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Genetic variation in common cattail (*Typha latifolia*) in southern Sweden

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Genetisk variation hos bredbladig kaveldun *Typha latifolia* i södra Sverige

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Summary

Typha latifolia and *T. angustifolia* are wide spread perennial aquatic plants that have long history of being used by humans as a food source (rich in starch) and for raw material but they are not used much today. *Typha* spp has potential to be used for bioremediation of polluted waste water as they tolerate high amounts of pollutants and are capable of taking up chemical pollutants and heavy metals. They also have some level of tolerance to salinity. Therefore, there is an increased interest in studying them further. And the molecular structure of the starch from in the rhizomes is known. There is a potential use of *T. latifolia* for production of active carbon. Some experiments with them as substrate for mushroom production have been made. Several microsatellite markers have been developed both for *T. latifolia* and *T. angustifolia* that can be used to study genetic variation and crossbreeding between different spp. In this study T. latifolia were collected from 16 different sites in southern Sweden and three different microsatellite markers developed for *T. latifolia* were used to analyze the genetic variation. The result from the 36 samples collected and analyzed were eight different genotypes, of which five were heterozygotes for one of the markers. But not all samples gave bands and some not for all of the markers used. So, further studies with samples from more locations across Sweden using more markers would be needed to get a more accurate assessment of the genetic variation of *T. latifolia* in Sweden.

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Abbreviations

- AFLP = amplified fragment length polymorphism
- ANOVA = analysis of variance
- bp = base pairs
- cpDNA = chloroplast DNA
- Fst = fixation index
- nDNA = nuclear DNA
- nSSR = nuclear simple sequence repeat
- RAPD = random amplified polymorphic DNA
- ptDNA = plastid DNA
- PCR = polymerase chain reaction
- STR = short tandem repeat
- SSR = simple sequence repeat
- VNTR = variable number tandem repeat

Introduction

Botany: Systematics, Morphology and Habitats

Common cattail (*Typha latifolia*) is an aquatic and perennial plant that propagates through seed and also vegetatively by rhizomes (Keane et al. 1999). It belongs to the Cattail family *Typhaceae* (Fernald and Kinsey 1943) that contains two genera and 51 species (Christenhusz and Byng 2016). Cattails belong to the genus *Typha* that includes 38 species according to the annual checklist of the Catalogue of Life (Roskov et al. 2014). A *Typha* plant has leaves with a strongly developed rounded flange at the connexion of the leaf blade and the sheathing base and they have creeping and branching rhizome and the flower spike is divided in to two parts (Fernald and Kinsey 1943). The flowering parts of *Typha* are on the top of a round straw (Lidman 1977). The upper part has the male flowers and the lower part has the female flowers (Fernald and Kinsey 1943). The female flowers form a cylindrical structure composed of many smaller flowers and the pollen from the male flowers is wind dispersed (Lidman 1977). The upper part is yellow before the pollen is released and thereafter dries up. The lower female part is green when young and at maturity it turns into brown (Fernald and Kinsey 1943).

The two species with the widest range in the Cattail family are *T. latifolia* and *T. angustifolia* (Kim and Choi 2011). *T. latifolia* and the related *T. angustifolia* can grow 2 to 3 m tall and both of them have narrow and long leaves and grow in nutrient rich shallow waters and waterlogged habitats (Lidman 1977). The habitats of all the *Typha* species are composed of bodies of fresh or brackish water of bogs, pond, quiet streams and marshes (Fernald and Kinsey 1943). T. latifolia is not as salt tolerant as some other species in the Cattail family like T. angustifolia that grows well in brackish water (Fernald and Kinsey 1943). *T. latifolia* is, however, still capable of growing well at salinity levels of 2.4% with a survival rate of 94% (Jesus et al. 2014). Its survival rate was 56% if treated with 2400 mgL-1 of NaCl when growing in a greenhouse (Koropchakand and Vitt 2013). Nevertheless, *T. latifolia* can unlike *T.* ×*glauca* and *T. angustifolia* grow in acidic soil (Hotchkiss and Dozier 1949).

The morphological differences between *T. latifolia* and *T. angustifolia* are that the former has wider leafs (Kim et al. 2003) and bigger female flower structure than the latter (Lidman 1977). The leaf width overlaps between *T. latifolia* (7-20 mm), *T. angustifolia* (3-12 mm) and *T.*

×*glauca* (3-14 mm) (Fassett and Calhoun 1952). Thus, the leaf width is not a suitable descriptor to distinguish between these *Typha* species (Fassett and Calhoun 1952). T. latifolia does not have a gap between staminate and pistillate inflorescence, thus favoring inbreeding. The

opposite is true for *T. angustifolia* in which there is a gap that favors outbreeding (Kim and Choi 2011). *T. latifolia* produces more rhizomes and fewer flowers than *T. angustifolia* (McNaughton 1966).

Historical and present usage of common cattail

Common cattails have a long history of being used by humans and new shoots and immature flowers can be cooked and eaten (Gaertner 1962). Leaves from cattail have been used by native Americans to produce mats and the down (the structure that allows seeds to be dispersed by wind) from the mature flower heeds have been used as padding for pillows (Coville 1897). All of the parts of the cattail plants are considered to be edible and their rhizomes are high in starch and protein content (Kurzawska et al. 2014; Vetayasuporn 2007). The sprouts can be used like asparagus (Liptay 1988) and as a vegetable after cooking, while its pollen can be used in baked and roasted products (Vetayasuporn 2007). The pollen grains are collected during the summer and autumn, whereas during the winter the rhizome can be collected (Liptay 1988). *T. latifolia* has been proven poisonous to humans in some cases but not very often (Woodcock 1925). Hurst (1942) –as cited by Morton (1975)–indicated that leaves and flower heads of *T. angustifolia* did not cause any damage to the testing animal.

Cattails can be established and survive in waste water of polluted wetlands (Calheiros et al. 2008). They are also capable of taking up many chemical pollutants and heavy metals (Kumari and Tripathi 2015). For those reasons it can be used for bioremediation of polluted wetlands (Keane et al. 1999; Kumari and Tripathi 2015). When grown together with the common reed (*Phragmites australis*), they are more efficient at bioremediation than either species alone because they complement each other (Kumari and Tripathi 2015). When grown in untreated urban sewage mixed with industrial efflux, Fe, Cr, Cu, Cd, Cr, Fe, Ni, Pb and Zn were the metal removed by *T. latifolia* (Kumari and Tripathi 2015). *T. latifolia* was better at removing Pb and Zn than *P. australis* (Kumari and Tripathi 2015). The amount of the metals that were examined had largest concentration in the roots of *T. latifolia* (Kumari and Tripathi 2015), while only small amounts of the metals taken up were allocated to the leaves and stems of the plants (Klink

et al. 2013). Another study found that *T. latifolia* accumulates Mg, Zn and Ni in higher concentration in shoots than in roots; and Cr, Fe, Mn and Cu were found mostly in the roots (Hazra et al. 2015). Cr, Cu and Fe had higher concentration in the roots than in the shoots, while for the highest concentration of other elements there are disagreements regarding their accumulation in the plant (Klink et al. 2013; Hazra et al. 2015). According to Klink et al. (2013),

T. latifolia has a potential use for biomonitoring Cu, Cd, Cr, Fe, Ni, Pb and Zn contamination through analyzing their concentration in the plants.

The potential use of common cattail

The rhizomes contain starch up to 70% of its dry weight (Kurzawska et al. 2014). The amylose/amylopectin ratio in the cattails starch is 31.7% amylose and 68. 3% amylopectin (Kurzawska et al. 2014). Common Cattail's cell walls were hard to break in the disintegration stage of the extraction, thus it is not suitable today for its use as an industrial source for starch (Kurzawska et al. 2014). Its amylose from the rhizome had molar weight higher than the one from potatoes, but slightly lower than the one from maize (Kurzawska et al. 2014). The amylopectin from the rhizome is more branched compared to the amylopectins from potato and maize, and its molar weight was between those from maize and potato (Kurzawska et al. 2014).

Common cattail has been examined as a potential substrate for growing gilled edible mushrooms of the genus *Pleurotus* (Vetayasuporn 2007). Although *Pleurotus* spp. were shown to grow on cattail substrate, their edible yield is lower as compared to when grown on sawdust substrates. Hence, common cattails are a less suitable growth medium for *Pleurotus* spp. production than other substrates commonly used (Vetayasuporn 2007).

Common cattail has also been tested as source for producing activated carbon (Song et al. 2015). Cattail-activated carbon can effectively remove Cu^{2+} and Zn^{2+} and it is cheaper than other sources used to produce activated carbon, e.g. wood (Song et al. 2015). The yields of activated carbon from dry leaves and stems were 48.56% and 38.25% respectively, while the pore volume and average diameters of the activated carbon were 13.29 ml g^{-1} and 4.14 nm, respectively (Song et al. 2015).

Cytology and Genetic Diversity

The sporophytic chromosome number of *T. latifolia* and *T. angustifolia* is 2n = 30 (Harada 1949). The complete *T. latifolia* plastid genome is 161,572 base pairs (bp) (Guisinger et al 2010).

Polymerase chain reaction – short tandem repeat (PCR-STR) probes were used to examine the genetic diversity in variable number of tandem repeat (VNTR) loci of *T. latifolia*. This assessment of diversity with PCR-STR was lower than those found in most species (Keane et al. 1999). Starch gel electrophoresis have also been used to examine the diversity of *T. domingensis*, *T. latifolia*, *T. angustifolia* and *T. glauca* from the USA by Sharitz et al. (1980) who were able to distinguish between these four species. Variation was lacking within the populations except for three enzyme systems in *T. glauca* and one in *T. latifolia* (Sharitz et al. 1980). *T. domingensis* and *T. angustifolia* were distinct for 6 of the 10 enzymes investigated, while only 1.4% of the 186 different individuals, with large geographic distribution, examined showed intraspecific variation (Mashburn et al. 1978).

T. ×*glauca* is the hybrid between *T. angustifolia* and *T. latifolia* and it is found in North America (Grace and Harrison 1986) and in Europe (Nowińska et al. 2014). Hybridization between *T. latifolia* and *T. angustifolia* has not been noticed in Asia even though the two species are found in this continent (Zhou et al 2015). Manual hybridization between *T. latifolia*, *T. domingensis* and *T. angustifolia* were made, and their derived-hybrids' fertility was investigated (Smith 1967). Meiosis was normal in hybrids derived from crossing *T. domingensis* and *T. angustifolia*, but not in the different hybrids with *T. latifolia*, which showed some abnormalities in their meiosis (Smith 1967). The percentage of normal pollen in the hybrids derived from crossing *T. domingensis* and *T. angustifolia* varied between 49 to 76%, while it was 47.5% and 38% in hybrids with *T. latifolia* although the seed production range for the hybrids was from 0 to 25% of that of normal cattail plants (Smith 1967). *T. angustifolia* and *T. latifolia* and their hybrids from two different areas were examined by Ball and Freeland (2013). The flowering time had a significant overlap between the different species and all hybrids had chloroplast DNA (cpDNA) sequences from *T. angustifolia*, thus confirming that cpDNA in *Typha* is inherited from the mother plant (Ball and Freeland 2013). Hence, it seems very likely that there are some barriers that prevents fertility in hybrids derived from crossing *T. angustifolia* and *T. latifolia* as the mother plant, but the flowering time is not the problem as it overlaps (Ball and Freeland 2013). Random amplified polymorphic DNA (RAPD) markers and cpDNA markers were used

to examine *T. latifolia*, *T. angustifolia*, *T. ×glauca*. and different crosses made with *T. latifolia* and *T. angustifolia* while only cpDNA from *T. angustifolia* was found in *T. ×glauca*

the plant from the crosses made had cpDNA from the species that were used as pollen recipients (Kuehn et al. 1999). The leaf width of *T. ×glauca* can reach 21 mm but it is often smaller than 16 mm (Kuehn et al. 1999).

Research on *T. latifolia* and *T. angustifolia* genetics was previously undertaken in Asia, North America and Ukraine (Na et al. 2010; Kim and Choi 2011; Tsyusko et al. 2005). Amplified fragment length polymorphisms(AFLP) markers were used to analyse four different types of *Typha* from East Asia by Na et al. (2010), who found that *T. latifolia* had the lowest level of genetic diversity among the *Typha* species included in their study. A relatively high difference between *T. latifolia* from North America and East Asia was, however, found with plastid DNA (ptDNA) sequencing (Kim and Choi 2011).

Eleven microsatellite or simple sequence repeat (SSR) loci were identified for *T. angustifolia* (Tsyusko-Omeltchenko et al. 2003). These SSR markers were used to evaluate genetic diversity of *T. latifolia* and *T. angustifolia* in Ukraine. *T. latifolia* showed low genetic diversity when analyzed with the 11 SSR markers. The genetic diversity was, however, greater than was previously noted for Typha with other DNA markers (Tsyusko-Omeltchenko et al. 2003). Microsatellite loci TA 5 and TA 21 developed by Tsyusko-Omeltchenko et al. 2003 had species specific alleles that did not overlap between *T. latifolia* and *T. angustifolia* (Snow et al. 2010).

Nine microsatellite loci (TL 45, TL146, TL 209, TL 213, TL 247, TL 305, TL 322, TL 368, and TL 442) were identified for *T. latifolia* (Ciotir et al. 2013). The markers at three of these nine loci (TL 146, TL 213 and TL 305) are different in *T. latifolia* and *T. angustifolia* (Ciotir et al. 2013), thus allowing their use to distinguish between these two species (Ciotir et. al. 2013). The allele richness was higher in plants from Europe than plants from America when assessing diversity using the nine SSR markers developed for *T. latifolia* by Ciotir et al. (2013).

Twenty diagnostic RAPD markers were used by Nowińska et al. (2014) to investigate if *T.×glauca* was present in Poland and to measure its genetic diversity along with its two parental species. Analysis of polymorphism using 116 RAPD markers revealed that in *T. latifolia* 59.48% of the RAPD markers were polymorphic, in *T. angustifolia* 71.55% and 87.07% in *T. glauca* (Nowińska et al. 2014). cpDNA and nuclear DNA (nDNA) sequences and nuclear microsatellites (nSSR) were used to determine the genetic variation in four different Typha

species found in Asia by Zhou et al. (2015), who also examined some morphological characters such as plant height, leaf width and inflorescences length.

The diversity found for *T. latifolia* with nDNA for the nuclear gene phytochelatin synthase, among populations was 67.78% and 32.22% within populations. Thus being the fixation index (Fst) equal to 0.678 (Zhou et al. 2015). The diversity with nSSR among populations was 72.25% and 27.52% within populations, while Fst was 0.723 (Zhou et al. 2015). The diversity with cpDNA for the nuclear gene phytochelatin synthase among populations was 96.54% (Fst = 0.965) while it was 3.46% within populations (Zhou et al. 2015).

Microsatellites are tandem repeats of 2 to 6 nucleotides found at high frequency in most species. The length of the locus often varies from 5 to 40 repeats. Dinucleotide, trinucleotide and tetranucleotide repeat are most frequently used for assessing diversity with the aid of molecular biology (Selkoe and Toonen 2006). The flanking region of the microsatellite locus are often highly conserved in individuals of the same species (Selkoe and Toonen 2006). Because of that primers can be designed to bind to the flanking region and guide the PCR amplification of the microsatellite locus (Selkoe and Toonen 2006). The alleles that differ in length can be distinguished by high-resolution gel electrophoresis (Selkoe and Toonen 2006).

The genetic diversity analysis of *T. latifolia* and *T. angustifolia* plants from Chernobyl area and reference population estimated using microsatellite markers showed that 48% of the total variation differentiated the two species, which is higher than the level of variation revealed both among populations within species and within populations (Tsyusko 2004). *T. latifolia* had lower expected heterozygosity at species level (0.37) and at population level (0.29); while in *T. angustifolia* it was 0.66 at species level 0.49 at population level, respectively (Tsyusko 2004). There was high diversity in the Chernobyl population but little effect was attributed to the radioactivity in the area (Tsyusko 2004).

A study in eastern North America found 136 unique genotypes identified from 215 ramets (i.e., individuals derived from a genet or clonal colony), which were divided into three distinct clusters by the first principal component (Kirk et al 2011). These clusters represented *T. latifolia*, *T. angustifolia* and *T. ×glauca* and accounted for 63% of the total variation in the data set of seven microsatellites used in their study. There were 53 unique genets of *T. latifolia*, 27 unique genets of *T. angustifolia*, and 56 unique genets of *T. ×glauca* which showed a

heterozygosity of 0.205%, 0.296% and 0.737% respectively (Kirk et al. 2011). These levels of heterozygosity were significantly different from the expected Hardy-Weinberg's equilibrium; i.e., they were lower for the parent species but not for their cross (Kirk et al. 2011).

Aim

The goal of this research was to investigate the genetic diversity of *T. latifolia* in southern Sweden. *T. latifolia* was chosen owing to its perennial plant habit and its use as a food source as its rhizomes contain starch. *T. latifolia* can also be used for bioremediation because it can accumulate heavy metals. Researching its genetic diversity will be interesting to get insights on the genetic distribution of *T. latifolia* in Sweden, which could be useful for future genetic research on this plant.

Materials and methods

A search of genetic markers available for *T. latifolia* and related species was helpful to determine those to be used for the genetic analyses of the plant material. Microsatellite markers were chosen because they can be highly polymorphic, thus needing few markers (Selkoe and Toonen 2006). The selected microsatellite markers were TL 146, TL 209 and TL 305, which were developed for *T. latifolia* by Ciotir et al. (2013). Shoots with rhizomes were collected from 16 different sites (Fig. 1) of roadside ditches, ponds, wetlands and lake shores. There were 36 different collected plants and the number of plants collected per site varied from one to five. The number of rhizomes collected from each site depended on the size and the number of stands at the site. Often, there was only one stand accessible as some could not be reached as they grew into deep water, swampy areas or inaccessible for other reasons. Hence, the collecting had a bias because the collected plants were those easiest to find and reachable. The coordinates and altitude for each site were recorded (Table 1). The leaves of the collected plant material (up to 5) were also measured (Table 1). Some of the above ground parts of the collected material were damaged by insect larva and aphids.

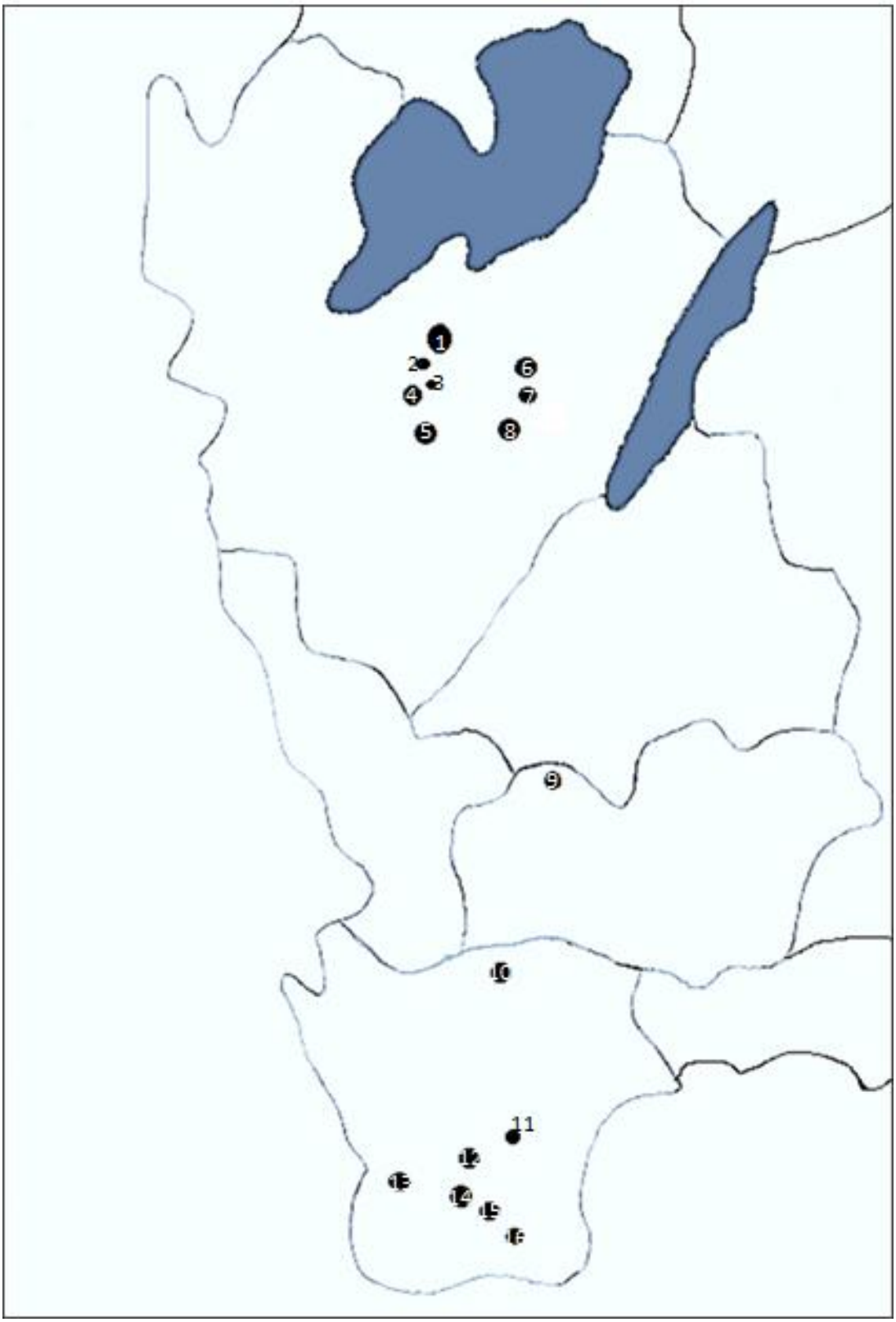

Figure 1. Map of southwestern Sweden showing the areas for collecting *T. latifolia* [black points]. (Fahlgren 2016)

Table 1. Coordinates, altitude (m) and habitat of 16 collecting sites, and measurement on up to 5 plants of their leaf width (cm).

Collecting area	Latitude	Longitude	Altitude	Habitat	A	B	C	D	E
1	N 58.42	E13.33	99	pond	1.9	1.6			
2	N 58.39	E 13.31	123	ditch	1.0	1.6			
3	N 58.33	E 13.15	109	ditch	1.0	0.7	1.3		
4	N 58.27	E 13.07	76	wetland	1.6	1.5	1.0		
5	N 58.16	E 13.28	229	wetland	1.4				
6	N 58.32	E 13.52	124	ditch	1.6	1.5			
7	N 58.29	E 13.47	209	ditch	2.2				
8	N 58.13	E 13.52	225	ditch	1.2				
9	N 56.97	E 14.01	179	ditch	1.4				
10	N 56.40	E 13.52	165	ditch	1.4				
11	N 55.91	E 13.77	199	Ditch. wetland	1.1	1.7	1.7	1.5	
12	N 55.80	E 13.56	95	lake	1.4	1.5	0.5	1.5	1.1
13	N 55.65	E 13.08	14	ponds	2.3	1.0	1.2	1.5	1.3
14	N 55.68	E 13.49	61	lake	1.5				
15	N 55.52	E 13.70	107	pond	1.4	1.0	1.3		
16	N 55.82	E 13.77	107	pond	1.4				

Fresh young leaf tissues (5–12 cm) were grounded in an Eppendorf tube after being frozen in liquid nitrogen. The resulting samples of each were then used for DNA extraction with a genomic DNA purification kit (Thermo Scientific). They were homogenized for 10 min at 65

°C in a mix of 300 µl TE buffer and 600 µl lysis solution. Afterwards 900 µl of chloroform were added and the tube were inverted 3-5 times and centrifuged at 10,000 rpm in the centrifuge for 2 min. The upper aqueous phase of the solutions was transferred to a new tube with 1200 µl precipitation solution and mix for 1 to 2 min at room temperature and thereafter centrifuged for 2 min and the supernatants were removed. About 450 µl of 70% cold ethanol were added and the DNA samples were allowed to precipitate for 10 min at –20 °C in the freezer, and afterwards centrifuged for 4 min. The ethanol was then removed and the pellets were washed with 450 µl of cold ethanol and dissolved in 50 µl TE buffer containing 0.1 mg ml-1 RNAse. They were thereafter allowed to dissolve at room temperature for at least 30 min. Afterwards the DNA concentration were examined using a Nanodrop machine. New DNA samples from the plants

were extracted for those showing DNA concentration below 10 ng µl-1. The DNA samples were

loaded on 1.2% agarose gel and electrophoresed (Table 2) to check for quality. The samples were treated with RNAse to remove the RNA that was detected and then stored at –20 °C. The 36 DNA samples (Table 3) were diluted with milli-Q water until reaching a concentration of 10 ng µl-1 prior to its use in the polymerase chain reaction (PCR).

Table 2. Gel composition.

Chemical	Amount 2% gel	Amount 1.2% gel
Agarose	8 g	4.8 g
TAE	400 ml	400 ml
Gelred	10 µl	10 µl

Table 3. DNA concentration (ng μl^{-1}) for the samples used for the polymerase chain reaction before dilution.

Sites of collection	samples				
	A	B	C	D	E
1	53.57	305.73			
2	42.35	12.46			
3	10.15	19.38	27.07		
4	27.12	34.3	60.54		
5	91.6				
6	10	73.36			
7	380.31				
8	125.32				
9	900.47				
10	634.86				
11	30.48	2716.95	227.78	2006.04	
12	22.52	15.54	10	14.66	18.87
13	525.45	38.36	45.64	38.62	89.11
14	47.24				
15	87.84	14.29			
16	29.84	31.47			

Touchdown PCR (Table 4) was used with two different PCR master mixes ((A) and (B) in Table 5) and were tested with three different DNA samples with 2 replications each and 2 controls without DNA. Two primer pairs were tested. The same PCR protocol with the temperature for denaturation lowered by 1°C following the specifics for the polymerase was also tried.

Table 4. Touchdown polymerase chain reaction protocol used.

Step	Temperature and time
1	2 min at 96°C
2	95°C for 20 sec
3	64°C for 50 sec
4	72°C for 60 sec, Repeat 5 times steps 2–4
5	95°C for 20 sec
6	64°C for 50 sec decreased by 0.5 °C $cycle^{-1}$ until 55 °C.
7	72°C for 60 sec, Repeat 21 times steps 5–7
8	96°C for 20 sec
9	55°C for 50 sec
10	72°C for 60 sec, Repeat 5 times steps 8–10
11	72°C for 10 min as final extension

Table 5. Polymerase chain reaction master mixes based on either (A) amount (in ul) used by Ciotir et al. (2013); or (B) from product protocol of Dream Taq DNA Polymerase (Thermo Scientific). The final master mix used in the PCR is given in column 4: (C)

Chemicals	(A)	(B)	(C)
dNTP Mix. 2 mM each	0.4	1	0.4
Dream Taq DNA Polymerase	0.5	0.05	0.25
10X Dream Taq Buffer	0.5	1	1
Forward primer	2	0.02	2
Revers primer	2	0.02	2
Milli-Q water	3.6	6.91	2.35
DNA	1	1	2
Final volume	10	10	10

Additionally, a protocol based solely on the recommended temperature profile for polymerase used was also tested; however, no amplification of the target loci was obtained. Hence, simple PCR program with a gradient was tested to determine the temperature to be used (Table 6), which also resulted in no amplifications.

Table 6. Polymerase chain reaction (PCR) protocols used.

Steps in PCR gradient protocol	Steps in final PCR protocol
1. 95°C. for 3 min	1. 95°C. for 3 min
2. 94°C for 30 sec	2. 94°C for 30 sec
3. Gradient 57–67°C for 30 sec	3. 57.5°C for 30 sec
4. 72°C for 1 min. Repeat steps 2–4 29 times	4. 72°C for 1 min. Repeat steps 2–4 29 times
5. 72°C for 10 min hold at 4°C	5. 72°C for 10 min hold at 4°C

Hence, a change in the master mix was therefore made to find any problem following the PCR troubleshooting protocol from Thermo Scientific. The PCR master mix used is described in column (C) in Table 5.

The temperature was then tested with all three primers to evaluate their suitable value for the final protocol. The working protocol was then used with primers TL 146, TL 209 and TL 305. About 2µl of loading dye was added to the PCR products amplified by each primer-pair. For the DNA ladder used to estimate fragment size, one microliter Thermo Scientific Gen Ruler 50 bp DNA ladder, l µl loading dye 4 µl and milli-Q water were mixed. Both the ladder and amplified PCR products were spun down and loaded on 2% agarose gel on separate wells for electrophoresis. The PCR products loading order from left to right on the gels was as follows: DNA ladder. 13A twice for TL 146, 13B, 13C, 13D, 13E, 11A, 11B, 11C, 11D, 9A, 7A, 1A, 1B, 10A, 4C, 2A, 2B, 3A, 3B, 3C, 16A, 16B, 15A, 15B, 12A, 12B, 12C, 12D, 12E, 4A, 5A, 6B, 14A, 4B, 8A, 6A and control. An extra ladder was also included in the gel for locus TL 305. Afterwards, the amplified DNA fragments were run at 100 amperes for 60 min. The results were visualized using UV light. The PCR products from TL 305 were loaded in the same way as the other except a ladder was also added in the second well from the right in the gel and it was run at 88 amperes for 70 min.

Results

All master mixes used gave primer dimers. The bands of the target loci were faint when the master mix recommended by the producer of the Taq polymerase was used. The test with more DNA and buffer gave some very faint bands (lane 3-4; Fig. 2) but the test with less Dream Taq

polymerase, more DNA and buffer gave the strongest band (Fig. 2; 13-14).

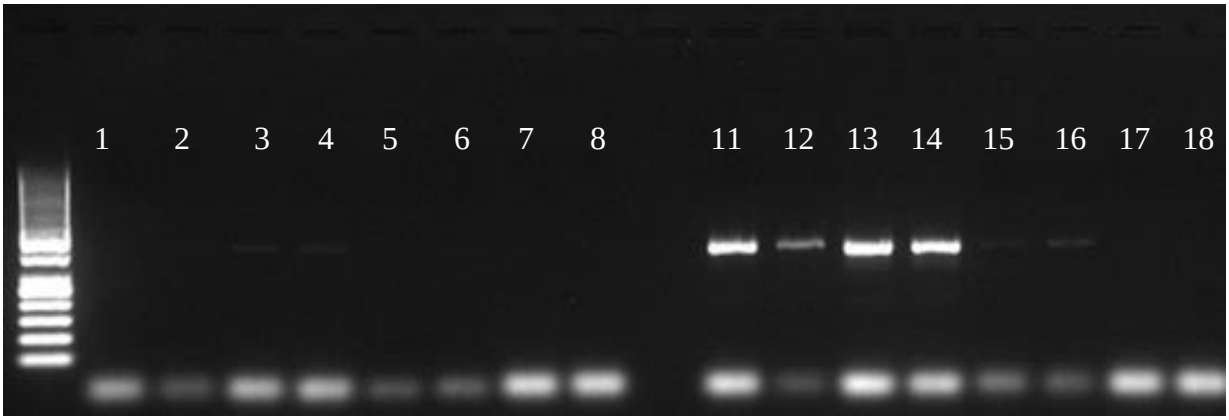

Fig. 2. PCR troubleshooting: DNA sample number 10 was used, and all had 1 ul buffer, 11-18 have 0.25 ul Taq polymerase, 3-4 and 13-14 had 2 ul DNA, 5-6 and 15-16 have 1 ul of forward and reverse primers. 7-8 and 17-18 were controls and had no DNA, apart from that they were the same as (A) in Table 5.

Samples with more DNA and less Taq polymerase were therefore tested on temperature gradient with primer TL 146. There were 4 samples showing good bands, 1 sample a weak band, and 3 samples did not get any band (Fig. 3). The other primers were tested thereafter. TL 209 got several good bands as well as TL 305 but theirs were not as strong as the former (Fig. 4).

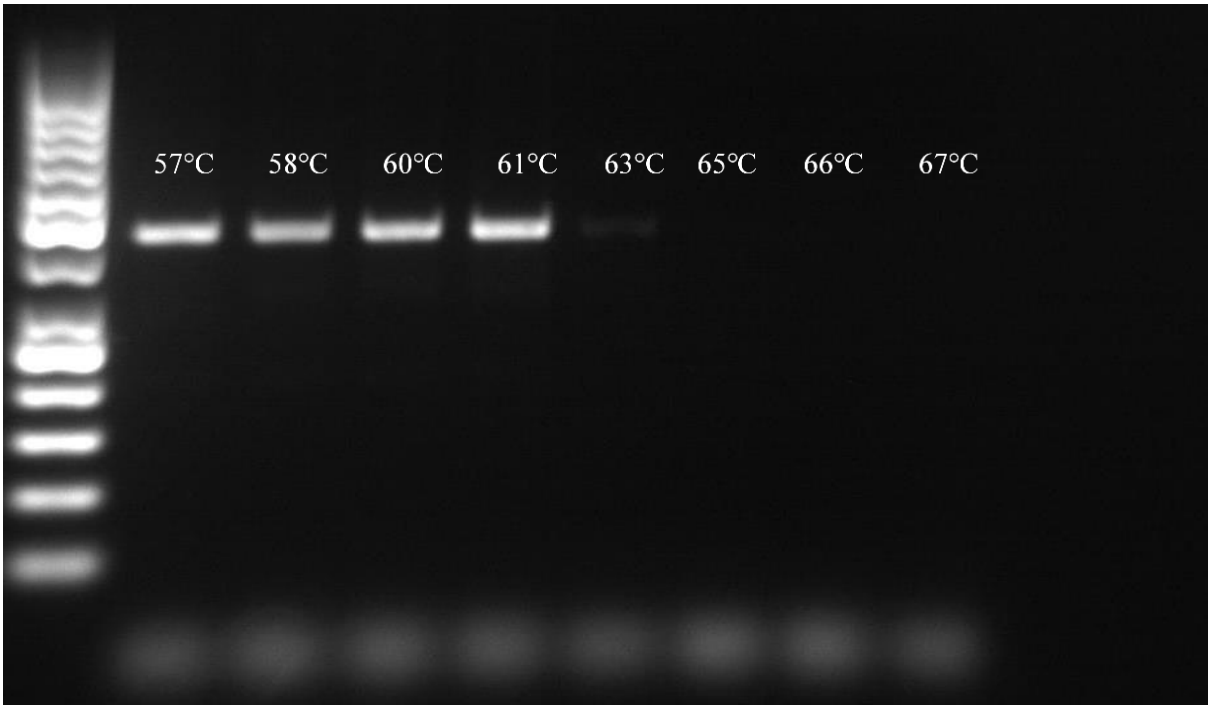

Fig. 3. DNA sample number 10 was tested for amplification using primer-pair TL 146 with temperature gradient ranging from 57 to 67 °C (melting temp 66,1°C as the amount of Mg2+ and dNTP affects the melting temperature).

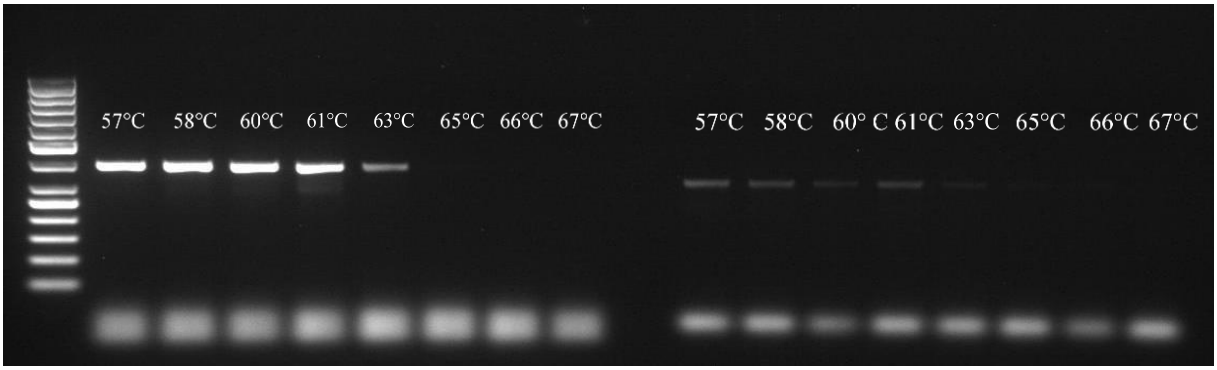

Fig. 4. Primer-pair TL 209 (melting temp 64,6°C) to the left and primer-pair TL 305 (melting temp 67,4 °C) to the right. The temperature gradient and the DNA sample used (sample number 10) were the same as in Fig. 4. The PCR mix used was the one shown in Table 5 under column (C).

The PCR products amplified for 36 DNA samples using three different primer-pairs TL 146 (Fig. 5), TL 209 (Fig. 6) and TL 305 (Fig. 7) were run on 2% agarose gel and then visualized using a machine with UV light source.

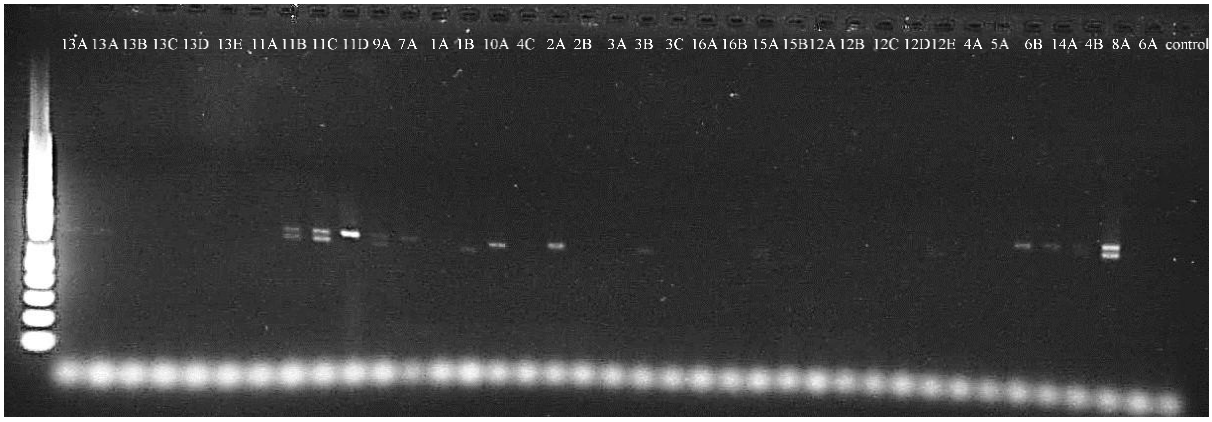

Fig. 5. The amplification of locus TL146 in 36 different DNA samples. Sample 13A was repeated, and one well was used as control. The amplification of 18 of the 36 samples revealed three alleles.

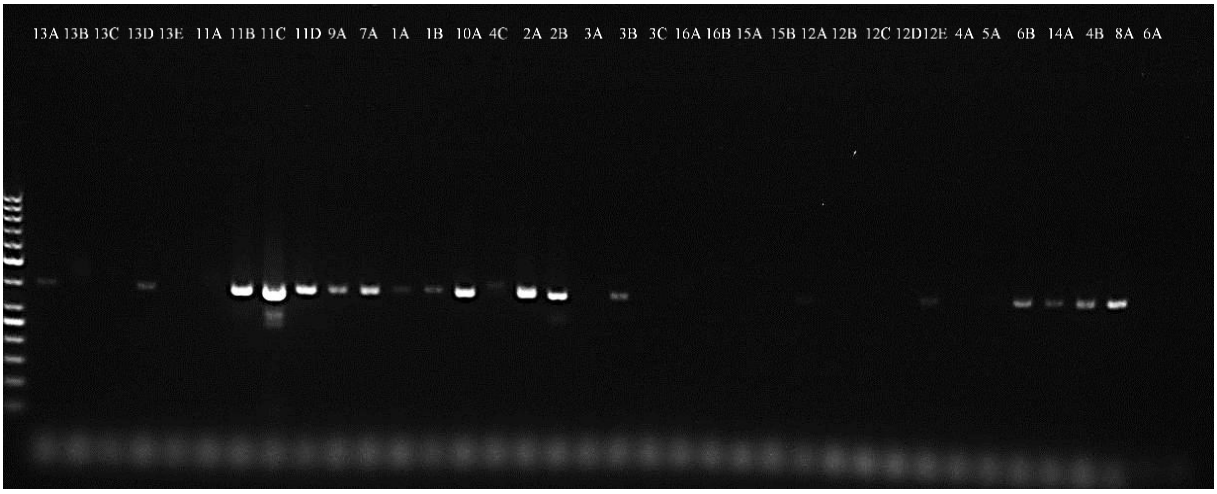

Fig 6. The amplification of locus TL209 in 36 different DNA samples and 1 control: The amplification of 20 of the 36 samples revealed two alleles.

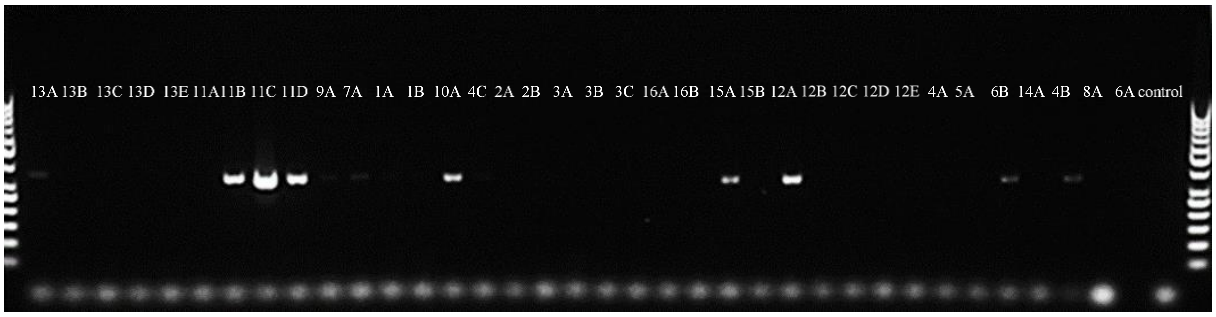

Fig 7. The amplification of locus TL305 in 36 different DNA samples and 1 control: The amplification of 14 of the 36 samples revealed three alleles.

Some samples got two bands with primer TL 146 and that indicates that they are heterozygotes for that specific locus. Two of those were from the same area whereas the rest were from different areas. Sample 3A, 3C, 4A, 5A, 6A, 11A, 12B, 12C, 12D, 13B, 13C, 13E, 15B, 16A gave no band for any of the loci, and some only for one or two of the loci. Sample 4C and12A gave no band for locus TL 146 but for the two other loci. Sample 15A gave no band for locus TL 209 but for the other two loci. Sample 12E and 14A, 8A, 3B, 2A gave no band for locus TL 305 but for the other two loci.

Table 7. Different bands per locus for various genotypes is represent by different later (A-D). For each locus, the pair of same letters represent homozygotes whereas the pair of different letters represent heterozygotes.

Different genotypes	Sample	TL 209	TL 305	TL 146
1	1A	BB	AA	AA
1	7A	BB	AA	AA
1	13A	BB	AA	AA
2	1B	BB	AA	CC
3	4B	AA	BB	AB
4	6b	BB	BB	AA
5	9A	BB	AA	AB
6	10A	BB	BB	BB
7	11D	BB	CC	AA
8	11B	BB	CC	AB
8	11C	BB	CC	AB

Eight different genotypes were detected from the samples that gave band on all primers (Table 7). There might be some more genotypes present amongst those having bands at only one or two loci, as some of those samples from the same area produced different fragments for the amplified loci (Table 8).

Table 8. Samples that did not give bands for al primers

Sample	TL 209	TL 305	TL 164
4C	AA	BB	0
2A	BB	0	BB
2B	BB	0	0
3B	BB	0	BB
15A	0	AA	BB
12A	BB	AA	0
12E	BB	0	AA
14A	AA	0	AA
8A	AA	0	AB
13D	BB	0	0

Discussion

Common cattails may be edible but they are also good at accumulating chemical pollutants. Those pollutants would be harmful for human if they were consumed. Hence, it would be good if there are genotypes that accumulates less pollutants or only in certain tissue(s) to allow common cattails to be used as food. For bioremediation the opposite would however be good. If they could accumulate more and higher concentration of chemical pollutants would increase its efficiency as bioremediation and for specific tissue could probably be good as well. Knowledge of how big variation exist would therefore be useful to improve the plant to get new cultivars. Their easy vegetative propagation facilitates getting many plants of the same genotype. It would have been better to collect later during the summer or early autumn as the morphological characters used for distinguishing between T. latifolia and T. angustifolia are all present, particularly when their leave width overlaps according to Fassett and Calhoun (1952).

Nevertheless, it would be possible to say that if the leaf width is less than 5 mm on fully grown plants it is most likely *T. angustifolia* and if it is more than 2 cm it is most likely *T. latifolia*. Hence, it is harder to distinguish between *T. angustifolia*, *T. latifolia* and *T.* ×*glauca* if the

plants collected were during spring and early summer as the flower structures are usually not present by then. Unfortunately, not all plants produce flowers every year.

The reason as to why the master mix and PCR based on Ciotir et al. (2013) only gave primer dimer before the master mix was modified was most likely related to the use of other polymerases and buffer that had a different working requirement compared to the one used in this work. Likewise, the working environment is different and that could affect the PCR. The samples that did not give band(s) for all of the primers could result from some error(s) when preparing the solutions for the PCR, some contamination in the tube, or a template that was hard to amplify so the amount amplified was undetectable. Those not giving band could be due to contamination by inhibitors in the DNA.

TL 164 produced three different amplification sizes from the samples and some had two bands, and TL 305 had three different fragments sizes but TL 209 had only two.

TL 146 11C had 2 bands and 11D had 1 band so there were at least two genotypes present at location 11 but for the other markers both had one band. Samples 1A and 1B had the same bands except for TL 146 in which they had different bands (Table 7). Our results were analyzed considering previous research by Ciotir et al. (2013) who got 16 different alleles for TL 146, 3 for TL 209 and 6 for TL 305 from 31 *T. latifolia* samples from Europe. They got more alleles especially for TL 146 (16 alleles) than what obtained in this study (3 alleles). The reason to this result is probably because they used samples from all over Europe while this study only included samples from southern Sweden and that the exact bp size for the PCR products were not analyzed in this study. It is more likely to find more different alleles in a larger sample area. The results were more similar for the other primers for which Ciotir et al. (2013) found lower number of alleles. This result could be due to low genetic diversity of the populations in southern Sweden. If they were equally common, then few samples would be needed to find them, while for TL 146 may need a larger collecting of sample to detect its various alleles.

Conclusions

It was hard to use solely morphology (e.g. leaf width) to identify if the materials collected belonged to *T. latifolia* or *T. angustifolia*. Furthermore, there were no flowers during the time of the collecting mission as the flower structure differs between the species and as already noted

for the leaf width, it may also vary significantly between plants at the same location (Fassett and Calhoun 1952; Kuehn et al. 1999). This study found some different genotypes but there are likely more genotypes present that were not detected. To detect them, further research by sampling more relevant locations throughout Sweden would be needed to get a more accurate assessment on the genetic diversity of the *Typha* population in this country. Plants should be collected from stands that are hard to access along with those having an easy access. They should be analyzed with all microsatellite primers available for *Typha* and not only a few (e.g. 3) to get a more accurate assessment on the whole genetic diversity of the *Typha* that grows in Sweden.

Reference list

Ball, D., & Freeland, J. R. (2013). *Synchronous flowering times and asymmetrical hybridization in* Typha latifolia *and* T. angustifolia *in northeastern North America.* Aquatic Botany, 104, 224-227. http://www.sciencedirect.com/science/article/pii/S0304377012001350 [2016-06-17]

Calheiros, C. S., Rangel, A. O., & Castro, P. M. (2008). *Evaluation of different substrates to support the growth of* Typha latifolia *in constructed wetlands treating tannery wastewater over long-term operation*. Bioresource Technology, 99(15), 6866-6877. http://www.sciencedirect.com/science/article/pii/S0960852408000813 [2016-06-13]

Christenhusz, M. J., & Byng, J. W. (2016). *The number of known plants species in the world and its annual increase*. Phytotaxa, 261(3), 201-217. N http://www.biotaxa.org/Phytotaxa/article/view/phytotaxa.261.3.1/20598 [2016-06-28]

Ciotir, C., Dorken, M., & Freeland, J. (2013). *Preliminary characterization of* Typha latifolia *and* T. angustifolia *from North America and Europe based on novel microsatellite markers identified through next-generation sequencing*. Fundamental and Applied Limnology/ArchivfürHydrobiologie, 182(3), 247-252. http://www.ingentaconnect.com/content/schweiz/fal/2013/00000182/00000003/art00005 [2016-5-30]

Coville, F. V. (1897). *Notes on the plants used by the Klamath Indians of Oregon.* Contributions from the United States National Herbarium, 5(2), I-II. http://www.jstor.org/stable/23490851 [2016-6-15]

Fassett, N. C., & Calhoun, B. (1952*). Introgression between* Typha latifolia *and* T. angustifolia. Evolution, 367-379. http://www.jstor.org/stable/2405699 [2016-18-06]

Fernald, M.L. Kinsey, A. C. (1943) *Edible wild plants of eastern North America*. Idlewild Press Cornwall-ON- Hudson, N.Y. http://hdl.handle.net/2027/uc1.b4313378 [2016-5-30]

Gaertner, E. E. (1962). *Freezing, preservation and preparation of some edible wild plants of Ontario*. Economic Botany, 16(4), 264-265. http://www.jstor.org/stable/4252377 [2016-5-14]

Guisinger, M. M., Chumley, T. W., Kuehl, J. V., Boore, J. L., & Jansen, R. K. (2010). *Implications of the plastid genome sequence of* Typha *(Typhaceae, Poales) for understanding genome evolution in* Poaceae. Journal of Molecular Evolution, 70(2), 149-166. http://link.springer.com/article/10.1007/s00239-009-9317-3 [2016-06-06]

Grace, J. B., & Harrison, J. S. (1986). *The Biology of Canadian Weeds. 73.* Typha latifolia *L.*, Typha angustifolia L. *and* Typha glauca Godr. Canadian Journal of Plant Science, 66(2), 361-379. http://www.nrcresearchpress.com/doi/abs/10.4141/cjps86-051#.V1r7f01f1aQ [2016-06-10]

Harada, I. (1949). *Chromosome numbers in* Pandanus, Sparganium *and* Typha. Cytologia, 14(3-4),214-218. https://www.jstage.jst.go.jp/article/cytologia1929/14/3-4/14_3-4_214/_article [2016-06-10]

Hazra, M. Avishek, K., & Pathak, G. (2015) *Phytoremedial potential of* Typha latifolia, Eichornia crassipes *and* Monochoria hastata *found in contaminated water bodies across Ranchi City (India).* International Journal of Phytoremediation, 17(9), 835-840, DOI: 10.1080/15226514.2014.964847 [2016-06-06]

Hotchkiss, N., & Dozier, H.L(1949). *Taxonomy and distribution of North American Cat-Tails*. The American Midland Naturalist, 41(1), 237-254. http://www.jstor.org/stable/2422028 [2016-06-14]

Jesus, J. M., Calheiros, C. S., Castro, P. M., & Borges, M. T. (2014). *Feasibility of* Typha latifolia *for high salinity effluent treatment in constructed wetlands for integration in resource management systems.* International Journal of Phytoremediation, 16(4), 334-346. http://dx.doi.org/10.1080/15226514.2013.773284 [2016-06-06]

Keane, B., Pelikan, S., Toth, G. P., Smith, M. K., &Rogstad, S. H. (1999). *Genetic diversity of* Typha latifolia *(Typhaceae) and the impact of pollutants examined with tandem-repetitive DNA probes.* American Journal of Botany, 86(9), 1226-1238. http://www.amjbot.org/content/86/9/1226.full.pdf+html[2016-06-13]

Klink, A., Macioł, A., Wisłocka, M., &Krawczyk, J. (2013). *Metal accumulation and distribution in the organs of* Typha latifolia *L.(cattail) and their potential use in bioindication*. Limnologica-Ecology and Management of Inland Waters, 43(3), 164-168. http://www.sciencedirect.com/science/article/pii/S0075951112000680 [2016-06-06]

Kim, C., & Choi, H. K. (2011). *Molecular systematics and character evolution of* Typha *(Typhaceae) inferred from nuclear and plastid DNA sequence data*. Taxon, 60, 1417-1428. http://www.jstor.org/stable/41317545?seq=1#page_scan_tab_contents [2016-5-6]

Kim, C., Shin, H., & Choi, H. K. (2003). *A phenetic analysis of* Typha *in Korea and far east Russia.* Aquatic Botany, 75(1), 33-43. http://www.sciencedirect.com/science/article/pii/S0304377002001523 [2016-5-6]

Kirk, H., Connolly, C., & Freeland, J. R. (2011). *Molecular genetic data reveal hybridization between* Typha angustifolia *and* Typha latifolia *across a broad spatial scale in eastern North America.* Aquatic Botany, 95(3), 189-193. http://www.sciencedirect.com/science/article/pii/S0304377011000866 [2016-06-17]

Koropchak S. and Vitt D. (2013) *Survivorship and growth of* Typha latifolia *L. across a NaCl gradient: a greenhouse study*, International Journal of Mining, Reclamation and Environment, 27(2), 143-150, DOI: 10.1080/17480930.2012.679860 http://dx.doi.org/10.1080/17480930.2012.679860 [2016-06-06]

Kuehn, M. M., Minor, J. E., & White, B. N. (1999). *An examination of hybridization between the cattail species* Typha latifolia *and* Typha angustifolia *using random amplified polymorphic DNA and chloroplast DNA markers*. Molecular Ecology, 8(12), 1981-1990. http://onlinelibrary.wiley.com/doi/10.1046/j.1365-294x.1999.00792.x/full [2016-06-19]

Kumari, M., & Tripathi, B. D. (2015*). Efficiency of* Phragmites australis *and* Typha latifolia *for heavy metal removal from wastewater.* Ecotoxicology and Environmental Safety, 112, 80-86. http://www.sciencedirect.com/science/article/pii/S0147651314005004 [2015-3-11]

Kurzawska, A., Górecka, D., Błaszczak, W., Szwengiel, A., Paukszta, D., & Lewandowicz, G. (2014). *The molecular and supermolecular structure of common cattail* (Typha latifolia) *starch.* Starch-Stärke, 66(9-10), 849-856. https://www.researchgate.net/profile/Grazyna_Lewandowicz/publication/264510193_The_molecular_and_supermolecular_structure_of_common_cattail_(Typha_latifolia)_starch/links/53ec98df0cf2233164945184.pdf [2016-5-14]

Lidman, C.A.M. (1977) *Nordens flora1*. Wahlström & Widstrand 1964, 1974. Esselte Herzogs, ISBN 91-46-12004-1.

Liptay, A. (1988). Typha*: Review of historical use and growth and nutrition.* In I International Symposium on Diversification of Vegetable Crops 242 (pp. 231-238). http://www.actahort.org/members/showpdf?session=18847 [2016-5-14]

Mashburn. S.J., Sharitz. R.R., & Smith M.H. (1978) *Genetic variation among* Typha *populations of the southeastern United States.* Evolution, 32(3), 681-685. http://www.jstor.org/stable/2407736 [2016-6-16]

McNaughton, S. J. (1966). *Ecotype function in the* Typha *community-type*. Ecological Monographs, 36(4), 298-325. http://www.jstor.org/stable/1942372?seq=1#page_scan_tab_contents [2016-6-13]

Morton, J. F. (1975). *Cattails (*Typha *spp.)—Weed problem or potential crop?* Economic Botany, 29(1), 7-29.http://www.jstor.org/stable/4253557?seq=1#page_scan_tab_contents [2016-06-14]

Na, H. R., Kim, C., & Choi, H. K. (2010). *Genetic relationship and genetic diversity among* Typha *taxa from East Asia based on AFLP markers.* Aquatic Botany, 92(3), 207-213. http://www.sciencedirect.com/science/article/pii/S0304377009001673 [2016-06-13]

Nowińska, R., Gawrońska, B., Czarna, A., & Wyrzykiewicz-Raszewska, M. (2014). Typha glauca *Godron and its parental plants in Poland: taxonomic characteristics.* Hydrobiologia, 737(1), 163-181. http://link.springer.com/article/10.1007/s10750-014-1862-0 [2016-5-14]

Roskov Y., Kunze T., Orrell T., Abucay L., Paglinawan L., Culham A., Bailly N., Kirk P., Bourgoin T., Baillargeon G., Decock W., De Wever A., &Didžiulis V., eds. (2014). *Species 2000 & ITIS Catalogue of Life, 2014 Annual Checklist*. Digital resource at www.catalogueoflife.org/annual-checklist/2014 [2016-6-15] Species 2000: Naturalis, Leiden, The Netherlands.

Selkoe, K. A., & Toonen, R. J. (2006). *Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers.* Ecology Letters, 9(5), 615-629. http://onlinelibrary.wiley.com/doi/10.1111/j.1461-0248.2006.00889.x/epdf [2016-06-12]

Sharitz R.R., Wineriter S. A, Smith M.H., & Liu E. H. (1980) *Comparison of isozymes among* Typha *species in eastern United States.* American Journal of Botany, Vol. 67(9),1297-1303. http://www.jstor.org/stable/2442132 [2016-6-16]

Smith, S. G. (1967). *Experimental and natural hybrids in North American* Typha *(Typhaceae).* American Midland Naturalist, 78, 257-287. http://www.jstor.org/stable/2485231 [2016-6-16]

Snow, A. A., Travis, S. E., Wildova, R., Fer, T., Sweeney, P. M., Marburger, J. E., Windels, S., Kubatova, B., Goldberg, D. E. & Mutegi, E., (2010). *Species-specific ssr alleles for studies of hybrid cattails* (Typha latifolia × T. angustifolia*; Typhaceae) in North America.* American Journal of Botany, 97, 2061– 2067. http://www.amjbot.org/content/97/12/2061.full.pdf+html [2016-6-16]

Song, J., Zhang, R., Li, K., Li, B., &Tang, C. (2015). *Adsorption of copper and zinc on activated carbon prepared from* Typha latifolia *L*. Clean Soil, Air, Water, 43(1), 79-85. http://onlinelibrary.wiley.com/doi/10.1002/clen.201300533/epdf[2016-06-06]

Tsyusko-Omeltchenko, O. V., Schable, N. A., Smith, M. H., & Glenn, T. C. (2003). *Microsatellite loci isolated from narrow-leaved cattail* Typha angustifolia. Molecular Ecology Notes, 3(4), 535-538. http://onlinelibrary.wiley.com/doi/10.1046/j.1471-8286.2003.00502.x/epdf [2015-2-18]

Tsyusko, O. V. (2004). *Radiation and genetics of cattail populations from Chernobyl* (Doctoral dissertation, Ph. D. dissertation, The University of Georgia, Athens, Georgia, USA).http://getd.libs.uga.edu/pdfs/tsyusko_olga_v_200412_phd.pdf [2016-6-16]

Tsyusko, O. V., Smith, M. H., Sharitz, R. R., & Glenn, T. C. (2005). *Genetic and clonal diversity of two cattail species,* Typha latifolia *and* T. angustifolia *(Typhaceae), from Ukraine*. American Journal of Botany, 92(7), 1161-1169. http://www.amjbot.org/content/92/7/1161.full.pdf+html [2016-06-06]

Vetayasuporn, S. (2007) *Using cattails (*Typha latifolia*) as a substrate for* Pleurotus ostreatus *(Fr.) Kummer cultivation*. Journal of Biological Sciences, 7, 218-221. http://www.scialert.net/qredirect.php?doi=jbs.2007.218.221&linkid=pdf [2016-5-14]

Woodcock E. F. (1925). *Observations on the poisonous plants of Michigan*. American Journal of Botany, 12(2), 116-131 http://www.jstor.org/stable/2435398 [2016-6-15]

Zhou, B., Yu, D., Ding, Z., & Xu, X. (2015). *Comparison of genetic diversity in four* Typha *species (Poales, Typhaceae) from China*. Hydrobiologia, 770, 117-128. http://link.springer.com/article/10.1007/s10750-015-2574-9 [2016-5-14]