Testing cross-amplification of microsatellites on European *Ranunculus* sect. *Batrachium* species

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Annotation:

Cross-amplification of microsatellites on European *Ranunculus* sect. *Batrachium* species was tested using primers that were designed for *R. bungei*. The main aim was to test whether these primers anneal successfully and the regions can be amplified using PCR in order to use them for population genetical studies.

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Abstract

Microsatellite markers are a valuable tool for genetic analysis in plants and animals. The objective of this study is to test if the microsatellite markers which were designed for *Ranunculus bungei* from China also work on European *Ranunculus* sect. *Batrachium* species. Therefore, 13 different primers were tested for amplification on 32 *Ranunculus* plants. Sanger sequencing and fragment analyses were used to gain information whether the primer annealed successfully or not and whether these methods yield enough variation. The results indicate that most of the primers annealed and the regions can be amplified using PCR. However, the method worked for most primers improperly which means that the *R. bungei* primers cannot be used for European species in order to perform population genetical studies. Still, the microsatellite patterns are occasionally species specific and therefore might be used for species identification.

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1. Introduction

1.1 *Ranunculus* sect. *Batrachium*

Ranunculus sect. Batrachium, hereafter *Batrachium,* water-crowfoots, is a taxonomically complicated group of aquatic plants. There are about 30 species worldwide, of which 14 – 18 can be found in Europe (Cook, 1966; Wiegleb *et al*., 2017). *Batrachium* easily form hybrids which enriches the taxonomic diversity. Some *Batrachium* species such as *Ranunculus trichophyllus* and *Ranunculus aquatilis* can be found all over the world, while others (e.g. *Ranunculus lobbi, Ranunculus pekinensis*) have a limited distribution (Cook, 1963; Lumbreras *et al*., 2011). The diversity of *Batrachium* is only weakly recognized, especially concerning regions outside Europe (Bobrov *et al*., 2015).

Batrachium species occupy different kinds of wetland habitats including lentic and lotic ecosystems (Spink, 1992). This means that they can grow in still waters such as lakes and ponds as well as in flowing waters. The occurrence of *Batrachium* strongly depends on the water quality (Spink *et al*., 1997; Lumbreras *et al*., 2009). Due to that, the different species may be used as bioindicators (e.g. Kopec *et al*., 2008; O'Hare *et al*., 2010; Lumbreras *et al*., 2013).

The morphological variability and the incomplete succession of leaf shape as well as the tendency towards hybridization often leads to identification problems of various species of *Batrachium* (Englmaier, 2016). The hybridogeneous species are often much better adapted to changing environments than their parent species (Englmaier, 2016). They are mostly sterile or poorly fertile and sometimes show allopolyploidy (Englmaier, 2016). Interspecific hybridization frequently occurs and it is presumed that all taxa are capable of crossing (Prančl *et al*., 2018). Hybrids are often overseen and are ascribed to the parent species which they resemble morphologically the most (Englmaier, 2016). Phenotypic plasticity, which means that the same genotype may have a different phenotype depending on the environmental conditions, and the frequent occurrence of polyploidy make investigations of this species complicated (Kaplan, 2002; Prančl *et al*., 2018). The basic chromosome number within *Batrachium* species is x=8 (Baltisberger & Hörandl 2016). Di- (2n=16), tetra- (2n=32) and hexaploids (2n=48) are the three most common ploidy levels of *Ranunculus* sect. *Batrachium* (Englmaier, 2016; Prančl *et al*., 2018). Other ploidy levels including 3x and 5x are rather rare,

where 5x can only be found in some hybrids.
Additional identification problems are often due to incomplete material such as damaged leaves or the presence of an incomplete set of leaves (Englmaier, 2016).

Batrachium taxa are known for the presence of two different kinds of leaves. They have capillary leaves which have a lamina that consists of branched capillary segments which are mostly submerged and some species have also laminar leaves which are floating on the water surface. The latter are laminar leaves with a flat, more or less lobate and dentate lamina (Prančl *et al*., 2018). The flower of *Batrachium* species usually has five white petals with a yellow spot at the base. The size of the flower is associated with the mode of reproduction. Taxa that are small flowered are regarded mostly autogamous or even cleistogamous. Species which have big flowers show various adaptions for outbreeding. Some taxa might even be self-incompatible (Turala-Szybowska, 1978; Hong, 1991).

In order to get a better understanding about the plant material used for this study, the characteristics of the species are briefly described. All pictures used below for describing the different species were obtained from Petr Koutecký, the supervisor of this thesis.

Figure 1: Ranunculus aquatilis

<u>*Ranunculus aquatilis (Fig. 1)*</u>: This aquatic plant which is also called common water crowfoot can be found all over Europe including North Africa (Lansdown, 2014a; Wiegleb *et al*., 2017). Additionally, it is known from West Asia (Wiegleb *et al*., 2017). The plant is mainly growing in mats on the surface of water (Blamey, 1989). The amphibious species has branching thread-like underwater leaves and toothed floating leaves (Cook, 1968; Blamey, 1989; Prančl *et al*., 2018). However, if growing in fast flowing water the floating may not develop (Blamey, 1989). The flowers are held above the water and are white petaled with a yellow centre (Blamey, 1989). There are two varieties of common water crowfoot namely var. *aquatilis* which possesses the floating leaves and var. *diffusus* which is lacking the laminate leaves (Lumbreras *et al*., 2014).

<u>*Ranunculus peltatus* (Fig. 2)</u>: This species, also known as pond water-crowfoot is closely related to the above mentioned *R. aquatilis* and is native to Europe, North Africa and West Asia (Lansdown, 2014b; Wiegleb *et al*., 2017). The plant is mostly associated with soft-water conditions in ditches, small streams and ponds (Wiegleb *et al*., 2017). It does not occur in large streams. There it is usually replaced by *R. penicillatus* (Wiegleb *et al*., 2017). *R. peltatus* has white flowers with a yellow centre (Blamey, 1989). The species has two different kinds of leaves. Broad, round floater leaves and finely divided thread-like submergent leaves which are mostly flaccid, densely branched and 3 – 8cm long (Blamey, 1989; Prančl *et al*., 2018).

Figure 2: Ranunculus peltatus

<u>*Ranunculus confervoides* (Fig. 3)</u>: This perennial or annual herb is weakly rooting or freely floating and mainly found in high alpine lakes in clear, well-buffered, carbonate rich waters (Englmaier, 2016). They are strictly homophyllous, only possess capillary leaves with filamentous segments. Laminar leaves are absent (Englmaier, 2016; Wiegleb *et al*, 2017). The flowers are very small and mostly found under water (Wiegleb *et al*, 2017). Since these characters of *R. confervoides* are also found in some forms of *R. trichophyllus*, this taxon is sometimes also included in the *R. trichophyllus* group (Wiegleb *et al.*, 2017). The correct name is then *R. trichophyllus* subsp. *eradicatus* (Wiegleb *et al.*, 2017).

Figure 3: Ranunculus confervoides

<u>*Ranunculus circinatus* (Fig. 4)</u>: This perennial or rarely annual herb grows in a variety of water bodies, mainly in eutrophic still waters, from ditches to deep lakes (Wiegleb *et al*., 2017, Englmaier, 2016). It can grow up to 0.5 – 2.5m long, dependent on the habitat (Englmaier, 2016). The plant is strictly homophyllous, only capillary leaves are present. Due to this capillary, rigid leave shape as well as their medium-sized petals with lunate nectar pit, the species is easily recognizable (Wiegleb *et al*., 2017). *Ranunculus circinatus* is distributed in most parts of meridional and boreal Europe eastwards to the Ural Mountains and Kazakhstan (Wiegleb *et al*., 2017). Additionally, the plant occurs in the meridional regions of North Africa and West Asia. *R. circinatus* do not occur in North America (Wiegleb *et al*., 2017).

Figure 4: Ranunculus circinatus

<u>*Ranunculus fluitans* (Fig. 5)</u>: This *Ranunculus* species is commonly named river water-crowfoot (Lansdown, 2011b). It is a perennial water plant that can grow up to 6 m in case of favourable conditions. Floating leaves are absent, instead it has long and narrow, tassel-like segments. The white flowers are held above the water (Rodwell, 1990; Wiegleb *et al*., 2017). River water-crowfoots are restricted to temperate and meridional regions in Europe (Wiegleb *et al*., 2017).

Figure 5: Ranunculus fluitans

<u>*Ranunculus trichophyllus* (Fig. 6 &7)</u>: *R. trichophyllus*, also named thread-leaf crowfoot or thread-leaved water-crowfoot is similar in form to *R. fluitans* and is native to Europe, Asia and North America (Lansdown, 2013). Usually this species is found in rivers, ponds or lakes, particularly in alkaline waters (Wiegleb *et al*., 2017). *R. trichophyllus* is strictly homophyllous. It has only underwater leaves with filamentous segments (Englmaier, 2016). Flowers are submergent or emergent (Englmaier, 2016).

Figure 7: Ranunculus trichophyllus A

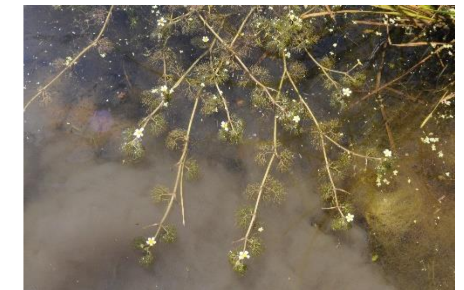
Figure 6: Ranunculs trichophyllus B

There are two different cytotypes *of R. trichophyllus*, namely *R. trichophyllus* A and *R. trichophyllus* B. They are both tetraploid but differ in genome size and have different habitat requirements, *R. trichophyllus* B is more thermophilous. The two cytotypes also slightly differ morphologically, *R. trichophyllus* B is more robust (Prančl *et al*., 2018).

<u>*Ranunculus penicillatus* (Fig. 8)</u>: This plant is also called stream water-crowfoot and it is a species which is entirely restricted to floating waters (Lansdown, 2011c). The large perennial plant can reach a length up to 5m and is characterized by laminar and intermediate leaves in addition to capillary leaves (Wiegleb *et al.,* 2017). Typically, this crowfoot occurs in small to medium-sized rivers and streams. However, it can occur in larger rivers too where it might form very extensive strands (Lansdown, 2011c). The plant appears to have a broad ecological tolerance since it can be found in calcareous as

Figure 8: Ranunculus penicillatus

well as acidic waters and in oligotrophic to mesotrophic or even in eutrophic waters. Stream water-crowfoots are endemic to Western and Central Europe (Lansdown, 2011c).

Figure 9: Ranunculus baudotii

Ranunculus baudotii (Fig. 9): The common name for this *Ranunculus* species is brackish water-crowfoot. The plant is endemic to Europe and can be mainly found in ditches, ponds and lagoons, either along the sea coast or more rarely in inland sites with high salt or calcium concentrations (Prančl *et al.*, 2018; Englmaier, 2016; Wiegleb *et al.*, 2017; Lansdown, 2011a). Other crowfoots growing in both sweet and brackish water are *R.confervoides* and *R. circinatus.*

Figure 10: Ranunculus rionii

Ranunculus rionii (Fig. 10): This species can be found in Europe, North Africa and West to Central Asia (Wiegleb *et al.*, 2017). Typically, the plant grows in seasonal pools (Cook, 1966). *R. rionii* has small flowers, laminar leaves are absent, capillary leaves are mostly flaccid and densely branched (Englmaier, 2016, Prančl *et al*., 2018). In some literature e.g. Englmaier, 2016, *R. rionii* is regarded as part of the *R. trichophyllus* group, where it is named *R. trichophyllus* subsp. *rionii.* Differentiation between *R. trichophyllus* and *R. rionii* is difficult and mainly done by comparison of the fruit size, since the fruits of the latter one are smaller.

When it comes to identification of species several methods are commonly used nowadays, including morphological taxonomy and molecular systematics (Friedheim, 2016).

While the first one is based on morphology and phenotypic characteristics, the latter one relies on molecular information to distinguish between one species and another (Friedheim, 2016). There are not a lot of studies which have dealt with the *Batrachium* group. Since the worldwide monograph of *Batrachium* (Cook, 1966) some supplemental studies appeared (Holmes, 1979; Wiegleb and Herr, 1983; Wiegleb, 1988; Hong, 1991; Webster, 1991; Pizarro, 1995, Dahlgren, 1995; Dahlgren and Cronberg, 1996; Tzvelev, 1998; Dahlgren and Jonsell, 2001; Lansdown, 2009). However, there are only few studies which use molecular markers to analyze genetic variation (Telford *et al.*, 2011, Zalewska-Gałosz *et al.,* 2014, Bobrov *et al*., 2015).

In this thesis, microsatellite analysis is tested in *Ranunculus* sect. *Batrachium*, which is one of the molecular methods and often used e.g. in taxonomy of plants or population genetics. For this reason the method is described below.

1.2 Microsatellites

Microsatellites or Simple Sequence Repeats (SSRs) are stretches of DNA which consist of short tandem repeats (STRs) of 1 – 6 base pairs in length (Weber & May, 1989; Weber, 1990; Katti *et al*., 2001, Dakin & Avise, 2004; Ellegren, 2004). They can be found in the genome of eukaryotes and prokaryotes and are even present in the smallest bacterial genomes (Field & Willis, 1996; Hancock, 1996). There was an exponential increase in the use of the markers during the 1990s and 2000s and nowadays they are the most popular choice for studies of kinship analysis, individual identification and population structure (Selkoe & Toonen, 2006).

Classification is done by the number of repeated copies of DNA segments (Abdul-Muneer, 2014). Satellites are made of units of several thousand base pairs which are repeated thousands or millions of times (Abdul-Muneer, 2014). Minisatellites have a length of 9 – 100 bp that are repeated from 2 to several 100 times at a locus (Abdul-Muneer, 2014). The shortest sequence repeats are microsatellites. Those consist of 1 – 6 bp and are repeated up to 100 times at each locus (Litt & Luty, 1989). Only microsatellites became common in population genetics studies (Abdul-Muneer, 2014).
There are several ways how microsatellites can be classified. Classification can be based on size, nature of the repeated unit or their position within the genome (Miah *et al*., 2013). Weber (1990) used the terms perfect $(CA)_n$, imperfect $(CCA)_n$ TT $(CGA)_n$ and compound $(CA)_n$ $(GA)_n$ to classify microsatellites depending upon arrangement of nucleotides within the repeat motifs. Wang *et al*., (2009) on the contrary chose the terms simple perfect $(CA)_n$, simple imperfect $(AAC)_n$ ACT $(AAC)_n$, compound perfect and compound imperfect $(CCA)_n$ TT $(CGA)_n$. Tandem arrays of a single repeat motif are perfect repeats. Repeats which are sometimes interrupted by non-repeat bases are imperfect repeats and if two basic repeat motifs are present together in various configurations, the term compound microsatellite is used (Miah *et al*., 2013). If microsatellites are classified with respect to the number of nucleotides per repeat unit these are called mono-, di-, tri-, tetra-, penta-, or hexanucleotide repeats (Miah *et al*., 2013).

Microsatellites occur in high numbers in the euchromatin of eukaryotes as well as in the coding and non-coding nuclear and organellar DNA (Pérez-Jiménez *et al*., 2013; Phumichai *et al*.,

2015). However, coding and non-coding regions differ significantly in their microsatellite distribution. The number of SSRs is lower in the former (Vieira *et al.,* 2016; Hancock, 1995). This is because microsatellites show high mutation rates (Vieira *et al.,* 2016; Goldstein & Schlötterer, 1999). They mutate at rates between 10^{-3} and 10^{-6} per cell generation, which is up to ten orders of magnitude greater than point mutations in normal coding sequences (Gemayel *et al*., 2012). Mutations in coding regions can lead to phenotypic changes and diseases, notably in triplet expansion diseases, in humans such as fragile X syndrome, Huntington's disease which is caused mainly by $(CAG)_n$ expansions (Warren & Nelson, 1993; Bates & Lehrach, 1994; Reddy & Housman, 1997), and cancer (Wooster *et al*, 1994; Arzimanoglou *et al.*, 1998). Therefore, these repeats are generally located in non-coding regions where mutations possibly do not interfere with the individual's fitness (Ellegren, 2004).

The SSRs are non-randomly distributed across UTRs (untranslated regions), introns and protein-coding regions (Garza *et al.* 1995). Additionally, the distribution patterns of various repeat types for different motifs in those regions were found to be highly taxon-specific (Tóth *et al*., 2000). Studies also revealed that the occurrence of microsatellites is relatively rare in butterflies, birds, bats and prokaryotes while most mammals as well as fishes tend to have a high frequency of repeat motifs (Shamjana *et al*., 2015). Also plant genomes are filled with SSRs, where a high density has been detected in the 5' UTR regions (Qin *et al*., 2015; Fujimori *et al*., 2003; Tranbarger *et al*., 2012; Zhao *et al*., 2014).

Historically, microsatellites were regarded as part of the non-functional DNA because they are very unstable (Gemayel *et al*., 2012). The repeat variation was believed to be neutral with no phenotypic consequences (Gemayel *et al*., 2012). However, with the detection of regulatory regions and transcripts, scientific interest in their possible biological function increased and more and more publications have presented evidence that microsatellites play a role in important processes including regulation of transcription and translation, organization of chromatin, genome size and the cell cycle (Nevo, 2001; Li *et al*., 2004; Gao *et al*., 2013). Generally, the consensus is that the biological function of microsatellites is related to its position in the genome (Vieira *et al.*, 2016). They may be important in genome organization, function and are associated with disease conditions (Shamjana *et al*., 2015).

There are several major advantages of microsatellites markers including codominant transmission and hypervariability as well we as high information content (Abdul-Muneer,

2014). Additionally, microsatellites are relatively abundant with uniform genome coverage and show a higher mutation rate than standard sequences (Abdul-Muneer, 2014). Those advantageous properties have led to modern developments such as digital storage and automated detection and scoring systems like automated DNA sequences and fluorescent-imaging devices (Abdul-Muneer, 2014). However, there are also various disadvantages regarding the usage of microsatellite-based methods, including technical challenges during the construction of enriched libraries, relatively high development costs and genus or species-specific primers (Miah *et al.,* 2013). Due to their very specific nature, primers have to be newly developed or at least transferred from a related species when a new species is studied. Still, even if some primers are already available those mostly do not work in a reasonable way.

Versatile characteristics of microsatellites such as their existence in genomes of all living organisms, high level of allelic variation, co-dominant way of inheritance have given rise to the rapid growth of molecular markers to tackle a range of purposes like ecological questions, disease diagnosis, personal identification, population genetic analysis and the construction of human evolutionary trees (Shamjana *et al*., 2015). More applications are summarized in Figure 11 below.

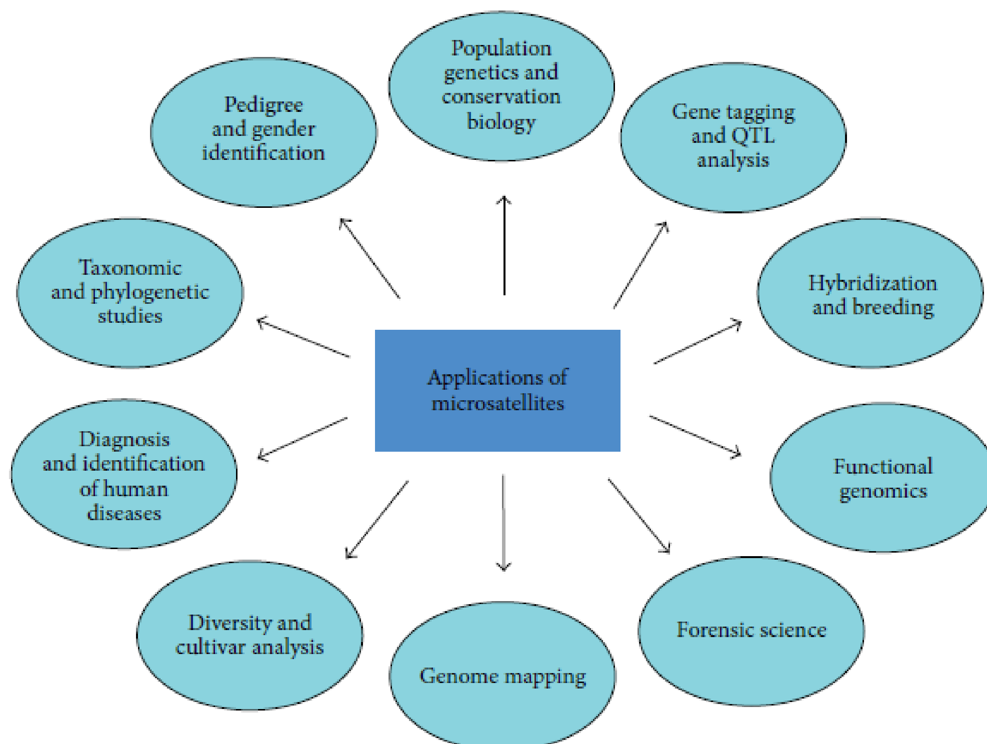

Figure 11:Application of microsatellite markers in different areas (Abdul-Muneer, 2014)

Another characteristic which makes microsatellites a powerful molecular marker is their PCR typeability (Shamjana *et al*., 2015). Microsatellite loci are amplified using specific primers, then the different alleles are separated along an electrophoretic gradient (Goldstein *et al.* 1999). Additionally, they can be genotyped on a sequencer (Schuelke, 2000).

During the last decade, microsatellites have become the marker of choice when it comes to plant genetics (Varshney *et al*., 2002). This is due to their genomic abundance and their ability to associate with many phenotypes (Filipe *et al*., 2011). Because of their importance microsatellites are used in a large number of plant species including major cereal species such as maize (Yu *et al.*, 2001), oat (Li *et al*., 2000), rice (Temnykh *et al.,* 2000 and 2001) and wheat (Roder *et al*., 1998; Varshney *et al.,* 2000). SSRs are useful for wild species as well as for cultivated species. For wild species they are used in studies of diversity measured on the basis of genetic distance, to estimate gene flow and crossing over rates as well as in evolutionary studies to get information about intraspecific genetic relations. For cultivated plants the molecular markers are used for the construction of linkage maps, for mapping loci which are involved in quantitative traits and for the estimation of the degree of kinship between genotypes (Vieira *et al*., 2016).

To get special knowledge of aquatic plants, microsatellites are widely used, including studies of taxonomy, hybridization as well as population genetics. Regarding hybridization, Snow *et al.* (2010) used species-specific microsatellites for identification between early-and later generation hybrids of cattails (*Typha* spp.). Shiga *et al.* (2017) focused on population genetics, where microsatellites were used to find differences in genetic variation between four populations of *Nuphar submersa* (Nymphaeaceae), a critically endangered freshwater macrophyte indigenous to central Japan. Fér and Hroudová (2008) used microsatellites as markers for population genetics. Here, these molecular markers were used to gain information about the dispersal of *Nuphar lutea* genotypes in rivers of Czech Republic. Interpretation of patterns in genetic variation lead to several conclusions about dispersal mechanisms, e.g that vegetative, long – distance dispersal is probably very limited in this species.

2. Aim of the thesis

As mentioned above, microsatellite primers are often species-specific. Recently, some were developed for *R. bungei,* a species of the *Batrachium* group occurring in China (Wu *et al.,* 2017) but not any for European species. The aim of this thesis was to find out if the primers developed for *R. bungei* also work on various, less related European *Ranunculus* sect. *Batrachium* species.

3. Materials and Methods

3.1 Plant material used for molecular analysis

Plant material from 32 different individuals representing nine *Ranunculus* species and several hybrids, which had been collected in various regions in Austria (8 localities), the Czech Republic (19 localities), Germany (1 locality), and Slovenia (1 locality) was studied (Table 1). The material was determined by the supervisor of this thesis based on morphology and genome sizes measured using flow cytometry (Prančl *et al*., 2018) Amplification of 13 different microsatellite loci which had been designed for *R. bungei* from China (Wu *et al*., 2017) was tested (Table 2). The loci were selected to show multiple alleles and high number of repeats in the original dataset of Wu *et al*. (2017), while invariable or little variable loci were omitted from testing.

3.2 DNA extractions

The total genomic DNA was extracted from leaves which had been airdried, then dried with silica gel and finally stored in a freezer.

The DNA of the samples 1 – 16 was isolated using the NucleoSpin Plant II Kit (Macherey-Nagel, Germany) following the manufacturer's protocol.

For samples 17 – 32 CTAB-method (Doyle and Doyle 1987; with minor modifications) was used. First, the dry leaf material (5 – 15 mg) was transferred into a 1.5 mL Eppendorf tube, two steel beads were added and the tubes were placed into a Retsch 400MM mill. The samples were ground for 1 min at maximum speed. Afterwards, 700 µL of the CTAB buffer [2% (w/v) cetyl trimethyl ammonium bromide (Sigma-Aldrich), 0.1M Tris-base (Serva), 0.02M Na_2-EDTA (Sigma-Aldrich), 1.4M NaCl, 2% (w/v) PVP-40 (Sigma-Aldrich)] and 20 µL of 2-mercaptoethanol (Sigma-Aldrich) were added. The tubes were mixed and incubated on a shaking incubator for 30 min at 50°C, then they were spun for 2 – 3s and the supernatant was transferred into a new 1.5 mL Eppendorf tube. The steel beads were removed and afterwards 500 µL chloroform-isoamyl alcohol mixture (24:1) was added. The tubes were inverted several times and incubated for 5 min at room temperature. Then they were centrifuged for 10 min at 10 000 rpm. Afterwards the aqueous (uppermost) phase was transferred into new 1.5 mL Eppendorf tubes. Next, 500 µL of ice-cold isopropanol (stored at -20°C) was added. After inverting the tubes several times, they were incubated for at least 30 min at -20°C. Then they were centrifuged for 5 min at 13 000 rpm. In the next step, the supernatant was discarded and 400 µL of ice-cold 96% ethanol (stored at -20°C) was added. In order to detach the DNA pellet

from the bottom of the tube, the tubes were flicked several times. Then the samples were incubated for 15 min at 37°C on a heat block, afterwards the samples were centrifuged for 5 min at 13 000 rpm. The supernatant was discarded and 200 μL of ice-cold 70% ethanol (stored at -20°C) was added. Again, the tubes were flicked several times to detach the DNA pellet from the bottom of the tubes. Then the samples were incubated for 5 min at room temperature. After that, they were centrifuged for 5 min at 13 000 rpm and the supernatant was discarded. Then the DNA pellets were dried by leaving the tubes open for ca 15 min at room temperature. When there were no drops of ethanol visible, 30 μL of sterile water were added. In order to dissolve the DNA pellets, the tubes were put in the fridge overnight. After this procedure, the DNA isolates were put in a freezer at -20°C for long-term storage.

Before preparing the samples for PCR amplification, the samples containing the genomic DNA were diluted with sterile water (1:10).

3.3 PCR amplification

The whole procedure was performed on ice. At first, all chemicals were defrozen slowly, then they were mixed gently and afterwards centrifuged briefly. The PCR mixture for each sample contained 1.85 μL sterile water, 0.45 μL of the forward primer (5 μM), 0.45 μL of the reverse primer (5 μM) and 3.75 μL of 2x Plain PP Master Mix (Top-Bio, Czech Republic). 1 μL of the diluted template DNA was added into each 0.2 mL tube except for the negative control. The tubes were gently mixed and quickly centrifuged.

PCR was run in a Biometra T3000 thermocycler. The cycling program was:
5 min initial denaturation step at 95°C, 35 cycles which included the cycle denaturation at 95°C for 30 s, primer annealing at temperature specific for each primer pair (Table 2) for 30 s and the cycle elongation at 72°C for 1 min, and final elongation for 10 min at 72°C as the last step. To enhance specificity, the program was started in advance and samples were put into the device after the temperature of the heating block reached 80°C.

3.4 Verification of amplification using gel electrophoresis

The amplification was verified by electrophoresis on 1.5% agarose gel in TBE buffer [89mM Tris-base, 89mM boric acid, 2.5mM Na_2-EDTA]. 0.7 μL of the staining solution [0.25% (w/v) Bromphenol blue (Sigma-Aldrich), 30% glycerol, 2 μL/mL of 10000x GelRed (Biotium)] was pipetted on a microtiter plate. Afterwards, 1.5 μL of the PCR products was added. The samples

were mixed with the staining solution by pipetting up and down three times and loaded onto the gel. To the first well (and optionally also last well, depending on the number of samples) 3 µL of the 100 bp ladder (New England Biolabs) was pipetted. Then the lid of the electrophoresis chamber was closed and voltage was set to 120V. After about 25 min, the run was completed and power supply was turned off. Afterwards the bands were visualized under UV light.

3.5 Sanger sequencing

Gel electrophoresis showed that the PCR amplification was successful for most loci (except B1) which meant that the primers were binding to the DNA. However, there could still be mutations in the amplified region. Therefore, it was necessary to check if the repeat (microsatellite) is still present and also if other length mutations including insertions or deletions in the flanking regions are absent. These mutations would have been unwanted since they can cause either low variation or variation that is not related to the number of repeats in the microsatellite. In order to ensure none of the above mentioned occurred, observation of the sequence was necessary, since neither gel electrophoresis nor fragment analysis gives sufficient information. Therefore, 2 – 4 samples for each locus were selected and sequenced.
Before sequencing, the PCR products were purified by enzymatic purification (Exo-AP). In this purification method, two enzymes are combined: (1) Exonuclease I cleaves single stranded DNAs from unincorporated primers, and (2) Fast Alkaline Phosphatase cleaves unincorporated dNTPs. The purification process was performed on ice and the enzyme mixture [Exonuclease I (Fermentas; 20 U/µL) and FastAP (Fermentas; 1U/µL) in a ratio 1:2] was stored at -20°C. 0.7 µL of the Exo-AP mixture was aliquoted in new 0.2 mL PCR strips and 2 µL of the PCR products were added. Afterwards the mixture was mixed gently and centrifuged briefly. The samples were then put on a thermocycler and kept 15 min at 37°C (activity of the enzymes) and 15 min at 85°C (deactivation of the enzymes). Then 2.3 µL sterile water and 5 µL of the forward primer (5 µM) were added to the PCR products and the samples were sent to SEQme company (Dobříš, Czech Republic) for sequencing.

The sequence data were visualised with FinchTV 1.4 software (Geospiza, USA).

3.6 PCR amplification for fragment analysis

After Sanger sequencing, the sequences were checked and problematic loci were discarded. Fluorescent colours were chosen in a way that pooling all loci into one fragment analysis was possible.

To add a fluorescent label into PCR products, modified PCR with 3 primers employing universal M13 fluorescent primers was used, following the protocol of Schuelke (2000).

Again, the whole procedure was performed on ice. The PCR mixture for each sample contained 1.025 µL PCR water, 0.6 µL M13 forward primer (2.5 µM, fluorescently labelled), 0.075 µL 'tailed' specific forward primer (5 µM), 0.3 µL specific reverse primer (5 µM) and 2.5 µL of 2x Plain PP Master Mix (Top-Bio). 0.5 µL of sample DNA was added. Strips were mixed and spun briefly.

Again, PCR was run in a Biometra T3000 thermocycler. The following cycling program was used: 2 min initiation step at 94°C, 33 cycles, each 30 s at 94°C, 30 s at primer specific annealing temperature (Table 2) and 60 s at 72°C, followed by 11 cycles, each 30 s at 94°C, 30 s at 46°C and 60 s at 72°C, and finally, one step cycle at 72° for 10 minutes and cooling down to 15°C.

When the PCR amplifications were finished, the strips were spun briefly and the PCR products were checked by agarose gel electrophoresis (see chapter 3.4).

3.7 Fragment analysis and evaluation using Gene Marker 1.80

After PCR amplifications (see chapter 3.6), PCR products from different loci of the same sample were pooled (0.5 µL each) and 4 µL of the mixture was sent to SEQme company for fragment analysis. Size standard GeneScan™-600 LIZ® was used (added by the company).

The data was evaluated using Gene Marker 1.80 software (SoftGenetics). Length (in bp) of each major peak was recorded for each primer and locus manually.

Table 1: Detailed information about the Ranunculus species included in this study. Ploidy level was obtained from Prančl et.al 2017

Sample no.	Country	Locality; habitat	Taxon	Ploidy
01	CZ	Lanžhot; Dědova pískovna; sand pit	*R.aquatilis*	6x
02	CZ	Choryně; artificial pool in a wetland	*R.aquatilis*	6x
03	SI	Julian Alps, Kriško jezero; alpine lake	*R.confervoides*	4x
04	CZ	Senožaty; Želivka River	*R.fluitans* 2x	2x
05	CZ	Senožaty; Želivka River	*R.fluitans* 3x	3x
06	CZ	Choryně; artificial pool in a wetland	*R.peltatus*	4x
07	CZ	Třísov; Vltava River	*R.peltatus*	4x
08	CZ	Vlkov; sand pit	*R.peltatus*	4x
09	DE	Erdmannsdorf; Zschopau River	*R.penicillatus* A	4x
10	CZ	Libočany; Ohře River	*R.penicillatus* A	4x
11	AT	Schloßhof; alluvial pool	*R.rionii*	2x
12	CZ	Andělská Hora; fishpond	*R.trichophyllus* A	4x
13	CZ	Vlkov; sand pit	*R.trichophyllus* A	4x
14	AT	Mattighofen; Kühbach brook	*R.trichophyllus* B	4x
15	CZ	Novosedly; periodic pool	*R.trichophyllus* B	4x
16	CZ	Přerov; gravel pit	*R.circinatus*	2x
17	CZ	Lišov; Čekal fishpond	*R.peltatus* x *R.trichophyllus* A	4x
18	CZ	Bukovsko; fishpond	*R.peltatus* x *?*	4x
19	CZ	Bukovsko; fishpond	*R.peltatus*	4x
20	CZ	Rychnov nad Malší; Malše River	*R.peltatus* x *R. trichophyllus s.l.*	4x
21	AT	Aigen-Schlägl; Große Mühl River	*R.fluitans* x *R. peltatus*	4x
22	AT	Sankt Georgen a. d. Gusen; Gusen River	*R.fluitans* x *R. trichophyllus.* B	3x
23	CZ	Karviná; gravel pit	*R.circinatus*	2x
24	AT	Totes Gebirge, Wildensee; alpine lake	*R.confervoides*	4x
25	CZ	Senorady; Jihlava River	*R.fluitans* 2x	2x
26	CZ	Harvraníky; Dyje River	*R.fluitans* 3x	3x
27	AT	Tadten; groundwater channel	*R.rionii*	2x
28	CZ	Jevišovice; artificial pond	*R.baudotii*	4x
29	AT	Angern an der March; sand pit	*R.baudotii*	4x

30	CZ	Lanžhot; Dědova pískovna; sand pit	*R.aquatilis*	6x
31	AT	Marchegg; Mühlbach brook	*R.aquatilis*	6x
32	CZ	Odrava; Odrava River	*R.penicillatus*	4x

Table 2: Detailed information about the microsatellite loci and primers included in this study. F and R – forward and reverse primer, respectively; T_a – annealing temperature. Information on repeat motif, primers, allele sizes and annealing temperatures are taken from Wu et al., 2017. The fluorescent dye used in this thesis is indicated in the last column.

Locus	**Repeat Motif**	**Primer sequences (5'-3')** (F – forward, R – reverse)	**Allele size range (bp)**	**T_a(°C)**	**Fluorescent dye for Fragment analysis**
B1	$(TC)_7$	F: GCAGTTGCCATAGATACC R: CAGGGAATGGAAATAGG	418-450	54	Not used
B2	$(GT)_7$	F: GCAAAGGGTAAGACTGCTAT R: ATCAAGTTCCGATTCTGGTT	408-410	52	Red (PET®)
B5	$(GA)_9$	F: AATTCTGCTGCCCCTAT R: TACTTCTTCTGCCTTGCTT	465-472	58	Not used
B6	$(CAG)_6$	F: CAGGGACTGGACAGATACAC R: CTCATAGGAGAGACGGTTGGT	345-366	56	Blue (6 FAM™)
B9	$(GA)_9$	F: ACCTGGTGATCTTGAAGTAAA R: CTAATCCGAAACAGTGTATCTAA	322-349	51	Green (VIC®)
B10	$(AG)_{10}$	F: GCCAAGCTCTTCTGCTCT R: GTGTCTTTGATTGATTTTACCG	297-313	54	Blue (6 FAM™)
B11	$(GA)_7$	F: TAGATGAAGAACTAGGGCAAA R: GCAAGCGAAGAAACCA	143-171	50	Yellow (NED™)
B12	$(TAT)_{11}$	F: GCAGCGGAGTAAAACCT R: CATTACAAAACATACCAGCAT	172-193	54	Green (VIC®)
B13	$(AG)_7$	F: GCTTCTATTCTACCCTTGTTC R: GCAGCACCTCCTACTTCG	107-109	56	Blue (6 FAM™)
B16	$(CTG)_{13}$	F: GGAAATGGCTGGCTGATA R: GATTCGGGAAGAGGTGGT	453-459	54	Blue (6 FAM™)
B17	$(TGG)_6$	F: CCAAGGCACCAGTTTCAG R: TTGTTGTGGAGAATGGACGA	430-445	54	Yellow (NED™)
B20	$(CAC)_6$	F: CCCTTCCCTTGTGCTTG R: GAATGCCCAGTTAGCCC	163-172	54	Blue (6 FAM™)
B21	$(TC)_{13}$	F: CAAAAGGACTTGGAGACG R: GTGGTGTTCAGAGCCATT	466-471	52	Green (VIC®)

4. Results

4.1 Gel electrophoresis

Gel electrophoresis, that was performed after the first PCR reactions, showed that most of the primer pairs annealed and loci amplified successfully (Figure 12). Locus B1 did not amplify and therefore was excluded for further studies (Figure 13). In locus B11 double bands were observed, i.e., non-specific PCR products occurred. However, this locus was retained in the dataset as the specific locus can be recognized based on the product size.

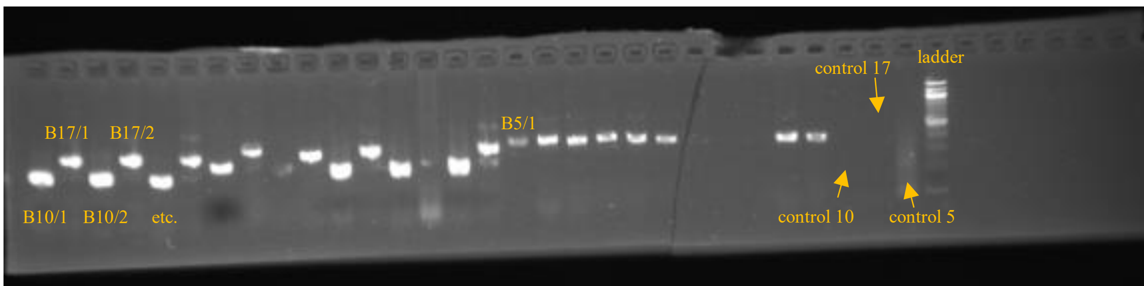

Figure 12: Gel electrophoresis samples 1-8, loci B10 and B17 alternating, middle: B5.

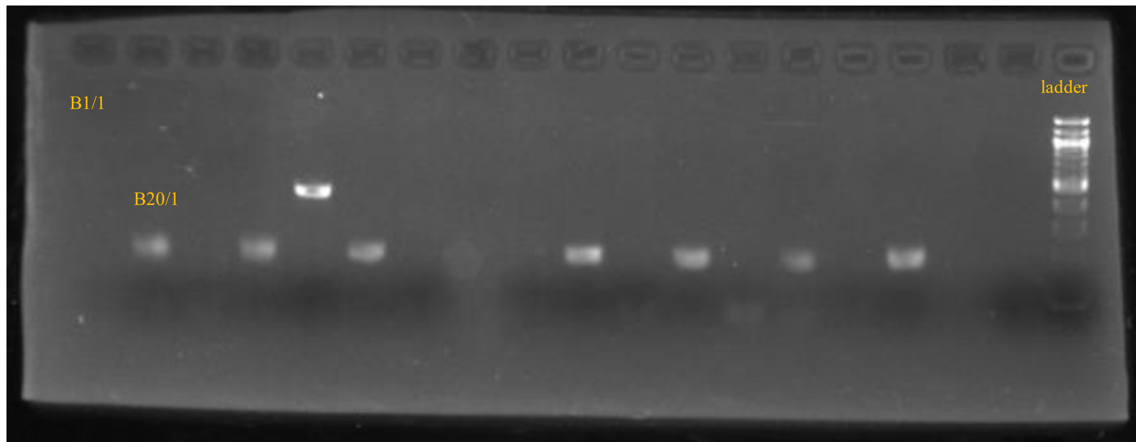

Figure 13: Gel electrophoresis sample 1-8, loci B1 and B20 alternating. Amplification of locus B1 was unsuccessful in most of samples (no bands visible).

4.2 Sanger sequencing

For each locus, name and type and number of repeats according to Wu *et al.* (2017) is given, and then own Sanger sequences of the respective locus are evaluated.

B2, $(GT)_7$

In sample 5 the repeat motif is repeated five times.
Regarding sample 7, GT is repeated eight times at the initial part of the sequence.

B5, $(GA)_7$

Only some $(GA)_2$ repeats and one $(GA)_3$ repeat are present in the sequence of sample 3. The sequence shows many overlaps and is quite ambiguous from the beginning, this might indicate length mutations (insertion or deletion) in the flanking region.
Similarly, sequence of sample 6 lacks any longer (GA) repeats and only a few $(GA)_2$ as well as one $(GA)_3$ repeat can be seen.
In summary, the repeat region was not resolved in this locus and the locus was therefore discarded from the subsequent fragment analysis.

B6, $(CAG)_6$

Sequences of samples 5 and 7 gave same results. In both one $(CAG)_3$ repeat and one $(CAG)_4$ repeat are present, separated by 9 bp of other nucleotides.

B9, $(GA)_9$

The sequence of sample 8 appears rather disordered, probably due to some indel occurring before the microsatellite region. One $(GA)_4$ repeat is present, afterwards another $(GA)_5$ repeat can be found.
Regarding sample 16, the sequence appears to be rather clear with low noise signals. However, towards the end, it gets more disordered which is also due to some indel occurring after the microsatellite in the flanking region. Similar to sample 8, the repeat of $(GA)_6$ can be found which is interrupted by a single adenine, after which the sequence continues with $(GA)_2$. Additionally, some $(GA)_2$ repeats are present.

B10, $(AG)_{10}$

Regarding sample 1, the repetition is present but significantly shorter, including only four repeats.

In the sequence of sample 3, several $(AG)_2$ repeats as well as multiple single AGs can be found. Concerning sample 8, one $(AG)_4$ repeat which is interrupted by two guanine bases and then followed by an $(AG)_3$ repeat is present. Furthermore, one single $(AG)_2$ repeat can be found in the sequence.

All the sequences from B10 are chaotic which might be due to technical problems or due to some indel in the flanking region. Another reason could be that the primer does not anneal completely specifically.

B11, $(GA)_7$

This primer was not used for Sanger sequencing due to the occurrence of unspecific PCR products (double bands visible in previously performed gel electrophoresis).

B12, $(TAT)_{11}$

In sequence 8 one $(TAT)_5$ repeat can be found. The repeat is then interrupted by a single adenine. Another microsatellite that is present in this sample as well as in sample 15 is a poly-T region in the flanking region. Moreover $(TAT)_2$ occurs in the latter sequence.

B13, $(AG)_7$

In sample 1 the AG repeat is present, however there are just three repeats. Regarding sample 5, $(AG)_5$ can be detected at the beginning.

B16, $(CTG)_{13}$

Results of the Sanger sequencing for sample 8 show a very chaotic sequence possibly due to either indel occurring early in the flanking region or imperfect annealing of the forward primer. Therefore, the sequence is hard to read. However, it is assumed that the microsatellite is present.

In the sequence of sample 16, one $(CTG)_6$ repeat and one $(CTG)_5$ repeat are present, separated by ca 80 bp.

B17, $(TGG)_6$

While at the beginning the signals of sample 1 are clear, the sequence gets more disordered towards the end which is due to indel in the flanking region. One $(TGG)_4$ repeat occurs at the beginning.

For sample 5 one $(TGG)_5$ repeat is present, the sequence is clear.

B20, $(CAC)_6$

In sample 5 the repeat motif is repeated five times.

Concerning sample 8, one $(CAC)_3$ repeat can be found at the beginning.

B21, $(TC)_{13}$

Sample 12 shows a sequence with very clear peaks (Figure 12). They are very well separated and resolved and the level of background noise is low. Starting around position 200, there is a long sequence of TC that is interrupted by several other bases. Problems might have occurred due to another repeat region (TCC) at around position 160 – 190. This TCC repeat could have caused problems because the repeat has different lengths than the requested TC repeat (3 vs. 2 bases). Also the TCC repeat is interrupted by several bases.

Sample 16 shows similar pattern as sample 12, a long sequence of the requested repeat is present which is interrupted by other bases. The repeat also starts at around 200 bp. Also in this sample the TCC repeat is present, similarly to sample 12.

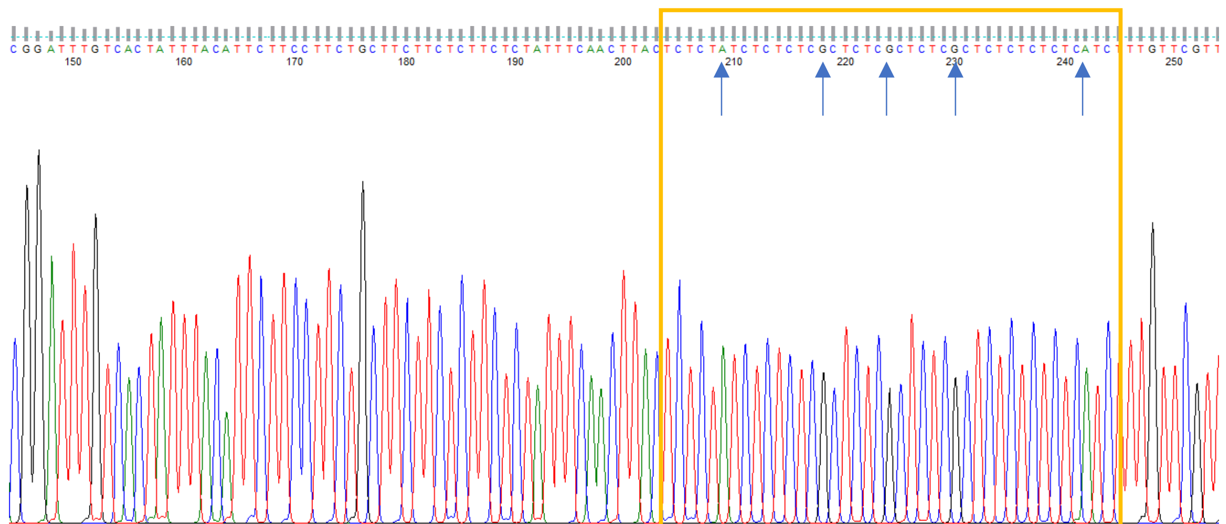

Figure 14: Sanger sequencing Primer 21/ sample 12, the repeat region is framed, the interruptions are indicated by arrows

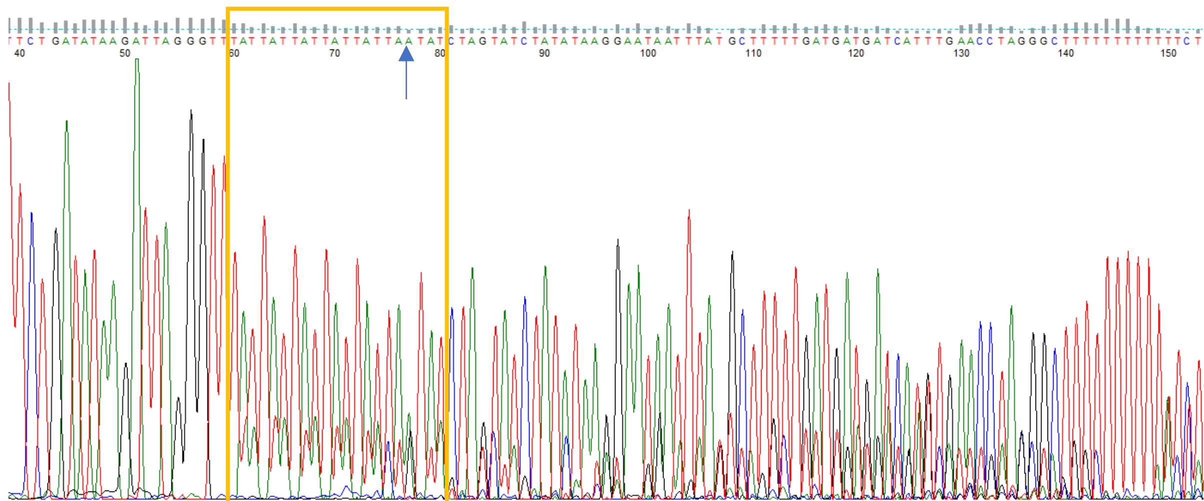

Figure 15: Sanger sequencing Primer B12/ sample 8

4.3 Fragment analysis

Results of fragment analysis are summarized in table 3 below. Since the primers B1 and B5 did not amplify sufficiently or lacked the microsatellite on Sanger sequences, those two were excluded from fragment analysis.

B2: Varying from 409 to 465 bps, locus B6 had a rather broad range of allele sizes. It did not work sufficiently on sample 10, 32 (both *R. penicillatus*) and 11 (*R. rionii*) as well as on one of two examined *R. baudotii* samples. In contrast, *R. aquatilis* (sample 1) and *R. aquatilis* (sample 2) showed high amount of similarity, whereas *R. aquatilis* (samples 30 and 31) seemed to be quite deviating from the previous ones but were also quite similar.

B6: This locus was ranging between 342 and 399 bp. Both *R. baudotii* samples, both R. rionii species and the two *R. trichophyllus* A showed the same pattern. Locus B6 did not work on two out of four *R. aquatilis* samples. The two *R. aquatilis* that worked revealed similar peaks.

B9: For this locus a lot of small peaks were detected which were not all included. Locus B9 was ranging between 344 and 375 base pairs. It worked successfully on almost all samples. Exceptions are sample 2 (*R. aquatilis*), sample 13 (*R. trichophyllus A*) and sample 21 (*R. fluitans x R. peltatus*). Under exclusion of *R. aquatilis* mentioned above, all other *R. aquatilis* samples showed very similar peaks. Both *R. baudotii*, and all *R. fluitans* 2x and 3x had identical peaks.

B10: Locus 10 was ranging from 286 to 313 bp. The primers did not work for five samples including two *R. aquatilis* samples, one *R. baudotii,* one hybrid between *R. fluitans* and *R. trichophyllus B* and one *R. fluitans* 3x. Apart from that, the two *R. aquatilis* samples that worked showed identical peaks. Patterns of both *R. circinatus*, both *R. trichophyllus* A, both *R. trichophyllus* B and both *R. rionii* samples are completely identical. Additionally, samples 6 and 7 (both *R. peltatus*) had identical peaks, the other two *R. peltatus* showed quite different pattern.

B11: For locus B11 there were a lot of small peaks which were not counted. The locus had a lenght range from 166 – 204. The primers didn't anneal on one out of two *R. fluitans* 2x and not on one of two *R. fluitans* 3x. It also failed on sample 20 which is a hybrid between *R. peltatus* and *R. trichophyllus s.l.* and on sample 22 which is a hybrid between *R.fluitans* and *R. trichophyllus* B. Additionally, it failed on sample 10 (*R. penicillatus* A). Concerning *R. aquatilis* the primer annealed successfully on three out of four species. Two out of those three have the same peaks. Peak patterns of all *R. baudotii*, all *R. circinatus* and all *R. confervoides* were the same. The same counts for both *R. rionii*, *R. trichophyllus* A and all *R. trichophyllus* B.

B12: The locus was ranging from 190 to 217 bp. There were a lot of small peaks, which were not all counted. Sample 1 and 2 (both *R. aquatilis*) had almost the same peaks. Sample 3 and 4 (both *R. aquatilis*) had identical peaks. A perfect match could be observed concerning the two *R. baudotii* samples which had the same peaks. The same counts for the two *R. fluitans* 2x as, for both *R. rionii* as well as for both *R. trichophyllus* A.

B13: Locus B13 had a range 124 – 131 bp. The primer annealed successfully at 30 out of 32 samples. It is remarkable that this primer showed almost identical peaks for all *R. aquatilis* species. Additionally, both *R.fluitans* 3x showed exactly the same peaks.

B16: In two out of four *R. aquatilis* samples the annealing did not work properly or failed completely. Furthermore, this locus did not work for sample 5 which has been a *R. fluitans* 3x sample and for sample 27 which is a *R. rionii*. Peaks of both examined *R. trichophyllus* A those of both *R. circinatus* and those of *R. baudotii* are identical. Locus 16 ranged between 462 – 495 bp.

B17: This locus had a range of 439 – 466 bp. It amplified on almost all samples. There was just one exception, namely one *R. trichophyllus* B sample. What is notable about this primer is that in a huge number of samples of the same species pattern was the same. So *R. aquatilis* samples 1 and 2 and *R. aquatilis* 3 and 4 were completely identical. The same is true for both *R. baudotii* samples and both *R. circinatus* samples. In addition to that, the peaks for both *R. fluitans* 2x and those of both *R. fluitans* 3x were completely identical. The *R. rionii* samples as well as the *R. trichophyllus* A samples had also exactly the same pattern.

B20: This locus amplified well at all samples except sample 11 which was taken from *R. rionii*. However, the other *R. rionii* sample analyzed did work. All four *R. aquatilis* samples and both *R. trichophyllus* B samples had almost identical peaks. Peaks of *R. fluitans* 2x were exactly the same.

B21: Considering all the loci used in fragment analysis, locus B21 (444 – 499 bp) was by far the least successful one. It did not work on 19 out of the 32 samples. A species on which the locus was amplified at all samples is e.g. *R. confervoides*.

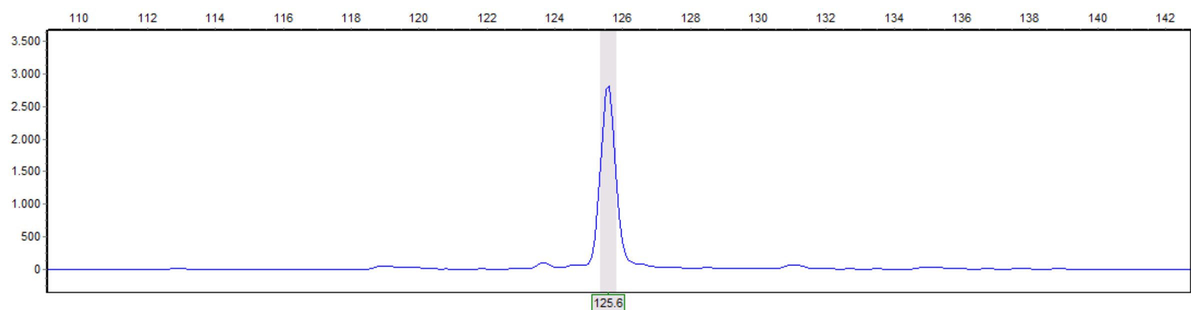

Figure 16: Locus B13/ sample 3 (blue dye). One allele can be found at 126 bp

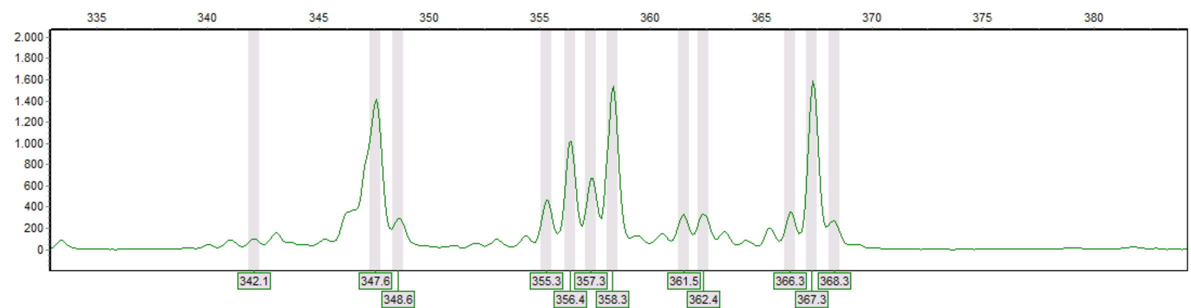

Figure 17: Locus B9/ sample 8 (green dye). Peaks at 348 bp, 356 bp, 358 bp and 367 bp were counted, the rest was regarded as stutter peaks, which are typical for microsatellites

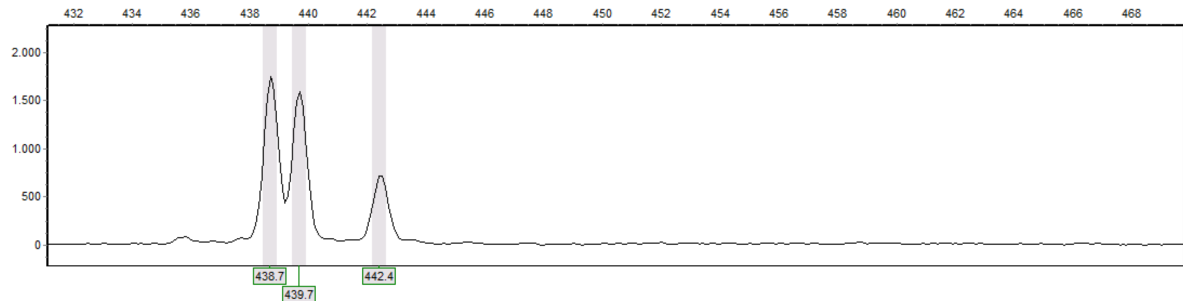

Figure 18: Locus B17/ sample 1 (yellow dye) Peaks at 439 bp, 440 bp and 442bp were counted

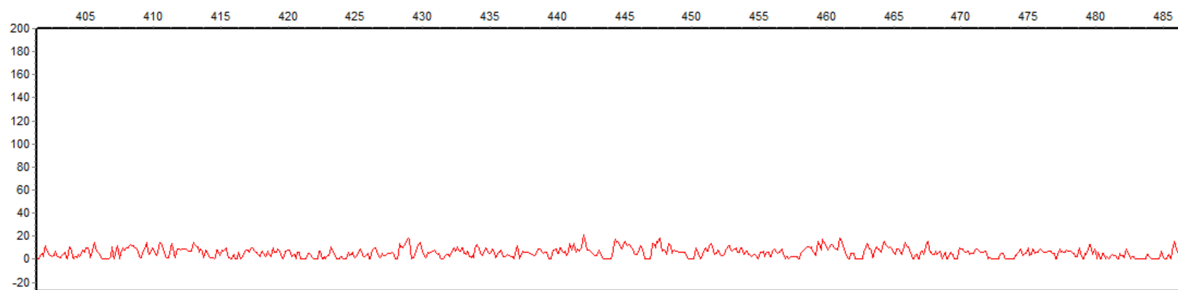

Figure 19: Locus B2/ sample 10 (red dye, as an example for a sample on which the locus did not work)

Table 3: Summary of the fragment analysis

Locus	Mean length [bp]	Min length [bp]	Max length [bp]	Mean allele count [-]	Max allele count [-]	No. of homozygotes	No. of failed samples
B2	444	409	465	2.32	5	9	4
B6	371	342	399	2.84	6	5	5
B9	356	344	375	2.21	5	8	5
B10	302	286	316	2.11	5	13	6
B11	182	166	204	1.81	4	12	6
B12	205	190	223	2.60	5	3	2
B13	128	124	131	1.80	3	12	2
B16	476	462	495	2.14	4	8	4
B17	447	438	466	1.94	3	8	1
B20	183	177	194	2.26	5	8	1
B21	482	444	499	1.43	4	11	18

Table 4: Detailed results of the fragment analysis. For each sample and locus, lengths of all alleles are listed [bp]. After locus names, the repeat motifs are indicated.

No.	Species	ploidy	B2 (GT)7					B6 (CAG)6						B9 (GA)9				
1	*R. aquatilis*	6x	409	426	429	463		378	381					356	358	368		
2	*R. aquatilis*	6x	409	426	429	455		378	381	384				-				
30	*R. aquatilis*	6x	409	419	463			-						358	368			
31	*R. aquatilis*	6x	409	419	465			-						358	368			
28	*R. baudotii*	4x	461	462				342	348	351	361	372	381	344	348			
29	*R. baudotii*	4x	-					342	348	351	361	372	381	344	348			
16	*R. circinatus*	2x	426					?						348	358			
23	*R. circinatus*	2x	425					375	381					358				
3	*R. confervoides*	4x	426	458				361	381					348	356			
24	*R. confervoides*	4x	425	457				360	366					347	363			
4	*R. fluitans* 2x	2x	465					348	378	399				348				
25	*R. fluitans* 2x	2x	464					?						348				
5	*R. fluitans* 3x	3x	465					378	399					348				
26	*R. fluitans* 3x	3x	464					347	369	390	399			348				
21	*R. fluitans* x *R. peltatus*	4x	457	460	464			361	364	378	381	384		-				
22	*R. fluitans* x *R. trichophyllus* B	3x	462	464				342	348	351	372	381		344	348			
6	*R. peltatus*	4x	446	448	455	457	459	384						355	357	358	367	
7	*R. peltatus*	4x	448	457	459	461		381	384	390				348	355	358	362	367
19	*R. peltatus*	4x	409	446				364						356	358			
8	*R. peltatus*	4x	457	459	461			378	381	384				348	356	358	367	
18	*R. peltatus* x ?	4x	411	426	446	457		360	381	384				348	367			
17	*R. peltatus* x *R. trichophyllus* A	4x	426	446	457			360	384					348	356	358		
20	*R. peltatus* x *R. trichophyllus s. l*	4x	409	446	459			378	381	389				348	362	363		
9	*R. penicillatus* A	4x	451	459	465			347	348					348				
10	*R. penicillatus* A	4x	-					347						348	358	363		
32	*R. penicillatus*	4x	-					-						345	346	356	375	
11	*R. rionii*	2x	-					375						370				
27	*R. rionii*	2x	445					375						366				
12	*R. trichophyllus* A	4x	426	457				361	384					348	356			
13	*R. trichophyllus* A	4x	426	457				361	384					-				
14	*R. trichophyllus* B	4x	419					378	381					358	360			
15	*R. trichophyllus* B	4x	426					375	378					352	358	370		

No.	Species	ploidy	B10	(AG)$_{10}$				B11	(GA)$_7$			B12	(TAT)$_{11}$			
1	*R. aquatilis*	6x	-					175	180			200	204	213		
2	*R. aquatilis*	6x	298	306				-				202	204	213		
30	*R. aquatilis*	6x	-					175	180			194	200	204	213	
31	*R. aquatilis*	6x	298	306				175	185			194	200	204	213	
28	*R. baudotii*	4x	-					176	193			194	204	207		
29	*R. baudotii*	4x	303	313				176	193			194	204	207		
16	*R. circinatus*	2x	304					189				213	214			
23	*R. circinatus*	2x	304					189				-				
3	*R. confervoides*	4x	304	310				189				214	220			
24	*R. confervoides*	4x	310					189				200	212			
4	*R. fluitans* 2x	2x	305					-				194	204			
25	*R. fluitans* 2x	2x	295	310	316			170				194	204			
5	*R. fluitans* 3x	3x	296	305				-				211	217			
26	*R. fluitans* 3x	3x	-					197				194	204	220		
21	*R. fluitans* x *R. peltatus*	4x	286	292	298	304		174	175	176	198	194	204	223		
22	*R. fluitans* x *R. trichophyllus* B	3x	-					-				194	204	207		
6	*R. peltatus*	4x	298	299	306			175	178	200		190	194	197	198	215
7	*R. peltatus*	4x	298	299	306			175				217				
19	*R. peltatus*	4x	288	298	304	305	306	177				191	194	199	204	213
8	*R. peltatus*	4x	298					171	174	175	204	213	216			
18	*R. peltatus* x ?	4x	292	298	304	310		177	197			194	206			
17	*R. peltatus* x *R. trichophyllus* A	4x	304	310				197				194	206			
20	*R. peltatus* x *R. trichophyllus s. l*	4x	289					-				194	215			
9	*R. penicillatus* A	4x	299	307				166	171	175	183	208	215	219	220	
10	*R. penicillatus* A	4x	289	292	307			-				?				
32	*R. penicillatus*	4x	292	297	306	308		174	175	178		194	204	211	214	
11	*R. rionii*	2x	308					188				194				
27	*R. rionii*	2x	308					188				194				
12	*R. trichophyllus* A	4x	304	310				197				206	217			
13	*R. trichophyllus* A	4x	304	310				197				206	217			
14	*R. trichophyllus* B	4x	306					175	180			200	204			
15	*R. trichophyllus* B	4x	306					175	180			194	204			

No.	Species	ploidy	B13 (AG)$_7$			B16 (CTG)$_{13}$				B17 (TGG)$_6$		
1	*R. aquatilis*	6x	126	127		469	471	473		439	440	442
2	*R. aquatilis*	6x	126	127		-				439	440	442
30	*R. aquatilis*	6x	126	127		-				441	466	
31	*R. aquatilis*	6x	126	127	130	468	471	473		441	466	
28	*R. baudotii*	4x	130			478				448	451	
29	*R. baudotii*	4x	126	130		478				448	451	
16	*R. circinatus*	2x	126	131		474				457		
23	*R. circinatus*	2x	126	131		474				457		
3	*R. confervoides*	4x	126			469	473			450	457	
24	*R. confervoides*	4x	124	126	131	465	473	474		445	457	
4	*R. fluitans* 2x	2x	126			485	491			447		
25	*R. fluitans* 2x	2x	126	128		484	493			447		
5	*R. fluitans* 3x	3x	126			-				447		
26	*R. fluitans* 3x	3x	126			484				447		
21	*R. fluitans* x *R. peltatus*	4x	126	128	131	462	473	493		442	446	
22	*R. fluitans* x *R. trichophyllus* B	3x	126	128	131	490				448	451	
6	*R. peltatus*	4x	126	131		484				441	443	445
7	*R. peltatus*	4x	126	130	131	462	473	495		443	465	
19	*R. peltatus*	4x	-			473	494			440	466	
8	*R. peltatus*	4x	126	130		462	473	484	495	438	443	~~444~~
18	*R. peltatus* x ?	4x	126	130		468	473	483		440	457	
17	*R. peltatus* x *R. trichophyllus* A	4x	126	130		468	473	483		440	457	
20	*R. peltatus* x *R. trichophyllus s. l*	4x	126			473	483			440	442	446
9	*R. penicillatus* A	4x	124	126		473	484	485	494	441	443	447
10	*R. penicillatus* A	4x	-			463	484			444	447	
32	*R. penicillatus*	4x	130			465	473	483		440	445	
11	*R. rionii*	2x	128			477				445		
27	*R. rionii*	2x	129			-				445		
12	*R. trichophyllus* A	4x	126			468	473			441	457	
13	*R. trichophyllus* A	4x	131			468	473			441	457	
14	*R. trichophyllus* B	4x	131			468	477			439	442	
15	*R. trichophyllus* B	4x	126	127	131	468	471			-		

No.	Species	ploidy	B20 $(CAC)_6$					B21 $(TC)_{13}$			
1	*R. aquatilis*	6x	180	183	186			-			
2	*R. aquatilis*	6x	180	183	186			-			
30	*R. aquatilis*	6x	180	183	186			-			
31	*R. aquatilis*	6x	180	183	186	189		-			
28	*R. baudotii*	4x	180	185	186	189		460	467		
29	*R. baudotii*	4x	180	186				-			
16	*R. circinatus*	2x	183					-			
23	*R. circinatus*	2x	183	187	188			493			
3	*R. confervoides*	4x	186	189				499			
24	*R. confervoides*	4x	186	189				491			
4	*R. fluitans* 2x	2x	180					-			
25	*R. fluitans* 2x	2x	180					464			
5	*R. fluitans* 3x	3x	180					-			
26	*R. fluitans* 3x	3x	177	180	181	183	187	-			
21	*R. fluitans* x *R. peltatus*	4x	180	186	189			-			
22	*R. fluitans* x *R. trichophyllus* B	3x	177	180	186			-			
6	*R. peltatus*	4x	177					-			
7	*R. peltatus*	4x	180	186				-			
19	*R. peltatus*	4x	177	186				493			
8	*R. peltatus*	4x	177	183				-			
18	*R. peltatus* x ?	4x	186	189				499			
17	*R. peltatus* x *R. trichophyllus* A	4x	183	189				499			
20	*R. peltatus* x *R. trichophyllus s. l*	4x	177	186				-			
9	*R. penicillatus* A	4x	177	180	183	186		-			
10	*R. penicillatus* A	4x	180	183				484			
32	*R. penicillatus*	4x	186					444			
11	*R. rionii*	2x	-					-			
27	*R. rionii*	2x	180					460	467	495	
12	*R. trichophyllus* A	4x	177	189				499			
13	*R. trichophyllus* A	4x	189					499			
14	*R. trichophyllus* B	4x	180	183				469	478	482	492
15	*R. trichophyllus* B	4x	180	183	194			-			

5. Discussion

In this study, primers for microsatellite loci designed for the species *Ranunculus bungei* (Wu *et al*., 2017) were tested on central European *Ranunculus* species. The aim was to figure out which primers anneal and which loci show sufficient variation in order to use them for further analysis of various European *Ranunculus* taxa. Therefore, a combination of Sanger sequencing and fragment analysis was performed. With Sanger sequencing information about presence of the microsatellite repeat was obtained. Fragment analysis gave additional knowledge about relative allele sizes and its variation in larger set of samples.

Most primers seemed to anneal and PCR reaction was usually successful. However, although the requested repeat motif was mostly found in Sanger sequences, numbers of repetitions were lower than reported by Wu *et al*. (2017) in nearly all cases. Generally, shorter repeats with low purity have a low mutation frequency (Vieria *et al*., 2016). An extreme example for this circumstance is locus B5, in which the repeat motif was not resolved (absent or very short) in both samples (Wu *et al*., 2017). Due to that result, this primer was excluded from further work (fragment analysis).

Another frequently occurring phenomenon was that the repeat was found using Sanger sequencing (and showed the required length) but was interrupted by a single or by multiple base pairs. This fact was observed for instance with locus B21. Furthermore, fragment analysis also revealed that locus B21 did not work properly on European *Ranunculus* species.

When it comes to fragment analysis, most of the loci were resolved in most of the samples. The obtained pattern for most taxa were comparable or even completely identical between different samples of the same species and therefore species-specific.

However, due to low within-species variation and several problematic features, the tested microsatellite loci cannot be used for usual purposes in central European *Batrachium* taxa (such as clone identification, population genetics, etc.). Those main problems include for example that the numbers of alleles are not corresponding to the ploidy levels. This means that in some cases more alleles had been found than is the number of chromosome sets (i.e., number of physical copies of a locus), which might have been caused by duplication of the locus or length variation in the flanking region or presence of another type of repeat. Additionally, the

difference between the alleles did sometimes not correspond to the length of the repeat which again shows that there is some other source of length variation in the flanking region.

Problems occurring in this study are quite common when one tries to cross-amplify microsatellites from less-related species. They are probably due to the large phylogenetic distance, which means that there was enough time for mutations to occur in various regions of the DNA including priming sites, the flanking regions or even within the microsatellite itself. Problems like that are well known in the literature, e. g. when testing primers designed for a moss *Scorpidium cossini* on the relatively closely related *Hamatocaulis vernicosus* (Manukjanová *et al.,* 2018). In this study, most samples of *H. vernicosus* were amplified successfully but there were various problems such as absent microsatellite motifs or the fact that the sequence contained another microsatellite which then interfered with the interpretation of the fragment analysis. Therefore, new species-specific SSR primers had to be designed for *H. vernicosus*. Since the same is true concerning the study about *Ranunculus* sect. *Batrachium*, new primers have to be designed for these plants in order to use them for population genetical studies in a reliable way.

Apart from that, this work revealed that sample 18, of which the species was previously not determined exactly, most probably is a hybrid between *R. peltatus* and *R. trichophyllus* A. This interesting aspect was found out by analyzing the data (fragment analysis) which showed that the pattern of sample 17 (*R. peltatus x R. trichophyllus* A) and sample 18 showed a high level of similarity.

6. Conclusion

In this study microsatellite markers which were obtained from *Ranunculus bungei* from China (Wu *et al*., 2017) were tested on various European *Ranunculus* sect. *Batrachium* species. The aim was to get information whether they amplify and are variable enough to use them for further population genetic studies. Results showed that most primers seem to anneal on most of the analyzed *Ranunculus* species and PCR reaction is successful. However, the variation is not sufficient and interpretation of the patterns in fragment analysis is not always clear-cut.

Therefore, the deployed primers cannot be used for studies on population genetics but some might be useful for species identification. For additional information and a deeper understanding further studies should be done.

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