0.75/0.15		

Table 7: Tailed primer and concentration of dyes used in multiplexes.

Tailed primer	Concentration nM
6-FAM	0.05
VIC	0.01
NED	0.065
PET	0.075

The labeled primers were amplified by PCR machine and tested on the Applied Biosystem 3130xl Genetic Analyzer for the genomic DNA of northern crested newt, and each microsatellite locus was tested individually. The results of individual PCR amplification for multiplex 1 is shown in figure 7. Figure EU760906, KF442196 and AJ292511 had the dye 6-FAM and EU760906 and KF442196 was observed to be homozygote and had the bp of 115 and 135, respectively, while AJ292511 was observed to be heterozygote and had the bp of 203 and 219. EU760902 had the dye VIC and the peak was observed to 174 bp and was a homozygote. The dye NED was on EU760908 and GU574495, where they were observer as homozygote and heterozygote, respectively. EU760908 had 125 bp and GU574495 had 145 and 148 bp. KF442205 had the dye PET, was observed to be heterozygote and had the peaks of 111 bp and 115 bp.

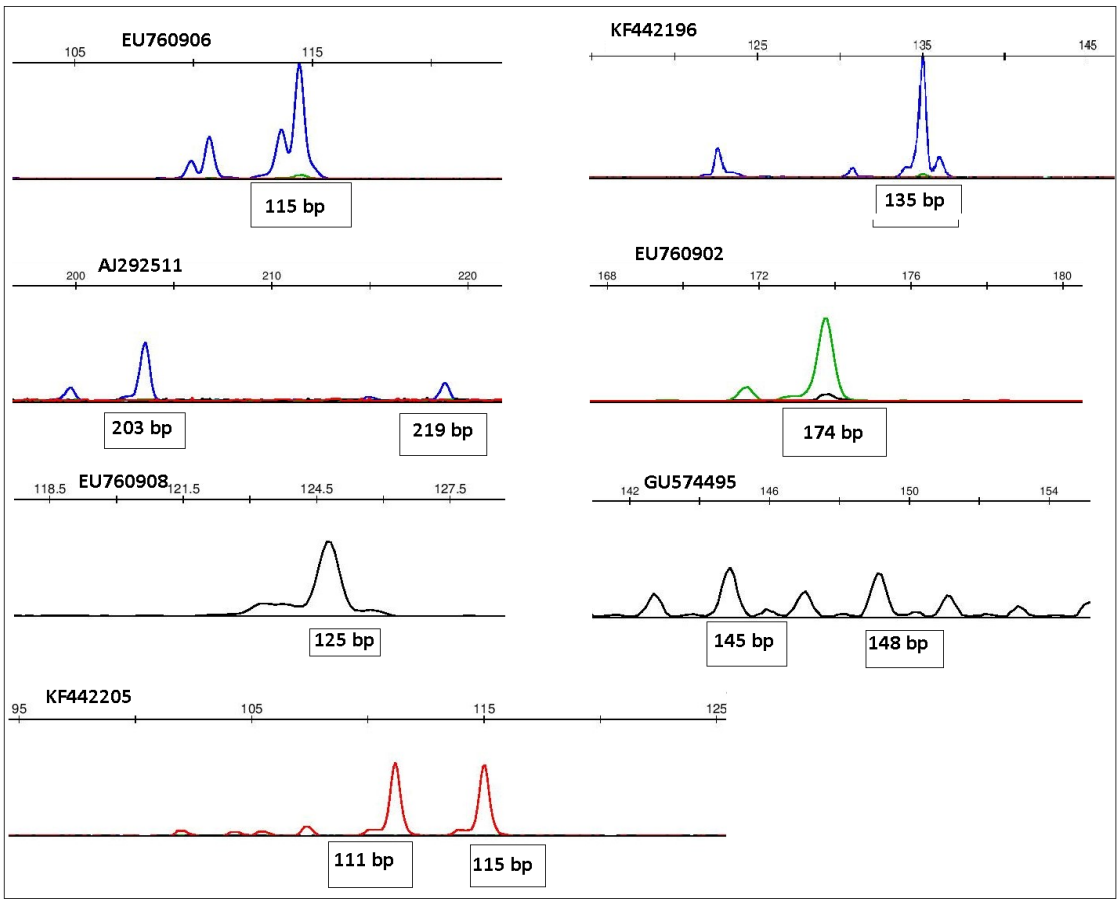

Figure 7: Development of the first multiplex PCR reaction was started with single PCR reaction where the PCR product was genotyped by Applied Biostystems 3130xl Genetic analyzer for each of the primer sets: EU760906, KF442196, AJ292511, EU760902, EU7600908, JU574495 and KF 442205. The X-axis shows size (base pair) while the Y-axis shows the intensity.

The results of PCR amplification for multiplex 2 are given in figure 8. KF442202, KF442203, AJ292517 and KF442197 are in multiplex 2 and have the dye 6-FAM, VIC, NED and PET, respectively. AJ292517 was observed to be heterozygote with the peaks of 227 and 231, while KF442202, KF442203 and KF442197 were observed to be homozygote and had the peaks 160 bp, 100 bp and 155 bp, respectively.

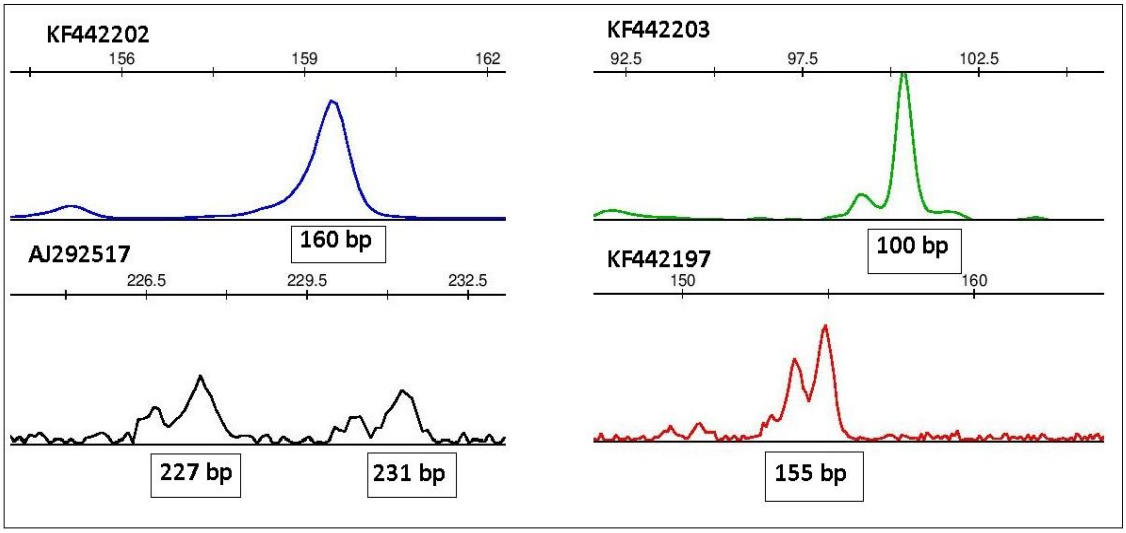

Figure 8: Development of the second multiplex PCR reaction was started with single PCR reaction where the PCR product was analyzed by Applied Biostystems 3130xl Genetic analyzer for each of the primer sets: KF442202, KF442203, AJ292517 and KF442197. The X-axis shows size (base pair) while the Y-axis shows the intensity.

The panel in figure 9 illustrates the bins that were given in the GeneMapper (v.5.0). Every frame is sorted in a way that it catches samples that are framed in a 0.9 frameshift from the original base pair value, for example EU760902 have 100 bp value, but if the value comes out as 100.5 it is the same as having a 100 bp value. As more alleles were discovered, bins were created and more samples were genotyped. The intensity (y-axis) varied, where especially GU574495, KF442205, KF442202, AJ292517, and KF442197 gave low signal intensity, while EU760906, KF442196, AJ292511, EU760902, EU760908 and KF442203 gave higher signal intensity.

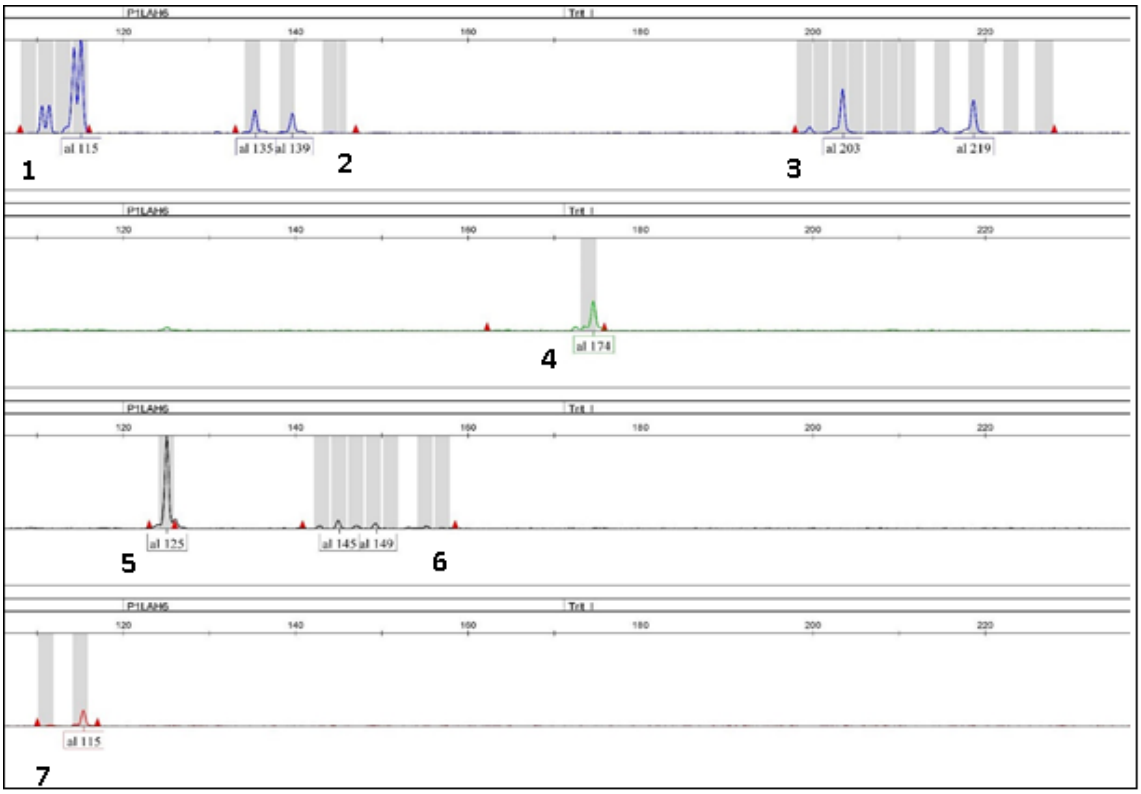

Figure 9: Multiplex 1 panel of a genotyping run of sample LAH 5. 1: EU760906, 2: KF442196, 3: AJ292511, 4: EU760902, 5: EU760908, 6:GU574495 7: KF442205.

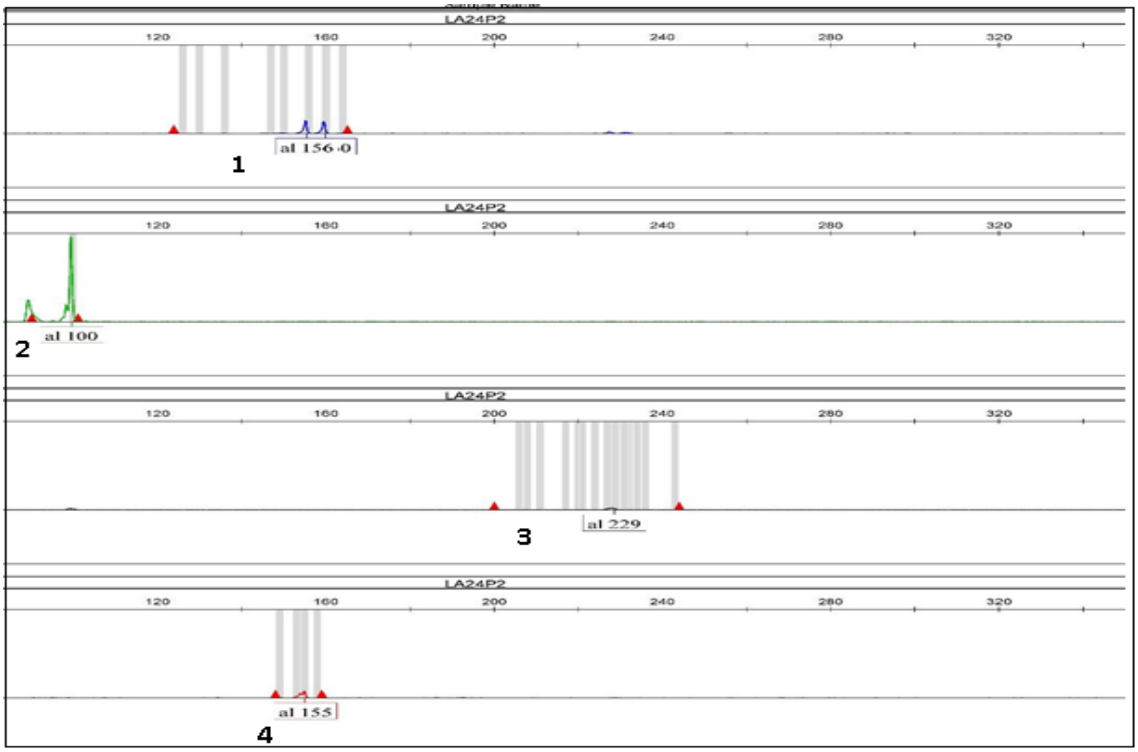

Figure 10: Multiplex 2 panel of genotype run of sample LAH24. 1: KF442202, 2: KF442203, 3: AJ292517, 4: KF442197.

3.4 Smooth newt sample on northern crested newt multiplex.

Figures 11 and figure 12 show isolated smooth newt DNA genotyped for multiplex 1 and 2, respectively. Multiplex 1 showed peaks at 115 bp for EU760906 and at 115 for KF442205. The peak of KF442205 was detected by GeneMapper with the low intensity of 50. Multiplex 2 showed a peak for KF442202.

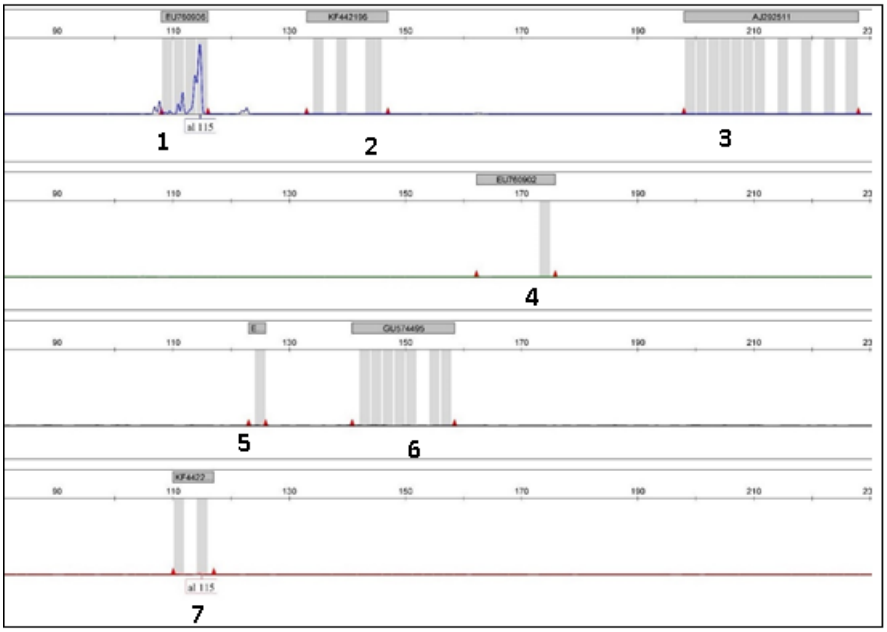

Figure 11: Genotyping of Smooth newt genome with multiplex 1 from northern crested newt. 1: EU760906, 2: KF442196, 3: AJ292511, 4: EU760902, 5: EU760908, 6:GU574495 7: KF442205.

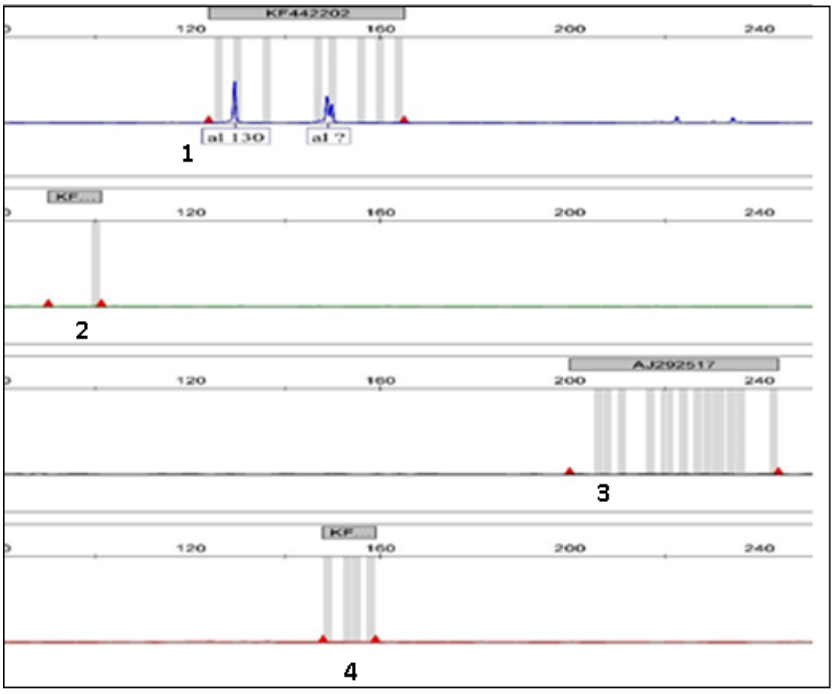

Figure 12: Genotyping of Smooth newt genome with multiplex 2 from northern crested newt. 1: KF442202, 2: KF442203, 3: AJ292517, 4: KF442197.

3.5 Genetic variation and characteristic of loci

3.5.1 Number of alleles and frequency

A total of 131 samples were genotyped of 11 microsatellite loci. The samples LAH3 and LAH4 were not analyzed (See table A2 in appendix for individual genotyping results). Samples with DNA concentration of 0 to 80 ng/µl were not adjusted to the recommended50 ng/µl. Samples with higher DNA concentration than this were adjusted to 50 ng/µl. Samples were taken from 4 different ponds. Genotyping had a success rate of 73 % on all the loci. Success rate applies the percentage of microsatellite markers that gave a signal in the form of peaks on the Applied Biosystems 3130 xl Genetic analyzer. LAH had the highest success rate of 95 % and SOL had the lowest with a success rate of 66 %. BJO and Viv had a success rate of 70 % and 69 %, respectively. Loci AJ292517 and KF442197 had the lowest success rate of 39 % and 38 %, respectively and none of the primer sets amplified at BJO.

Allele frequency in a geographic way is shown in figure 13. The results showed that loci EU760902, EU760908 and KF442203 had one allele that amplified each and are not shown, which had a bp of 174, 125 and 100, respectively. The loci with the most number of alleles was AJ292517 (15 alleles). Over the 11 microsatellite loci the average were 5.2 alleles per where AJ292517 had he most alleles with 15 and KF442202 had the biggest allele range of 38 bp.

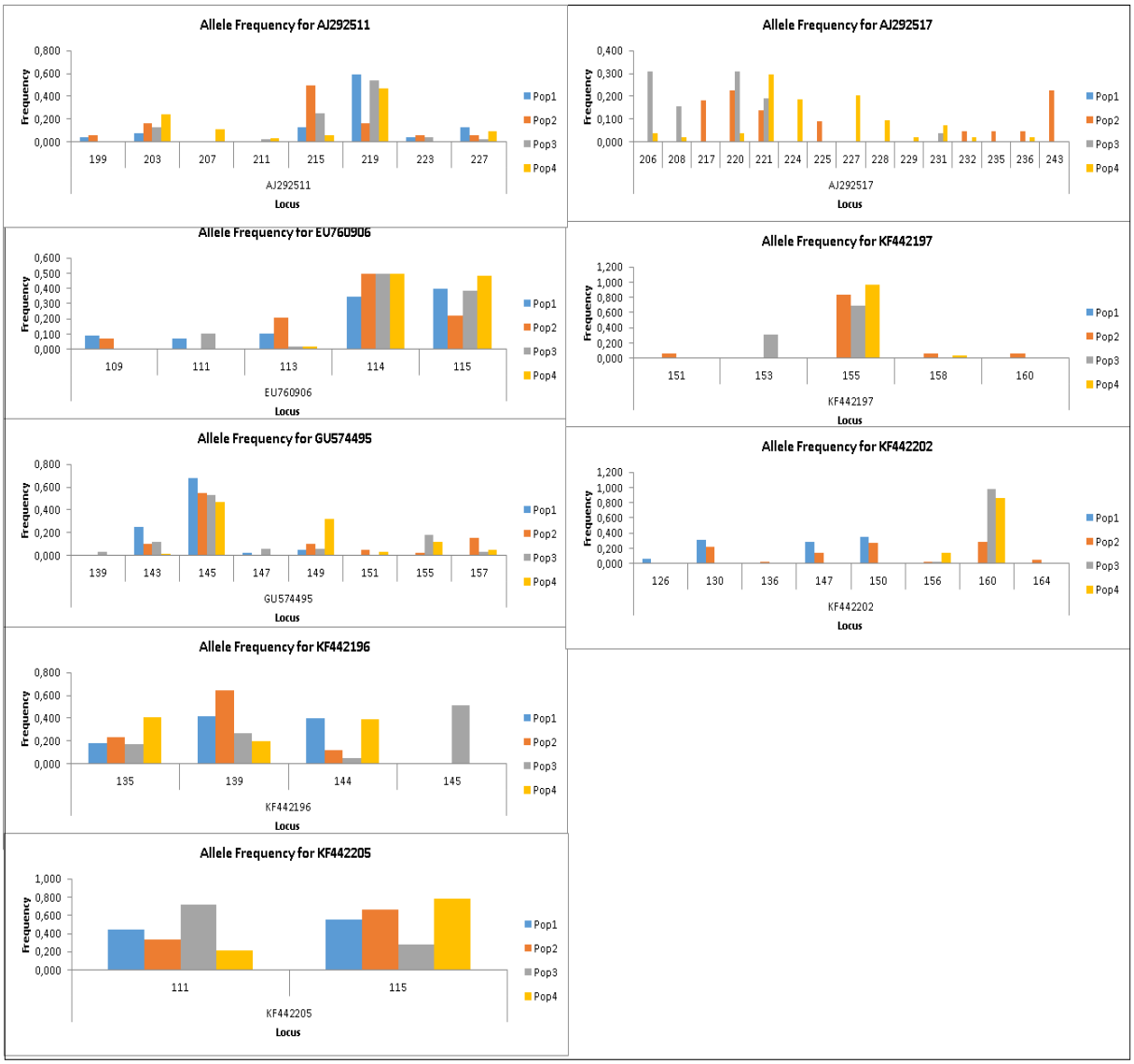

Figure 13: Allele frequency for every locus where pop1:Bjørsrud, pop2: Solheim, pop3: Vivelstad and pop4: Lahell.

Calculated expected and observed heterozygosity for each loci over every population are shown in figure 14. It shows that AJ292511, KF442196, KF442205,AJ292517, KF442197 and KF442202 or 6 out of 8 microsatellite loci had a higher expected heterozygosity than observed heterozygosity, while EU760906 and GU574495 had a higher observed heterozygosity than expected heterozygosity.

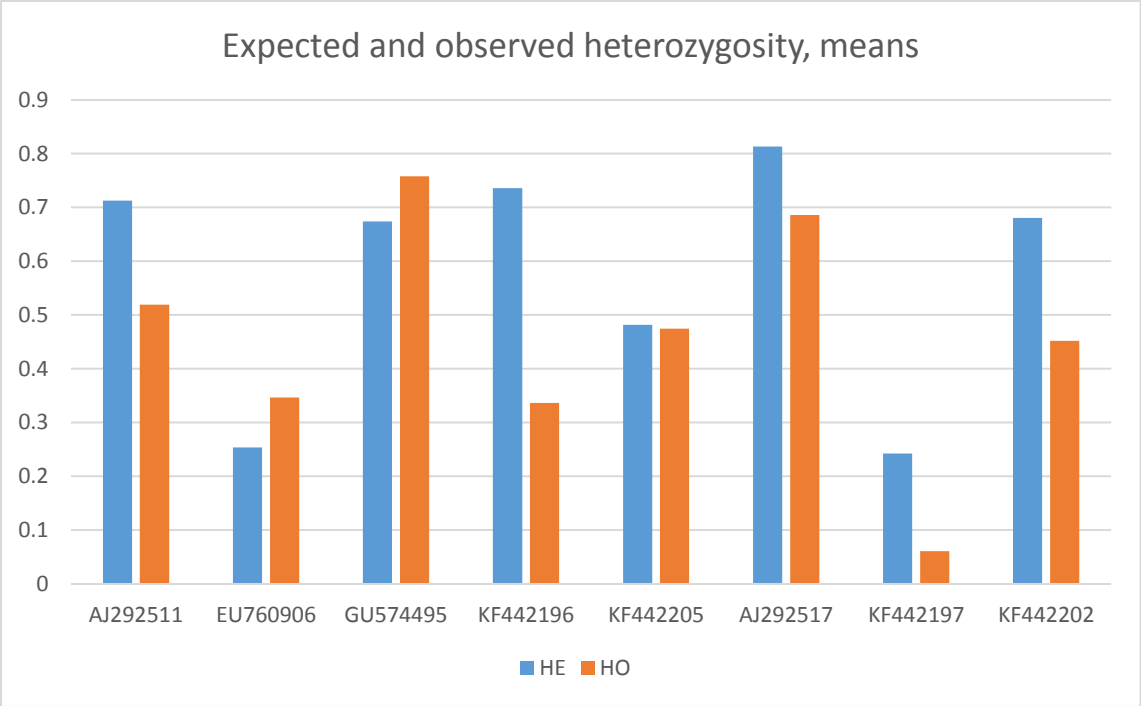

Figure 14: Means expected heterozygosity (He) and observed heterozygosity (Ho) in the 4 investigated populations.

Due to the low success rate of AJ292517 and KF442197, they were taken out of further results because population BJO had no amplification on these locus. Total number of alleles for every population in the remaining 9 microsatellite loci are given in figure 12. BJO, SOL, VIV and LAH had respectively 27, 29, 28 and 24 different alleles.

Genotypic results were further analyzed by GENEPOP and ARELQUIN (table 8). Alleles that were monomorphic or had low success rate, were not included. Significant deviation from Hardy-Weinberg equilibrium (H-W equilibrium), which means deviation from expected heterozygote (He and observed heterozygote (Ho) at $p<0.05$, were observed in 10 out of 24 cases. For BJO, SOL and VIV, significant derivation from (H-W equilibrium) was observed in 2 out of 6 cases and for LAH it was observed in 4 out of 6 cases.

Table 8: Collection of data from 6 loci analyzed in 4 different populations of Northern crested newt in Norway. Number of samples (N), Number of different alleles (Na), number of effective alleles (Ne), observed heterozygote (Ho), expected heterozygote (He) and Inbreed coefficient (F_{IS})

		BJO	SOL	Viv	LAH
AJ292511	**N**	27	18	24	33
	Na	6,000	6,000	6,000	6,000
	Ne	2,545	3,176	2,673	3,295
	Ho	0,333	0,611	0,333	0,758
	He	0,618	0,705	0,639	0,707
	F_{IS}	0,466	0,136	0,484	-0,072
EU760906	**N**	29	29	35	33
	Na	5,000	3,000	3,000	2,000
	Ne	2,170	2,010	1,258	1,062
	Ho	0,517	0,379	0,229	0,000
	He	0,549	0,511	0,208	0,060
	F_{IS}	0,058	0,261	-0,099	1,000
GU574495	**N**	22	19	17	33
	Na	4,000	6,000	8,000	6,000
	Ne	1,887	2,809	3,590	3,175
	Ho	0,455	0,737	0,824	0,939
	He	0,480	0,661	0,743	0,696
	F_{IS}	0,056	-0,117	-0,112	-0,358
KF442196	**N**	25	17	32	33
	Na	3,000	3,000	4,000	3,000
	Ne	2,711	2,050	2,716	2,767
	Ho	0,200	0,235	0,219	0,606
	He	0,644	0,527	0,642	0,648
	F_{IS}	0,694	0,561	0,663	0,066
KF442205	**N**	28	21	18	32
	Na	2,000	2,000	2,000	2,000
	Ne	1,977	1,800	1,670	1,519
	Ho	0,750	0,476	0,222	0,375
	He	0,503	0,455	0,412	0,347
	F_{IS}	-0,504	-0,047	0,469	-0,081
KF442202	**N**	26	28	21	29
	Na	4,000	7,000	2,000	2,000
	Ne	3,322	4,480	1,049	1,312
	Ho	0,808	0,607	0,048	0,276
	He	0,713	0,791	0,048	0,242
	F_{IS}	-0,136	0,236	0,000	-0,143

Means values of the inbreed coefficient (F_{IS}) for the different populations are shown in figure 15. The values of F_{IS} are 0.03, 0.24, -0.20 and 1.00 for BJO, SOL, VIV and LAH, where every locus are included in the calculations, respectively.

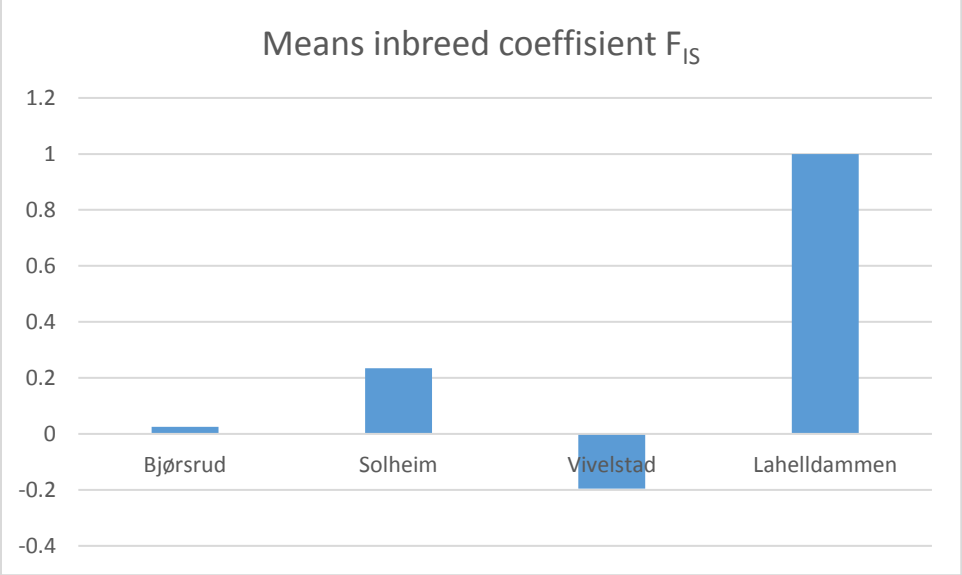

Figure 15: Means inbreed coefficient (F_{IS}) calculated for 6 loci in 4 different populations.

3.5.2 Genetic variation between populations

Analyze of pairwise genetic distance (F_{ST}) between populations in the 4 different ponds showed that both VIV and LAH (F_{ST} 0.02-0.26) were significantly differentiated from the other populations, while SOL and BJO was not significantly differentiated from each other (0.00) at P=0.05 (table 8).

Table 8: Matrix of population pair wise F_{st} values where significance level=0.05 using distance method (* indicates significance).

	BJO	**SOL**	**VIV**	**LAH**
BJO	-			
SOL	0.00	-		
VIV	0.02*	0.16*	-	
LAH	0.19*	0.26*	0.08*	-

Percentage of variation from AMOVA results is shown in table 9 done by ARLEQUIN. The genetic variation within all the individuals was predominant to the individuals among the populations and within the populations. The genetic variation within individuals were 73.88

%, among the populations were 17.02 % and within the populations were 9.11 %. The average F_{IS} and F_{ST} were calculate to 0.10974 and 0.17018.

Table 9: AMOVA results from ARLEQUIN software (6 loci) illustrating the genetic variation.

Source of variation	Sum of squares	Variance components	Percentage value
Among population	55.410	0.32374	17.01755
Among individuals within populations	175.417	0.17323	9.10608
Within individuals	143.000	1.40541	73.87637
Total	373.827	1.90237	
Average F-Statistics over all loci			
F_{IS}	0.10974		
F_{ST}	0.17018		
F_{IT}	0.26124		

STRUCTURE analysis suggested K = 4 as the most likely number of clusters due to maximum ΔK (figure 16).

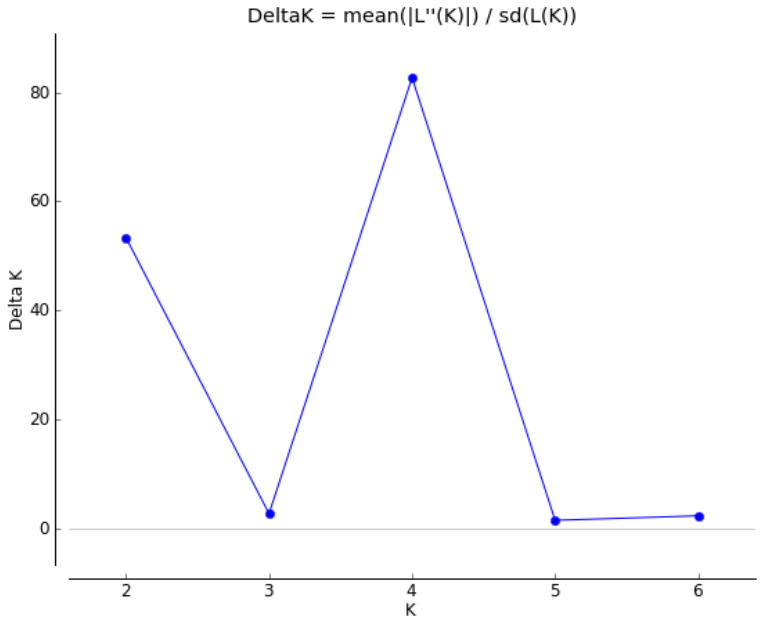

Figure 16: Probability of the data for a tested number of clusters (K = 1-6).

STRUCTURE analysis show that cluster 1 is dominated by samples from BJO and from SOL and cluster 4 is dominated by samples from LAH. Cluster 2 are evenly spread out and cluster 3 mostly resides within the VIV domain. (figure 17)

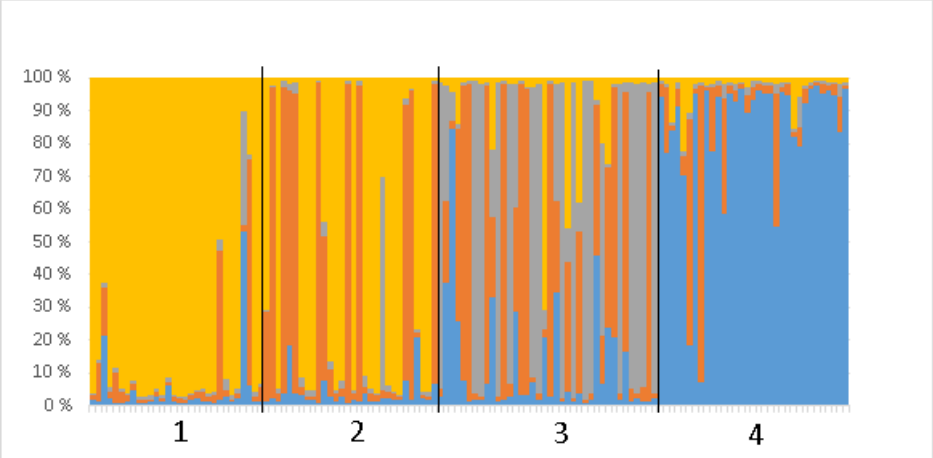

Figure 17: Percentage of individuals of each sample assigned to the four clusters identified by STRUCTURE. 1: Bjørsrud 2: Solheim 3: Vivelstad 4: Lahell.

Linkage disequilibrium test suggested that some of the loci had significant linkage disequilibrium: AJ292511-GU574495, AJ292511-KF442202, EU760206-AJ292517, GU574495-KF442205, KF442196-KF442202, AJ292517-KF442197, AJ292517-KF442202 and KF442197-KF442202 (table 9). KF442202 caused the most significant linkage disequilibrium with 4 different loci.

Table 9: Significant linkage disequilibrium (significance level = 0.0500) for all populations in the form of histogram and matrix. 0: AJ292511, 1: EU760902 2: EU760206, 3: EU760208, 4: GU574495 5: KF442196, 6: KF442205, 7: AJ292517, 8: KF442197, 9: KF442202, 10: KF442203.

Locus:	0	1	2	3	4	5	6	7	8	9	10
	2	0	1	0	1	2	1	3	2	4	0

Locus #	0	1	2	3	4	5	6	7	8	9	10
0	-					+				+	
1		-									
2			-					+			
3				-							
4					-		+				
5	+					-				+	
6					+		-				
7			+					-	+	+	
8								+	-	+	
9	+					+		+	+	-	
10											-

ML-relate results shows a large number of unrelated samples, with the exception of VIV that had more samples of full siblings than the other populations. Table 10 have the means of each matrix of the ML-relate (raw data not shown).

Table 10: Calculated maximum likelihood estimates of relatedness and relationship taken from matrix (ML-relate) where each population is set up individually. Unrelated (U), full sibling (FS) and half sibling (HS).

	BJO	SOL	VIV	LAH
U	391	320	302	422
FS	44	52	76	177
HS	47	33	10	23

3.5.3 Analysis of isolation by distance

Results from Principal Coordinates (PCoA) suggest that population SOL and BJO are relatively closely related, while VIV and LAH are further away (figure 18).

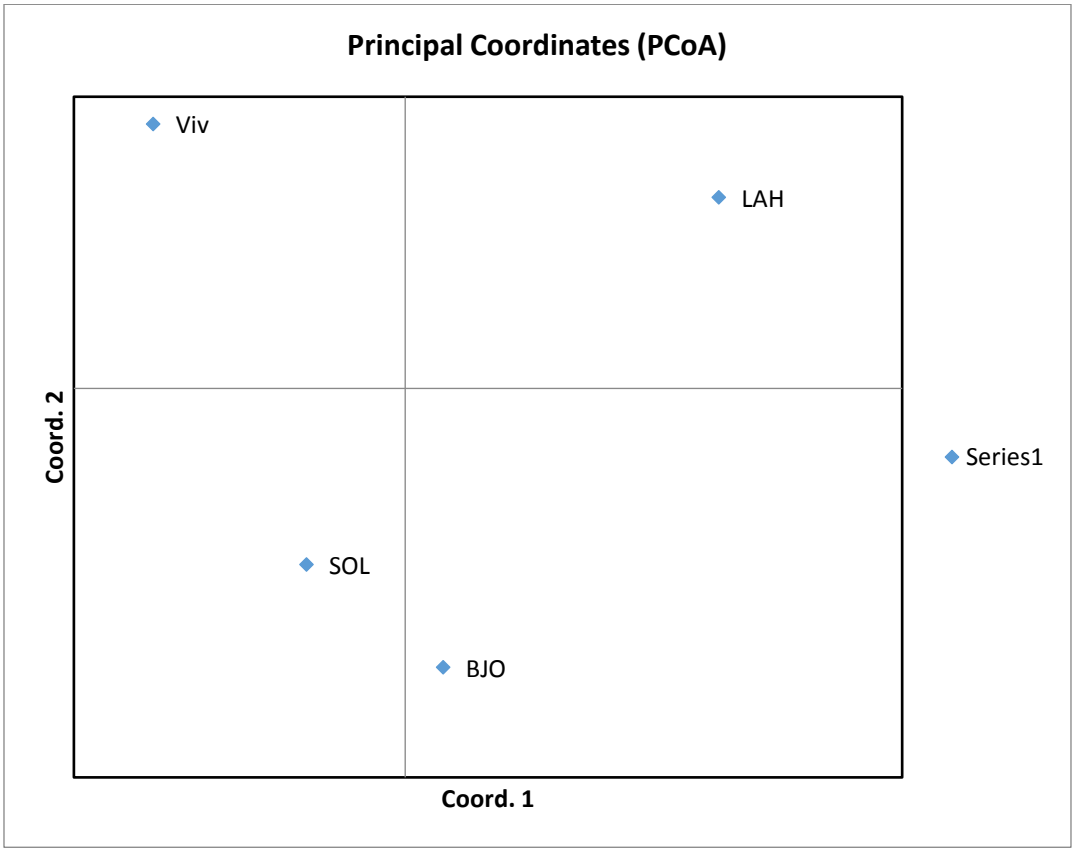

Figure 18: Principal coordinates analysis done by GenAlEx6.

4 Discussion

This study's aim was to develop multiplexes to genotype 4 different populations of northern crested newt (*Triturus cristatus*) and also test these multiplexes with a few samples of smooth newt (*Lissotriton vulgaris*). Differences between these two species are very hard to see in the early stages of egg and larvae stage life cycle. Established microsatellite loci was gathered from different articles and primers were designed using MPprimer. Primers were tested in various PCR reaction and then put into two different PCR multiplexes with 7 and 4 primers, respectively. 131 samples over 4 populations were genotyped.

4.1 Isolation of DNA

DNA was successfully isolated from tissue, egg and larvae tissue samples from the northern crested newt. Isolation of DNA from smooth newt gave a higher DNA concentration compared to DNA isolation of northern crested newt. A high variance of concentration of DNA was observed in the studied populations of northern crested newt, where samples from SOL had an exceptionally high concentration. Based on the concentration observed in isolating DNA from samples of smooth newt, it is strongly believed that some of the samples from Solheim were in fact samples of smooth newt. DNA concentration of samples from VIV were lower than the other 3 populations. There were used isolation methods from Bioline and OMEGA bio-tek, where both worked and small derivations were observed when using either.

The samples that were isolated were not identical, where samples from SOL were observed to be a mixture of larvae tissue and tail. The samples with tissue had some complications with being isolated, where an extra step was required to remove the solids after lysis.

Figure 13 shows a few private alleles. One specifically which was special was the one with 145 bp on FK442196 on the population VIV, while other populations had 144 bp the loci and also some from VIV as well. The difference of this one base pair is shown in appendix (figure 2 A in appendix).

4.2 Microsatellite markers

Established markers were used to design microsatellite primers. 25 primers from Northern crested newt (*Triturus Cristatus*) were designed and tested on tissue sample with the software MPprimer, and 3 primers from smooth newt were taken from a similar study and tested on tissue sample. The sequences were found by searching articles (Drechsler et al., 2013; Krupa

et al., 2002) where it was found by 454-sequencing (Drechsler et al., 2013). Developing of new microsatellite loci for the endangered species would allow a more detailed identification of population structure, dispersal, and migration rates across geographic scales and estimates of effective population sizes of subpopulations or even populations from single ponds can now be identified with much higher resolution. (Drechsler et al., 2013) Out of the 25, there were 10 primers that worked with fluorescently labeled primers and 1 out of the 3 for smooth newt worked. Primers that did not work was not redesigned and retested.

Different concentrations of $MgCl_2$, primer concentration and annealing temperature was tested, but did not give any better results. $MgCl_2$ concentrations can inhibit the reaction at higher concentrations, but generally it will increase the specificity of the target to be amplified(Henegariu, Heerema, Dlouhy, Vance, & Vogt, 1997). Bovine serum albumin (BSA) was added to the reaction, but optimization of the concentration was not attempted. BSA has the known effect of relieving interference in PCR(Kreader, 1996). Extending the annealing has been shown to not have much effect, but altering the annealing temperature have shown to have effects in another study, hence there must be an optimal annealing temperature(Henegariu et al., 1997). The annealing temperature remained on 58 $^{\circ}$C after several trials with different temperature.

Out of the starting 25 primers, 18 of the samples gave result in gel electrophoresis, and 10 of them gave results with fluorescently labeled primers. The results did not seem to be random because the samples were tested more than once and there were consistencies of which primers gave results. Gel electrophoresis was done before ordering fluorescently labeled primers due to a relative difference in price.

One of the marker, KF442195, was a heterozygote with huge allele difference (175 and 238 bp) shown in appendix: figure 1 A. When it was put into a multiplex, there were no peaks given in this range.

4.3 Developing of 2 microsatellite multiplexes

Primers were successfully grouped into two different multiplexes of 7 and 4 primers each. Development, testing and optimization of multiplex PCR for genotyping is a time consuming work, but the cost and time to develop it should outweigh the amount of runs that are needed for testing the microsatellite markers one by one and is should be a good investment (Lerceteau-Köhler & Weiss, 2006). After the first few test runs, the loci's with the best results were used in one multiplex, and the few that did have worse results were taken in the other

multiplex. There was struggles with the “off-scale” parameter on the Applied Biosystems 3130xl genetic analyzer, were the dye VIC caused a high peak, which caused other dyes to show peaks at the same bp which was expected with the labelled dye VIC, but not for other dyes(not shown). Concentrations of the dyes were changed to the extent that the results could be read and analyzed. Concentration of the primer sets were adjusted, but only small effect were noticed so only a few changes were made. When many specific loci are simultaneously amplified, the more efficiently amplified loci will negatively influence the yield of the product from the less efficient loci (Henegariu et al., 1997). The multiplexes did not need the same concentrations of universal primers, but for simplicity, it was decided to use the same concentration for both.

4.4 Genotyping results

Genotyping were successfully done on 131 individuals and their data was analyzed by GeneMapper (v 5.0) and bins were made as new alleles were discovered. Out of the 11 microsatellite loci that were markers, 8 were shown to be polymorphic loci,2 had a low success rate of genotyping and were 3 were considered monomorphic loci.

Two of the loci, AJ292517 and KF442197 gave no results in the genotyping of samples from population BJO. They were also not present in a lot of the samples from VIV and SOL compared to the other loci that was analyzed. In most cases when one of the loci was not present, the other was neither. The low rate of successful genotyping caused them to be excluded of some genetic analyses by standards of the software. AJ292517 was the loci with the most amount of varied alleles, even though it had the least amount of peaks shown in that range of bp.

EU760908, KF442203 and EU760902 have been shown to be monomorphic loci, which are often excluded from both evolutionary and population genetics approaches due to their apparent lack of genetic variability (Nazareno & dos Reis, 2011). In another study it has showed that KF442203 have a number of different alleles of 5 from two different populations (Drechsler et al., 2013). A study done by Macedonian crested newt (*Triturus macedonicus*) had different number of alleles on loci EU760902 and EU76090, which had an allele number of 4 and 5, respectively from a single pond of 40 individual samples (Sotiropoulos et al., 2008).

A population study of northern crested newt from two different population had an average alleles per loci of 10.7 (Drechsler et al., 2013). This study had a value of 5.2 alleles per loci.

A comparison of concentration and genotyping could been drawn, where DNA concentrations of 200 ng/µl and more have shown a genotype figure similar to the smooth newt. Sample SOL11, SOL12 and SOL13 (appendix 2) are examples, were genotyping are similar to the genotyping done on Smoot newt (figure 10 and 11). It cannot be certain that it is smooth newt in the sample size, but it is highly likely.

4.5 Genetic analysis

It is important to note that Lahell is isolated with long distance from other ponds. VIV got 3 ponds within 500 meter. The eggs from LAH was collected all at once and the water level sank so that the "egg collectors" were dried hence the samples from LAH could be the offspring of limited selection of females, while samples from VIV was gathered over 2 weeks. This could also explain why the inbreed coefficient is so high in LAH, and the high number of siblings compared to VIV and the others. The collection of egg might give more chance of closely related individuals. BJO are 5 years old and near neighbor of SOL, which is older. (A. Linløkken, personal communication).

The population pairwise F_{ST} showed valued from 0.00 to 0.26. A F_{ST} of zero indicated no divergence between populations (Lewontin, 1972). SOL and BJO had a F_{ST} value of zero. The other populations have a significant value of F_{ST}, which means they are not significant greater than random distribution of sequences among populations (Excoffier, Laval, & Schneider, 2005).

The structure program computed that the populations were best divided into 4 different clusters (figure 13). Each individual had one line that could have 4 different colors based on the calculations of the program, based on their F_{st} (figure 14). The final figure was made from 5 simulations on the STRUCTURE program and composed to one figure to have less randomness to the simulations. Cluster 3 (VIV) had the most chaotic cluster, where there were components from the other clusters. BJO and LAH had a more clear clusters without sharing components, while BJO and SOL shared components.

Linkage disequilibrium did happen for 8 of the loci matches. Most linkage equilibrium holds for most loci because of balancing factors like genetic drift, random mating and distance between markers, but equilibrium is reached when random mating and recombination should ensure that mutations spread from original haplotype to all haplotypes in the population (Spielman, McGinnis, & Ewens, 1993).

The Principal coordinates suggested that BJO and SOL were close in distance, which was accurate based on the distance geographically (figure 4). Viv and LAH were further apart and not close to each other. Parent/Offspring in ML-relate was taken away because our study was with one generation.

4.6 Recommended future studies

DNA was successfully isolated from tissue, egg and larvae tissue from northern crested newt, but the slime samples (not shown) were not successful. A way to extract DNA from slime samples of northern crested newt could be designed.

In this study, 11 established microsatellite loci were put into multiplexes. A greater number of microsatellite loci could be designed. MPprimer program eliminated potential 5 primers in the early stage, and gel electrophoresis eliminated 7 more. These primers could be re-designed and retested.

Due to a large focus on not getting the “off-scale” from gene mapper, the optimization of different parameter $MgCl_2$ concentration and annealing temperature can be done better.

Due to the fact smooth newt and northern crested newt can live in the same ponds, a genotype test can be designed to separate them. In this study there were forebodings of samples from smooth newt, but it could not been certain.

5 Conclusion

The genotypic on northern crested newt (*Triturus cristatus*) was successfully accomplished with the help of 11 microsatellite markers. It was developed two multiplex PCR reactions based on established microsatellite markers. 131 samples of northern crested newt from 4 different ponds were analyzed and the population diversity, genetic variation and structure were observed. Three of the eleven microsatellite markers were observed to be monomorphic loci, while the remaining eight were observed to be polymorphic loci, where 5.2 alleles per locus were observed.

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Appendix

Table 1 A: Concentration and absorbed values of northern crested newt individuals.

SAMPLE ID	NG/µL	260/280	260/230
BJO1	48.95	1.95	1.56
BJO2	60.4	2.05	2.09
BJO3	42.7	1.9	0.98
BJO4	54.1	1.9	1.34
BJO5	54	1.72	0.91
BJO6	35.7	2.19	1.69
BJO7	72.0	1.95	1.60
BJO8	33.4	2.26	1.97
BJO9	38.4	1.96	2.35
BJO10	45.4	2.24	2.46
BJO11	47.4	2.02	1.87
BJO12	23.6	2.02	2.09
BJO13	56.7	2.33	2.33
BJO14	26.3	2.08	1.82
BJO15	41.0	1.84	2.25
BJO16	117	2.07	2.10
BJO17	63.9	2.10	2.07
BJO18	88.4	2.16	2.25
BJO19	74.8	1.90	1.27
BJO20	93.1	1.81	1.15
BJO21	133.4	1.72	0.91

BJO22	236.3	2.01	2.29
BJO23	24.8	1.84	1.87
BJO24	30.71	1.81	1.84
BJO25	49.3	2.08	1.77
BJO26	36.5	1.97	1.93
BJO27	64.8	2.11	2.07
BJO28	36.4	2.12	1.96
BJO29	70.3	1.77	1.16
BJO30	58.1	2.27	2.19
LAH1	22.2	2.13	2.09
LAH2	63.6	2.09	1.48
LAH3	25.8	2.08	1.05
LAH4	22.2	1.92	1.10
LAH5	62.5	1.69	2.08
LAH6	38.6	1.82	1.94
LAH7	19.4	1.65	2.15
LAH8	5.4	0.99	0.74
LAH9	32.1	1.15	2.00
LAH10	9.7	1.15	2.00
LAH11	15.3	1.41	1.06
LAH12	93.5	1.80	2.10
LAH13	19.11	1.9	0.71
LAH14	25.28	1.85	1.07
LAH15	18.08	1.94	1.07
LAH16	29.73	1.92	0.74
LAH17	19.44	1.99	1.27
LAH18	7.16	1.59	0.32
LAH19	60.26	2.01	1.31
LAH20	16.71	1.76	0.62
LAH21	26.9	2.09	1.26
LAH22	25.2	1.99	1.14
LAH23	10.9	1.86	0.72
LAH24	25.6	1.93	1.19
LAH25	12.3	2.18	0.81
LAH26	21.5	1.78	1.03
LAH27	55	1.74	0.94
LAH28	23.5	1.89	1.04
LAH29	12.0	1.49	0.54
LAH30	12.8	1.73	1.0
LAH31	32.2	2.02	1.49
LAH32	11.1	2.65	0.93
LAH33	18.0	2.07	1.00
LAH34	11.3	2.08	0.99
LAH35	8.80	1.28	0.73
VIV1	4.8	1.28	0.94
VIV2	11.7	2.34	1.21
VIV3	9.6	1.74	1.26
VIV4	104.8	2.01	1.91
VIV5	84.9	2.04	2.15
VIV6	45.4	1.97	1.47
VIV7	9.0	2.20	0.89
VIV8	6.1	2.05	0.68
VIV9	85.5	2.03	1.90

VIV10	5.3	2.33	0.37
VIV11	29.2	2.03	1.02
VIV12	51.7	1.93	1.76
VIV13	16.0	2.03	1.04
VIV14	1.6	1.17	0.18
VIV15	62.1	2.01	1.66
VIV16	109.8	1.96	1.95
VIV17	13.5	2.22	1.13
VIV18	2.1	2.21	0.22
VIV19	10.9	2.37	1.14
VIV20	2.0	1.46	0.33
VIV21	14.1	2.20	0.96
VIV22	5.1	1.71	1.55
VIV23	15.3	2.15	0.49
VIV24	1.8	2.07	0.29
VIV25	32.8	2.14	1.63
VIV26	11.5	2.04	0.84
VIV27	11.8	1.96	0.85
VIV28	11.3	1.79	1.20
VIV29	28.2	2.16	1.25
VIV30	15	2.13	1.21
VIV31	30.3	2.00	1.21
VIV32	1.0	0.72	0.12
VIV33	95.8	1.93	1.94
VIV34	7.4	1.77	0.93
VIV35	108.1	1.61	0.77
VIV36	3.3	1.67	0.46
VIV37	13.30	2.11	1.20
VIV38	3.1	1.25	0.27
VIV39	1.15	2.11	0.81
VIV40	7.6	2.04	0.98
SOL1	78.3	2.13	2.33
SOL2	85.4	2.21	2.70
SOL3	210	1.96	2.38
SOL4	268	1.97	2.36
SOL5	85.6	1.94	2.36
SOL6	269	1.94	2.34
SOL7	157.4	1.95	2.36
SOL8	143	1.94	2.42
SOL9	275	1.93	2.30
SOL10	42.7	1.82	2.26
SOL11	332.6	1.96	2.38
SOL12	562.6	1.93	2.31
SOL13	483.6	1.96	2.35
SOL14	198.1	1.99	2.31
SOL15	77.5	1.93	2.31
SOL16	139.6	1.97	2.25
SOL17	57	1.94	1.6
SOL18	401.2	1.95	2.34
SOL19	180.1	1.99	2.34
SOL20	115.0	2.01	2.32
SOL21	270.4	1.98	2.32
SOL22	31.4	2.13	2.26

SOL23	38.8	2.11	1.99
SOL24	34	1.94	1.78
SOL25	104	1.99	2.25
SOL26	95.75	2.02	2.10
SOL27	31.3	2.13	1.83
SOL28	105.5	2.04	2.27
SOL29	46.64	2.03	1.85
SOL30	20.33	2.09	1.85

Table 2 A: raw results of genotype of 131 samples between 4 populations.

Sample	AJ292511		EU760902		EU760906		EU760908		GU574495		KF442196		KF442205		AJ292517		KF442197		KF442202		KF442203	
BJO 1	227	227	174	174	109	115	125	125	145	149	139	139	111	115	0	0	0	0	147	147	100	100
BJO 2	199	199	0	0	109	115	125	125	145	147	139	144	111	115	0	0	0	0	130	150	100	100
BJO 3	219	219	174	174	115	115	125	125	145	145	139	144	111	115	0	0	0	0	0	0	0	0
BJO 4	219	219	0	0	115	115	125	125	145	145	0	0	111	115	0	0	0	0	147	147	0	0
BJO 5	215	215	0	0	115	115	125	125	143	145	139	139	111	115	0	0	0	0	130	150	100	100
BJO 6	219	219	174	174	111	115	125	125	145	145	139	139	111	115	0	0	0	0	130	150	0	0
BJO 7	219	219	174	174	109	115	125	125	0	0	135	135	111	111	0	0	0	0	147	147	100	100
BJO 8	0	0	0	0	0	0	125	125	145	145	135	135	115	115	0	0	0	0	126	150	0	0
BJO 9	215	219	0	0	111	115	125	125	145	145	144	144	111	115	0	0	0	0	130	150	100	100
BJO 10	215	219	174	174	113	115	125	125	143	145	139	144	111	115	0	0	0	0	130	150	100	100
BJO 11	219	219	0	0	115	115	125	125	143	143	144	144	111	115	0	0	0	0	147	147	100	100
BJO 12	219	227	174	174	115	115	125	125	145	145	135	135	111	115	0	0	0	0	130	150	100	100
BJO 13	203	219	174	174	111	113	125	125	143	145	135	135	111	115	0	0	0	0	130	150	100	100
BJO 14	203	219	174	174	115	115	125	125	0	0	144	144	111	115	0	0	0	0	126	147	0	0
BJO 15	219	219	174	174	113	115	125	125	145	149	139	144	111	115	0	0	0	0	147	147	100	100
BJO 16	227	227	174	174	113	115	125	125	143	145	135	139	111	115	0	0	0	0	130	150	100	100
BJO 17	219	227	174	174	111	115	125	125	143	145	139	139	111	115	0	0	0	0	147	150	100	100
BJO 18	219	219	174	174	115	115	125	125	145	145	144	144	111	115	0	0	0	0	130	150	100	100
BJO 19	223	227	0	0	113	115	125	125	0	0	144	144	115	115	0	0	0	0	126	150	0	0
BJO 20	0	0	0	0	113	115	125	125	0	0	0	0	111	111	0	0	0	0	130	150	100	100
BJO 21	219	219	174	174	109	115	125	125	0	0	0	0	0	0	0	0	0	0	130	150	100	100
BJO 22	0	0	174	174	113	115	125	125	143	145	139	139	111	115	0	0	0	0	130	150	100	100
BJO 23	219	223	174	174	115	115	125	125	143	145	139	139	111	115	0	0	0	0	0	0	100	100
BJO 24	219	219	174	174	115	115	125	125	0	0	0	0	115	115	0	0	0	0	130	147	100	100
BJO 25	219	219	174	174	115	115	125	125	143	143	144	144	111	115	0	0	0	0	147	150	100	100
BJO 26	215	219	0	0	115	115	125	125	0	0	144	144	0	0	0	0	0	0	130	150	100	100
BJO 27	203	203	174	174	115	115	125	125	145	145	0	0	115	115	0	0	0	0	0	0	0	0
BJO 28	219	219	174	174	115	115	125	125	145	145	139	139	111	115	0	0	0	0	0	0	0	0
BJO 29	215	215	174	174	109	115	125	125	0	0	144	144	115	115	0	0	0	0	130	150	100	100
BJO 30	219	219	174	174	115	115	125	125	145	145	139	139	111	115	0	0	0	0	130	147	100	100
SOL 1	215	223	174	174	109	115	125	125	145	157	139	139	111	115	0	0	0	0	130	150	100	100
SOL 2	215	223	174	174	113	113	125	125	145	157	135	135	111	115	221	243	155	155	160	160	100	100
SOL 3	0	0	0	0	113	115	0	0	0	0	0	0	0	0	0	0	0	0	130	150	0	0
SOL 4	215	215	174	174	115	115	125	125	145	155	139	139	115	115	220	243	151	155	160	160	100	100
SOL 5	215	219	174	174	115	115	125	125	145	149	139	139	115	115	0	0	0	0	160	160	100	100

SOL 6	215	215	174	174	115	115	125	125	145	145	139	139	115	115	243	243	155	155	160	160	100	100
SOL 7	0	0	0	0	115	115	0	0	0	0	0	0	0	0	0	0	0	0	150	150	0	0
SOL 8	0	0	0	0	109	115	0	0	0	0	0	0	115	115	0	0	0	0	147	147	100	100
SOL 9	0	0	0	0	109	115	0	0	0	0	0	0	115	115	0	0	0	0	130	150	0	0
SOL 10	215	215	174	174	115	115	125	125	145	157	139	139	111	111	220	225	155	155	160	164	100	100
SOL 11	0	0	0	0	113	115	125	125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SOL 12	0	0	0	0	113	115	125	125	0	0	0	0	0	0	0	0	0	0	136	150	100	100
SOL 13	0	0	0	0	113	115	0	0	0	0	0	0	0	0	0	0	0	0	130	150	0	0
SOL 14	0	0	174	174	115	115	0	0	0	0	0	0	0	0	0	0	0	0	130	150	100	100
SOL 15	199	215	174	174	115	115	125	125	145	157	139	139	111	115	220	220	155	158	160	164	100	100
SOL 16	0	0	0	0	113	115	125	125	0	0	0	0	0	0	0	0	0	0	130	150	100	100
SOL 17	215	215	174	174	115	115	125	125	145	145	139	139	111	115	225	235	155	155	160	160	100	100
SOL 18	0	0	0	0	115	115	0	0	0	0	0	0	115	115	0	0	0	0	130	150	100	100
SOL 19	0	0	0	0	113	115	125	125	0	0	0	0	0	0	0	0	0	0	130	150	100	100
SOL 20	203	203	174	174	109	115	125	125	145	145	139	139	111	111	0	0	0	0	130	150	100	100
SOL 21	0	0	0	0	0	0	125	125	143	143	0	0	0	0	0	0	0	0	0	0	100	100
SOL 22	215	219	174	174	115	115	125	125	143	145	135	135	115	115	0	0	0	0	147	147	100	100
SOL 23	215	219	174	174	113	113	125	125	145	149	135	144	115	115	0	0	0	0	130	150	100	100
SOL 24	219	227	174	174	113	113	125	125	145	149	135	144	111	115	0	0	0	0	147	147	100	100
SOL 25	215	227	174	174	113	113	125	125	149	157	139	139	115	115	232	236	155	155	160	160	100	100
SOL 26	199	215	174	174	113	113	125	125	145	151	139	139	111	115	221	221	155	160	160	164	100	100
SOL 27	203	203	174	174	115	115	125	125	145	151	135	144	111	115	217	217	0	0	130	150	100	100
SOL 28	203	219	174	174	115	115	125	125	143	145	135	144	111	115	0	0	0	0	147	147	100	100
SOL 29	203	219	174	174	113	115	125	125	145	145	0	0	111	115	217	217	0	0	130	150	100	100
SOL 30	215	215	174	174	115	115	125	125	145	157	139	139	111	115	220	243	155	155	156	160	100	100
Viv 1	219	219	0	0	115	115	125	125	0	0	145	145	0	0	221	221	155	155	160	160	100	100
Viv 2	219	219	0	0	115	115	125	125	0	0	139	145	0	0	220	231	155	155	156	160	100	100
Viv 3	203	211	0	0	115	115	125	125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Viv 4	219	219	174	174	113	115	125	125	145	147	135	144	111	111	208	220	153	153	160	160	100	100
Viv 5	215	219	174	174	115	115	125	125	145	149	135	139	111	111	206	221	153	153	160	160	100	100
Viv 6	215	219	174	174	115	115	125	125	145	155	139	139	111	111	206	220	153	153	160	160	100	100
Viv 7	0	0	0	0	115	115	125	125	0	0	145	145	0	0	0	0	0	0	160	160	100	100
Viv 8	219	219	0	0	111	115	125	125	0	0	145	145	115	115	0	0	0	0	0	0	100	100
Viv 9	219	219	174	174	115	115	125	125	145	149	139	139	111	111	206	206	155	155	160	160	100	100
Viv 10	219	219	0	0	115	115	125	125	0	0	0	0	0	0	0	0	0	0	0	0	100	100
Viv 11	0	0	0	0	111	115	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 12	219	223	174	174	115	115	125	125	145	157	139	139	111	115	208	220	0	0	160	160	100	100
Viv 13	0	0	0	0	111	115	125	125	145	145	145	145	0	0	0	0	0	0	160	160	100	100
Viv 14	0	0	0	0	0	0	125	125	0	0	0	0	0	0	0	0	0	0	160	160	100	100
Viv 15	215	219	174	174	115	115	125	125	145	155	135	139	111	111	206	220	155	155	160	160	100	100
Viv 16	219	219	174	174	115	115	125	125	145	155	139	139	111	111	206	220	153	153	0	0	0	0
Viv 17	203	227	0	0	0	0	125	125	0	0	145	145	0	0	0	0	0	0	160	160	100	100
Viv 18	0	0	0	0	0	0	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 19	203	203	0	0	115	115	125	125	143	145	139	144	111	115	0	0	0	0	0	0	100	100
Viv 20	219	219	174	174	115	115	125	125	145	155	139	139	111	111	208	220	155	155	160	160	100	100
Viv 21	0	0	0	0	115	115	125	125	0	0	0	0	0	0	0	0	0	0	160	160	100	100

Viv 22	0	0	0	0	111	115	125	125	0	0	145	145	115	115	0	0	0	0	0	0	100	100
Viv 23	219	219	0	0	111	115	125	125	0	0	0	0	111	111	0	0	0	0	0	0	100	100
Viv 24	203	203	0	0	115	115	125	125	143	143	145	145	0	0	0	0	0	0	160	160	100	100
Viv 25	215	215	0	0	115	115	125	125	143	145	135	139	0	0	0	0	0	0	0	0	100	100
Viv 26	0	0	0	0	115	115	125	125	145	145	145	145	115	115	0	0	0	0	160	160	100	100
Viv 27	0	0	174	174	115	115	125	125	0	0	145	145	0	0	0	0	0	0	160	160	100	100
Viv 28	219	219	174	174	115	115	125	125	145	147	135	144	111	111	206	221	155	155	160	160	100	100
Viv 29	0	0	0	0	111	115	125	125	0	0	0	0	0	0	0	0	0	0	0	0	100	100
Viv 30	0	0	174	174	115	115	125	125	139	145	135	135	111	111	0	0	0	0	0	0	100	100
Viv 31	219	223	174	174	115	115	125	125	145	155	135	135	111	115	206	221	155	155	160	160	100	100
Viv 32	0	0	0	0	115	115	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 33	215	215	0	0	115	115	125	125	0	0	135	135	111	115	0	0	155	155	160	160	100	100
Viv 34	215	215	0	0	115	115	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 35	0	0	0	0	115	115	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 36	215	215	0	0	115	115	0	0	0	0	145	145	0	0	0	0	0	0	0	0	100	100
Viv 37	215	219	174	174	111	115	125	125	145	155	139	139	111	111	208	220	155	155	160	160	100	100
Viv 38	0	0	0	0	115	115	125	125	0	0	145	145	0	0	0	0	0	0	0	0	100	100
LAH 1	203	219	174	174	115	115	125	125	149	155	135	135	111	115	221	224	155	155	160	160	100	100
LAH 2	219	219	174	174	115	115	125	125	145	145	135	135	111	115	227	227	155	155	160	160	100	100
LAH 5	219	219	174	174	115	115	125	125	145	149	144	144	115	115	0	0	0	0	0	0	100	100
LAH 6	203	219	174	174	115	115	125	125	145	149	135	139	115	115	0	0	0	0	160	160	100	100
LAH 7	219	219	174	174	115	115	125	125	145	149	139	144	115	115	0	0	0	0	0	0	100	100
LAH 8	219	219	0	0	113	113	125	125	145	145	135	135	115	115	208	220	155	155	160	160	100	100
LAH 9	203	227	174	174	115	115	125	125	145	145	135	144	111	115	206	221	155	155	160	160	100	100
LAH 10	219	219	174	174	115	115	125	125	145	149	139	139	111	111	206	220	155	155	160	160	100	100
LAH 11	207	219	174	174	115	115	125	125	145	149	144	144	115	115	0	0	0	0	0	0	100	100
LAH 12	219	219	174	174	115	115	125	125	149	155	139	144	111	115	0	0	0	0	160	160	100	100
LAH 13	203	219	174	174	115	115	125	125	145	155	135	135	115	115	224	227	155	155	160	160	100	100
LAH 14	203	219	174	174	115	115	125	125	145	155	139	139	115	115	221	231	155	155	160	160	100	100
LAH 15	207	219	174	174	115	115	125	125	145	149	139	144	115	115	224	227	155	155	160	160	100	100
LAH16	215	219	174	174	115	115	125	125	145	149	135	144	115	115	221	224	155	155	156	160	100	100
LAH 17	211	219	174	174	115	115	125	125	145	149	135	144	111	115	227	231	155	155	156	160	100	100
LAH 18	215	219	174	174	115	115	125	125	145	145	135	144	115	115	221	221	155	155	156	160	100	100
LAH 19	207	215	174	174	115	115	125	125	145	149	135	135	115	115	227	231	155	155	160	160	100	100
LAH 20	211	219	174	174	115	115	125	125	145	155	135	144	115	115	221	231	155	155	160	160	100	100
LAH 21	203	219	174	174	115	115	125	125	145	149	135	144	111	115	221	224	158	158	160	160	100	100
LAH 22	207	219	174	174	115	115	125	125	145	149	139	144	115	115	224	229	155	155	160	160	100	100
LAH 23	203	219	174	174	115	115	125	125	145	157	135	139	0	0	224	232	155	155	160	160	100	100
LAH 24	207	215	174	174	115	115	125	125	145	149	144	144	115	115	228	228	155	155	156	160	100	100
LAH 25	219	227	174	174	115	115	125	125	149	155	135	144	111	115	228	236	155	155	160	160	100	100
LAH 26	203	227	174	174	115	115	125	125	145	151	135	144	111	115	0	0	0	0	0	0	0	0
LAH 27	203	219	174	174	115	115	125	125	143	149	135	135	111	115	221	221	155	155	160	160	100	100
LAH 28	219	219	174	174	115	115	125	125	145	151	144	144	111	115	228	228	155	155	160	160	100	100
LAH 29	203	227	174	174	115	115	125	125	145	149	135	144	111	115	221	224	155	155	156	160	100	100
LAH 30	203	207	174	174	115	115	125	125	149	155	135	144	115	115	227	227	155	155	160	160	100	100
LAH 31	203	219	174	174	115	115	125	125	149	157	135	144	115	115	227	227	155	155	160	160	100	100

LAH 32	219	227	174	174	115	115	125	125	145	155	135	144	115	115	221	221	155	155	156	160	100	100
LAH 33	203	207	174	174	115	115	125	125	145	157	139	144	115	115	224	227	155	155	156	160	100	100
LAH 34	203	203	174	174	115	115	125	125	145	149	139	139	115	115	221	224	155	155	160	160	100	100
LAH 35	203	227	174	174	115	115	125	125	145	149	135	144	111	115	221	221	155	155	156	160	100	100

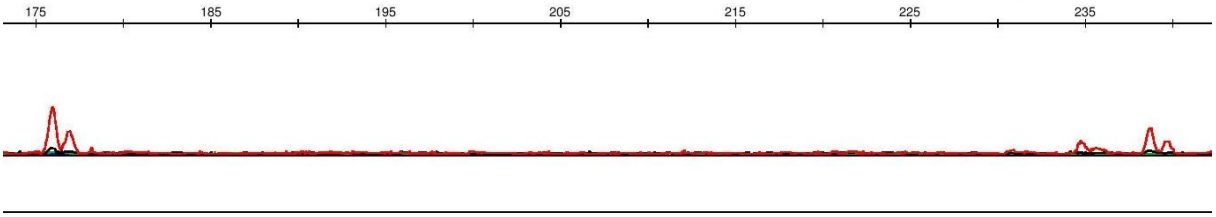

Figure 1 A: Genotype of KF442195.

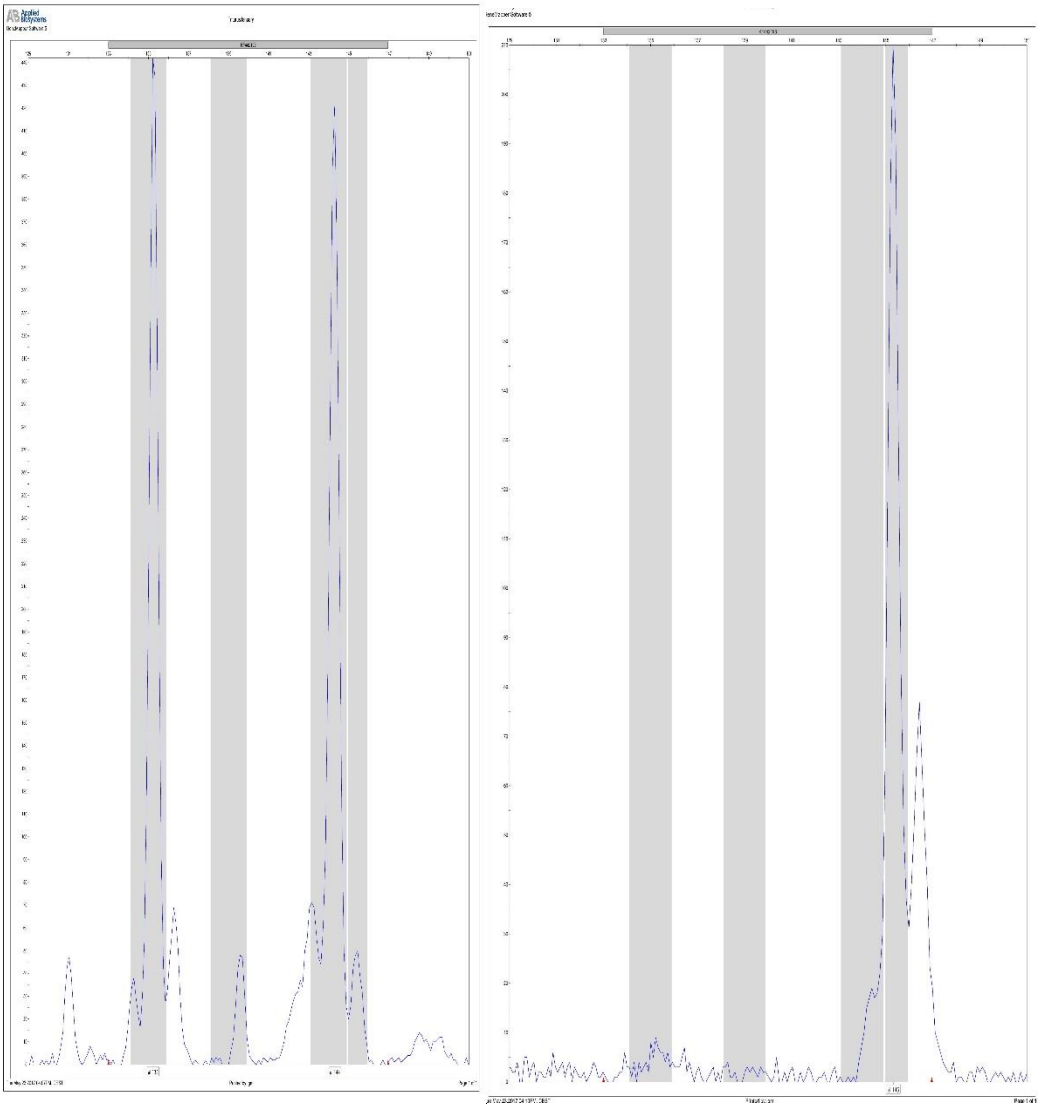

Figure 2 A: Indicates a difference between the alleles 144 and 145 on loci KF442196. Left: Viv39 Right:Viv37

Figure 3 A: Pond of Bjørsrud.

Figure 4 A: Pond of Solheim.

Figure 5 A: Pond of Lahell.

Figure 6 A: Pond of Vivelstad.