RADIATION AND GENETICS OF CATTAIL POPULATIONS FROM CHORNOBYL

by

OLGA VASYLIVNA TSYUSKO

(Under the Direction of Cham E. Dallas)

ABSTRACT

Genetic diversity was studied in cattail populations (*Typha angustifolia* and *T. latifolia*) from areas around Chornobyl, site of a nuclear accident, and other locations in Ukraine. My overall objective was to evaluate radiation effects on genetics of Chornobyl *Typha* populations. Two types of genetic markers, microsatellites and sequences from non-coding DNA, were used. *Typha's* biology contributed to high spatial variability estimated from data for multiple reference populations, and this complicated the detection of radiation effects. Chornobyl populations of both species showed higher microsatellite variability for most diversity measures when compared to those of reference populations. Several characteristics showed positive correlations with radionuclide concentrations. Five percent of the total variance was observed between Chornobyl and reference populations of *T. latifolia*, but less than 1% of *T. angustifolia* which was the more variable species. Chornobyl populations of *T. latifolia* had a tendency to cluster together in the phylogeographic analysis. Isolation by distance was shown for *T. latifolia* but not *T. angustifolia.*

Results for sequence data of *T. latifolia* were inconsistent with those of microsatellites. The amount of inter-population variation was higher (45%) than that for microsatellite loci (23%). Less than one percent of variation occurred between Chornobyl and reference

populations, and Chornobyl populations did not cluster together on a dendrogram. Diversity measures were not different between Chornobyl and reference populations, and there were no significant differences in the number of haplotypes between the two groups. However, the number of unique haplotypes was significantly smaller in Chornobyl populations. The individual nucleotide differences of the Chornobyl samples correlated positively with radionuclide concentrations. Thus, genetic differences of Chornobyl populations are at least partially due to radiation. However, many other factors including geography, biology, ecology, and climate in addition to radiation contributed to genetic variability within and among *Typha* populations.

INDEX WORDS: Cattails, Chernobyl, Chornobyl, Genetic diversity, Genetic markers, Heterozygosity, Isolation by distance, Microsatellites, Mutations, Non-coding DNA, Nuclear accident, Phylogeography, Radiation, Sequences, *Typha angustifolia, Typha latifolia,* Ukraine

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THIS WORK IS DEDICATED TO

MY PARENTS:

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CHAPTER 1

INTRODUCTION

Overview

Potential exposure to genotoxic contaminants is a serious concern for society (Shugart and Theodorakis, 1998). Genotoxicity which is a genetic response caused by a contaminant involves DNA alteration (Newman, 2001). Genotoxicants can interrupt normal cellular processes and cause structural modifications of DNA (Shugart, 2000). DNA damage may occur because of different processes including DNA alkylation and production of free radicals. Exposure to ionizing radiation results in the production of free radicals and may cause irreversible DNA damage. Efficiency of DNA repair processes depends on the damage, and mutations can be sustained through DNA replication.

Radiation-induced mutations were discovered in 1926 by Muller (Carlson, 1981). Muller (1950) thought that most mutations, which he termed genetic load, are detrimental to an organism and can decrease individual fitness. If every mutation was deleterious, effected populations would not persist with a large genetic load. In some cases, increased fitness of exposed populations is possible due to mutations and may help populations to persist in variable environments (Wallace 1991). However, relatively few mutations are adaptive, most have no effect at all, and some are almost always deleterious (Drake et al. 1998). Most mutations are actually "silent" and occur mostly in non-coding, "junk" DNA (Crow 1997), but because of their neutrality, they can accumulate and persist for a long time in a population and eventually influence its dynamic.

Multiple studies have been conducted on the genetic effects of radiation, but mostly in the laboratory. There are relatively few studies that are directly relevant to understanding the responses of plant and animal populations to radionuclides in their natural environments. Most studies have emphasized individual rather than population responses, acute rather than chronic irradiation, and primary rather than secondary effects (Whicker and Hinton 1996). Studying responses of natural populations to chronic radiation requires a contaminated environment, such as at Chornobyl, Ukraine. The Chornobyl accident was one of the worst nuclear accidents and resulted in a massive release of radioactivity, $1.8 – 3.7 * 10^{18}$ Bq, into the environment (Powers et al. 1987). Several consequences of the radiation exposure at Chornobyl have been reported for humans including increases in thyroid cancer in children, malformations in newborns from Belarus, and a higher frequency of chromosomal aberrations (Dubrova et al. 1996). Genetic effects of chronic radiation exposure, specifically to low-dose radiation, on plant and animal populations are still understudied.

A variety of molecular genetic markers available to researchers provide powerful tools to investigate genetic effects of radiation in natural populations. Among these markers are DNA breakage, allozymes, microsatellites, sequencing of coding and non-coding DNA, and gene expression. One of the most obvious genotoxic effects of radiation is DNA strand breaks. Breakages are either the result of free-radical mechanisms, or of compromises to the normal DNA repair mechanisms (Hartwig 1994; Shugart et al. 1990; Snow 1994). Double strand breaks (DSBs) are not as easily repaired as single strand breaks and are more significant, because they cause DNA fragmentation (Theodorakis et al. 2000; Ward 1988). DSBs could not only lead to altered gene function affecting the present health of individuals (e.g., carcinogenesis), but heritable changes from DSBs may ultimately have evolutionary consequences for natural

populations (Blocher et al. 1989; Fox 1995; Hartwig 1994). DNA strand breaks are among the most easily detected and quantified types of DNA damage (Sugg et al. 1995; Theodorakis et al. 1994). Single-cell gel electrophoresis or Comet assay is a sensitive and rapid method for detecting DNA double- and single stranded breaks and has been widely used to detect DNA damage (Cotelle and Férard 1999). Increase in DNA breakage due to radiation exposure has been shown for several organisms, such as turtles (Meyers-Schone et al. 1993), sunfish (Shugart and Theodorakis 1998), and mosquitofish (Theodorakis and Shugart 1997). Individual mosquitofish with more DNA breakage also had lower fecundity suggesting the potential for a contaminant-induced selection for resistant phenotypes (Shugart and Theodorakis 1994). Thus, DNA breakage occurs as a biological response to exposure to genotoxicants and can be used as a biomarker of exposure (Shugart 2000).

Allozymes have also been used extensively in studies of the effects of environmental contaminants on populations. Theodorakis and Shugart (1997) demonstrated evidence for selection of certain genotypes and elimination of sensitive genotypes from a radioactively contaminated population of mosquitofish. Heterozygous individuals from the contaminated site also had higher reproductive success and less DNA breakage (Shugart and Theodorakis, 1998). Correlation between some allozyme genotypes and the presence of contaminants was shown for a stoneroller population (Gillespie and Guttman 1989). Tolerant genotypes are also generally found in plants exposed to heavy metals (Shaw 1994). Even though surveying allozymes is relatively inexpensive and methodologically simple (D'Surney et al. 2001), there are disadvantages to their use such as a limited number of polymorphic loci available and the level of variability varies depending on species. Some species like *Typha* show almost no allozyme

variation (Sharitz et al. 1980) and large samples are needed to document the small amount of variability.

Microsatellites, which are tandem arrays of simple DNA sequences with core repeat units of 1-6 base pairs are common in all eukaryotic genomes (Ellegren 2000; Tautz 1989; Tautz 1993) and are becoming widely used in genotoxicology studies. Most microsatellite loci usually undergo step-wise mutations as indicated experimentally in a variety of organisms (Ellegren 2000), but there is evidence for more complex patterns of mutations such as found in wheat plants growing at Chornobyl (Kovalchuk et al. 2003). There are several reasons for using microsatellites in studies of environmental insults (Brown et al. 2001). First, the high number of alleles at these loci makes detailed studies of genetic variation within and among groups of individuals possible (Queller et al. 1993). Second, large numbers of individuals and loci can be genotyped quickly (Tautz 1989; Weber and May 1989). Third, these loci have high mutation rates (ca. 1×10^{-3}; Ellegren, 2000) making it possible to measure rates directly from pedigrees (e.g., Weber and Wong 1993; Davis et al. 2001). When microsatellites are used to calculate mutation rates, they act as biomarkers of exposure and effect. Biomarkers are by definition characteristics of a biological response to a contaminant, and they can be used as a measure of exposure and sometimes of toxic effects (Peakall and Walker 1994). Thus, microsatellites are among the most promising genetic markers for studying genetic effects of contaminants.

Several studies have supposedly demonstrated increased mutation rates at mini- and microsatellite loci. Studies of captive mice have clearly demonstrated that radiation increases mutation rates of expanded simple tandem repeat (ESTRs, Dubrova et al. 1993, 1998a, 2000a; Fan et al. 1995; Sadamoto et al. 1994) which can be inherited by unexposed offspring (Dubrova et al. 2000b; Dubrova and Plumb 2002). In addition, irradiation of one of the parents can induce

genomic instability in another non-exposed parent and results in increased minisatellite mutations (Niwa and Kominami 2001). Increase in germline mutation rates using mini- and microsatellite loci have been reported for humans, barn swallows, and wheat (Baker et al. 1999a; Dubrova et al. 1996, 1997, 1998b; Ellegren 2000; Kovalchuk et al. 2000, 2003). However, these studies have serious problems recognized by the authors.

There are a limited number of studies that compare genetic diversity of Chornobyl and reference populations. A common flaw of these studies is a lack of an adequate number of reference populations analyzed. There is only one study, which included more than four reference populations and showed increases in microsatellite variability in contaminated *Apodemus flavicollis* populations closest to the failed reactor (Oleksyk 2001). Other studies used different genetic markers and documented significant genetic changes, such as increases in DNA heteroplasmy (Baker et al. 1999), and slight or no changes in mammal populations at Chornobyl (Baker and Chesser 2000; DeWoody 1999; Livshits et al. 2001; Rodgers and Baker 2000). Sequencing of the control region of mitochondrial DNA (D-loop), which is highly polymorphic DNA in vertebrates, was used in studies of Matson et al. (2000) and Baker et al. (2001). They estimated genetic diversity for *Clethrionomys glareolus* from Chornobyl but did not demonstrate significant overall genetic changes in these populations. Sequencing allows direct assessment of the DNA changes (Newman 2001). The high cost and consequent limitations on sample sizes and number of DNA regions that can be analyzed are major disadvantages of this technique. Differences in population genetic structure of plants and animals from contaminated environments, such as Chornobyl, can be used as biological indicators of contaminant exposure (Bickham et al. 2000; Peles et al. 2003). Care should be taken in choosing genetic markers, and determining which and how many reference populations

are necessary to establish a baseline for evaluation of effects. Stochastic processes can have a strong influence on the genetic composition of effected populations and result in the need for many reference populations in the study of effects of contaminants on genetic diversity.

Subject of Study and Objectives

Cattails, which are aquatic plants with world-wide distributions, were chosen for this study. There are two species (*Typha angustifolia* and *T. latifolia*), that are common in Ukraine and also occur in the area around the Chornobyl Nuclear Power Plant (ChNPP). There were several reasons why cattails were studied. Cattails are easily collected. They can remove chemical contaminants from wastewater (Lan et al. 1992) and have been used as indicator species for radiocesium concentration in foodwebs around nuclear facilities (Brisbin et al. 1989). In addition, cattails at Chornobyl are growing in sediments with a high percentage of clay to which radiocesium binds and may become available for plant uptake. Aquatic sediments contain much of the radioactivity in contaminated environments. Finally, cattails have shown an increase in genetic diversity at sites contaminated with organic pollutants (Keane et al. 1999). Thus, cattails are an excellent choice for the study of the genetic effects of radiation in plants from Chornobyl.

The overall objective of this study is to evaluate genetic effects induced by long-term radiation exposure of populations of two cattail species, *Typha angustifolia and T. latifolia*, from Chornobyl. Two types of highly variable specific genetic markers, microsatellites and non-coding DNA sequences, will be used. Radiation is not expected to be the only factor responsible for observed genetic variability of Chornobyl populations. Thus, a baseline for overall genetic variability will be established by examining genetic variability and phylogeographic patterns in multiple reference populations from Ukraine. Secondly, genetic diversity characteristics will be compared between Chornobyl and reference populations. Because differences in the measured

characteristics among *Typha* populations can be expected by chance alone or can be due to phylogeographic patterns that predated the Chornobyl accident, relationships between the characteristics and distance from the failed reactor will be examined. Correlations between diversity measures and radionuclide concentrations will be calculated. Significant relationships ($\alpha \leq 0.05$ for each test) will be considered as supporting radiation effects. Third, total genetic variance will be determined between Chornobyl and reference populations. Finally, studying two species will allow examination of differences in species responses to various factors including radiation.

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CHAPTER 2

MICROSATELLITE LOCI ISOLATED FROM NARROW-LEAVED CATTAIL *TYPHA ANGUSTIFOLIA*[1]

[1] Olga V. Tsyusko-Omeltchenko, Nancy A. Schable, Michael H. Smith and Travis C. Glenn. Published in Molecular Ecology Notes 2003. 3, 535-538

Abstract

We present 11 dinucleotide microsatellite DNA loci isolated from the narrow-leaved cattail (*Typha angustifolia*) and describe conditions for their amplification. The PCR primers were tested on at least 20 individuals of *Typha angustifolia* and *T. latifolia* from two Ukrainian populations per species. The primers amplify loci with relatively high numbers of alleles (averaging 7.22 and 4.95 alleles per locus in *T. angustifolia* and *T. latifolia* respectively), and polymorphic information content (averaging 0.61 and 0.46 in *T. angustifolia* and *T. latifolia* respectively).

Cattails are widespread wetland plants that invade and colonize aquatic and marsh habitats in temperate and tropical regions of the world (Liu *et al*. 1978). Cattails are distributed across a broad range of environmental conditions including some contaminated sites. Contaminants may increase the rate of mutation, and highly variable, codominant and specific genetic markers are needed for determining whether the contaminant has increased the amount of mutation. Microsatellites are good markers for this purpose and have been shown to have elevated mutation rates in wheat grown in contaminated Chornobyl soils (Kovalchuk *et al*. 2000). Cattail populations occur naturally in radioactively contaminated areas around the Chornobyl Nuclear Power Plant in Ukraine. Microsatellites can be used to test for differences in genetic variation between *Typha* populations from the contaminated Chornobyl environment and reference sites in Ukraine. Our objective was to develop primers for the amplification of microsatellite DNA loci in two *Typha* species and to test them for polymorphism in cattails from Ukraine.

DNA was extracted from dry plant material using a DNeasy Plant Mini Kit from Qiagen (http://www.qiagen.com). DNA was serially enriched twice for $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AC)_{13}$, $(AATC)_6$, and $(AATG)_6$ following a protocol modified from Hamilton *et al*. (1999). The detailed protocol of microsatellite isolation is available from TCG. Briefly, the DNA was digested with *HaeIII*, and simultaneously ligated to double stranded SuperSNX linkers (SuperSNX24 Forward 5'-GTTTAAGGCCTAGCTAGCAGCAGAATC and SuperSNX24 Reverse 5'-GATTCTGCTAGCTAGGCCTTAAACAAAA; modified from the SNX linkers of Hamilton *et al.* [1999]). Ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotides, which were then captured on streptavidin coated paramagnetic beads (Dynal). DNA unhybridized to the captured DNA was washed away and the remaining DNA eluted from the beads. The eluted DNA was amplified in polymerase chain reactions (PCR) using the SuperSNX-24 primer. The product was ligated into pCR®2.1 Vector (Invitrogen), and plasmid ligations were transformed into Top 10 Chemically Competent *E. coli* (Invitrogen). The bacterial clones were screened for inserts using the β-galactosidase gene following the procedure from TOPO TA cloning kit (Invitrogen). Approximately 60 positive clones were amplified with M13 forward and reverse primers and sequenced on ABI 377-96 sequencer using BigDye Terminator 2.0 (Applied Biosystems). Sequences from both strands were assembled and edited in Sequencer 4.1. (Genecodes, Ann Arbor, MI) and exported to Ephemeris 1.0 (available at http://www.uga.edu/srel/DNA_Lab/dnacomputer_programs.htm) for microsatellite searching. PCR primers were designed using Oligo 6.67 (Molecular Biology Insights). The tag that produced least secondary structure, M13Reverse (5'-GGAAACAGCTATGACCAT-3') or CAG tag (5'-CAGTCGGGCGTCATCA-3'), was added to the 5' end of one of each primer pair. Addition of the 5' tag allows use of a 3rd primer in the

PCR (M13R or CAG) that is fluorescently labeled for detection on the ABI 377 (Boutin-Ganache *et al.* 2001). M13 and CAG universal primers were labeled with a FAM or HEX fluorescent dye.

PCR amplifications were performed in a 25 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/mL BSA, 0.4 μM unlabeled primer, 0.04μM tag labeled primer, 0.36μM universal dye labeled primer, 3 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and 50ng DNA template) using an Eppendorf Mastercycler Gradient thermal cycler. Twenty four primer pairs were tested using touchdown thermal cycling programs (Don *et al*. 1991) encompassing a 10° span of annealing temperatures ranging between 65-55°C, 60-50°C or 55-45°C. Cycling parameters were 5 cycles of 96°C for 20 s, the highest annealing temperature for 30 s, and 72°C for 1 min; 21 cycles of 96°C for 30 s, the highest annealing temperature minus 0.5°C per cycle for 30 s, and 72 °C for 1 min; and lastly 10 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min (Schable *et al.* 2002). PCR products were initially scored for amplification on agarose gels, and successful PCR products were run on an ABI-377-96 sequencer and sized using Gensize Rox 500 ladder (Genpak). Results were analyzed using Genescan and Genotyper software (PE Applied Biosystems). Eleven of the tested primer pairs were successfully optimized by adjusting the $MgCl_2$ concentration (4 mM) and re-synthesized by incorporating a fluorescent tag on the 5'end of one of the primers in each pair.

Samples from at least 20 individual stems from each of the two Ukrainian *Typha angustifolia* populations including one from Chornobyl and one from reference sites were used to test for microsatellite variability across the 11 loci. PCR amplifications were carried out in a final volume of 25μL with 10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/mL BSA, 4 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and 5-20 ng DNA template. The primer (reverse and forward) concentrations were adjusted and vary from 0.3 to

0.5μM so that primers can be amplified in multiplex PCR reactions, combining two or three loci (Table 2.1). The characteristics of the 11 working primer pairs developed from *T. angustifolia* are given in Table 2.1. All 11 microsatellite primer pairs were cross amplified in the closely related species *T. latifolia* and tested for microsatellite variability using at least 20 plants from each of the two Ukrainian populations.

Table 2.2 demonstrates the primer characteristics for both *Typha* species. The two main modes of *Typha* reproduction are self-pollination and vegetative propagation. To account for clonal reproduction and to prevent biases in estimation of the population genetic parameters in our calculations, we included genotypes from clonal individuals only once. We estimated the number of alleles per locus (N_A), observed and expected heterozygosity (H_o and H_e), Polymorphic Information Content (PIC) using Cervus 2.0 (Marshal, 1998) and tested for deviations from Hardy-Weinberg equilibrium (HWE) and for genotypic linkage disequilibrium using Genepop (Raymond and Rousset 1995). Five loci in *T. angustifolia* and four loci in *T.latifolia* deviate significantly from expectations of Hardy-Weinberg equilibrium ($p<0.001$). The estimated frequency of null alleles was also relatively high (0.110-0.786) at those loci. The breeding system of *Typha* is likely to lead to extensive inbreeding and thus heterozygote deficiency, causing deviation from (HWE) and high estimates of null alleles. It should be noted, however, that the presence of null alleles may also be influenced by radiation-induced mutations. Of 110 paired loci comparisons for each species, 10 in *T. angustifolia* and 2 in *T. latifolia* yielded significant linkage disequilibrium ($p<0.0001$). Tests for linkage disequilibrium assumes HW proportions of genotypes (Excoffier and Slatkin 1998), thus the presence of the loci in linkage disequilibrium can be due to departure from HWE.

The high numbers of alleles per locus (up to 14) and high PIC values (up to 0.81) demonstrate the potential application of the developed microsatellite primers for a variety of purposes, including tests for differences in genetic variability between *Typha* populations from contaminated environments versus populations from reference sites. It is important to note that although variation is high in both species, variation is higher in the species from which these loci were developed.

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Table 2.1. Characterization of 11 primer pairs that amplify microsatellites from *Typha angustifolia and T. latifolia*. Clone Size is the size of the PCR product amplified from the clone used to develop each locus which was identical to the size predicted from the DNA sequence. The multiplex PCR reactions A+E and C+D can be combined prior to electrophoresis, thus all 11 loci can be scored from three lanes.

Locus	Primer Sequence 5' ---> 3'	Genbank Accession Number	Touchdown Temperature	Repeat Sequence	Clone Size (bp)	Multi PCR	Primer Concentration (μM)
TA 3U TA 3L	TGGATACGGCAGTGTTA Fam GAGTTGGGAAGAAGGGATTA	AF536553	60	(AC)12...(AG)13	219	A	0.3
TA 5U TA 5L	ACTGCCATCAATAGAA Fam GAAGGAACCAAAATCTAA	AF536555	60	(AG)21	298	E	0.5
TA 7U TA 7L	Ned ATTCAACCCAAACTCTAACAA CACCCAAAGGACCACATT	AF536556	60	(AC)9...(AG)17	218	A	0.3
TA 8U TA 8L	Ned TCTTCGCTGAAAGTGACATAC ATTGGCTTCGTTGGATT	AF536557	60	(AC)11	286	E	0.3
TA 13U TA 13L	Fam ATTTTTGGAGCACTAT CACCTTTTATTAATTCAC	AF536549	55	(AC)10	94	C	0.5
TA 15U TA 15L	Hex GAATAAAGCTGCCCTAACT TCCACCACCTCAACC	AF536552	60	(AG)7...(AC)8	178	B	0.5
TA 16U TA 16L	GCCAAAGTTCATAAGAT Hex CCGTAACTTCTTTAATATC	AF536550	55	(CT)17	195	C	0.3
TA 18U TA 18L	Hex CAACTCCACTCTGTGAG TTGGCTCCTAGTTAGTCT	AF536551	60	(CT)29	309	A	0.5
TA 19U TA 19L	CGGAGGGAAGTATGA Hex ATCTACTCAATCCGAGTT	AF536546	60	(AG)9	108	D	0.5
TA 20U TA 20L	Ned ATGCCTAGTGAGGATTC CACACTTATTTTCGAACAA	AF536547	60	(AG)10	98	D	0.3
TA 21U TA 21L	Fam GTATGCCACCAATAGC ATACCTGAGAGGGAATAAG	AF536548	60	(AC)8...(AG)7	283	B	0.3

Table 2.2. Characteristics of 11microsatellite loci for *Typha angustifolia and T. latifolia.* N is sample size. N_A is the number of alleles. H_e and H_o are expected and observed heterozygosity and PIC is polymorphic information content. *N, N_A, H_e, H_o, PIC*, and null allele frequency are given for two populations per species.

Locus	*Typha angustifolia*							*Typha latifolia*						
	N	N_A	Size Range	H_O	H_e	*PIC*	Null freq	*N*	N_A	Size Range	H_o	H_e	*PIC*	Null freq
TA 3	16/20	10/10	177-231	0.813 0.800	0.819 0.851	0.767 0.810	-0.009 +0.02	20/14	6/7	177-211	0.650 0.643	0.821 0.817	0.769 0.759	+0.095 +0.110
TA 5	16/20	8/10	282-308	0.813 0.750	0.760 0.794	0.715 0.756	-0.052 +0.0002	20/14	4/4	282-296	0.150 0.143	0.517 0.323	0.466 0.292	+0.532 +0.429
TA 7	16/20	7/8	188-234	0.563 0.200***	0.651 0.472	0.581 0.445	+0.082 +0.457	20/14	7/5	186-210	0.300 0.214	0.400 0.384	0.379 0.354	+0.217 +0.308
TA 8	16/20	7/8	268-296	0.688 0.650	0.839 0.818	0.789 0.770	+0.075 0.098	20/14	4/3	270-280	0.050*** 0.071	0.314 0.140	0.326 0.131	+0.713 +0.456
TA 13	16/19	6/8	80-106	0.500 0.211***	0.738 0.563	0.670 0.532	+0.171 +0.488	20/14	6/3	76-106	0.500 0.500	0.638 0.500	0.590 0.395	+0.123 -0.004
TA 15	16/19	3/2	174-182	0.125 0.263	0.232 0.309	0.210 0.255	+0.280 +0.066	20/13	2/3	178-188	0.050 0.769	0.224 0.517	0.171 0.374	+0.614 -0.215
TA 16	16/20	6/12	171-229	0.438 0.500***	0.760 0.824	0.694 0.786	+0.259 +0.254	20/14	6/5	183-201	0.650 0.357	0.656 0.627	0.576 0.557	-0.001 +0.263
TA 18	16/20	8/14	269-315	0.375*** 0.600***	0.774 0.835	0.725 0.793	+0.355 +0.155	20/14	12/7	263-311	0.650*** 0.786	0.891 0.741	0.855 0.673	+0.110 -0.080
TA 19	16/20	4/8	102-130	0.563 0.800	0.647 0.788	0.556 0.733	+0.047 -0.033	20/14	7/4	106-132	0.300*** 0.643	0.732 0.722	0.671 0.644	+0.413 +0.019
TA 20	16/20	4/8	94-128	0.625 0.450***	0.506 0.751	0.454 0.698	-0.162 +0.266	20/14	6/4	96-106	0.150*** 0.143	0.428 0.206	0.395 0.193	+0.494 +0.277
TA 21	16/17	3/5	265-287	0.188 0.412	0.179 0.661	0.166 0.595	-0.041 +0.258	20/14	3/2	153-287	0.700 0.071	0.535 0.198	0.409 0.173	-0.148 +0.445

Significant deviations from Hardy-Weinberg equilibrium are indicated (***) at P < 0.001

CHAPTER 3

POPULATION GENETIC VARIABILITY, STRUCTURE, AND CLONAL DIVERSITY OF TWO CATTAIL SPECIES, *TYPHA LATIFOLIA AND T. ANGUSTIFOLIA* (*TYPHACEAE*)[1]

[1]Olga V. Tsyusko, Michael H. Smith, Rebecca R. Sharitz, and Travis C. Glenn. Submitted to American Journal of Botany on 08-23-2004

Abstract

Genetic and clonal diversity can vary among closely related plant species. These diversities were calculated from microsatellite data and compared in Ukrainian populations of two cattail species (*Typha angustifolia* and *T. latifolia*). Genetic variability was higher than previously reported for allozymes and VNTRs. There was 48% of the total variation partitioned between the species. *Typha angustifolia* has higher heterozygosity at both species (H_{es} = 0.66) and population (H_{ep} = 0.49) levels than does *T. latifolia* (H_{es} = 0.37 and H_{ep} = 0.29, respectively). Significant differences among populations between and within species were shown for most genetic and genotypic characteristics. Clonal diversity was relatively high for both species. Population differentiation occurred in both species with F_{ST} equal 0.24 and 0.29 for *T. angustifolia* and *T. latifolia*, respectively. *Typha*'s mating system was probably responsible for deviations from Hardy-Weinberg equilibrium in several populations. *Typha angustifolia* had higher number of alleles and more of them had low frequency suggesting larger effective population sizes in this species than *T. latifolia*. Genetic and geographic distances were correlated for *T. latifolia* but not *T. angustifolia*; however, there were no distinct geographic clusters within dendrograms for either species.

Introduction

Asexual reproduction occurs widely in plants and is also found in lichens, fungi and some animal groups (Stuefer et al., 2002). Many plants use both sexual and clonal reproductive modes (Richards, 1986), and the extent to which these modes are used can vary widely within and among species. This variation in the mode of reproduction has significant consequences for the ecology, genetics, and evolution of plants (Eckert, 2002). Clonal and population genetic

diversities may be independent and influenced by different factors (Hangelbroek et al. 2002). Several studies show that genetic variation in clonal plants can be similar to that of non-clonal plants (Hamrick and Godt, 1989). This may partially explain the ability of widely distributed clonal plants to adapt to a variety of environmental conditions.

Widespread plants are likely to have relatively high genetic diversity. However, geographic range can be a poor predictor of genetic structure (Loveless and Hamrick, 1984; Hamrick and Godt, 1989) as indicated by different levels of genetic diversity among widespread plants. The breeding system is an important factor in determining population genetic structure. Many clonal plants have a mixed mating system with both outcrossing and selfing. Self-pollination can occur not only within the same flower (autogamy) but also between different ramets of the same genet (geitonogamy). Selfing, which is mostly occurs through geitonogamy, may lead to inbreeding depression (Eckert, 2000) and can explain why progeny from outcrossing are about twice as fit as those from selfing (Eckert and Barrett, 1994). A low frequency of outcrossing events may be sufficient to maintain genetic diversity and fitness in clonal plants.

Depending on the rates of seedling recruitment clonal species can show one of two strategies: “initial seedling recruitment” (ISR) and “repeated seedling recruitment” (RSR, Eriksson, 1997, Eckert, 2002). These types of recruitment can determine genetic structure at the genet or ramet level (Travis et al. 2004). Species with RSR consistently produce genets of different sizes, while new genets may get established only when open spaces are available for the offspring of ISR species. Cattails, which are primarily an ISR species, are emergent wetland plants that combine both sexual and vegetative modes of reproduction. When a cattail stand of ramets is established, seedlings cannot contribute much to its maintenance because of competition with older ramets (McNaughton, 1966; Grace, 1985). Disturbance can be important

for preserving or introducing genetic variability in cattail populations by creating open spaces for seedlings resulting in more fragmented populations. Populations of different or even the same species may show very wide ranges of genetic and clonal diversities depending on different ecological and biological factors.

Variation in the amount of sexual and asexual reproduction may partially explain rapid colonization and persistence of cattails in many diverse habitats. *Typha* shows ecotypic variation at morphological, physiological, and biochemical levels (McNaughton, 1966, 1967). McNaughton (1967) suggested that this variation has a genetic basis, and Suda et al. (1977) indicated that genotypic flexibility and plasticity may be critical for *Typha*'s adaptation to stressful environments. Different genetic markers show varying levels of variation for widely distributed plants, including cattails. Even though allozymes have been used successfully to study breeding structure of many clonal plants (Ellstrand and Roose, 1987; Widen et al., 1994), almost no polymorphism was found in populations of *T. latifolia* and *T. domingensis* from the eastern United States (Mashburn et al., 1978; Sharitz et al., 1980). Keane et al. (1999) demonstrated variation among *T. latifolia* populations using a variable number of tandem repeats (VNTRs). His heterozygosity estimates were the lowest detected for plants using VNTR loci (Keane et al., 1999). Microsatellites, which are co-dominant neutral loci with high mutation rates of 10^{-3} to 10^{-4}, are often used as genetic markers (Jeffries and Gottlieb, 1988). Microsatellite loci in *T. angustifolia* and *T. latifolia* have relatively high numbers of alleles (Tsyusko-Omeltchenko et al., 2003) making them useful for the study of *Typha*'s population genetic structure. Despite the high level of variation for microsatellites, there have been few studies of population structure of clonal plants using them (Reusch et al., 2000).

Our primary objective was to describe genetic variation for two *Typha* species from Ukraine. Specifically, we wanted to examine genetic and clonal diversity of populations of *T. angustifolia* and *T. latifolia*. Comparisons between genet and ramet data were made to investigate the effects of clones on *Typha*'s genetic diversity. Secondly, we tested for differences in genetic and genotypic diversity among populations between species. Finally, differentiation among populations and correlation between pairwise genetic and geographic distances among intraspecific populations were calculated.

Materials and Methods

Study species

Typha latifolia and T. angustifolia are aquatic plants with world-wide distributions from the Arctic Circle to about 30°S (Sculthorpe, 1967). *Typha* is a perennial plant that grows successfully in a variety of habitats including fresh and brackish water, deep marshes or shallow road ditches. When found together, the two species are often segregated by water depth with *Typha latifolia* preferring shallow and *T. angustifolia* deeper water. *Typha latifolia* is a much better competitor in shallow water in dense cattail stands due to its greater ability for light capture and tolerance for shading. When they occur together in shallow water, *T. latifolia* may completely replace *T. angustifolia* (Grace & Wetzel, 1981). While reproducing through rhizomes, *Typha* forms dense stands where other plants are excluded. This form of cattail growth is called "phalanx". The rhizomes stay viable for 17-22 months and keep physiological connection between ramets for about two years (Dickerman & Wetzel, 1985). *Typha latifolia* produces more rhizomes then *T. angustifolia* and therefore vegetative reproduction maybe more enhanced in the first than the second species. Male and female inflorescences are located on the

same plant, and selfing is one of the reproductive modes. Selfing (autogamy and geitonogamy) occurs in up to 70% of the matings in *Typha* populations (Kratinger, 1975). The seed production is extensive with 117,000 to 268,000 small seeds from a single ramet. *Typha angustifolia* produces more seeds than *T. latifolia*, and some of them should be distributed more widely than in *T. latifolia*. Each fruit is equipped with hairs for wind dispersal and has a one seed inside, which is released as soon as the fruit touches the water. Several conditions (warm temperature, moisture, shallow water, and decreased oxygen concentration) are critical for seed germination and the percentage of seeds successfully germinated is very low.

Sampling sites

We sampled 13 and 11 Ukrainian populations of *T. angustifolia* and *T. latifolia*, respectively (Fig. 3.1). Samples ($N = 659$) were collected for both species at distances of at least 1 m or more apart during July - August of 2001 and 2002. Populations were chosen from five geographical regions: Transcarpathia, Cherkasska, Zhitomirska, Chernigovska, and Kievska. A geographical positioning system was used to determine latitude and longitude at each location. There were five sites in Transcarpathia (Borony, 49º6'N, 23º5'E; Batevo, 48º44'N, 21º58'E; Zarichevo, 48º46'N, 22º4'E; Glubokoe, 48º47'N, 22º8'E; Chop, 48º44'N, 21º59'E), two sites in the Cherkasska region (Buzivka, 50º20'N, 26º46'E; Uman, 50º13'N, 26º24'E), one site in Zhitomirska region (Brusilov, 50º51'N, 28º17'E), three sites in Chernigovska region (Mekshunovka ,51º13'N, 29º21'E; Gubichi, 51º10'N, 29º13'E; Chernigov, 51º08'N, 29º07'E), and five sites in Kievska region (Kiev, 50º57'N, 28º36'E; Belaya Tserkov , 50º40'N, 27º45'E; Ivankovo, 51º12'N, 29º19'E; Atashev, 51º15'N, 29º28'E; and Paryshev, 51º16'N, 29º30'E). There were three sites with relatively small areas of approximately 40 m^2 (Borony, Zarichevo, and Glubokoe) and three (Brusilov, Kiev, and Ivankovo) with large areas, up to 500 m^2. Most of

the sites were located in disturbed areas except Ivankovo, Brusilov, and Chernigov. The other sampling sites occupied areas of 200-300 m^2. Ivankovo, Atashev, and Paryshev, populations of *T. angustifolia* were located close, 35-40 km, to the failed Chornobyl nuclear reactor. *Typha latifolia* populations were located at least 90 km away from Chornobyl. Species co-occured together at 8 sites (Fig. 3.1).

DNA techniques

The tops of leaves (about 20 cm in length) were placed in plastic bags with silica gel. Dry samples were crushed in liquid nitrogen, and DNA was isolated with a Qiagen DNeasy Kit. Multiplex Polymerase Chain Reactions (PCR) with 11 and 9 pairs of microsatellite primers for *T. angustifolia* and *T. latifolia*, respectively were used to amplify DNA. Detailed primer descriptions, conditions of their amplification, and allele scoring are given in Tsyusko-Omeltchenko *et al*. (2003). A Gensize Rox 500 ladder (Genpak) was mixed with PCR product, and the mixture was run on an ABI 377 sequencer to determine microsatellite allele sizes. Allele scoring was conducted using Genescan and Genotyper software (PE Applied Biosystems). The data were transformed into Genepop and Arlequin formats using a genotyper-genepop converter program (www.today.myip.org).

Data analyses

Genetic diversity characteristics including percentage of polymorphic loci (*Pp*), mean allele number (*MAN*), average observed and expected heterozygosities (H_o and H_e, respectively), mean allele size (*MAS*), and its variance for each population were calculated. Genepop version 3.1 (Raymond and Rousset, 1995) was used for calculation of H_o and H_e, and RSTCALC (Goodman, 1997) for *MAS* and its variance. Effective number of alleles (*Ae*) was calculated for each locus as $Ae = 1/\Sigma p_i^2$, where p_i is frequency of the ith allele, and averaged across loci for each

population. Tests for deviations from Hardy-Weinberg expectations and for linkage disequilibrium were conducted using Genepop version 3.1. Sequential Bonferroni corrections were applied to estimate significance where appropriate (Rice, 1988).

Cattail reproduction is primarily vegetative, and several samples (ramets) can have the same genotype and belong to one genetic individual (genet). To differentiate between ramets and genets, we calculated expected frequencies of multilocus genotypes that occurred in more than one sample. These frequencies were used to calculate *Pgen*, the probability that two samples have the same genotype by chance (Reusch et al., 2000). Ramets with the same genotype were considered as one genet when $Pgen < 0.05$. Genetic characteristics were calculated for each population for both genets and ramets to detect differences between them. Comparisons between estimates based on ramet and genet data for each population were conducted using paired *t*-tests. Three basic genotypic diversity characteristics were calculated for each population. The proportion of distinguishable genotypes was calculated as a ratio of number of genets (*Ng*) to that of ramets (*Nr*). Simpson's diversity index (*D*) corrected for sample size were calculated according to Pielou (1969). Fager's (1972) evenness index (*E*) was used to estimate the distribution of genets in a population.

Individual H_{oi}, MAN_i, MAS_i, and its variance were calculated for each genet. These data were not normally distributed, had heteroscedastic variances, and none of the applied transformations (square root, log, or arcsine) solved these problems; therefore, the values were ranked, and Duncan's (1955) multiple comparisons test was used on the ranked values to test for differences among populations. Wilcoxon rank sum tests (Sokal and Rohlf, 1995) were used to compare genetic and genotypic characteristics between species. Histograms of number of alleles for all populations for each species were constructed using 11 frequency intervals. Chi square

was used to test if number of alleles per frequency class was the same in both species. The first two categories, which had the lowest allele frequencies, were sequentially removed from these analyses to determine if distributional differences between the species were mostly due to these categories. Most statistical tests were conducted using SAS 8.1 (2000).

Analyses of Molecular Variance (AMOVA; Excoffier et al. 1992) were applied to partition variance between and within species using ARLEQUIN (Schneider et al., 2000). AMOVA accounts for either frequency or size differences between pairs of different haplotypes and was performed using both options (F_{ST} or R_{ST}, respectively). Only genet data were used to test for differentiation among populations. Regression analyses of diversity characteristics with latitude or longitude were conducted to test for clinal relationships. Genetic distances (F_{ST} and Rh_{oST}) were calculated using FSTAT (Goudet, 1995) and RSTCALC (Goodman, 1997), and matrices of pairwise genetic distances were constructed for each species. Geographical distances were determined from longitudinal and latitudinal data (Viard et al., 1997). Correlations between genetic and geographic matrices were calculated with Mantel's (1967) procedure using 10,000 permutations. Mantel tests were also used to calculate correlations between F_{ST} and Rh_{oST} matrices for each species. Five subprograms of PHYLIP, version 3.5 (Felsenstein, 1993) were used to generate the trees. Each allele frequency matrix was resampled 1000 times with SEQBOOT; GENDIST was used to calculate Cavalli-Sforza and Edwards genetic distances(D_C,1967) for each bootstrap matrix; unrooted neighbor-joining trees were generated with NEIGHBOUR. Consensus trees were created using CONSENSE and DRAWGRAM.

Evidence of population bottlenecks within both species was tested using Bottleneck 1.2.02 (Cornuet and Luikart, 1997) and AGARst (Harley, 2001). Distributions of individual heterozygosities expected from the observed allele frequencies for each population and locus

were calculated with Bottleneck using the assumptions of three different mutation models: Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two-Phase Model (TPM). Wilcoxon sign-rank tests were used in the program to detect deviations from expected heterozygosities. To detect reductions in population size, the mean ratio of the number of alleles to the range in allele size (M; Garza & Williamson, 2001) for each locus was calculated with AGARst (Harley, 2001). A recent bottleneck was indicated when $M < 0.68$.

Statistical significance was indicated when $p \leq 0.05$.

Results

Species (s) diversity level calculated from the pooled data overall was higher for *T. angustifolia* than *T. latifolia* (P_{ps} = 0.89 vs 0.65; H_{es} = 0.66 vs 0.37; H_{os} = 0.50 vs 0.25 and MAN_s = 11.64 vs 6.22, respectively). Characteristics of within population variability (H_e, H_o, *Ae*, *MAN*, mean allele size, and mean variance of allele size) calculated from ramet and genet data for both species are listed in Tables 3.1 and 3.2. Probabilities for multilocus genotypes occurring more than once (*Pgen*) were always < 0.05, and this facilitated differentiation between genets and ramets. Intermingled clones that consisted of at least two identical ramets were frequently observed within stands. Both species demonstrated significant differences between data for ramets and genets for H_e and mean variance of allele size ($p < 0.05$), and *T. latifolia* also showed significant differences for H_o.

A summary of genotypic diversity (*Ng*/*Nr*, *D*, and *E*) for 13 populations of *T. angustifolia* and 11 populations of *T. latifolia* is given in Table 3.3. There were significant differences in the number of distinguishable genotypes among populations of *T. angustifolia* ($\chi^2 = 22.0$, $p < 0.05$), but not *T. latifolia* ($\chi^2 = 9.9$, $p > 0.05$). Simpson's *D* was relatively high for nine populations of

T. angustifolia (0.827 - 0.975) and for all populations of *T. latifolia* (0.842 - 0.981). Genotypes were evenly distributed in all but one population (Batevo, $E = 0.064$) of *T. latifolia* and three populations (Gubichi, Kiev, and Batevo) of *T. angustifolia* ($E = 0.042 – 0.065$).

There were five populations for which means of individual genetic characteristics were significantly different from those of other populations (Fig. 3.2). Among them were three populations of *T. angustifolia*, Mekshunovka, Ivankovo, and Atashev, with the first showing significantly lower and second significantly higher values for two individual genetic characteristics (H_o and MAN_i). Atashev had the highest values for MAN_i and mean variance of allele size. Two *T. latifolia* populations (Buzivka and Uman') were significantly different from other populations; Buzivka had the highest and Uman' the lowest values for H_{oi} and MAN_i. There were significant differences among populations between and within species for individual H_{oi}, MAS_i, MAN_i and mean variance of allele size ($p < 0.01$). There were significant differences between species when compared for their population genetic and genotypic characteristics ($p < 0.01$) except for E. The number of alleles per frequency class was not the same in both species ($\chi^2 = 23$; df = 7; $p < 0.005$, Fig. 3.3). Significant differences were also observed after data from the first and second frequency categories were removed from the analyses ($\chi^2 = 17$; df = 6; $p < 0.01$ and $\chi^2 = 22$; df = 5; $p < 0.001$, respectively).

Four populations of *T. angustifolia* and three of *T. latifolia* showed significant deviations from HW equilibrium ($p < 0.001$, Table 3.1 and 3.2). These populations showed deviations on average for two loci. Of 395 paired locus comparisons for *T. latifolia* and 714 for *T. angustifolia*, 11 and 53 respectively yielded significant linkage disequilibrium ($p < 0.0001$). None of the loci were consistently linked across all populations in either species, so linkage disequilibrium is not likely to produce serious bias in the other analyses of genetic and genotypic

characteristics. The two tests for the occurrence of bottlenecks produced different results. Bottlenecks were detected in two populations of *T. latifolia* (one per test) and 10 populations of *T. angustifolia* (six were indicated by both tests).

More variance was partitioned between species (48% with F_{ST} and 76% with R_{ST}) than among or within populations of each species. There was more variance in each species distributed within than among populations when F_{ST} or R_{ST} options of AMOVA were used (Table 3.4). The percent of variation within and among populations calculated with F_{ST} was different from those calculated with R_{ST} for both species (Table 3.4). Rh_{oST} and F_{ST} matrices correlated significantly in *T. angustifolia* ($r = 0.82$ and $p = 0.01$) and *T. latifolia* ($r = 0.61$ and $p = 0.02$). Pairwise F_{ST} and Rho_{ST} values varied from 0.07 to 0.51 for *T. angustifolia* (with averages of 0.24 for F_{ST} and 0.26 for Rho_{ST}) and from 0.06 to 0.51 for *T. latifolia* (with averages of 0.29 for F_{ST} and 0.22 for R_{ST}). There was significant positive correlation between matrices of pairwise genetic (F_{ST} or Rho_{ST}) and geographic distances for *Typha latifolia* ($r_m = 0.26$ with $p = 0.04$ for F_{ST} or R_{ST}) but not for *T. angustifolia* (Fig. 3.4). Longitudinal and latitudinal clines were observed only for one characteristic (*MAS*, $r^2 = 0.45$ and 0.46, respectively) in *T. angustifolia*, but *T. latifolia* failed to demonstrate any clines. Relationships among populations in both species are given in unrooted trees (Fig. 3.5). Neither species demonstrated distinct clades according to the location of their populations when Cavalli-Sforza distances were used. However, there was a tendency for some adjacent populations to occur together in the tree. Bootstrapping values generated from 1000 bootstrap runs were below 70.

Discussion

Within population variability

The amount of variation detected for microsatellite loci in both *Typha* species is higher than previously demonstrated for allozyme (Mashburn et al., 1978; Sharitz et al., 1980) or VNTR loci (Keane et al., 1999). In *T. latifolia*, the higher polymorphism was observed for such population characteristics as *MAN* (1.28 for VNTR's vs 2.63 for microsatellites), proportion of polymorphic loci (0.16 vs 0.66), and H_o (0.08 vs 0.26). Various markers have different mutation rates and are expected to show differences in their polymorphism. However, mutation rates of VNTR loci should be similar to those of microsatellites, because unlike allozymes they are both non-coding, selectively neutral markers (Jeffreys et al., 1988). The microsatellites used in this study were dinucleotides, while VNTRs in Keane's study contained tetra repeats. In addition, the samples in this and Keane's studies were collected from two different geographic regions (Ukraine and United States, respectively) and many more samples were taken from a larger geographical area in this study, so levels of variability may or may not vary between these regions. Microsatellites, because of their high polymorphism, are good markers for examining genetic and clonal diversities in plants like *Typha* in which allozymes showed almost no polymorphism.

Genotypic diversity of both *Typha* species is relatively high for the proportion of distinguishable genotypes (ca. 0.58) and Simpson's *D* (ca. 0.90) but not *E* (ca. 0.40) when compared to the allozyme results for 21 (Ellstrand and Roose, 1987) and 47 (Widen et al., 1994) clonal plant species (Ng/Nr = 0.17 and 0.27, D = 0.62 and 0.75, and E = 0.68 and 0.75, respectively). Genotypic diversity of *T. latifolia* calculated for VNTR loci (Keane et al., 1999) has lower proportions of distinguishable genotypes (0.39) than that calculated for microsatellite

loci (0.61). The average proportion of distinguishable genotypes calculated from microsatellite data is lower for *Typha* than for *Zostera marina* (0.74, Reusch et al., 2000) and *Elymus athericus* (0.92, Bockelmann et al., 2003). Thus, genotypic characteristics calculated from microsatellite data for both *Typha* species were overall higher than allozyme and VNTR's estimates but lower than microsatellite estimates for other clonal plants probably because of *Typha*'s reproductive characteristics. The over- and underestimations of *Typha* population genetic diversity may occur because of significant differences between ramets and genets, and this results in an additional problem for these types of comparisons.

Significant differences for individual genetic characteristics were common among populations of both species. Individual genetic characteristics of *T. angustifolia* were lower in the eastern population (Mekshunovka) but higher in the northern populations (Ivankovo and Atashev), which were located in close proximity to Chornobyl. Radiation induced mutations may be partially responsible for its higher values (Kovalchuk et al., 2003). Differences in genetic variability among populations could also be due to their biogeography. However, there were no distinct clades associated with geographical regions observed in the species' dendrograms (Fig. 3.5). Since the clines over longitude and latitude were observed only for *MAS* in *T. angustifolia*, and there was a large gap in the geographical data, the effect may be due to chance. Biogeography also does not explain the patterns of variation of the southern populations of *T. latifolia*, because Buzivka showed higher variability while Uman' was lower. The high interpopulational differences in genetic variability between adjacent populations of both species make it unlikely that geographical area can be used to explain these differences.

Hybridization of *Typha* species could have contributed to the high variability of Buzivka, one of the sites where the species co-occurred. Samples were taken only from plants with clearly

identified species-specific morphological characteristics, thus the probability of collecting hybrids was low. There were also no heterozygotes involving species-specific alleles at this site or other locations where both species co-occurred. Hybridization is unlikely to have occurred in the studied *Typha* populations. Establishment of populations by a few founders from several populations with different gene frequencies followed by breeding among genets could also cause high genetic diversity. Different factors are probably responsible for changing the genetic diversity, and population heterogeneity seems to be the rule for many plants including *Typha*.

The degree to which plant species are sexual and asexual varies among species and even populations of the same species (Eckert et al., 2003). In Widėn's et al. (1994) survey of clonal plants about 91% of populations were multiclonal and 48% showed some monoclonality. A substantial difference in proportion of distinguishable genotypes was detected between estimates for two *Saggitaria* species that were based on the same allozymes (Edwards and Sharitz, 2003). Both *Typha* species also showed large variation among populations in clonal diversity. Different factors such as age and level of disturbance could have influenced clonal diversities of these populations. Clonal diversity varies with population age in *Spartina alterniflora* (Travis et al., 2004). Disturbance may introduce new open spaces for seed recruitment and may increase genetic and clonal diversity in ISR plants like *Typha*. The amount of geitonogamy, which may lead to inbreeding depression (Eckert and Barrett, 1994), is higher in larger than smaller fragmented clones (Travis et al. 2004), and the occurrence of the latter may be more frequent with an increase in disturbance level.

Close inbreeding occurs commonly in *Typha* (McNaughton, 1966). Inbreeding increases homozygote frequency relative to that expected with random mating (Hartl, 2000). Significant homozygote excesses were observed in three populations of *T. angustifolia* and two of *T.*

latifolia. Wahlund effect could also be responsible for the heterozygote deficiencies, but the distances between *Typha* samples within a stand were relatively small, and the existence of two subpopulations over this short distance is unlikely. Intermingled clones also occur frequently within stands. Inbreeding rather than Wahlund effect probably accounts for the deviations from Hardy-Weinberg equilibrium in our study. Without knowledge of the history of the populations, it is not possible to completely explain the differences in genetic variation between these two species.

The two closely related *Typha* species showed similarity in evenness of their genotypic distributions, but they differed significantly in all other genetic and genotypic characteristics with *T. angustifolia* having higher genetic diversity than *T. latifolia*. Higher variability of *T. angustifolia* is at least partially due to an ascertainment bias (Ellegren et al., 1995): microsatellite alleles tend to be longer in the species for which the primers were first developed, which was *T. angustifolia* (Tsyusko-Omeltchenko et al., 2003). However, differences among biological characteristics of the two *Typha* species could also be important in explaining their variability. The species demonstrated significant differences between their distributions of the number of alleles per frequency class. *Typha angustifolia* has more alleles and more of them have low frequency (Fig. 3.3) suggesting larger effective population sizes in this species than *T. latifolia* and is probably a consequence of their reproductive characteristics. *Typha angustifolia* has greater seed production and lower production of rhizomes than *T. latifolia* (McNaughton, 1966), which may be important for dispersal and colonizing new habitats.

Among population variability

There was more variability of differences in haplotype frequency within (ca. 75%) than among populations (ca. 25%) in both species. The variation among populations for *Typha* was similar

to that for *Zostera marina* (29%; Reusch et al., 2000), but higher than estimates for *Elliottia racemosa* (18%; Godt and Hamrick, 1999) or *Elymus athericus* (14%; Bockelmann et al., 2003). The high variation among *Typha* populations is probably due to their high selfing rates (Keane et al., 1999), which reduces gene flow among populations. Decreased gene flow has been documented for selfing plants (Hamrick and Godt, 1989; Schoen and Brown, 1991) and animals (Jarne and Charlesworth, 1993; Viard et al., 1997). Most pairwise population-genetic distances in both *Typha* species were relatively high, as expected with limited gene flow. Vegetative reproduction also likely contributes to the reduced gene flow among *Typha* populations.

Population differentiation was relatively high among *Typha* populations in both species. The F_{ST} and Rho_{ST} values could have been underestimated depending on the level of polymorphism of the microsatellite loci used (Hedrick, 1999). In *T. angustifolia* six loci had heterozygosity estimates larger than 0.8 and in *T. latifolia* there were three loci with such high heterozygosities (Tsyusko-Omeltchenko et al., 2003). Since level of differentiation cannot exceed the level of homozygosity estimated with the same markers (Hedrick, 1999), the F_{ST} and Rho_{ST} values were closer to saturation in *T. angustifolia* than *T. latifolia*. The occurrence of bottlenecks was higher in the first than second species, and population differentiation may have increased substantially because of the bottlenecks (Hedrick, 1999). Thus, care should be taken while interpreting population differentiation, because it may depend on level of the marker's polymorphism, recent bottlenecks and constraints on allele size and back mutations (Hedrick, 1999; Nauta and Wessing, 1996).

The combination of population differentiation and gene flow normally produces positive correlation between genetic and geographic distances (Reusch et al., 2000; Oleksyk, 2001; Bockelmann et al., 2003), which is used as evidence for isolation by distance. The correlation

between genetic and geographic distances although significant was relatively low for *T. latifolia* and even lower and non-significant for *T. angustifolia* (Fig 3.4). Higher gene flow is expected among populations located close to each other when IBD occurs, and it decreases as the distance among them increases. The lack of correlation between genetic and geographic distances in *T. angustifolia* is unexpected given the species biology: *T. angustifolia* because of higher seed production presumably has more founder events than does *T. latifolia*. Founder events interacting with asexual reproduction may be one of the major causes for creating more scatter about the trend with geographical distance. The positive slope of these relationships is similar for both species, but the scatter of the points is larger in *T. angustifolia* than *T. latifolia* (Fig. 3.4). The IBD observed in *T. latifolia* may also be partially explained by chance variation among the smaller number of its populations included in the analysis. Relatively isolated founding populations that expand through asexual reproduction can produce high differentiation even among adjacent *Typha* populations.

In conclusion, the within population variability is higher than found in previous studies of *Typha* using allozymes and VNTRs. Despite its clonal nature, *Typha*'s genotypic diversity is relatively high. Even though *Typha* can be wind pollinated, there is a limited amount of gene flow among populations. Limited gene flow among adjacent populations and strong spatial heterogeneity can explain the lack of or low correlation between genetic and geographic distances. Higher genetic diversity is expected in *T. angustifolia* due to its greater reproductive potential. The higher number of alleles with many of them having low frequency in *T. angustifolia* may be an indication of larger effective population sizes in this species. There were more bottlenecks indicated for *T. angustifolia* than *T. latifolia*. There are no consistent trends for

genetic variation across latitude or longitude in either species despite the large areas that were sampled in Ukraine.

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Table 3.1. Characteristics of within population variability for *Typha angustifolia* where H_e is expected heterozygosity and H_o is observed heterozygosity for 11 microsatellite loci. A_e is effective allele number. Mean allele size is given in number of base pairs.

Population	H_e[a]		H_o		A_e		Mean Allele Number	Mean Allele Size		Mean Variance of Allele Size[b]	
	Ramets	Genets	Ramets	Genets	Ramets	Genets		Ramets	Genets	Ramets	Genets
1. Atashev	0.63	0.65	0.51	0.51*	3.21	3.52	6.45	203.75	203.54	37.92	42.77
2. Paryshev	0.47	0.48	0.51	0.50*	2.42	2.28	3.36	205.39	205.44	34.72	37.13
3. Ivankovo[c]	0.65	0.65	0.68	0.67*	3.99	3.89	5.45	205.33	205.81	37.41	38.02
4. Mekshunovka[c]	0.15	0.23	0.16	0.19	1.20	1.36	1.82	204.45	204.30	0.58	2.04
5. Gubichi	0.21	0.41	0.31	0.37	1.45	1.87	2.18	202.84	203.38	3.65	14.18
6. Chernigov	0.36	0.42	0.55	0.55*	1.76	2.11	2.27	203.78	203.67	2.11	2.94
7. Kiev	0.37	0.54	0.45	0.45	1.56	2.22	2.73	204.11	204.53	5.13	14.86
8. Brusilov	0.48	0.53	0.48	0.48	2.19	2.47	3.55	204.11	202.81	5.13	23.77
9. Belaya Tserkov	0.57	0.62	0.49	0.47	3.34	3.35	4.55	203.05	204.04	17.67	29.34
10. Buzivka	0.49	0.56	0.43	0.45	2.23	2.20	3.45	204.30	204.41	35.09	40.42
11. Zarichevo	0.49	0.47	0.68	0.65	2.03	2.35	3.00	203.49	202.96	27.51	18.32
12. Chop	0.39	0.42	0.52	0.50	1.75	1.75	2.09	202.70	202.74	0.36	0.42
13. Batevo	0.32	0.39	0.40	0.40	1.60	1.79	3.00	201.46	201.78	5.24	9.23
Mean	**0.43**	**0.49**	**0.47**	**0.44**	**2.21**	**2.40**	**3.38**	**203.75**	**203.80**	**16.35**	**21.03**

[a]The H_e for genets was significantly greater than the value for ramets at $p < 0.05$ but this was not true for H_o

[b]Variance for genets was significantly greater than that for ramets at $p < 0.05$, but there was no difference for mean allele size

[c]These two populations were significantly different from others in their individual genetic characteristics

*Significant deviations from Hardy-Weinberg equilibrium at two or more loci are indicated at $p < 0.05$

Table 3.2. Characteristics of within population variability for *Typha latifolia* where H_e is expected heterozygosity and H_o is observed heterozygosity for 9 microsatellite loci. A_e is effective allele number. Allele size is given as number of base pairs.

Population	H_e[a]		H_o		A_e		Mean Allele Number	Mean Allele Size		Mean Variance of Allele Size[b]	
	Ramets	Genets	Ramets	Genets	Ramets	Genets		Ramets	Genets	Ramets	Genets
1. Mekshunovka	0.25	0.27	0.23	0.24	1.49	1.41	2.56	196.86	197.27	3.04	5.55
2. Gubichi	0.14	0.18	0.17	0.22	1.25	1.07	2.22	197.44	196.95	9.75	16.63
3. Chernigov	0.31	0.35	0.29	0.30*	1.80	1.74	2.79	197.19	196.77	11.17	16.49
4. Kiev	0.29	0.30	0.32	0.31*	1.89	1.74	2.78	196.74	196.71	2.63	2.81
5. Brusilov	0.28	0.30	0.32	0.32*	1.71	1.63	2.89	196.71	196.57	5.86	7.89
6. Belaya Tserkov	0.31	0.32	0.30	0.30	1.72	1.56	2.56	197.18	197.20	7.10	7.53
7. Buzivka[c]	0.40	0.45	0.34	0.34	1.94	1.71	3.67	196.70	196.78	15.59	15.97
8. Uman'[c]	0.16	0.22	0.12	0.15	1.32	1.23	1.89	196.92	196.39	9.83	14.00
9. Borony	0.26	0.30	0.21	0.25	1.42	1.31	2.44	197.10	197.02	3.63	5.48
10. Glubokoe	0.30	0.31	0.24	0.25	1.61	1.36	2.78	196.83	196.86	2.45	2.75
11. Batevo[c]	0.17	0.23	0.08	0.12	1.29	1.29	2.33	196.81	196.87	3.37	4.86
Mean	**0.26**	**0.29**	**0.24**	**0.26**	**1.59**	**1.46**	**2.63**	**196.95**	**196.85**	**6.77**	**9.09**

[a]The H_e and H_o for genets was significantly greater than the value for ramets at $p < 0.05$

[b]Variance for genets was significantly greater than that for ramets at $p < 0.05$, but there was no difference for mean allele size

[c]These three populations were significantly different from others in their individual genetic characteristics

*Significant deviations from Hardy-Weinberg equilibrium at two or more loci are indicated at $p < 0.05$

Table 3.3. Summary of genotypic diversity for *Typha angustifolia* (13 populations) and *T. latifolia* (11 populations). *Nr* is number of ramets or number of samples analyzed; *Ng* is number of genets; *D* is Simpson's diversity index; and *E* is Fager's evenness index for genet distribution.

Populations	***Nr***	***Ng***	***Ng/Nr***	***D***	***E***
T. angustifolia					
1. Atashev	26	18	0.69	0.975	0.819
2. Paryshev	25	17	0.68	0.973	0.453
3. Ivankovo	25	12	0.48	0.933	0.403
4. Mekshunovka	30	7	0.23	0.747	0.305
5. Gubichi[a]	25	5	0.20	0.567	0.042
6. Chernigov	27	10	0.37	0.650	0.314
7. Kiev[a]	29	8	0.28	0.628	0.063
8. Brusilov	25	14	0.56	0.920	0.737
9. Belaya Tserkov	30	8	0.27	0.864	0.422
10. Buzivka	25	6	0.24	0.827	0.302
11. Zarichevo	26	12	0.46	0.911	0.191
12. Chop	30	10	0.33	0.876	0.434
13. Batevo[a]	25	11	0.44	0.847	0.065
Mean	**26.77**	**10.62**	**0.40**	**0.82**	**0.35**
T. latifolia					
1. Mekshunovka	30	15	0.50	0.894	0.179
2. Gubichi	30	14	0.47	0.855	0.124
3. Chernigov	30	17	0.57	0.940	0.222
4. Kiev	29	18	0.62	0.956	0.324
5. Brusilov	31	29	0.65	0.963	0.407
6. Belaya Tserkov	31	20	0.58	0.963	0.332
7. Buzivka	21	18	0.86	0.981	0.891
8. Uman'	25	15	0.60	0.970	0.750
9. Borony	25	14	0.56	0.973	0.683
10. Glubokoe	30	22	0.73	0.986	0.895
11. Batevo[a]	29	15	0.52	0.842	0.064
Mean	**28.27**	**17.91**	**0.61**	**0.94**	**0.44**

[a]One or both species showed clustered distributions of their genotypes at these sites

Table 3.4. Analysis of molecular variance calculated from haplotype differences using F_{ST} / R_{ST} options for *Typha angustifolia* and *T. latifolia*.

Groups	Source of variation	df	Percent variation*
T. angustifolia			
	Among populations	12	24.82 / 35.00
13 populations	Within populations	264	75.18 / 65.00
	Total	276	
T. latifolia			
	Among populations	10	24.57 / 15.10
11 populations	Within populations	384	75.43 / 84.90
	Total	394	
Both species	Between species	1	48.30 / 76.36
	Among populations within species	22	12.98 / 7.51
24 populations	Within populations	647	38.73 / 16.12
	Total	670	

*$p < 0.05$ for all sources

Fig. 3.1. Sampling sites for populations of *Typha angustifolia* and *T. latifolia* in Ukraine. Both species co-occur at eight sites where the circles are in contact. Sites for *T. angustifolia* (open circles): 1 – Atashev, 2- Paryshev, 3 – Ivankovo, 4 – Mekshunovka, 5 – Gubichi, 6 – Chernigov, 7 – Kiev, 8 – Brusilov, 9 - Belaya Tserkov, 10 – Buzivka, 11 – Zarichevo, 12 – Chop, 13 – Batevo and for *T. latifolia* (closed circles): 1- Mekshunovka, 2 – Gubichi, 3 – Chernigov, 4 - Kiev, 5 – Brusilov, 6 – Belaya Tserkov, 7 – Buzivka, 8 – Uman', 9 – Borony, 10 – Glubokoe, 11 – Batevo.

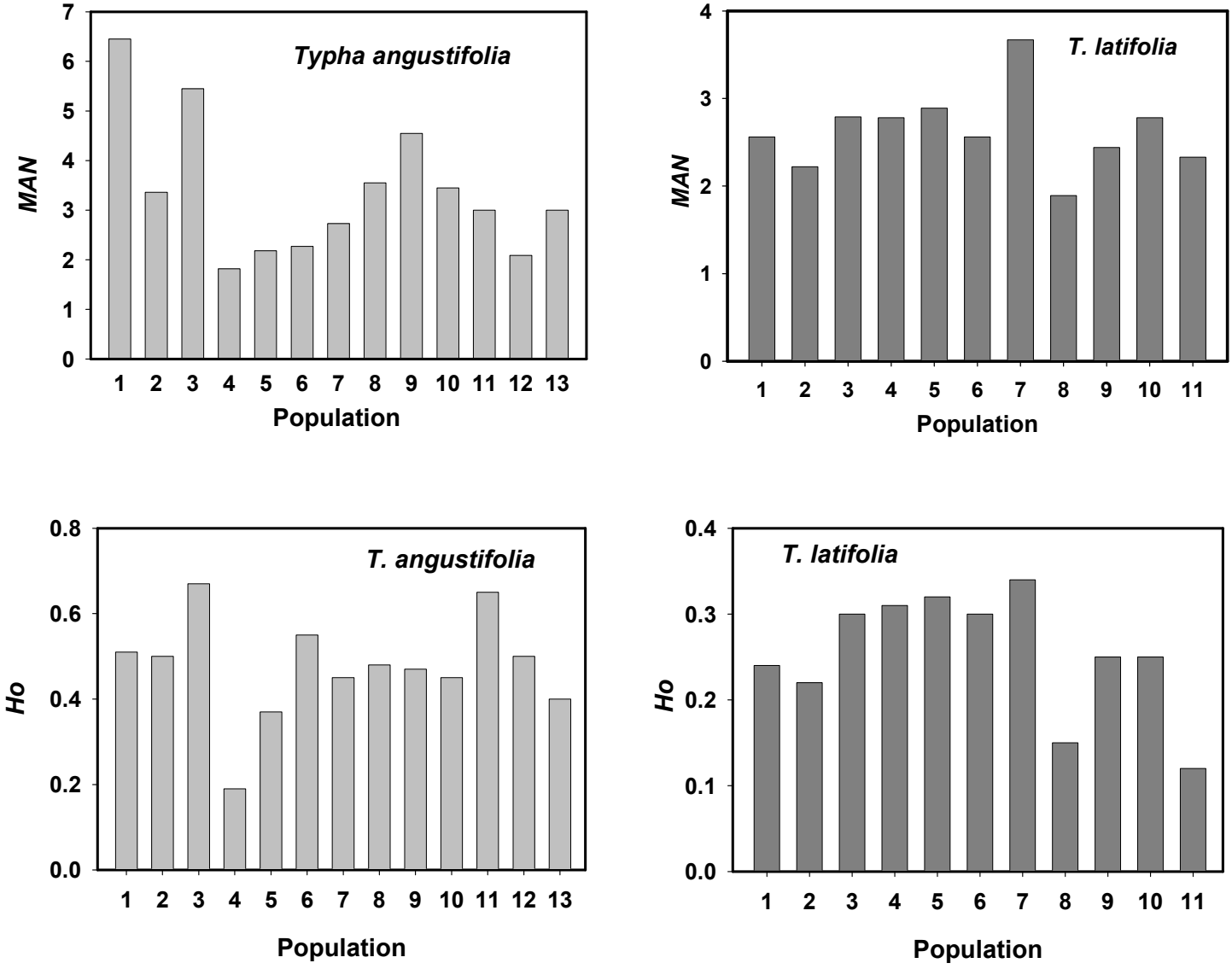

Fig 3.2. Mean allele number (*MAN*) and observed heterozygosity *(H_o)* in 13 populations of *Typha angustifolia* and 11 populations of *T. latifolia*. For population names see Fig.3.1.

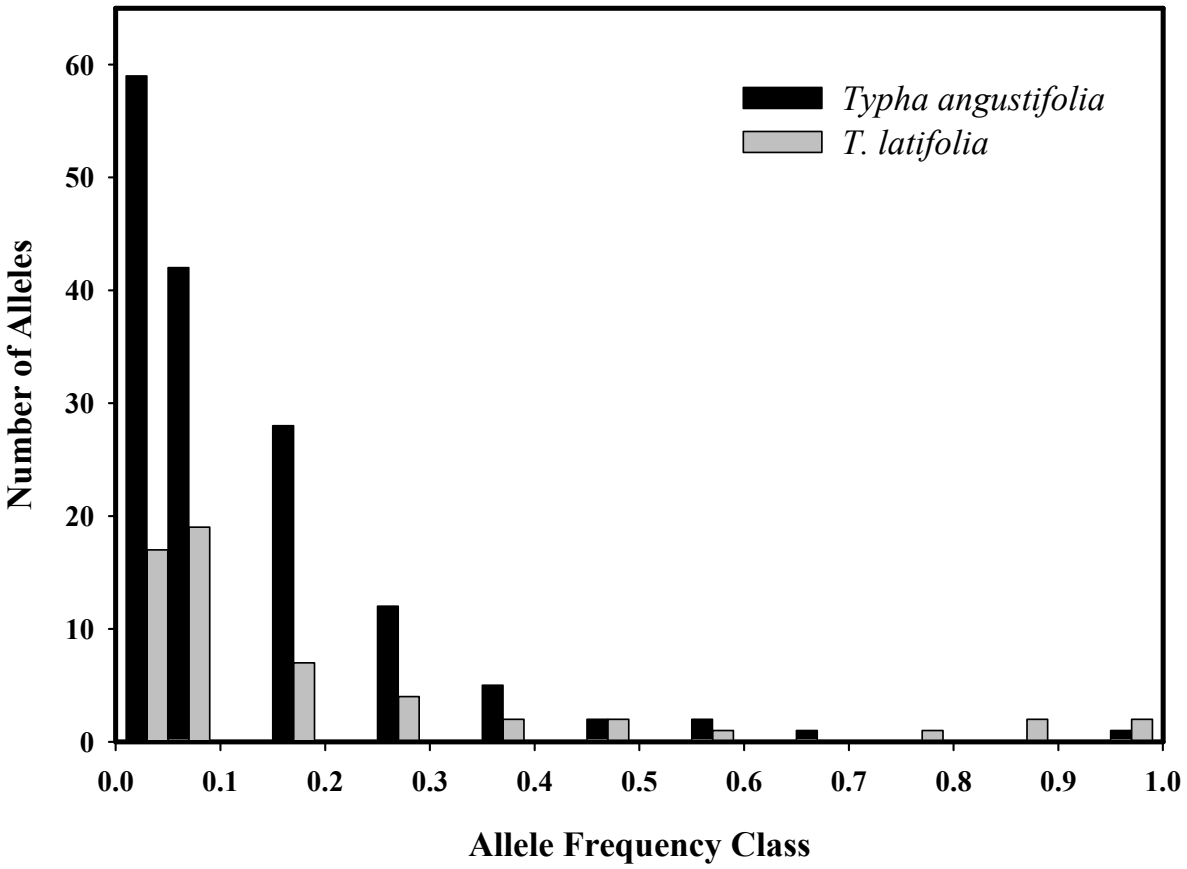

Fig 3.3. Distributions of the number of alleles per allele frequency class for both *Typha* species. Number of alleles is calculated across microsatellite loci and populations.

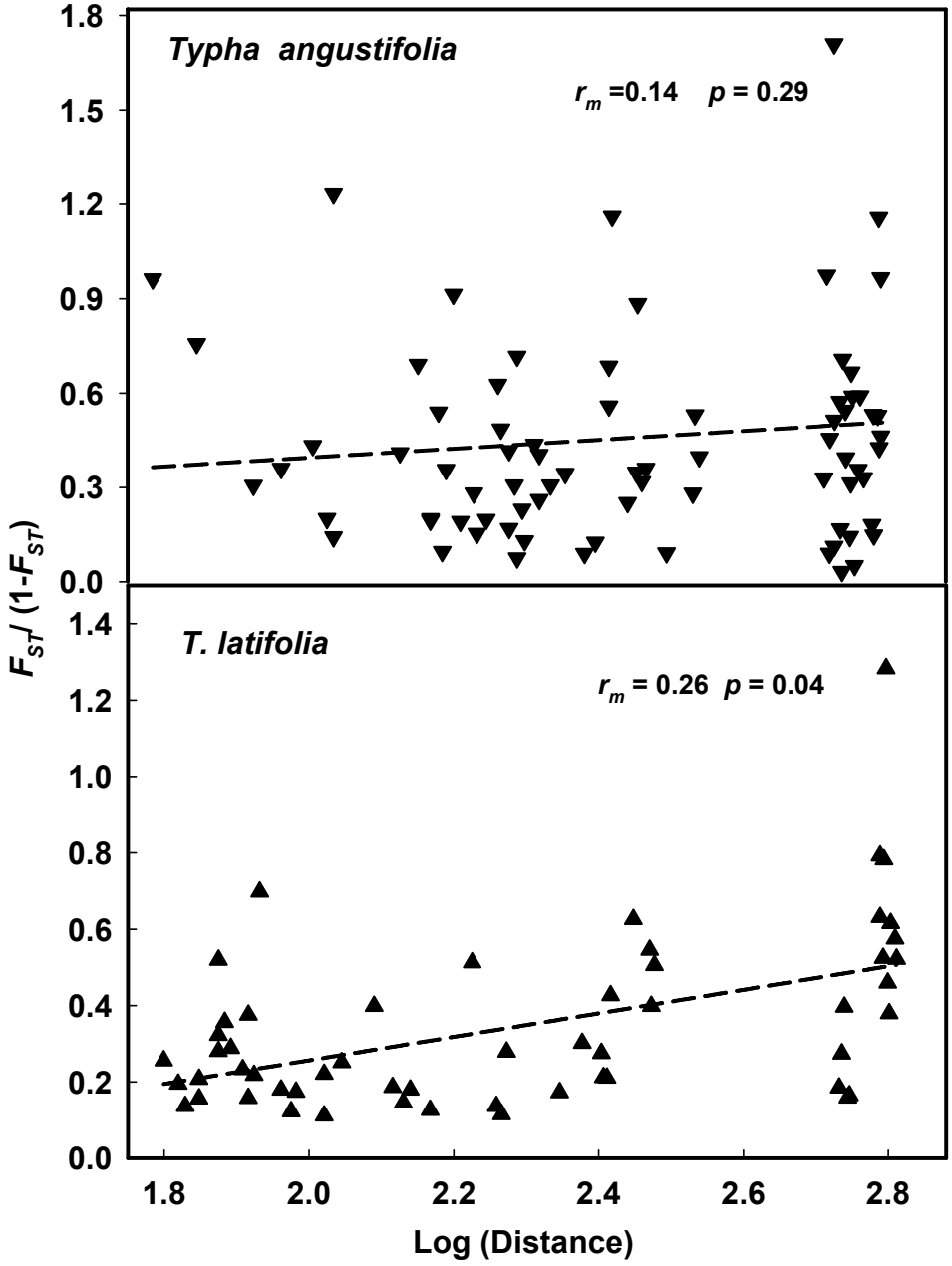

Fig. 3.4. Genetic differentiation expressed by relationships between pairwise genetic ($F_{ST}/(1-F_{ST})$ and log of pairwise geographic distances among 13 populations of *Typha angustifolia* and 11 populations of T. *latifolia*. The correlation coefficient (r_m) and *p*-vales are given from Mantel test.

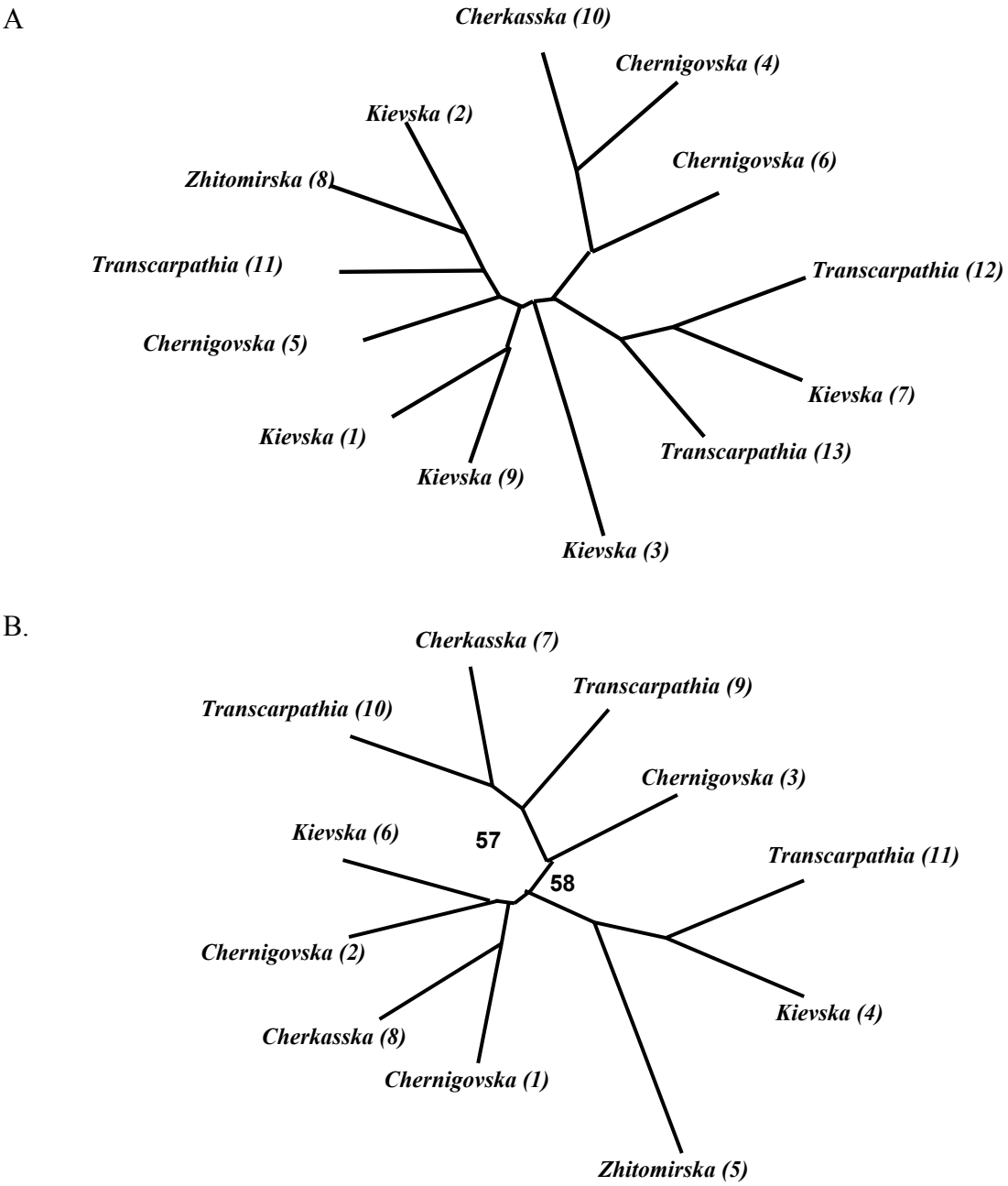

Fig. 3.5. Unrooted neighbor-joining trees for relationships among populations of *Typha angustifolia* (A) and *T. latifolia* (B) generated with Cavalli-Sforza and Edwards distances. Bootstrap values above 50 are given (based on 1000 bootstrap runs). The numbers in parenthesis refer to populations of *T. angustifolia* (A, 1-13) and *T. latifolia* (B, 1-11) (see Fig. 3.1)

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CHAPTER 4

RADIATION EFFECTS OF THE CHORNOBYL ACCIDENT ON GENETICS OF CATTAILS[1]

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Abstract

Variability of microsatellites was studied in two cattail species, *Typha angustifolia* and *T. latifolia*, from radioactively contaminated and reference areas in Ukraine. Differences for five genetic and genotypic characteristics were observed between Chornobyl and reference populations in each species. Populations closest to the failed reactor had the highest genetic variability, which decreased with increasing distance from the site of the accident. Positive correlations of several population characteristics with mean of radionuclide concentrations (^{137}Cs and ^{90}Sr) in plants also indicated radiation effects in both *Typha* species. There were no significant correlations of these characteristics with external dose rate or soil isotopic concentrations. When the total genetic variance was partitioned between Chornobyl and reference populations, a small significant difference was found for *T. latifolia* but not *T. angustifolia.* Chornobyl populations of *T. latifolia* clustered together in a dendrogram, which shows relationships among all intraspecific populations. Geographic distances among populations from Chornobyl were less than those from reference populations of both species, but evidence for isolation by distance was seen only for *T. latifolia*. Bottlenecks and deviations from Hardy-Weinberg equilibrium occurred in reference as well as Chornobyl populations. Many genetic and genotypic characteristics of *Typha* were different in radioactively contaminated areas, but factors other than radioactivity also contributed to these differences.

Introduction

Mutations caused by environmental contaminants have been the focus of extensive research (Belfiore & Anderson, 2001). At the down of nuclear age, Hermann Muller (1950) defined the concept of "genetic load" emphasizing the tendency for mutations to be deleterious and accumulate in populations. He indicated that problems directly linked to the deleterious

mutations would arise in populations from the release of radioactivity by nuclear bombs during the 1950-1960s. Since that time, nuclear accidents and atomic bomb testing released considerable amounts of radioactivity into the environment. One of the largest such accidents occurred at the Chornobyl Nuclear Power Plant (ChNPP) in Ukraine during 1986, released 1.8 - $3.7*10^{18}$ Bq of radioactivity (Medvedev, 1994). This massive amount of radiation should have had a significant impact on the genetics of organisms living around the ChNPP.

Elevated mutation rates at mini and microsatellite loci have been demonstrated in populations living in contaminated environment around the ChNPP (Dubrova *et al.*, 1996; 1997; 1998; Ellegren, 1997, 2000; Kovalchuk *et al.*, 2000). However, other studies at this site had contradictory results or failed to show any genetic effects (Baker & Chesser, 2000; Baker *et al.*, 1999; DeWoody, 1999; Livshits *et al.*, 2001; Matson *et al.*, 2000; Rodgers & Baker, 2000). Unfortunately, most of these studies had small numbers of reference populations (from 1 to 4), a fact that may have contributed to the significant results. Moreover, a large amount of genetic variation was shown among nine reference populations of *Apodemus flavicollis* from Ukraine emphasizing the need for sampling many reference populations to establish a baseline for evaluating radiation effects in studies like these (Oleksyk, 2001; Oleksyk *et al*., 2002).

It is important to understand radiation effects in complex natural environments, and more emphasis has recently been placed on long-term studies of exposed populations including those at Chornobyl. Contaminants can affect individual survivorship, recruitment, reproductive success, and dispersal, and these factors complicate interpretations of cause and effect relationships for radiation (Bickham & Smolen, 1994). Radiation can influence demographic and genetic characteristics and affect partitioning of genetic variation among and between contaminated and reference populations. Most genetic studies conducted at Chornobyl have

involved humans or animals, but plants because of their importance in ecosystem should also be examined in this regard. Most radionuclides were deposited in soils and sediments where plants grow and uptake radioisotopes. Radioactive sediments become more important over long time intervals as erosion moves soils to the aquatic environments. Thus, aquatic plants are particularly interesting, yet there have been no studies conducted on genetic effects concentrating on plants in aquatic environments.

Two cattail species *(Typha latifolia* and *T. angustifolia*) that occur worldwide are also abundant in the area around the ChNPP. Brisbin *et al.* (1989) suggested the use of cattails as indicator species for radiocesium concentration in foodwebs around nuclear facilities, such as Savannah River Site in South Carolina. Cattails can remove chemical contaminants from wastewater (Lan *et al.*, 1992). In the recent decade, it has been suggested that *Typha* shows genetic changes in response to organic contaminants (Keane *et al.*, 1999). Different methods have been used to assay genetic diversity of *Typha*. Allozymes show almost no polymorphism for *T. latifolia* populations from the Southeastern United States (Mashburn *et al.*, 1978; Sharitz *et al.*, 1980), whereas more polymorphism has been revealed using RAPDs (Marcinko-Kuehn *et al.*, 1999), VNTRs (Keane *et al.*, 1999), and microsatellites (Tsyusko-Omeltchenko *et al*., 2003; Chapter 3). In fact, microsatellites have a higher level of polymorphism for *Typha* than found in other studies using different genetic markers. This makes *Typha* excellent candidate for the study of contaminated environments.

Microsatellites are highly variable markers with mutation rates of 10^{-3} to 10^{-4} per locus (Weber & Wong, 1993), which is several orders of magnitude higher than that of allozymes. The Stepwise Mutation Model (SMM), which is commonly used for microsatellite loci, involves the loss or gain of one repeat at a time (Schlötterer & Tautz, 1992). Mutational changes for

microsatellites can be also complex involving deletion or insertion of several repeats or a complete loss of loci (Ellegren, 2000; Kovalchuk *et al.*, 2003). Most microsatellite mutations occur in non-coding regions called "junk DNA" and have no or slightly deleterious effects (Crow, 1997). Those with slight effects can accumulate and persist, affecting a large number of individuals and eventually decreasing fitness and population size (Gabriel *et al.*, 1993). Thus, microsatellites in Chornobyl populations are expected to express higher amounts of variation then elsewhere (Dubrova *et al*., 1996; 1997; 1998; Oleksyk *et al*., 2002).

Our overall objective was to test for differences in genetic diversity between radioactively contaminated and reference *Typha* populations. Eight diversity measures were used for the comparisons. Establishing relationships between measures of genetic diversity and distance from the reactor were used to test for possible radiation effects. Two species were included to test for differences in their responses to radiation. We calculated correlations of genetic diversity variables with means of radiocesium (^{137}Cs) and radiostrontium (^{90}Sr) concentrations in the plants and soil, and external dose rates in both species. Finally, we evaluated spatial genetic heterogeneity and partitioned genetic variance between Chornobyl and reference populations.

Materials and Methods

Sampling sites

A total of 708 and 277 samples from 24 reference and 10 radioactively contaminated populations, respectively, were collected (Fig. 4.1). Both species were found together at two contaminated and eight reference sites. Contaminated populations (five/species) were located within the Chornobyl Exclusion Zone, a 30-km zone around the failed reactor where public

access is not allowed. The two most radioactive sites were Gorodijna (51º25'N, 29º59'E) and Gluboky Lake (51º27'N, 30º04'E). Four other sites (Emerald Camp, 51º20'N, 30º08'E; Pripyat, 51º25'N, 29º58'E; Zimovishe, 51º26'N, 30º02'E, and River, 51º27'N, 30º03'E) had less radioactivity. The least contaminated site, Dityatki (51º17'N, 29º36'E), was located at the edge of the Zone, 35 km from the reactor. Reference sites (N = 13 and 11 for *T. angustifolia* and *T. latifolia*, respectively) were located 32 - 700 km to the west, south and northeast from ChNPP (reference sites are described in Chapter 3). Individual samples were taken at least 1 m and usually farther apart during July - August of 2001 and 2002.

^{137}Cs and ^{90}Sr concentrations

At least fifteen samples were taken from each contaminated population to measure individual ^{137}Cs and ^{90}Sr concentrations. For each reference population two samples were formed by pooling half of the samples, dried at 50°C, ground, and counted for ^{137}Cs and ^{90}Sr. If the radioactivity level for a reference sample was above detectable, radionuclide concentrations were measured in individual 15 samples from each of these populations. ^{137}Cs and ^{90}Sr concentrations are given in Bq/g (dry weight). Radiocesium concentrations were measured using a Minaxi γ-5000 counter. Counting times were set to achieve $\sigma \leq 5\%$ of the estimate for each sample. Detector efficiency was calculated from low activity standards with a counting geometry similar to the plant samples. Concentrations of ^{90}Sr were determined non-chemically using a beta-gamma spectrometer. This beta-spectrometer has a scintillation film detector (ca. 1mm thick), with efficiency sufficient to register gamma quantum with energy of 661 keV, one twentieth of that for an electron with similar energy (Bondarkov *et al*., 2001). Observed combined spectra of energy levels from electrons, gamma-quantum, and X-rays were partitioned into contributions of ^{137}Cs and ^{90}Sr/^{90}Y. External doses were estimated using thermo-luminescent dosimeters (TLDs).

Twelve TLDs were placed for 20 days at each contaminated site. TLDs were attached to cattails about 20 cm above the water and at least 3m apart. Twenty four TLDs (12 per site) were left as controls at the Savannah River Ecology Laboratory in South Carolina and at the International Radioecology Laboratory in Slavutych, Ukraine. A Solon-Harshaw QS 3500 was used to read TLDs. Six soil samples from each but one contaminated site, which was flooded during sampling, were collected, dried at 100°C, ground, and counted for ^{137}Cs and ^{90}Sr.

DNA Technique

Plant samples, about 20 cm long, were taken from the top of leaves and placed in bags with silica gel. Dry samples were crushed in liquid nitrogen, and DNA was isolated using a Qiagen DNeasy Kit. Extracted DNA was amplified in multiplex Polymerase Chain Reactions (PCR) using 11 and 9 pairs of microsatellite primers for *T. angustifolia* and *T. latifolia*, respectively. Primers were developed for *T. angustifolia,* and nine of the loci were consistently cross-amplified in *T. latifolia* (Tsyusko-Omeltchenko *et al*., 2003). PCR amplifications were performed in a final volume of 25µL with 10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/mL BSA, 4 mM $MgCl_2$, 0.15 mM dNTPs, 0.3-05µM of each primer (10µM), 0.5 units JumpStart Taq DNA Polymerase (Sigma), and 5-20 ng DNA template. The primer concentrations were adjusted for amplification in multiplex PCR reactions (more detailed description is in Tsyusko-Omeltchenko *et al*., 2003). PCR products were mixed with a Gensize Rox 500 ladder (Genpak) to determine allele sizes and run on an ABI 377 Sequencer. Genescan and Genotyper (PE Applied Biosystems) were used for allele scoring. A data converter (www.today.myip.org) was used to convert data from Genotyper to Genepop and Arlequin formats.

Data Analyses

Basic indices of genetic diversity including mean allele number (*MAN*), mean allele size and its variance, average observed and expected heterozygosity (H_o and H_e) for each population were calculated for genet and ramet data of each population using Genepop version 3.1 (Raymond & Rousset, 1995). To differentiate between ramets and genets, we used expected frequencies of multilocus genotypes that occurred in more than one sample to calculate *Pgen*, the probability that two samples have the same genotype by chance (Reusch *et al*., 2000). Ramets with the same genotype were considered as one genet when $Pgen < 0.05$. Differences between estimates of genetic characteristics calculated from genet and ramet data were evaluated using paired-sample t-tests (Zar, 1999). The estimates of genetic characteristics of genets were used to make comparisons between Chornobyl and reference groups. Deviations from Hardy-Weinberg (HW) equilibrium and linkage disequilibrium between loci were tested with Genepop. Sequential Bonferroni corrections were used to estimate significance levels for the tests (Rice, 1988). Differences between Chornobyl and reference groups in the number of populations deviating from HW were analyzed using Chi-square tests.

Genotypic characteristics including proportions of distinguishable genotypes, Simpson's *D*, and Fager's (1972) evenness (*E*) of distribution of clones were calculated for each population. The proportion of distinguishable genotypes was calculated as a ratio of the number of genets to that of ramets (*Ng*/*Nr*). Simpson's diversity index (*D*) corrected for sample size were calculated according to Pielou (1969). Wilcoxon rank sum tests (Sokal & Rohlf, 1995) were used to compare genetic and genotypic characteristics between the Chornobyl and reference populations including data from all populations and after removing data from outlier populations. Data were

tested for outliers using Grubbs' test (Grubbs, 1969). Chi square was used to test for differences between Chornobyl and reference groups in the number of genets adjusted for number of ramets.

Simple regressions were calculated for each species between each characteristic and the population's log-transformed geographical distance from ChNPP including and excluding data from outlier populations. Correlation coefficients (*r*) for these relationships were compared between species using *t* – tests after transformation of *r*s to *z*s (Zar, 1999). Multiple regressions were calculated to test for relationships of population genetic and genotypic characteristics with means of internal log-transformed ^{137}Cs and ^{90}Sr concentrations in plants and with external dose rates (Genetic effect = $\beta o + \beta_1{}^{137}Cs + \beta_2{}^{90}Sr + \beta_3$External Dose + e). The reference populations included in these analyses represented samples with slightly above detectable radioactivity levels. These populations were located no further than 100 km from the reactor. Data from a total of 11 populations of *T. angustifolia* (5 Chornobyl and 6 reference) and 9 of *T. latifolia* (5 Chornobyl and 4 reference) were analyzed. Simple linear regressions for the same relationships used in multiple regressions were also calculated.

Total variance of haplotype differences were partitioned within and among populations, and between Chornobyl and reference groups for each species using Analysis of Molecular Variance (AMOVA). AMOVA was performed with Arlequin (Schneider *et al*., 2000) using two options, F_{ST} or R_{ST}. F_{ST} (Weir & Cockerham, 1984) was calculated from differences in haplotype frequencies and R_{ST} (Slatkin, 1995) from squared differences between haplotype sizes. Genetic distances (F_{ST} and Rho_{ST}) between pairs of populations were calculated using FSTAT (Goudet, 1995) and RSTCALC (Goodman, 1997). Correlations between matrixes of genetic and geographic distances were calculated using Mantel's (1967) test by including all populations or removing those from the reference or Chornobyl group. PHYLIP, version 3.5 (Felsenstein,

1993) was used to generate dendrograms using Cavalli-Sforza genetic distances that were calculated from allele frequencies with GENDIST. Each allele frequency matrix was resampled 1000 times with SEQBOOT. Unrooted neighbor-joining trees were generated with NEIGHBOUR, consensus trees were created using CONSENSE, and dendrograms were plotted with DRAWGRAM.

The occurrence of recent bottlenecks in exposed and reference populations was tested using Bottleneck 1.2.02 (Cornuet & Luikart, 1997) and AGARst (Harley, 2001). Heterozygosity data were analyzed under the assumptions of three mutation models including the infinite allele model (IAM), stepwise mutation model (SMM), and two-phase model (TPM). The ratio of the number of alleles to the range in allele size (M) and its variance for each locus and population were calculated using AGARst. According to Garza and Williamson (2001), a recent bottleneck has occurred when $M < 0.68$. Bottleneck program was also used to test for fit of data to IAM, SMM, or TPM.

Statistical significance was indicated when $p \leq 0.05$.

Results

Within population diversity

Genetic and genotypic characteristics calculated from genet data for reference and Chornobyl populations of both species are given in Table 4.1 and 4.2. Probabilities for multilocus genotypes occurring more than once (*Pgen*) were always < 0.05, and this facilitated differentiation between genets and ramets. There were significant differences between characteristics calculated from genet and ramet data (Chapter 3), and only genet data were used in further analyses. Significant deviations from HW equilibrium were found on average at two

loci in each of eight populations of *T. angustifolia* and six populations of *T. latifolia* after applying Sequential Bonferroni corrections (Table 4.1 and 4.2; $p < 0.01$ for both species). Three and four of these were Chornobyl populations of *T. angustifolia* and *T. latifolia*, respectively. There were no significant differences between reference and Chornobyl groups in the number of populations deviating from HW equilibrium.

Two outlier populations Gorodijna and Buzivka were identified using Grubbs' test. Gorodijna, the outlier population of *T. angustifolia*, showed significant differences in H_e, *MAN*, Simpson's *D*, and the ratio of number of genets to that of ramets ($p < 0.01$ for all tests) when data from two sequential years were compared. There was a significant decrease in its genetic variability: fewer alleles were detected and the proportion of genets decreased from 0.28 to 0.08. Thus, Gorodijna, which was new to the area, had a significant decrease in genetic variability after one year. Repeated microsatellite analyses from several samples from both years gave the same result.

Comparisons between Chornobyl and reference populations revealed significant differences in *MAN*, mean variance of allele size, proportion of distinguishable genotypes, Simpson's *D*, and *E* for *T. angustifolia* ($p < 0.01$ for each case) but only after data from the outlier population, Gorodijna, were deleted from the analyses. In *T. latifolia* H_o ($p = 0.04$), H_e ($p = 0.03$), *MAN* ($p = 0.05$), the proportion of distinguishable genotypes ($p = 0.04$), and *E* ($p < 0.01$) were significantly different for this comparison when data from the outlier population (Buzivka) were excluded. In both species Fager's *E* for Chornobyl were significantly different from that of the reference populations even when all data were analyzed ($p \leq 0.04$ for both species). Overall, *T. angustifolia* and *T. latifolia* each showed significant differences between Chornobyl and reference populations for five diversity measures after data from the outliers were removed.

Regression analyses

Both species had significant negative relationships for H_e, *MAN*, ratio of the number of genets to that of ramets, and Fager's *E* with the log-transformed distance from ChNPP after outlier data were removed (examples given in Fig. 4.2; Appendix A, Fig. A-1). Mean allele size and its variance significantly decreased with distance from ChNPP in *T. angustifolia* but not *T. latifolia*. Simpson's *D* of *T. angustifolia* also demonstrated significant decreases with distance from the failed reactor after data from the outlier population were removed. Thus, after excluding data from the outliers there were four of eight characteristics of *T. latifolia* (H_o, *MAS* and its variance, and Simpson's *D*) that failed to demonstrate significant relationships with distance and only one of *T. angustifolia* (H_o). The correlations for *T. latifolia* were significantly lower than those for *T. angustifolia* after outlier data from each species were removed ($p = 0.01$).

Concentrations of ^{90}Sr in plants and sediment were higher than those of ^{137}Cs for all but one population per species (Table 4.3), and correlation between the concentrations of the isotopes was significant ($r = 0.88$ and $p < 0.001$). Concentration ratios varied from 0.05 to 8.09 (Table 4.3). *Typha angustifolia* and *T. latifolia* showed significant multiple correlation coefficients for five (H_e, *MAN*, variance of mean allele size, *Ng/Nr*, and *E*) and four (*MAN*, *D*, *Ng/Nr*, and *E*) characteristics of genetic diversity, respectively with means of radioisotope concentrations ($p < 0.05$). Simple regression plots for *MAN* with means of log-transformed radionuclide concentrations for both species are shown in Fig. 4.3. A trend toward increasing genetic characteristics with increasing radioactivity was shown for all eight characteristics of *T. angustifolia* and six of *T. latifolia*. *MAS* and its mean variance decreased for *T. latifolia*. There were no significant correlations of population genetic or genotypic characteristics (except mean variance of allele size in *T. latifolia*) with means of soil radionuclide concentrations. Only *MAN*

of the genetic and genotypic characteristics showed significant correlation with external radioactivity in *T. angustifolia*.

Population Differentiation

There was a large amount of variability in haplotype differences among populations for both species (ca. 23%; Table 4.4). There was a small but significant difference between Chornobyl and reference populations for *T. latifolia* when AMOVA was calculated using the F_{ST} or R_{ST} options (5.3% with $p < 0.01$ and 3.44% with $p < 0.05$, respectively, Table 4.4). *Typha angustifolia* failed to demonstrate a significant difference (< 1%) between these groups. There was no regional phylogeographic structure observed among populations of *T. angustifolia*; *Typha latifolia* however showed one cluster defined by four Chornobyl populations (Fig. 4.4).

The values of F_{ST} and Rho_{ST} for each pair of populations for each species are given in Appendix A, Tables A.1 and A.2. There were significant differences for F_{ST} and Rho_{ST} in Chornobyl (0.16 and 0.07, respectively) vs reference populations (0.29 and 0.12, respectively) of *T. latifolia* but not *T. angustifolia* where F_{ST} and Rho_{ST} in Chornobyl were 0.24 and 0.19, respectively and 0.24 for both variables in reference populations. There was a significant positive correlation between genetic (F_{ST}) and geographic distances for *T. latifolia* when data for all populations were included in the Mantel analysis ($r_m = 0.49$; $p = 0.01$). The correlation was not significant when Rho_{ST} values were used ($r_m = 0.08$; $p = 0.09$), but it was for both F_{ST} and Rho_{ST} when reference populations were considered alone ($r_m = 0.30$; $p = 0.04$ and $r_m = 0.27$; $p = 0.05$, respectively). There were negative correlations for the Chornobyl populations ($r_m = -0.47$; $p = 0.04$ for F_{ST} and $r_m = -0.72$; $p = 0.01$ for Rho_{ST}), which was mainly due to data from Dityatki. *Typha angustifolia* failed to demonstrate significant correlation between genetic (F_{ST} or Rho_{ST}) and geographic distances when data from all populations or from only reference populations

were analyzed ($p > 0.05$ for both tests). The correlation for Chornobyl populations of *T. angustifolia* was negative but not significant.

Mutation Models and Bottlenecks

Populations of both species demonstrated a better fit with SMM than the other two models (Appendix A, Table A.3). *Typha latifolia* populations were consistent with expectations of IAM, SMM, or TPM after Bonferroni correction was applied. Only three reference populations of *T. angustifolia* significantly deviated from the expectations of IAM ($p \leq 0.001$) and two from TPM ($p = 0.001$) after Bonferroni correction. None of the Chornobyl populations of both species deviated from expectations of IAM or TPM. Two ways of testing for the effects of bottlenecks gave inconsistent results. Two Chornobyl and nine reference populations of *T. angustifolia* had heterozygosity excess at 50% or more loci (the examples are given in Appendix A, Tables A.4 and A.5). Garza and Williamson's M test indicated bottlenecks for six of these populations ($M < 0.68$). In addition, three Chornobyl and one reference populations not indicated by the first test had $M < 0.68$. These populations demonstrated heterozygosity excess for two mutation models (IAM and TPM) but not SMM, and this was the reason why they were not indicated by the first test. Four populations of *T. latifolia* (three Chornobyl and one reference) showed heterozygosity excesses at more than 50% of their polymorphic loci under expectations of each of the three mutation models. When Garza and Williamson's test was used, only the results for one reference population indicated a recent bottleneck with $M = 0.56$.

Discussion

Differences in genetic diversity between Chornobyl and reference populations were observed for both species. Chornobyl populations closest to the reactor showed the highest level of variability

for most genetic and genotypic characteristics. Our results are consistent with the findings of other studies that show elevated mutations in humans, animals, and plants from Chornobyl (Dubrova *et al.*, 1996, 1997, 1998; Ellegren, 1997, 2000; Kovalchuk *et al*., 2000; Sadamoto *et al.*, 1994). Genetic diversities of *Apodemus flavicollis* populations within the Chornobyl exclusion zone were also highest near the reactor and decreased with distance from it (Oleksyk *et al*., 2002). Even though many of *Typha*'s diversity characteristics increase in populations around the reactor, some do not including H_o which is unexpected. In addition, significant percent of variation in haplotype differences between Chornobyl and reference populations occur only in *T. latifolia* but not *T. angustifolia*. This inconsistency is surprising given the high mutation rates for microsatellites and the long exposure times for the Chornobyl populations. There was so much radioactivity released at Chornobyl that noticeable genetic effects should have been observed in exposed populations. Six out of ten Chornobyl populations inhabited areas with relatively high amounts of radioactivity. Kovalchuk et al. (2000) documented a six-fold increase in microsatellite-mutation rates of wheat plants grown for one year in an area with lower levels of radioactivity than *Typha* was exposed to for more than a decade. The observed effects of radiation were relatively slight despite massive contamination at Chornobyl. Several factors in addition to radiation likely account for changes in genetic variation of the contaminated populations.

One of the explanations for the high diversity of Chornobyl populations may be greater gene flow among them than among reference populations. Geographic distances among Chornobyl populations are generally shorter than those among reference populations. Smaller distances usually lead to increased gene flow, which results in increased genetic variability within and similarity among populations. There was a positive correlation between genetic and

geographic distances for *T. latifolia* but not *T. angustifolia* suggesting isolation by distance and more gene flow among close populations in the first species but not the latter. However, some of the Chornobyl populations had large differences in genetic variability even when geographical distances were quite short. Unfortunately, samples were not collected as intensively in most of the reference areas as in the Chornobyl exclusion zone, so the different effects of gene flow within the two groups cannot be determined. The distinct cluster of Chornobyl populations of *T. latifolia* on a dendrogram (Fig. 4.4B) most probably is also due to this sampling effect. Increased gene flow may be partially responsible, but is probably not sufficient to explain the high genetic and genotypic diversity of the contaminated populations.

The northern location of Chornobyl populations in Ukraine could also have influenced their genetic diversity. The populations could have been genetically more diverse than other ones even before the accident. High genetic diversity of the three northern populations located just outside the Exclusion zone supports this hypothesis. However, diversity also varies among reference populations within different geographical regions. Thus, the location of Chornobyl populations alone may not explain their high variability. While their histories before the accident are unknown and reasons for their current high diversities may be complex, higher mutation rates due to radioactivity are probably involved in the explanation.

Significant increases in the proportions of distinguishable genotypes in Chornobyl populations may be due to accumulated mutations. Radiation could also have increased the frequency of sexual reproduction and/or seed bank germinations. Outcrossing and seed bank germination often increase population genetic variability (Eckert & Barrett, 1993; Edwards & Sharitz, 2003). Production and recruitment of different proportions of sexual and clonal progeny also vary in response to ecological factors and may change population genetic structure (Eckert,

2002). Factors such as flooding or drought may impose stress on populations and produce similar effects. There were significant fluctuations in precipitation between the years of sampling with a prolonged drought during 2002 that could have affected some populations (OT, pers. comm.). Thus, geographical and ecological factors may be responsible for the high genetic diversity of contaminated *Typha* populations. Even after the detailed consideration of other possible factors, radiation may still be a primary determining factor.

Radiation is known to cause mutations. Since microsatellite mutation rates are high (from10^{-3} to 10^{-5}; Shlotterer & Tautz, 1992; Weber & Wong, 1993), they are good markers for studying genetic effects in *Typha* (Tsyusko-Omeltchenko *et al*., 2003) where allozyme failed previously to show any polymorphism (Sharitz *et al*., 1980). Even though Valdes *et al*. (1993) found that allele frequencies at 108 human microsatellite loci were consistent with SMM, mutations do not always occur in this way. A study of wheat plants at Chornobyl revealed a complex pattern of mutations at microsatellite loci (Kovalchuk *et al*., 2003). Most population data for *Typha* have a good fit to all three-mutation models but a better to SMM (Appendix A, Table A.3). This finding indicates an occurrence of mutations in *Typha* populations, but their time of origin is unknown and not necessarily due to radiation. So these models cannot be used to document the radiation effects at Chornobyl.

Correlations of genetic and genotypic characteristics with radionuclide concentrations provide a test for radiation induced genetic effects. These correlations were often observed when data from outliers were excluded. There were two outlier populations (one per species) among the Chornobyl and reference samples: the most contaminated population of *T. angustifolia* (Gorodijna) and the reference population of *T. latifolia* (Buzivka). The Gorodijna population was assumed to be new to the area, because it was not observed during the two years prior to the

first sampling. Decreased genetic variability due to founder effects are often found in new populations. The decreased genetic diversity can also be due to occurrence of bottlenecks, which are common in *Typha angustifolia* (Chapter 3). The most southern reference population, Buzivka, of *T. latifolia* has the highest values for H_e and *MAN* and its high variability may be due to the establishment of a population by founders from multiple sources (Chapter 3). There are a number of factors that can cause a population to be an outlier, but they should be excluded only when they can be statistically identified as in this study.

The observed correlations support the idea that radiation had the expected effects for some but not all of the characteristics. Significant positive correlations of several population genetic and genotypic characteristics with means of radionuclide concentrations occur in both species (Fig. 4.3). Some reference populations of *T. angustifolia* located in close proximity to the Chornobyl exclusion zone had similarly high levels of variability as in contaminated populations (Fig. 4.3). In contrast, samples from Dityatki, the population of *T. latifolia* located near the border inside the zone, had very low concentrations of both isotopes, and genetic characteristics of this population were similar to those of many reference populations. Reference and contaminated populations occasionally have similar levels of genetic variability indicating that many reference samples chosen carefully are required for this type of comparison.

Concentrations of radionuclides in Chornobyl sediments vary over 100% in samples collected only a few meters apart (Jagoe *et al*., 1997). There were no significant correlations (except one) for the diversity characteristics with means of ^{137}Cs and ^{90}Sr soil concentrations. There probably would have been more correlations if individual measures of ^{137}Cs and ^{90}Sr for soil samples associated with each plant were used to test for correlations between genetic characteristics and soil radioactivity (Pinder & Smith, 1975). Soil properties influence

radionuclide distributions, their retention, and uptake by plants (Melin *et al*., 1994). Chornobyl sediments have a high percentage of clay, but radiocesium can be readily taken up by *Typha* as indicated by concentration ratios as high as eight (Table 4.3); thus emphasizing the importance of doses due to internal contamination. External dose after the accident was due to many isotopes, and although many of them decayed rapidly, they probably caused some genetic changes because of their temporarily high concentrations. There were no more genetic and genotypic characteristics that correlated with external dose rates than expected by chance. Concentrations of ^{137}Cs and ^{90}Sr and external dose rates were measured during short time intervals, but plants were exposed to radiation over longer time periods. Accumulative dose for radioactively exposed plants is not well represented by one-time measurements, and unfortunately it is impossible to accurately estimate cumulative dose for each plant.

Typha latifolia has significantly higher concentrations and concentration ratios for both isotopes than does *T. angustifolia*, but it shows fewer correlations of genetic characteristics with radionuclide concentrations; this complicates the explanation of the responses of each species to radiation. However, the higher concentrations in the former species and/or lack of reference samples adjacent to the zone may help explain the significant difference in the amount of variation between contaminated and reference populations of this species but not *T. angustifolia* (Table 4.4). The pattern of spatial heterogeneity but not the amount of variation among populations (23% in both species) probably accounts for the lack of difference between contaminated and reference populations in *T. angustifolia*. The amount of spatial heterogeneity is large enough to confound interpretation of contaminant effects on genetic variation among populations. High spatial genetic heterogeneity indicates the presence of a series of metapopulations in Ukraine for both *Typha* and *Apodemus* (Oleksyk, 2001; Chapter 3). The

existence of genetically distinct metapopulations makes it difficult to document the genetic effects of contaminants and necessitates a larger sampling program to do so.

Conclusions

Several factors influence the genetic diversity of *Typha* populations at Chornobyl. These were more variable for several of their genetic and genotypic characteristics than reference populations indicating the importance of radiation effects. Enhanced interpopulation gene flow at Chornobyl and/or increased germination from the seed bank may have contributed to this high diversity. The isolation by distance model loosely fit the data for *T. latifolia* but not *T. angustifolia*. High spatial heterogeneity among populations makes it difficult to detect significant genetic differences between Chornobyl and reference samples. *Typha latifolia* but not *T. angustifolia* shows a small but significant percent of variation between the two groups, and Chornobyl populations also cluster together on a dendrogram for the first species.

Exposure of Chornobyl populations has continued for more than a decade at a relatively high level, and a lot of genetic damage was expected. The higher genetic and genotypic diversity of Chornobyl populations may be partially due to accumulation of mutations. Various measures of genetic diversity of both species are higher in populations closest to the reactor. These characteristics show positive correlations with concentrations of radioisotopes, but the pattern differed between species that respond differently to radioactivity and its interactions with other factors. There is a lack of correlation between genetic and genotypic characteristics with concentrations of both radionuclides in soil and/or external radioactivity. Population histories are unknown but likely important in determining genetic diversities.

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Table 4.1. Characteristics of genetic and genotypic characteristics calculated from genet data using 11 microsatellite loci for *Typha angustifolia*.

Populations	***Nr***	***Ng***	**Nr/Ng**	H_e	H_o	***MAN***	***MAS***[b]	**Mean Variance of Allele Size**	***D***	***E***
Gorodijna[a]	25	7	0.28	0.35	0.49	1.91	207.43	5.88	0.72	0.34
Gluboky Lake 1	28	27	0.96	0.64	0.43*	6.36	206.09	35.65	1.00	1.00
Gluboky Lake 2	33	23	0.70	0.68	0.51*	7.64	203.53	61.44	0.97	0.65
Zimovishe	27	27	1.00	0.62	0.50*	5.82	204.05	36.50	1.00	1.00
Emerald	32	23	0.72	0.56	0.45*	5.00	204.71	57.93	0.97	0.73
Mean (Chornobyl)	**29.0**	**21.4**	**0.73**	**0.57**	**0.49**	**5.35**	**205.16**	**39.48**	**0.93**	**0.74**
Atashev	26	18	0.69	0.65	0.51*	6.45	203.54	42.77	0.98	0.82
Paryshev	25	17	0.68	0.48	0.50*	3.36	205.44	37.13	0.97	0.45
Ivankovo	25	12	0.48	0.65	0.67*	5.45	205.81	38.02	0.93	0.40
Mekshunovka	30	7	0.23	0.23	0.19	1.82	204.30	2.04	0.75	0.31
Gubichi	25	5	0.20	0.41	0.37	2.18	203.38	14.18	0.57	0.04
Chernigov	27	10	0.37	0.42	0.55*	2.27	203.67	2.94	0.65	0.31
Kiev	29	8	0.28	0.54	0.45	2.73	204.53	14.86	0.63	0.06
Brusilov	25	14	0.56	0.53	0.48	3.55	202.81	23.77	0.92	0.74
Belaya Tserkov	30	8	0.27	0.62	0.47	4.55	204.04	29.34	0.86	0.42
Buzivka	25	6	0.24	0.56	0.45	3.45	204.41	40.42	0.83	0.30
Zarichevo	26	12	0.46	0.47	0.65	3.00	202.96	18.32	0.91	0.19
Chop	30	10	0.33	0.42	0.50	2.09	202.74	0.42	0.88	0.43
Batevo	25	11	0.44	0.39	0.40	3.00	201.78	9.23	0.85	0.07
Mean (Reference)	**26.8**	**10.6**	**0.40**	**0.49**	**0.44**	**3.38**	**203.80**	**21.03**	**0.82**	**0.35**
Total Mean	27.5	14.0	0.51	0.52	0.45	4.00	204.23	26.86	0.86	0.47

*Significant deviations from Hardy-Weinberg equilibrium at two or more loci are indicated at $p < 0.01$

[a]Outlier population

[b]Some loci showed higher variances in MAS than others

Nr and *Ng* is number of ramets and genets, H_e and H_o are expected and observed heterozygosity, *MAN* is mean allele number. Allele size is given as number of base pairs; *D* is Simpson's diversity index; and *E* is Fager's evenness index for genet distribution. *Nr/Ng, MAN*, variance of *MAS*, *D*, and *E* are significantly different between Chornobyl and reference populations after the outlier data are excluded. Populations are arranged in descending order as distance from the reactor increased. Means are given in bold for Chornobyl and reference populations.

Table 4.2. Characteristics of genetic and genotypic characteristics calculated from genet data for nine microsatellite loci of *Typha latifolia*.

Populations	*Nr*	*Ng*	*Ng/Nr*	H_e	H_o	*MAN*	*MAS*	Variance of *MAS*[a]	*D*	*E*
Gluboky Lake	20	14	0.70	0.37	0.29	3.22	196.90	7.72	0.99	0.92
River site	26	22	0.85	0.34	0.36*	2.89	196.60	2.40	0.98	0.90
Pripyat	30	28	0.93	0.42	0.36*	2.67	196.97	4.66	0.99	0.89
Emerald	30	25	0.83	0.34	0.25*	3.00	196.88	5.10	0.98	0.86
Dityatki	26	15	0.58	0.34	0.30	2.33	196.89	4.18	0.91	0.48
Mean (Chornobyl)	**26.4**	**20.8**	**0.78**	**0.36**	**0.31**	**2.82**	**196.85**	**4.81**	**0.97**	**0.81**
Mekshunovka	30	15	0.50	0.27	0.24	2.56	197.27	5.55	0.89	0.18
Gubichi	30	14	0.47	0.18	0.22	2.22	196.95	16.63	0.86	0.12
Chernigov	30	17	0.57	0.35	0.30*	2.79	196.77	16.49	0.94	0.22
Kiev	29	18	0.62	0.30	0.31*	2.78	196.71	2.81	0.96	0.32
Brusilov	31	29	0.65	0.30	0.32*	2.89	196.57	7.89	0.96	0.41
Belaya Tserkov	31	20	0.58	0.32	0.30	2.56	197.20	7.53	0.96	0.33
Buzivka[b]	21	18	0.86	0.45	0.34	3.67	196.78	15.97	0.98	0.89
Uman	25	15	0.60	0.22	0.15	1.89	196.39	14.00	0.97	0.75
Borony	25	14	0.56	0.30	0.25	2.44	197.02	5.48	0.97	0.68
Glubokoe	30	22	0.73	0.31	0.25	2.78	196.86	2.75	0.99	0.89
Batevo	29	15	0.52	0.23	0.12	2.33	196.87	4.86	0.84	0.06
Mean (Reference)	**28.3**	**17.9**	**0.61**	**0.29**	**0.26**	**2.63**	**196.85**	**9.09**	**0.94**	**0.44**
Total Mean	27.6	18.9	0.67	0.32	0.25	2.70	196.85	7.58	0.95	0.57

*Significant deviations from Hardy-Weinberg equilibrium at two or more loci are indicated at $p < 0.01$

[a]Some loci showed higher variances in MAS than others

[b]Outlier population

Nr is number of ramets and *Ng* is number of genets; H_e and H_o are expected and observed heterozygosity; *MAN* is mean allele number; MAS is mean allele size given as number of base pairs; *D* is Simpson's diversity index; and *E* is Fager's evenness index for genet distribution. H_o, H_e, *MAN*, *Ng/Nr*, and *E* are significantly different between Chornobyl and reference populations after the othier data are excluded.
Populations are arranged in descending order with increasing distance from the reactor. Means are given in bold for Chornobyl and reference populations.

4.3. Means of concentrations of ^{137}Cs and ^{90}Sr in plants and soil, range for concentration ratios (CR), means of percentage of clay, and means of external dose rates for Chornobyl populations of *Typha angustifolia* and *T. latifolia*.

Populations	^{137}Cs in plants[b]	^{90}Sr in plants[b]	^{137}Cs in soil[b]	^{90}Sr in soil[b]	External dose rates (mrad/hour)	CR[a] (range)	Clay %
Typha angustifolia							
Gorodijna	118.7	306.8	-	-	323.1	-	-
Gluboky Lake2	4.2	61.9	13.2	33.8	189.7	0.06-0.29	64.45
Gluboky Lake1	2.0	60.9	41.4	77.4	118.5	0.07-0.16	65.56
Zimovishe	3.1	12.7	4.2	2.3	104.3	0.06-0.16	63.15
Emerald	1.3	6.0	0.6	0.6	98.2	0.05-0.19	70.48
T. latifolia							
Gluboky Lake	83.1	562.6	123.0	210.3	153.6	0.05-7.74	65.78
River	9.9	104.2	15.3	169.8	102.5	0.90-8.09	66.85
Pripyat	5.5	13.6	23.5	104.8	87.7	0.04-0.99	60.44
Emerald	5.5	6.0	11.4	6.0	65.2	0.41-3.06	68.89
Dityatki	0.5	5.1	5.2	43.5	55.9	0.09-0.25	65.78

[a]Ratio of concentrations on a dry weight basis of radionuclides in plants to that in soil
[b]Bq/g
Soil samples were not available for Gorodijna.

4.4. Analyses of Molecular Variance of allele length calculated with the F_{ST}/R_{ST} option for *Typha angustifolia* (TA) and *T. latifolia* (TL) using Arlequin

Source of Variation	**DF**		**Percentage of Variation**	
All populations (18 for TA and 16 for TL)	TA	TL	TA	TL
Among populations	17	15	22.49^{**} / 27.77^{**}	23.63^{**} / 10.96^{**}
Within populations	472	564	77.51^{**} / 72.23^{**}	76.37^{**} / 89.04^{**}
Total	489	579		
Chornobyl and reference populations				
Among groups	1	1	$< 1^{ns}$ / $< 1^{ns}$	5.33^{*} / 3.44^{*}
Among populations within groups	16	14	22.94^{**} / 30.73^{**}	20.37^{**} / 9.07^{**}
Within populations	472	564	77.77^{**} / 73.75^{**}	74.30^{**} / 87.48^{**}
Total	489	579		

**Statistical significance is indicated at $p < 0.001$, *at $p \leq 0.05$, and $^{ns}p > 0.05$

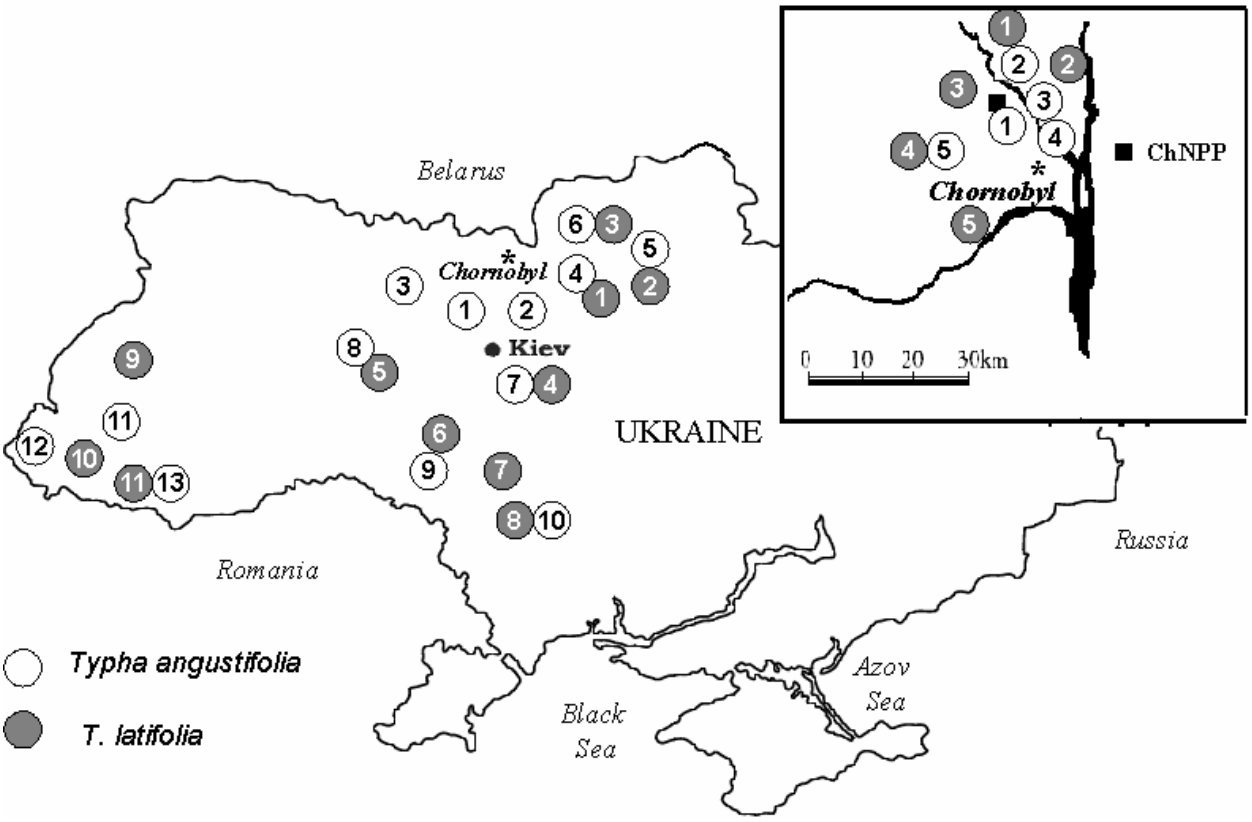

Fig. 4.1. Sampling sites for populations of *Typha angustifolia* and *T. latifolia* in Ukraine. Both species co-occur at eight reference and two Chornobyl sites. Reference sites for *T. angustifolia* (open circles): 1 – Atashev, 2- Paryshev, 3 – Ivankovo, 4 – Mekshunovka, 5 – Gubichi, 6 – Chernigov, 7 – Kiev, 8 – Brusilov, 9 - Belaya Tserkov, 10 – Buzivka, 11 – Zarichevo, 12 – Chop, 13 – Batevo and Chornobyl sites: 1 – Gorodijna, 2 – Gluboky lake2, 3 – Gluboky Lake1, 4 – Zimovishe, 5 – Emerald. Reference sites for *T. latifolia* (closed circles): 1- Mekshunovka, 2 – Gubichi, 3 – Chernigov, 4 - Kiev, 5 – Brusilov, 6 – Belaya Tserkov, 7 – Buzivka, 8 – Uman', 9 – Borony, 10 – Glubokoe, 11 – Batevo and Chornobyl sites: 1 – Gluboky Lake, 2 – River, 3 – Pripyat, 4 – Emerald, 5 – Dityatki.

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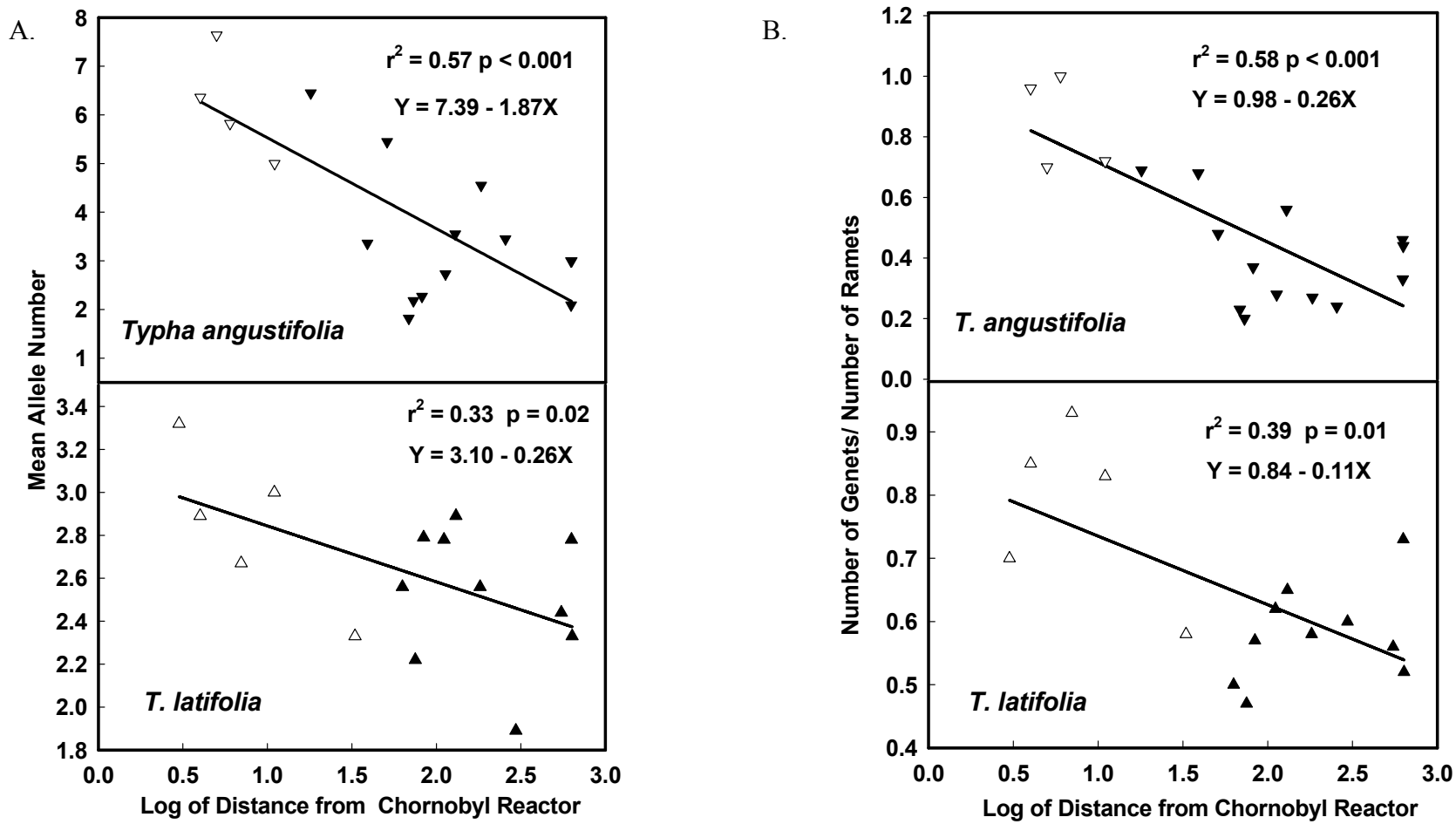

Figure 4.2. Simple regressions for mean allele number (A) and *Ng/Nr* (B) with the distance from the Chornobyl Nuclear Power Plant for *Typha latifolia* and *T. angustifolia. Ng/Nr* is ratio of the number of genets to the number of ramets. Probability for a significant relationship, *p* value, is given for each case. Populations in the Chornobyl exclusion zone are indicated with open triangles. The relationships in B were significant after data from one outlier population per species was removed.

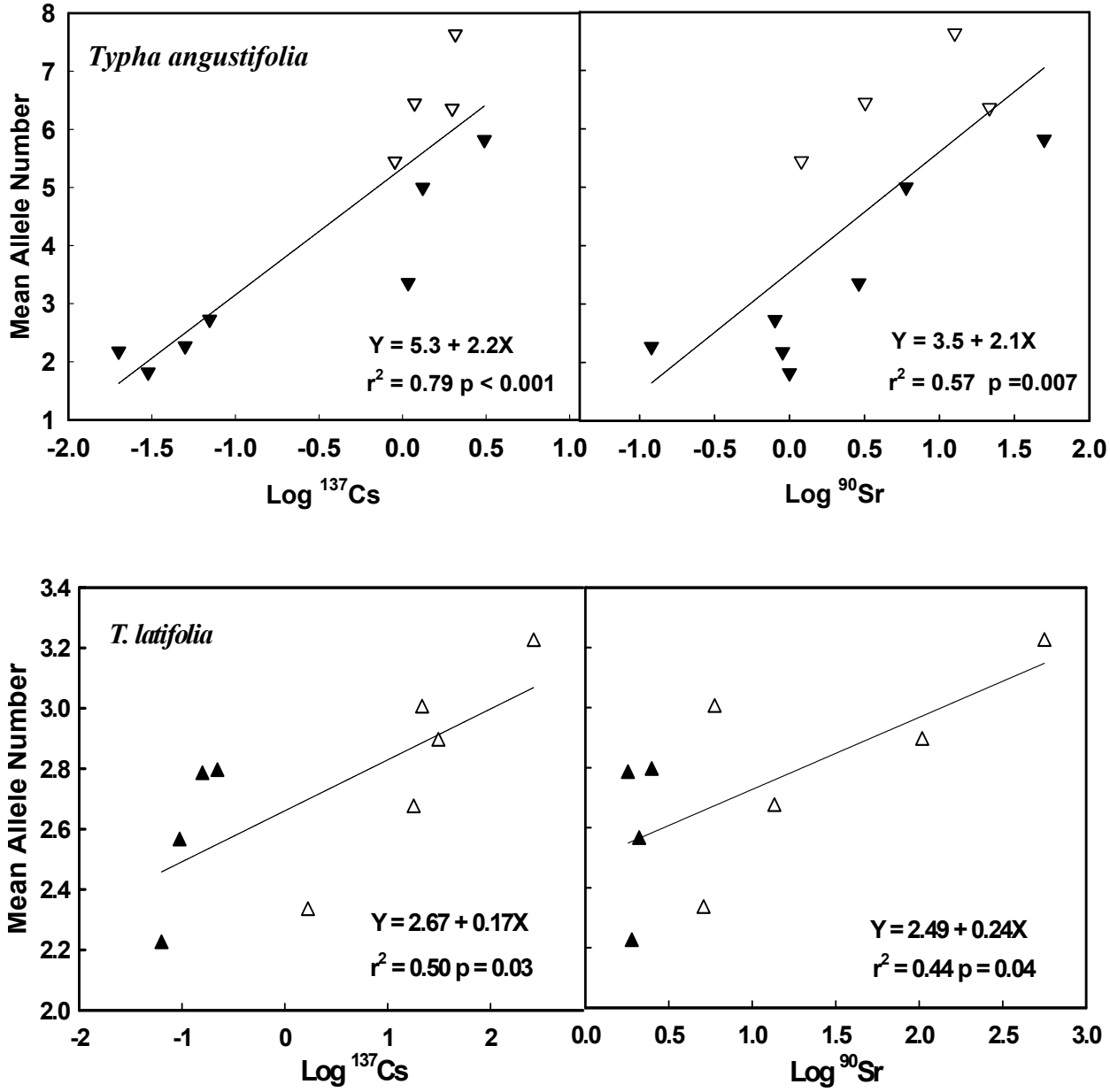

Figure 4.3. Simple regressions of *MAN* with means of log-transformed ^{137}Cs and ^{90}Sr concentrations (Bq/g) for *Typha angustifolia* and *T. latifolia*. The regressions are shown after data from outliers were removed. Probability for a significant relationship, *p* value, is given for each case.

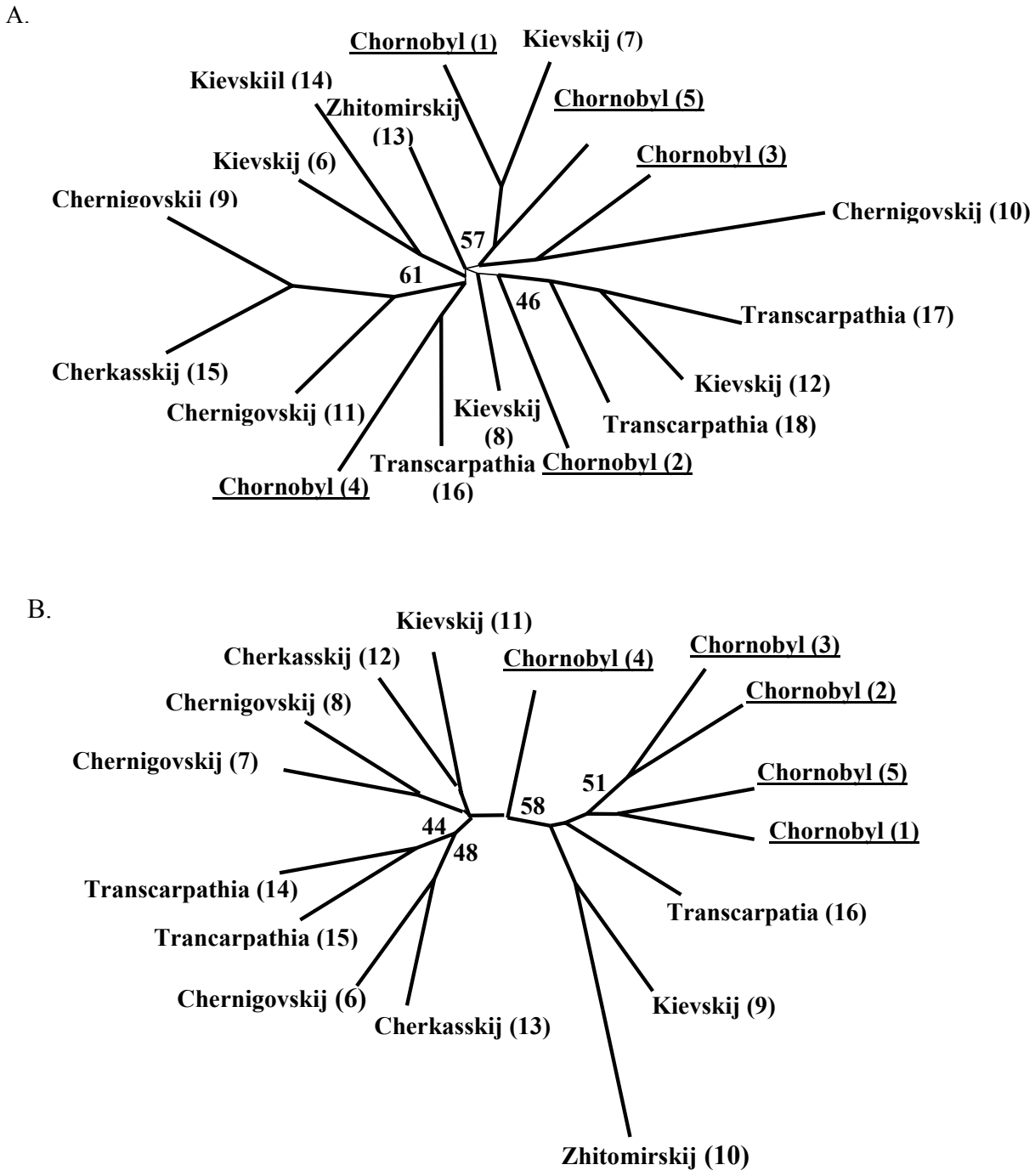

Figure 4.4. Regional phylogeographic relationships among populations of *Typha angustifolia (A) and T. latifolia* (B) based on Cavalli-Sforza genetic distances. Bootstrapping values were calculated from 1000 runs. Chornobyl populations are underlined. The population names are given in Fig. 4.1

APPENDIX A

Table A.1. Values of F_{ST} and Rho_{ST} calculated for each pair of populations from allele frequency data of 11 microsatellite loci in *Typha angustifolia*. F_{ST}s are shown above the diagonal and Rho_{ST}s are below. The names and numerical designations for Chornobyl populations are in bold.

Populations[a]	**1**	**2**	**3**	**4**	**5**	6	7	8	9	10	11	12	13	14	15	16	17	18
Gorodijna		0.280	0.235	0.312	0.207	0.255	0.268	0.188	0.641	0.462	0.430	0.330	0.327	0.233	0.321	0.408	0.393	0.508
GlubokyLake2	0.373		0.097	0.191	0.165	0.104	0.215	0.124	0.383	0.157	0.262	0.148	0.173	0.124	0.170	0.187	0.237	0.269
GlubokyLake2	0.279	0.158		0.167	0.087	0.068	0.138	0.067	0.307	0.177	0.213	0.088	0.094	0.051	0.119	0.171	0.147	0.127
Zimovishe	0.383	0.137	0.055		0.198	0.155	0.277	0.142	0.356	0.295	0.228	0.207	0.217	0.125	0.168	0.215	0.278	0.348
Emerald	0.194	0.164	0.079	0.094		0.138	0.189	0.105	0.390	0.247	0.267	0.143	0.189	0.107	0.132	0.223	0.235	0.265
Atashev	0.314	0.124	0.087	0.085	0.144		0.166	0.067	0.298	0.215	0.192	0.114	0.100	0.033	0.084	0.098	0.197	0.251
Paryshev	0.258	0.286	0.188	0.308	0.139	0.243		0.132	0.481	0.352	0.362	0.251	0.159	0.141	0.273	0.301	0.267	0.375
Ivankovo	0.147	0.168	0.106	0.142	0.093	0.070	0.190		0.329	0.231	0.191	0.099	0.144	0.034	0.087	0.194	0.169	0.284
Mekshunovka	0.441	0.483	0.295	0.330	0.330	0.283	0.394	0.268		0.607	0.480	0.417	0.432	0.333	0.349	0.452	0.500	0.511
Gubichi	0.349	0.183	0.278	0.298	0.245	0.221	0.404	0.271	0.651		0.442	0.312	0.289	0.252	0.342	0.394	0.450	0.470
Chernigov	0.383	0.298	0.172	0.178	0.246	0.130	0.410	0.105	0.319	0.394		0.340	0.285	0.214	0.212	0.327	0.352	0.394
Kiev	0.264	0.193	0.086	0.222	0.132	0.141	0.204	0.052	0.452	0.289	0.265		0.220	0.087	0.160	0.288	0.215	0.295
Brusilov	0.323	0.242	0.082	0.257	0.189	0.140	0.200	0.176	0.370	0.291	0.321	0.214		0.102	0.223	0.220	0.232	0.294
Belaya Tserkov	0.235	0.166	0.057	0.163	0.079	0.071	0.165	0.050	0.368	0.291	0.179	0.099	0.104		0.081	0.139	0.152	0.285
Buzivka	0.322	0.261	0.098	0.121	0.191	0.113	0.324	0.079	0.289	0.406	0.059	0.241	0.255	0.100		0.235	0.295	0.307
Zarichevo	0.385	0.168	0.031	0.095	0.190	0.079	0.237	0.154	0.342	0.275	0.212	0.211	0.120	0.109	0.121		0.322	0.368
Chop	0.299	0.267	0.110	0.276	0.161	0.210	0.229	0.118	0.551	0.410	0.298	0.156	0.231	0.099	0.303	0.242		0.350
Batevo	0.471	0.351	0.074	0.315	0.240	0.219	0.339	0.264	0.394	0.416	0.248	0.255	0.048	0.200	0.227	0.100	0.287	

[a]The numbers from 1 to 18 indicate the populations listed in the first column from Gorodijna to Batevo

Table A.2. Values of F_{ST} and Rho_{ST} calculated for each pair of populations from allele frequency data of nine microsatellite loci in *Typha latifolia*. F_{ST}s are shown above the diagonal and Rho_{ST}s are below. The names and numerical designations for Chornobyl populations are in bold.

Populations[a]	**1**	**2**	**3**	**4**	**5**	6	7	8	9	10	11	12	13	14	15	16
Gluboky Lake		0.140	0.197	0.124	0.090	0.204	0.120	0.263	0.181	0.152	0.218	0.174	0.336	0.137	0.315	0.365
River	0.090		0.089	0.240	0.081	0.163	0.172	0.219	0.100	0.127	0.103	0.175	0.285	0.141	0.275	0.343
Pripiat	0.092	0.084		0.307	0.043	0.135	0.342	0.179	0.201	0.157	0.120	0.216	0.353	0.284	0.439	0.381
Emerald	0.015	0.102	0.095		0.241	0.224	0.273	0.411	0.148	0.285	0.339	0.232	0.385	0.156	0.442	0.562
Dityatki	0.002	0.051	0.031	0.014		0.136	0.244	0.152	0.189	0.109	0.112	0.147	0.299	0.215	0.387	0.344
Mekshunovka	0.017	0.179	0.146	0.040	0.059		0.283	0.116	0.213	0.235	0.206	0.173	0.342	0.237	0.421	0.370
Gubichi	0.084	0.232	0.266	0.141	0.133	0.183		0.358	0.235	0.234	0.266	0.271	0.375	0.078	0.327	0.470
Chernigov	0.060	0.207	0.140	0.126	0.102	0.119	0.059		0.343	0.289	0.244	0.258	0.399	0.364	0.493	0.341
Kiev	0.016	0.181	0.184	0.068	0.098	0.079	0.125	0.104		0.199	0.198	0.220	0.315	0.176	0.236	0.444
Brusilov	0.003	0.127	0.130	0.051	0.047	0.067	0.110	0.060	0.006		0.044	0.306	0.361	0.202	0.342	0.359
Belaya Tserkov	0.071	0.193	0.243	0.154	0.132	0.195	0.112	0.132	0.123	0.087		0.313	0.369	0.234	0.362	0.329
Buzivka	0.039	0.154	0.131	0.059	0.063	0.046	0.160	0.118	0.081	0.063	0.064		0.267	0.251	0.369	0.496
Uman'	-0.002	0.096	0.145	0.030	0.029	0.042	0.111	0.105	0.038	0.045	0.103	0.065		0.315	0.438	0.562
Borony	0.071	0.178	0.110	0.101	0.088	0.101	0.213	0.058	0.095	0.076	0.174	0.114	0.113		0.349	0.487
Glubokoe	0.025	0.196	0.151	0.087	0.065	0.118	0.192	0.061	0.082	0.017	0.142	0.083	0.088	0.077		0.519
Batevo	0.099	0.161	0.229	0.146	0.156	0.175	0.204	0.191	0.124	0.127	0.224	0.176	0.141	0.217	0.190	

[a]The numbers from 1 to 18 indicate the populations listed in the first column from Gorodijna to Batevo

Table A.3. Probabilities for heterozygosity excess (H. exc.) and heterozygosity deficiency (H. def.) at 9 and 11 microsatellite loci in populations of *Typha latifolia and T. angustifolia,* respectively.

Populations	IAM		SMM		TPM	
	H. def.	H.exc.	H. def.	H.exc.	H. def.	H.exc.
T. angustifolia						
Gorodijna	1.000	0.004	0.996	0.008	1.000	0.004
Glub2	0.949	0.062	0.003	0.998	0.517	0.517
Glub1	0.289	0.740	0.006	0.995	0.051	0.959
Zimovishe	0.793	0.232	0.103	0.913	0.618	0.416
Emerald	0.884	0.138	0.161	0.862	0.615	0.423
Atashev	0.650	0.382	0.003	0.998	0.139	0.880
Paryshev	0.997	0.005	0.674	0.367	0.981	0.024
Ivankov	0.993	0.009	0.688	0.348	0.920	0.097
Mekshunovka	0.406	0.656	0.234	0.813	0.344	0.711
Gubichi	0.986	0.020	0.844	0.191	0.963	0.098
Brusilov	0.998	0.004	0.998	0.004	0.998	0.004
Kiev	1.000	**0.0002**	0.973	0.034	0.999	**0.002**
Brusilov	0.966	0.042	0.650	0.382	0.840	0.183
Bel. Tserkov	1.000	**0.001**	0.545	0.500	0.990	0.014
Buzivka	0.981	0.024	0.500	0.545	0.787	0.248
Zarichevo	0.988	0.016	0.652	0.385	0.935	0.080
Chop	1.000	**0.001**	0.998	0.006	0.999	**0.001**
Batevo	0.461	0.577	0.065	0.947	0.216	0.813
T. latifolia						
Gluboky	0.289	0.766	0.027	0.980	0.039	0.973
River	0.781	0.281	0.578	0.500	0.719	0.344
Pripyat	0.674	0.367	0.019	0.986	0.150	0.875
Emerald	0.594	0.469	0.406	0.656	0.531	0.531
Dityatki	0.945	0.078	0.781	0.281	0.781	0.281
Mekshunovka	0.891	0.313	0.891	0.312	0.891	0.312
Gubichi	0.438	0.844	0.094	0.938	0.094	0.938
Chernigov	0.969	0.047	0.406	0.688	0.688	0.406
Kiev	0.891	0.313	0.406	0.688	0.406	0.688
Brusilov	0.656	0.422	0.016	0.992	0.423	0.656
Bel. Tserkov	0.977	0.039	0.078	0.945	0.718	0.344
Buzivka	0.367	0.674	0.064	0.976	0.150	0.875
Uman'	0.688	0.406	0.313	0.891	0.500	0.594
Borony	0.406	0.656	0.019	0.988	0.234	0.813
Glubokoe	0.473	0.578	0.230	0.809	0.320	0.727
Batevo	0.344	0.719	0.078	0.945	0.344	0.719

H.def. and H.exc. were calculated under expectations of the Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two-Phase Model (TPM). After Bonferroni correction only p-values $\leq$ 0.001 were significant, and they are indicated in bold.

Table A.4. Measures for data from two populations of *Typha angustifolia* indicating occurrence of genetic bottleneck at least at 50% of microsatellite loci.

			IAM					TPM				SMM			
Locus	***n***	**ko**	***H_o***	***H_e***	**SD**	**DH/SD**	**Prob.**	**Heq**	**SD**	**DH/SD**	**Prob**	**Heq**	**SD**	**DH/SD**	**Prob**
Gorodijna															
3	14	2	0.440	0.306	0.147	**0.907**	0.326	0.322	0.152	**0.772**	0.373	0.349	0.145	**0.622**	0.447
5	14	4	0.670	0.635	0.103	**0.340**	0.410	0.667	0.093	**0.034**	0.454	0.696	0.076	-0.336	0.329
7	14	2	0.538	0.305	0.148	**1.579**	0.047	0.332	0.148	**1.398**	0.055	0.337	0.149	**1.348**	0.059
8	14	2	0.440	0.311	0.148	**0.871**	0.343	0.329	0.149	**0.74**	0.378	0.346	0.142	**0.658**	0.42
13	14	1	Monomorphic												
15	14	1	Monomorphic												
16	14	2	0.538	0.3	0.146	**1.633**	0.036	0.332	0.146	**1.417**	0.057	0.35	0.15	**1.264**	0.065
18	14	3	0.670	0.505	0.131	**1.266**	0.095	0.544	0.12	**1.056**	0.156	0.562	0.112	**0.968**	0.201
19	14	1	Monomorphic												
20	14	2	0.527	0.299	0.15	**1.523**	0.135	0.332	0.146	**1.341**	0.158	0.349	0.148	**1.211**	0.187
21	14	1	Monomorphic												
Ivankov															
3	24	7	0.855	0.762	0.079	**1.173**	0.049	0.797	0.057	**1.008**	0.120	0.822	0.043	**0.786**	0.244
5	24	8	0.877	0.806	0.062	**1.147**	0.061	0.835	0.046	**0.910**	0.156	0.854	0.031	**0.750**	0.265
7	24	8	0.855	0.809	0.059	**0.777**	0.241	0.834	0.044	**0.492**	0.382	0.854	0.031	**0.049**	0.467
8	24	6	0.754	0.721	0.086	**0.376**	0.433	0.752	0.069	**0.025**	0.434	0.782	0.052	-0.542	0.257
13	24	3	0.583	0.438	0.154	**0.945**	0.220	0.479	0.141	**0.744**	0.282	0.521	0.123	**0.511**	0.383
15	24	2	0.083	0.260	0.157	-1.125	0.282	0.294	0.16	-1.32	0.225	0.3	0.158	-1.375	0.206
16	24	6	0.797	0.715	0.09	**0.914**	0.178	0.756	0.065	**0.626**	0.304	0.783	0.052	**0.281**	0.489
18	20	9	0.853	0.862	0.041	-0.231	0.318	0.877	0.035	-0.698	0.18	0.889	0.024	-1.496	0.084
19	24	4	0.728	0.559	0.134	**1.268**	0.054	0.6	0.119	**1.073**	0.096	0.649	0.088	**0.909**	0.18
20	24	3	0.627	0.437	0.152	**1.248**	0.100	0.476	0.144	**1.048**	0.151	0.525	0.123	**0.827**	0.21
21	24	1	Monomorphic												

The calculations were conducted under assumptions of Infinite Allele Model (IAM), Stepwise Mutation Model (SPM), and Two-Phase Model (TPM). H_o and H_e are observed and expected heterozygosities, respectively. SD is standard deviation. DH = H_o-H_e. Probability is the probability that $H_o > H_e$. Values in bold indicate heterozygosity excess.

Table A.5. Measures for two populations of *Typha latifolia* indicating occurrence of genetic bottleneck at least at 50% of polymorphic microsatellite loci.

			IAM					TPM				SMM			
Locus	***n***	**ko**	***H_o***	***H_e***	**SD**	**DH/SD**	**Prob.**	***H_e***	**SD**	**DH/SD**	**Prob**	***H_e***	**SD**	**DH/SD**	**Prob**
River															
3	44	4	0.701	0.494	0.155	**1.334**	0.053	0.554	0.133	**1.104**	0.083	0.615	0.102	**0.843**	0.184
5	40	1	Monomorphic												
7	44	2	0.169	0.216	0.163	-0.29	0.526	0.248	0.166	-0.473	0.446	0.262	0.164	-0.566	0.402
8	44	1	Monomorphic												
13	44	6	0.452	0.649	0.119	-1.657	0.076	0.704	0.092	-2.743	0.023	0.754	0.058	-5.155	0
15	42	2	0.418	0.232	0.166	**1.121**	0.23	0.248	0.165	**1.027**	0.245	0.271	0.163	**0.904**	0.288
16	44	4	0.61	0.497	0.152	**0.743**	0.275	0.55	0.13	**0.461**	0.38	0.614	0.099	-0.043	0.411
20	42	5	0.731	0.581	0.139	**1.072**	0.113	0.648	0.108	**0.766**	0.212	0.696	0.078	**0.447**	0.385
21	44	1	Monomorphic												
Emerald															
3	50	4	0.68	0.476	0.162	**1.26**	0.087	0.545	0.137	**0.988**	0.139	0.608	0.103	**0.697**	0.284
5	50	2	0.15	0.205	0.162	-0.337	0.53	0.244	0.164	-0.57	0.41	0.259	0.164	-0.661	0.381
7	50	5	0.26	0.564	0.145	-2.101	0.055	0.633	0.106	-3.514	0.007	0.692	0.077	-5.605	0
8	50	1	Monomorphic												
13	50	5	0.705	0.562	0.146	**0.981**	0.156	0.626	0.11	**0.724**	0.269	0.688	0.083	**0.211**	0.513
15	50	2	0.372	0.214	0.163	**0.972**	0.249	0.246	0.167	**0.757**	0.31	0.255	0.165	**0.711**	0.317
16	50	4	0.663	0.479	0.16	**1.151**	0.128	0.546	0.13	**0.9**	0.185	0.607	0.101	**0.555**	0.359
20	50	3	0.153	0.37	0.173	-1.256	0.171	0.418	0.161	-1.638	0.107	0.485	0.136	-2.434	0.035
21	50	1	Monomorphic												

The calculations were conducted under assumptions of Infinite Allele Model (IAM), Stepwise Mutation Model (SPM), and Two-Phase Model (TPM). H_o and H_e are observed and average expected heterozygosities, respectively. SD is standard deviation. DH = H_o -H_e. Probability is the probability that $H_o > H_e$. Values in bold indicate heterozygosity excess.

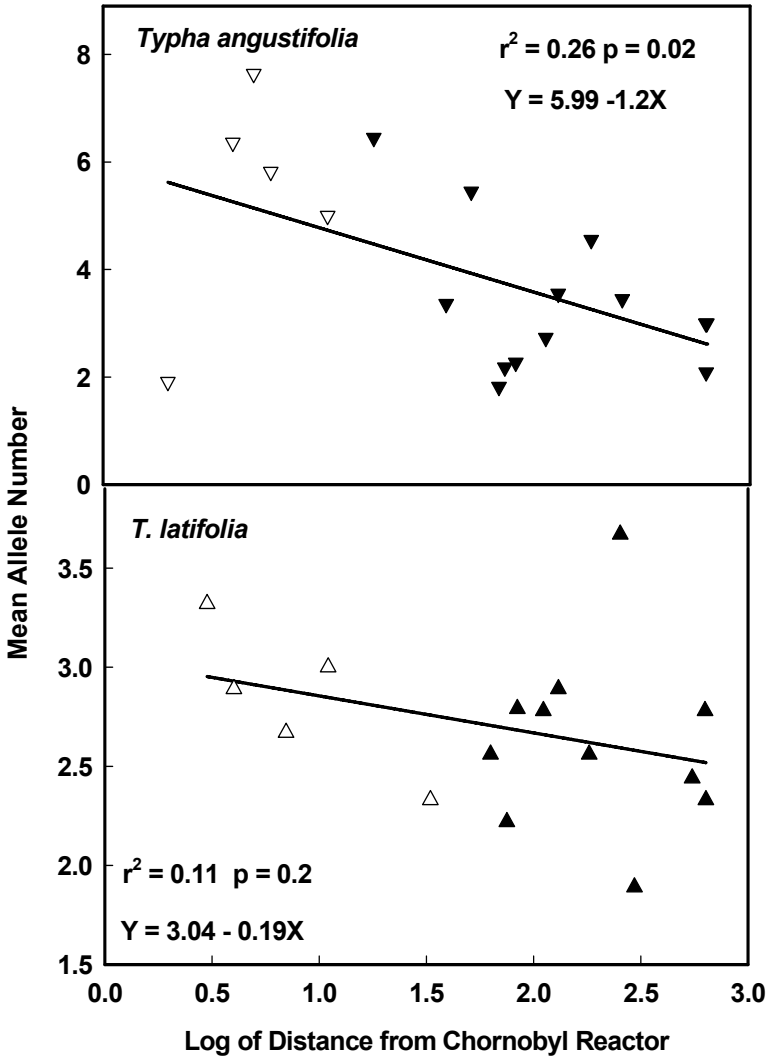

Figure A-1. Non-linear regressions between mean allele number (*MAN*) and the distance from the Chornobyl Nuclear Power Plant for *Typha latifolia* and *T. angustifolia*. The relationships are significant after data from outlier populations (in circle) excluded. Probability for a significant relationship, *p* value, is given for each case. Populations in the Chornobyl exclusion zone are indicated with white triangles.

CHAPTER 5

SEQUENCE VARIATION AT NON-CODING DNA IN CHORNOBYL PLANT POPULATIONS[1]

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Abstract

Radiation induced DNA changes are expected for plants and animals living in radioactively contaminated sites, such as Chornobyl. Sequence variability in non-coding DNA was examined in cattail (*Typha latifolia*) populations from five radioactively contaminated and 11 reference sites from Ukraine. There were 22 haplotypes observed in Chornobyl and reference populations, which had 10 and 21 haplotypes, respectively. Nine haplotypes were shared between Chornobyl and reference populations. There were no significant differences between Chornobyl and reference populations for genetic diversity characteristics except of the number of unique haplotypes, which was lower in Chornobyl populations. There was no correlation between genetic and geographic distances among populations probably due to high spatial heterogeneity, which was also indicated by a lack of phylogeographic structure. This level of heterogeneity made it difficult to document significant differences between Chornobyl and reference populations. Total genetic variance partitioned among populations was very high (45%), but the variation detected between Chornobyl and reference populations was only 3%. The genetic differences were calculated for each individual from contaminated populations by comparing its sequence to a consensus sequence for the reference populations. The number of differences increased significantly with increasing concentrations of radiocesium and radiostrontium. The two most contaminated populations showed significantly higher numbers of individual genetic differences than the other contaminated populations. The correlations between individual genetic changes and the concentrations of radioactive isotopes is an evidence for a radiation effect on these plants. This effect is demonstrated within the contaminated populations and is in contrast to the lack of effect between contaminated and reference populations.

Introduction

Individuals, populations, and species express different levels of sensitivity to contaminants (Woodward et al., 1996). Exposure to contaminants can cause changes in genetic composition of populations and affect their ecology and evolution (Theodorakis and Shugart, 1998). Plant populations growing in environments contaminated with metals frequently evolve a high tolerance level (Shaw, 1994). Contaminants may change genetic diversity of populations by selecting tolerant and eliminating sensitive genotypes (Guttman, 1994; Shugart and Theodorakis, 1998). Studying genetic diversity of populations from contaminated environments, such as Chornobyl, is important for understanding radiation effects. Radiation causes "genetic load" by accumulation of deleterious mutations (Muller, 1950; Wallace, 1991), which eventually decreases fitness and survival of individuals in a population.

The accident at the Chornobyl Nuclear Power Plant (ChNPP) in 1986 released from 100 to 200 million Ci into the atmosphere (Sich, 1994). Even though animal and plant species living around the failed reactor are diverse and abundant (Baker et al., 1996), several studies have observed elevated mutation levels in some of them (Dubrova et al., 1996, 1997, 1998; Ellegren, 2000; Kovalchuk et al., 2000, 2003; Matson et al., 2000). However, other studies failed to show any significant radiation effects (Baker and Chesser, 2000; Baker et al., 1999; DeWoody, 1999; Livshits et al., 2001; Rodgers and Baker, 2000). Comparing genetic structure of contaminated populations to that of reference populations may reveal sensitive indicators for the effects of stress on populations (Guttman, 1994). Changes in genetic diversity in radioactively contaminated populations are expected and have been shown in several studies with some demonstrating decreases (Baker et al., 2001; Oleksyk, 2001; Shugart and Theodorakis, 1998;

Theodorakis and Shugart, 1997) or increases in genetic diversity (e. g., Nevo et al., 1986; Strittholt et al., 1988; Chapter 4).

Comparisons of contaminated and reference populations need to be made by taking potential confounding factors into the account. There are many biogeographical factors that influence genetic variability of populations, and they must be considered in addition to the type and level of contamination. Studies that examine genetic effects of contaminants at the population level must define the structure and naturally occurring variation within and among populations (Belfiore and Anderson, 1998). Comparisons between multiple reference and radioactively contaminated populations are necessary to document genetic differences due to radiation.

Highly variable genetic markers with relatively high mutation rates, such as non-coding DNA sequences and microsatellites, are necessary to study the effects of radiation on natural populations using a relatively small number of samples. A limited number of studies have used DNA sequences to examine genetic diversity in Chornobyl animal populations (Baker et al., 2001; DeWoody, 1999; Matson et al., 2000). These studies showed no convincing evidence for genetic effects of radiation partly because of the markers chosen and the limited number of reference populations used. However, microsatellite diversity was higher in *Typha* from Chornobyl than from reference populations (Chapter 4). Non-coding DNA has much higher variability than coding DNA and can be used as a genetic marker to characterize diversity of contaminated populations. Our study is the first to use non-coding DNA sequence to examine genetic diversity in radioactively contaminated natural plant populations.

Our objective was to compare genetic variation in the non-coding DNA region between contaminated cattail populations (*Typha latifolia)* around the Chornobyl failed reactor and

reference populations from Ukraine. The reference populations were necessary to establish a baseline to statistically test for radiation effects. To achieve the objective, we need to establish the patterns of partitioning of genetic variance within and among populations and between Chornobyl and reference groups. Phylogeographic relationships among populations were established since they can have important effects on this partitioning. Finally, possible relationships of individual or population genetic characteristics with individual or mean radionuclide concentrations needed to be tested. We expected for the Chornobyl populations to have higher levels of genetic variability and show relationships between the genetic changes and the amount of radioactive contamination.

Materials and Methods

Sampling and DNA techniques

A number of samples ($N = 227$) were collected from 11 reference and 5 Chornobyl populations of *T. latifolia*. The sampling locations and population sample sizes are given (Appendix B, Fig. B-1). The five radioactively contaminated sites were located within the Chornobyl Exclusion Zone. Reference sites were located to the west, east, and south from the reactor. A geographical positioning system was used to determine coordinates for each location, and their exact coordinates and general sampling procedures are given in Chapter 4. The leaf tops (about 20 cm in length) were placed in silica gel, dried, crushed in liquid nitrogen, and DNA was isolated using Qiagen Kit. Ten primer pairs were designed using Oligo 6.67 (Molecular Biology Insights) from the sequences obtained while developing microsatellite primers (Tsyusko-Omeltchenko et al., 2003). The sequences contained no more than four dinucleotide repeats or sequences located before or after microsatellite repeats were used for developing primers. PCR

amplifications were performed in a 25 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/mL BSA, 0.5 μM of each primer, 3 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 U JumpStart Taq DNA Polymerase (Sigma), and 50ng DNA template). The cycling conditions for the reactions were described in Tsyusko-Omeltchenko et al. (2003). Amplified products were sequenced using a capillary Sequencer on ABI PRISM® 310 Genetic Analyzer according to the manufacturer's instructions, and some sequences were generated on a ABI 377-96 sequencer using BigDye Terminator 2.0 (Applied Biosystems). Sequences from forward and reverse strands were assembled and edited in Sequencer 4.1. (Genecodes, Ann Arbor, MI). Of 10 developed primer pairs polymorphism was detected only for one pair for *T. latifolia*. The primers used for amplification were: *Typha*1U (TCG CTG AAA GTG ACA TAC) and *Typha*1L (TCA TTA TGG CAT TTA ACT TC). DNA regions amplified with these primer pairs were used for further analyses.

Radioactivity measurements were conducted as described in Chapter 4.

Data analyses

Edited sequences were aligned using Sequence Navigator 1.0.2 (Applied Biosystems). DNASP ver. 2.2 (Rozas and Rozas, 1999) was used to calculate nucleotide diversity (*Pi*; global, population, and Chornobyl vs reference), haplotype diversity (*Hd*), average number of nucleotide differences between whole sequences (*K*), and the number of polymorphic sites (*S*). To solve the problem of heterozygous base pairs and make it possible to analyze the data with a program DNASP, 240 sequences were duplicated giving a total of 480 haplotypes. If a sample had heterozygous sites, then duplicated sequences differed at those sites. Wilcoxon rank sum test (Sokal and Rohlf, 1995) was used to compare the genetic diversity characteristics between Chornobyl and reference populations. Series of non-linear regressions were conducted to test for

relationships between the diversity characteristics of each population and its geographical distance from the reactor. A consensus sequence was generated from combined samples from reference populations using Sequencher 4.1, and each individual sample from Chornobyl populations was compared to the consensus sequence to calculate the individual differences between the sequences. Non-parametric Spearman's correlation (r_S) was used to assess a level of association between the number of differences and radionuclide concentrations for 45 individuals from Chornobyl populations. The number of differences fell into five categories defined as categories with 2-3, 4, 5, 6, and 7 differences. Kruskal-Wallis test was used to test for differences in radionuclide concentrations among the categories. Data for individual radiocesium and radiostrontium concentrations were not normally distributed, and none of the applied transformations made the data normally distributed. These data were ranked, and Duncan's test (1955) was used to determine which of the categories were significantly different for mean radionuclide concentrations. Most of the statistical analyses were conducted in SAS 8.1 (2000).

Variance of haplotype data among populations and between Chornobyl and reference groups was partitioned using analyses of molecular variance (AMOVA; Excoffier et al., 1992) in Arlequin (Schneider et al., 2000). A Kimura two-parameter model (Kimura, 1980) was used to conduct AMOVA. Arlequin program accepts and can analyze input file containing heterozygous sequences; therefore only one haplotype per sample was included in the analyses. Variance was partitioned among population groups defined by their latitude, longitude, and geographical locations. The effects of longitude and latitude on genetic diversity were tested using a Wilcoxon rank sum statistic. Phylogeographic relationships for all populations were examined using the maximum parsimony algorithm in PAUP 4.1. (Swofford, 1999). Genetic distances (F_{ST})

between each population pair were calculated using DNASP (Rozas and Rozas, 1999), and Mantel test (1965) was used to calculate correlations between genetic and geographic pairwise distances. Mantel tests were conducted when all populations were included in the analysis and after Chornobyl populations were removed.

Statistical significance was indicated by $p \leq 0.05$.

Results

The length of the non-coding DNA region analyzed in *T. latifolia* was 424 bp. A total of 12 polymorphic sites were observed in this region. There were four transitions, two transversions, and four indels (two of two bp, and two of one bp in length). Each indel was considered as a single mutation. Heterozygous individuals showed ambiguous base pairs at one or two sites (87 and 394). Of 22 distinct haplotypes, there were two that were common with frequencies of 21 and 16%, 14 with frequency less than 10%, and 6 with frequency less than 1% (Fig. 5.1). Diversity characteristics are given in Table 5.1 and Fig. 5.2. Overall total haplotype diversity was relatively high, but total nucleotide diversity was low. Chornobyl and reference populations had a total of 10 and 21 haplotypes, respectively with one unique in Chornobyl and nine in reference populations (Fig 5.1B). Reference unique haplotypes occurred mostly in two of the southern populations (Uman' and Buzivka) and in one western population (Borony). *K*, *Pi,* and H_o were slightly lower in reference than in contaminated populations. When Chornobyl and reference groups were compared, there were no significant differences between them for any of the five characteristics ($p > 0.05$). Diversity characteristics varied widely among populations (Table 5.1).

There were significant correlations between the number of individual differences and radiocesium or radiostrontium concentrations ($r_S = 0.35$ and $p = 0.01$ for ^{137}Cs; $r_S = 0.43$ and $p = 0.003$ for ^{90}Sr). Number of differences varied significantly among contaminated populations with two (Gluboky Lake and River) having the highest (6 - 7) and one (Emerald) the lowest (2 - 3) number of differences. Individual plant concentrations of ^{137}Cs and ^{90}Sr varied significantly among five categories of the individual differences (Fig. 5.3; $p < 0.001$). Range of radiostrontium concentration was larger than that for radiocesium. Duncan's test showed that category of 6 differences had the highest and category of 2-3 differences the lowest mean radioactivity for both radionuclides.

There were no significant relationships between the five population characteristics and distance from the reactor ($p > 0.05$). Visual inspection of the regression plots did not reveal consistent patterns of the increasing or decreasing of the characteristics with the distance from the reactor. Pairwise F_{ST} varied from 0.011 to 0.99 with an average of 0.26. Chornobyl populations had lower F_{ST} (average of 0.055) than did reference populations (average of 0.24). Mantel test failed to show significant relationships between pairwise genetic and geographic distances when all populations were analyzed ($r = -0.11$ $p = 0.22$). There were significant negative relationships when data from reference ($r = -0.26$ $p = 0.04$) but not Chornobyl ($r = 0.04$ $p = 0.32$) populations were analyzed.

AMOVA results are given in Table 5.2. There was a high amount of variation for haplotype differences among populations (45%). The percentage of variation within populations was 55% ($p < 0.001$). There was 3% of the variation partitioned between Chornobyl and reference groups, but it was not significant. There was less than one percent of the variation among groups defined by latitude or area, but it was higher (5 % and $p = 0.04$) when defined by

longitude (seven groups). However, there were no significant effects of latitude or longitude on genetic diversity characteristics when tested with Wilcoxon rank sum test. Dendrograms failed to demonstrate a phylogeographic structure among *Typha* populations: there were no distinct clades according to the locations of the populations (Appendix B, Fig. B-2). Some populations that were geographically adjacent occurred together in the tree but others did not. Values calculated from 10,000 bootstrap runs were below 70.

Discussion

Typha latifolia showed relatively high sequence variability in the non-coding DNA, which was also found for the microsatellite data (Chapter 3). There was a large amount of differentiation detected for non-coding DNA among *Typha* populations. Species like *Typha*, which use selfing as part of their reproductive process, normally show high spatial heterogeneity among populations. Another self-compatible plant, *Solanum pimpinellifolium*, has high population differentiation in non-coding DNA (Caicedo and Schaal, 2004). Spatial heterogeneity was also high among *Typha* populations when microsatellites were used (Chapter3). Both of the highly variable neutral genetic markers show a lot of spatial heterogeneity in contrast to that of invariant allozymes (Sharitz et al., 1980).

Limited gene flow is expected in selfing plants (Hamrick and Nason, 1996) and those with vegetative reproduction. Isolation by distance would normally result in higher pairwise F_{ST} for populations located far apart but low F_{ST} for those close together (Wright, 1978). F_{ST} were smaller when distances between populations were small as in the Chornobyl Exclusion zone (average F_{ST} = 0.055). However, there was a lack of correlation between genetic and geographic distances when data for both Chornobyl and reference populations were analyzed, and the

correlation was even negative for the reference populations alone. This can only happen when some distant populations have haplotypes in common. For instance, Mekshunovka and Glubokoe were located 740 km apart and shared four widely distributed haplotypes, while Buzivka and Uman', two southern populations, located 20 km apart did not have even one haplotype in common. The latter two populations had the highest number of unique haplotypes. The most likely reason for the negative correlation between genetic and geographic distances was high stochastic variation that created similarities among some distant populations. Some haplotypes could have been more widely distributed in the past.

A similar percent of variation was distributed among (45%) and within (55%) populations. Variation among groups of populations was significant when the groups were defined by longitude but not by latitude or area. The longitude effect was not clinal and did not account for much variation (5%), and the effect is probably due to the occurrence of similar populations within some groups by chance. High spatial heterogeneity and lack of phylogeographic structure may explain why only 3% of the variation was observed between Chornobyl and reference populations. The sample sizes and number of reference populations were insufficient to statistically document an effect this small given the variability of the reference populations, which was partly due to the relatively high polymorphism of the genetic marker used. The results of this study are not consistent with those of other studies of Chornobyl plant and animal populations using microsatellites, such as *Typha* (Chapter 4) and *Apodemus flavicollis* (Oleksyk, 2001). The differences in the nature of the markers, breeding biology, and radiosensitivity of the species probably account for the inconsistencies.

Even though there were no significant differences in mean haplotype number and haplotype diversity between contaminated and reference *Typha* populations (Table 5.1), the total

number of haplotypes was smaller for Chornobyl than reference populations (10 vs 21, respectively, Fig. 5.1). The smaller number of Chornobyl populations included into the analysis may be part of the reason for this difference. The difference was not due to geographic location, because area did not account for a significant amount of variation. Selection against some haplotypes in the contaminated populations could have contributed to this difference. Exposure to contaminants can act as a selective force for non-coding regions only if they are linked to coding regions. Selection for heterozygotes with higher fecundity was observed for radiated mosquitofish (*Gambusia affinis*, Shugart and Theodorakis, 1998).

The absence of significant relationships between genetic diversity characteristics and distance from the failed reactor is inconsistent with the microsatellite results, which showed significant increases in genetic diversity in populations located closest to the reactor (Chapter 4). Some microsatellite loci contributed more than did others to the differences between Chornobyl and reference populations, and having nine loci and larger sample sizes made it possible to detect small but significant differences between Chornobyl and reference populations (Chapter 4). Using sequences from only one polymorphic DNA region is a definite disadvantage of this study.

There were enough data to document relationships for an increase in the number of genetic differences between the reference consensus sequence and individual sequences from Chornobyl samples with increasing radionuclide concentrations (Fig. 5.3). The relationship was more significant for strontium than for cesium most likely due to the wider range of strontium concentrations. The number of differences was higher for the two most contaminated populations, Gluboky Lake and River, but the lowest for one of the less contaminated populations, Emerald. Because of the skewed distributions of radionuclides in plants and

animals from areas around the failed reactor (Oleksyk et al., 2002; Smith et al., 2002), the variances of radionuclide concentrations in a population can be large. The stochastic nature of populations also increases the variance among them. The small number of Chornobyl populations, scatter in the data, and lower confidence in the genetic estimates from a non-coding region make it difficult to detect relationships between the means of radionuclide concentrations and genetic characteristics.

Spatial heterogeneity may be the rule for reference plant and animal populations in studies of radiation effects (e. g., Chapters 3 and 4, Oleksyk, 2001; Baker et al., 2001). This is especially true for organisms with reproductive traits like those of *Typha* (Chapter 3). This makes the questions of which and how many reference sites to choose important for establishing an adequate baseline for natural population variability. Depending on the chosen reference populations, the conclusions concerning radiation effects can include strong positive or negative effects, weak effects with no significant difference between contaminated and reference populations, or no effects at all. A general principle for the study of effects of any contaminant on genetic diversity of plant or animal populations is that most samples should come from reference populations because of the high level of spatial heterogeneity in the genetic data.

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Table 5.1. Genetic diversity within populations of *Typha latifolia*

Population	Distance from the reactor (km)	*N*[a]	Number of Haplotypes (*Nh*)	Haplotype diversity (*Hd*)	Nucleotide diversity (*Pi*)	*Ho*[d]	*K*[b]
1. Gluboky Lake	3	30	6	0.713	0.0022	0.73	0.487
2. River	4	30	3	0.579	0.0019	0.93	1.030
3. Prypiat	7	30	5	0.604	0.0021	0.20	0.430
4. Emerald	11	34	6	0.808	0.0025	0.33	0.667
5. Dityatki	33	30	2	0.516	0.0013	0.93	0.516
Chornobyl (Mean)	**-**	**31.6**	**4.4**	**0.644**	**0.0020**	**0.62**	**0.626**
Chornobyl (Total[c])	-	158	10	0.867	0.0059	0.48	1.928
6. Mekshunovka	63	32	5	0.714	0.0020	0.88	0.681
7. Gubichi	75	32	1	0.000	0.0000	0.00	0.000
8. Chernigov	84	32	3	0.339	0.0013	0.13	0.016
9. Kiev	111	32	5	0.806	0.0023	0.13	0.411
10. Brusilov	130.5	16	3	0.600	0.0006	0.00	0.233
11.Bel. Tserkov	181.5	32	5	0.635	0.0014	0.06	0.504
12. Buzivka	253.5	18	7	0.850	0.0041	0.44	1.340
13. Uman'	295.5	28	6	0.775	0.0030	0.70	1.017
14. Borony	549	26	7	0.893	0.0024	0.77	0.806
15. Glubokoe	630	30	6	0.532	0.0015	0.29	0.267
16. Batevo	636	32	2	0.726	0.0020	0.38	0.798
Reference (Mean)	**-**	**28.2**	**4.5**	**0.625**	**0.0019**	**0.34**	**0.552**
Reference (Total)	-	296	21	0.872	0.0025	0.33	0.787
Total	-	454	22	0.880	0.0026	0.43	1.056

[a]Number of sequences
[b]Average number of nucleotide differences
[c]Total was calculated from individual data combined across populations
[d]Observed heterozygosity

Table 5.2. Analyses of Molecular Variance calculated from haplotype data for *Typha latifolia* using a Kimura two-parameter model (Kimura, 1980).

Groups	Source of variation	df	Percentage of variation
16 populations	Among populations	15	45.1**
	Within populations	210	54.9**
	Total	225	
Chornobyl and Reference groups	Between groups	1	3.1ns
	Among populations within groups	14	42.9**
	Within populations	210	54.0**
	Total	225	
Groups defined by area[a]	Among groups	4	< 1ns
	Among populations within groups	11	54.1**
	Within populations	210	55.3**
	Total	225	
Groups defined by longitude[b]	Among groups	6	5.2*
	Among populations within groups	9	40.3**
	Within populations	210	54.5**
	Total	225	
Groups defined by latitude[c]	Among groups	3	0.95ns
	Among populations within groups	12	44.3**
	Within populations	210	54.7**
	Total	225	

**Significance level indicated at $p < 0.001$, *$p < 0.05$, and ns$p > 0.05$ for associated Fs

[a]Five groups

[b]Seven groups

[c]Four groups

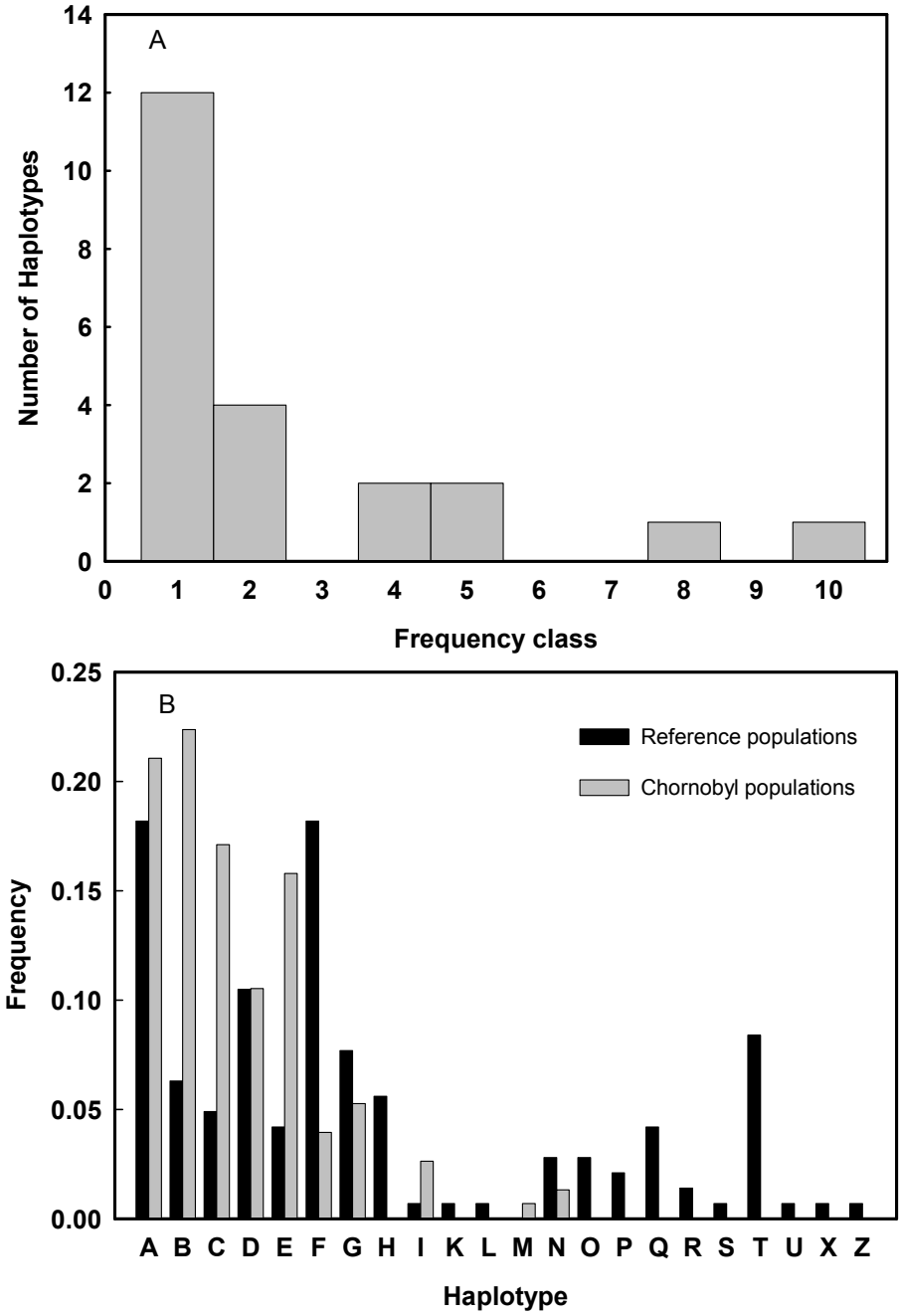

Fig 5.1. Distributions of haplotypes in *Typha latifolia* populations. A. Number of haplotypes combined across populations per frequency class (from the lowest to the highest frequency). B. Distribution of 22 haplotypes (A – Z) in reference (black bars) and Chornobyl (gray bars) populations.

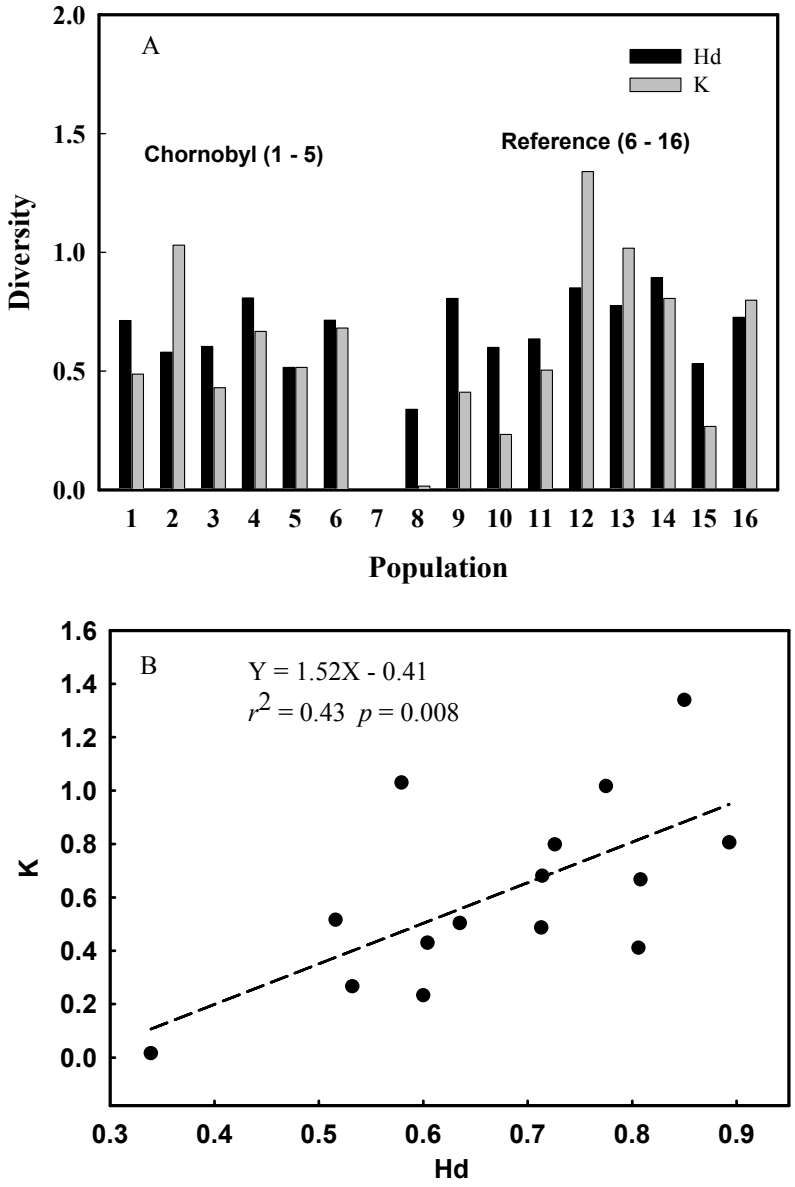

Fig. 5.2. Haplotype diversity (*Hd*) and average number of nucleotide differences (*K*) for each population (A) and relationships between these variables (B). Population names (1-16) are given in Table 5.1 following the same order.

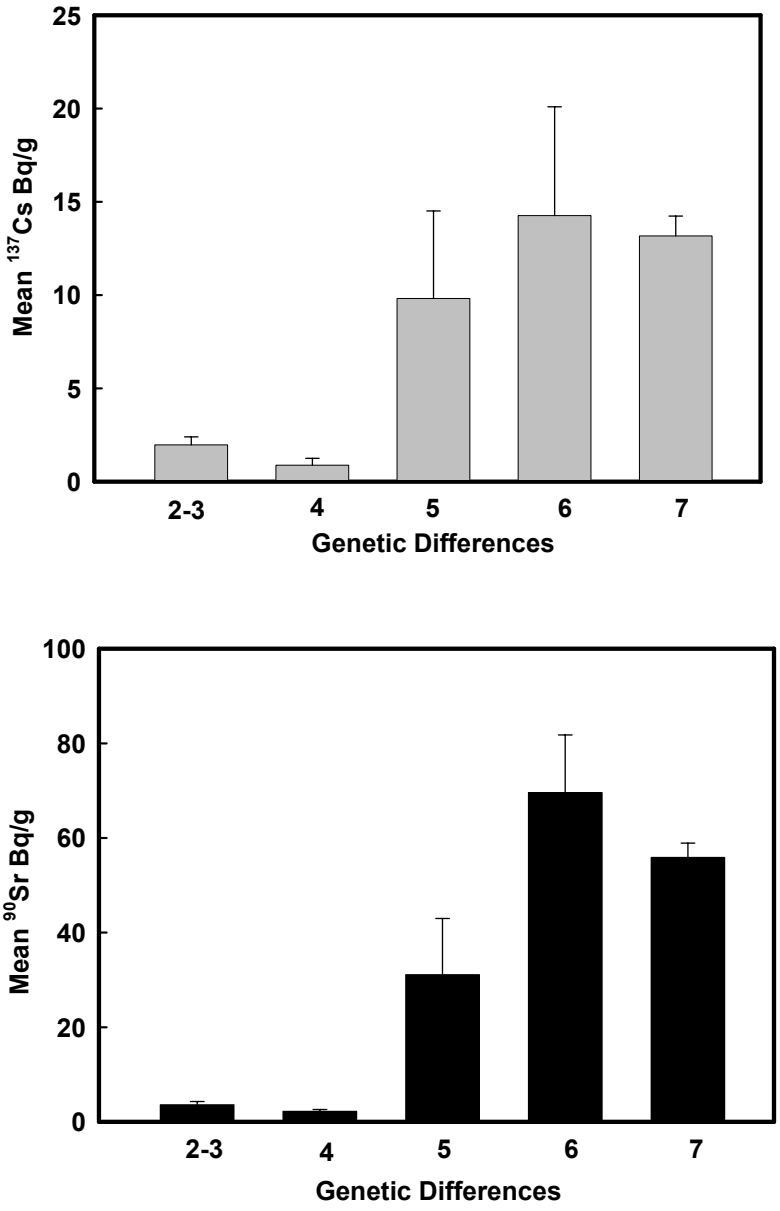

Fig 5.3. Mean radiocesium (A) and radiostrontium (B) concentrations for each category of individual genetic differences. The number of the differences was calculated from comparisons of reference consensus sequence with sequence of each individual sample from Chornobyl. There are 5 categories with 2-3, 4, 5, 6, and 7 individual genetic differences.

APPENDIX B

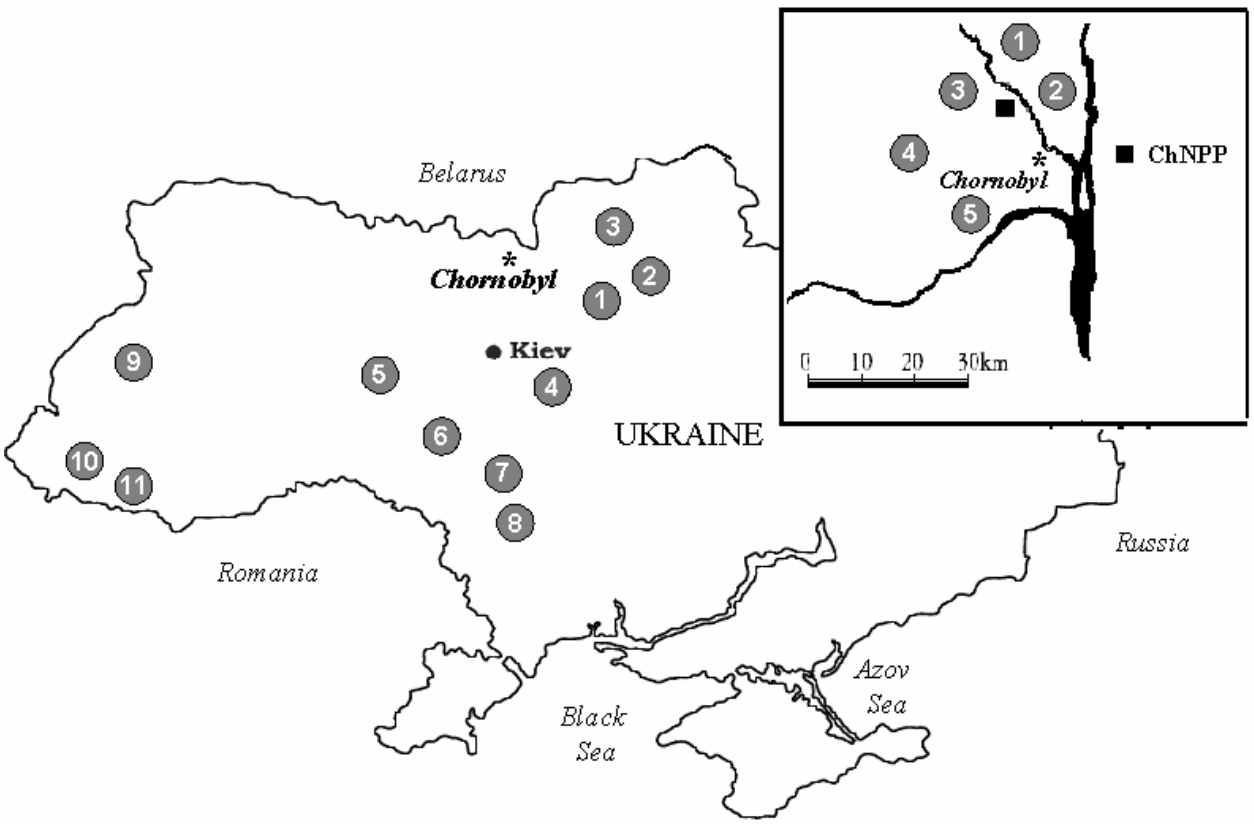

Fig. B-1. Sampling sites for 16 populations of *Typha latifolia* in Ukraine. The square indicates locations of the 5 Chornobyl sites: 1 – Gluboky Lake, 2 – River, 3 – Prypiat', 4 – Emerald, and 5 – Dityatki. There are 11 reference sites: 6 – Mekshunovka, 7 – Gubichi, 8 – Chernigov, 9 – Kiev, 10 – Brusilov, 11 – Bel. Tserkov, 12 – Buzivka, 13 – Uman', 14 – Borony, 15 – Glubokoe, and 16 – Batevo.

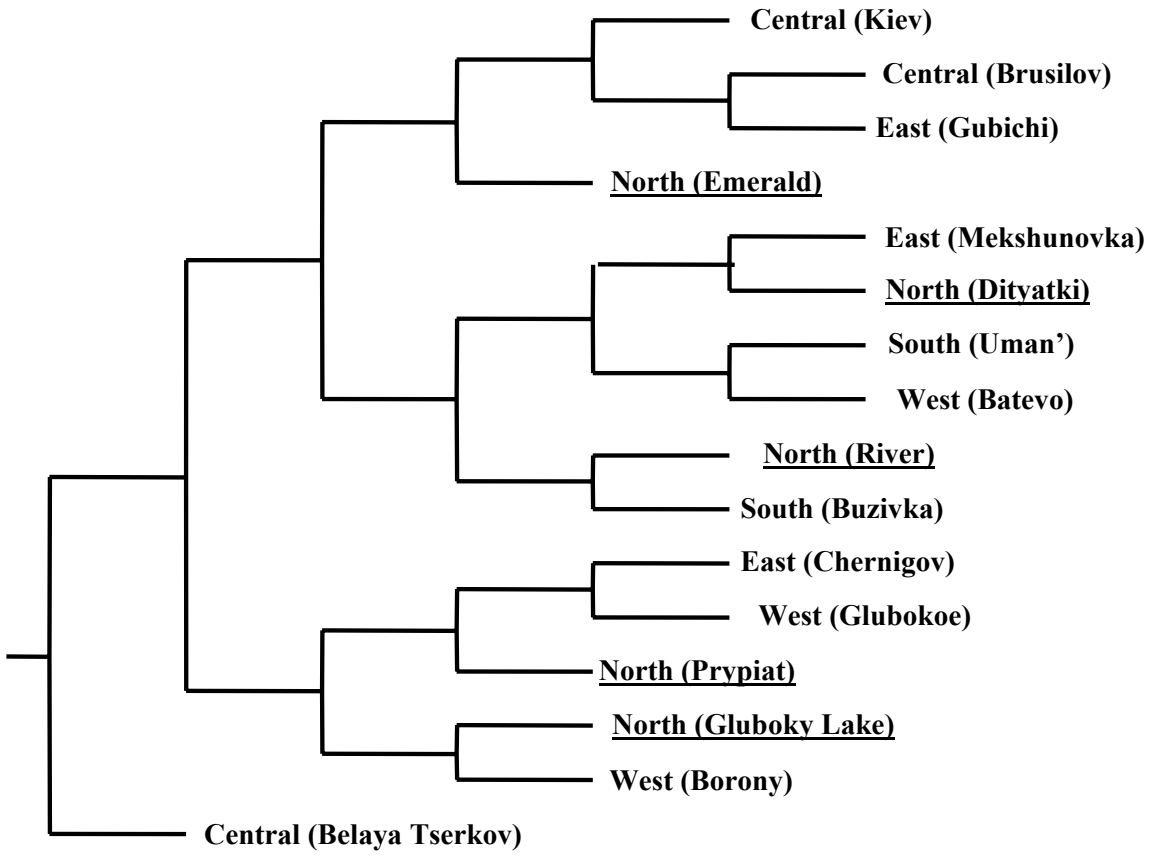

Fig. B-2. Phylogeographic relationships among 16 populations of *Typha latifolia* from Ukraine. The tree is generated based on non-coding DNA region. Samples underlined are representatives from the Chornobyl Exclusion Zone.

SUMMARY

Overall objective of this study was to estimate radiation effects on genetics of cattail populations (*Typha angustifolia and T. latifolia*) from areas around the Chornobyl Nuclear Power Plant in Ukraine. First, genetic variation in multiple reference populations from Ukraine was described to establish a baseline for their comparisons with that of Chornobyl populations. Both *Typha* species were characterized by high spatial heterogeneity among populations and lack of phylogeographic structure. *Typha*'s breeding system is likely to be primary factor responsible for the observed high variation among populations. Selfing and vegetative propagation of cattails reduce gene flow among populations, increase their spatial isolation, and partially explain low or no correlation between genetic and geographic distances. The amount of variation between species was higher than that within or among populations. *Typha angustifolia* showed more variability than *T. latifolia* as indicated by *MAN*, H_o, and H_e. In addition, *T. angustifolia* had more alleles and many of them had low frequency. This species probably experience more founder events than *T. latifolia*. Isolation by distance was shown by *T. latifolia* but not *T. angustifolia*. The established baseline for variability was relatively high for reference populations of each *Typha* species complicating the evaluation of radiation effects.

Despite the high background variability, several genetic effects that were most likely due to radiation were observed in Chornobyl populations. Contaminated populations showed significant differences for most of their genetic and genotypic characteristics when compared to reference populations. Populations closest to the failed reactor demonstrated the highest amount of variability. There were also significant positive correlations of several diversity indices with

mean radionuclide concentrations in both species. More diversity characteristics showed these correlations in *Typha angustifolia* than *T. latifolia* even though the latter had overall higher radionuclide concentrations. A small but significant amount of variation (ca. 5%) in haplotype differences between Chornobyl and reference populations was detected in *T. latifolia*, and its Chornobyl populations created a distinct group in the dendrogram. However, not all genetic diversity characteristics of Chornobyl populations showed positive correlations with mean radionuclide concentrations. Isolation by distance was still observed in *T. latifolia* when data from Chornobyl populations were added to the analyses. Chornobyl populations due to smaller geographic distances among them had smaller genetic distances than did reference populations suggesting increased gene flow, which may have resulted in the increased variability. Ecological stress factors such as extreme drought could have stimulated gene bank effects and also contributed to the genetic changes of Chornobyl populations. Thus, some of my microsatellite results indicate occurrence of radiation effects in Chornobyl populations, but this conclusion can be questioned when other factors are taken into account.

Another genetic marker in addition to microsatellites used in this study was a non-coding DNA. A higher amount of spatial heterogeneity among *T. latifolia* populations was detected with this marker than with microsatellites. The amount of variation among (45%) was similar to the one within populations (55%), and there was no phylogeographic structure. The percent of variation between Chornobyl and reference populations was not significant due to the high spatial heterogeneity and limited number of samples. There were also no significant differences in number of haplotypes between Chornobyl and reference populations. However, the number of unique haplotypes was significantly smaller in Chornobyl than reference populations. Correlations between individual genetic changes and concentrations of radionuclides most

probably indicate occurrence of radiation effects. Number of genetic differences for each individual from Chornobyl was calculated by comparing its sequence to a consensus sequence for the reference populations. The number of these differences correlated positively with radioisotope concentrations. There were some inconsistencies in the results obtained with the two genetic markers, and these are likely due to the larger number of loci and sample sizes used with microsatellites.

In conclusion, this study provides insight into complicated pattern of spatial variation and the effects of radiation on natural plant populations from Chornobyl. Increased genetic variability of Chornobyl populations and positive correlations of their diversity characteristics with radionuclide concentrations are evidence for radiation effects. However, these effects occur in a background of high natural variability emphasizing the importance of other factors including ecology, biology, and geography. These factors must always be considered when studying the influence of any contaminant on genetic variability. The results of any study that fails to include these factors have to be suspect.