1 Introduction

1.1 General Introduction

The rate of species extinctions due to anthropogenic activities has dramatically increased during the past few centuries (Dirzo & Raven, 2003; Novacek & Cleland, 2001). Although the mechanisms and ultimate causes leading to the extinction of species remain largely unclear (Frankham et al., 2002), five threats to global biodiversity have frequently been referred to as the most important: habitat destruction and fragmentation, global climate change, hunting and overuse of food resources, biological invasions and environmental pollution (Dudgeon et al., 2006; Lewis, 2006; Novacek & Cleland, 2001). Different research fields, as conservation biology, ecology and ecotoxicology, aim to investigate the effects of these factors on organisms and found strong evidence for their negative impact on regional and global biodiversity.

In most cases, natural populations will be impacted not only by one threat, but rather a combination of them (Buckley & Roughgarden, 2004; Kappelle et al., 1999). Multiple environmental stress factors might have cumulative negative effects on the survival of populations (Sih et al., 2004). Revealing how natural populations respond to combinations of different stress factors is thus of crucial importance in order to understand our present and future impact on all scales of biodiversity (Warren et al., 2001).

The effects of anthropogenically introduced chemicals on organisms and ecosystems are investigated in the field of ecotoxicology. Research in this area has led to a large body of information concerning the impact of chemical stress on the fitness of model species in the laboratory. In contrast to this, there is an obvious lack of knowledge on the effects of contaminants on natural populations and communities (Bickham et al., 2000; Bourdeau et al., 1990). For instance, ecotoxicologists have just started to investigate the impact of environmental pollution on the genetic variability of natural populations (Bickham et al., 2000; Whitehead et al., 2003). Genetic variation provides the raw material for populations in order to adapt to changing environmental conditions and is thus the substrate for the evolution and long-term survival of populations and species (Frankham, 2005). The amount of genetic variation in populations is positively correlated with its effective population size (Frankham, 1996). Habitat destruction and fragmentation has divided the ranges of many species into small and isolated refuges. Without migration from adjacent habitats, isolated populations will decrease in their level of genetic diversity through random loss of alleles

(Hedrick, 2000). Frankham (1995) for instance, showed that 32 of 37 endangered species (which occur in small populations per definition) of different animals and plant taxa display reduced levels of heterozygosity compared to closely related and more widespread species. In strongly human impacted landscapes, both factors, environmental pollution and habitat destruction, can be expected to occur frequently together. It is thus of crucial importance to investigate the impact of reduced genetic diversity and inbreeding on the reaction to chemical stress. In addition, chemical exposure has frequently been discussed to have an impact on the extent of genetic variability in exposed populations (Guttman, 1994; Staton et al., 2001; van Straalen & Timmermans, 2002). However, evidence for this 'genetic erosion hypothesis' remained scarce to date, most likely because of the difficulty to single out the impact of pollution stress from a background of multiple factors influencing patterns of genetic variability in natural populations (Belfiore, 2001; Staton et al., 2001; van Straalen & Timmermans, 2002).

1.2 Outline of the Study

The general scope of this thesis was to investigate if genetic variation decreases in populations exposed to xenobiotics and if reduced genetic variation affects tolerance towards chemical exposure. In detail, this work is focusing on the following questions:

i) Does environmental pollution reduce genetic variability of populations in the laboratory and in the wild?

ii) Are genetically impoverished populations more susceptible towards chemical stress?

iii) Do genetic variation and inbreeding affect the outcome of ecotoxicological exposure tests?

Genetic tools were developed and applied in order to investigate the effects of reduced genetic variation and chemical stress on populations in the laboratory and in the field. The choice of a suitable model species is of crucial importance in ecological genetics and ecotoxicology. The chosen model organisms should ideally be of great ecologically importance, widely distributed, and easy to culture under laboratory conditions (Lowe et al., 2004). For the experiments conducted in this study, the non-biting midge *Chironomus riparius* Meigen 1831 was chosen as a model organism based on the following reasons: Chiromids are a worldwide distributed family of nematocerans that occupy a wide range of

fresh water environments. They dominate many limnic habitats both in biomass and species richness (Armitage et al., 1995). Furthermore, chironomids play a key role in these environments of high detritus consumption rates. In addition, chironomids are an important nutrition source for a variety of predators, as fishes, water foul and many invertebrate taxa (Armitage et al., 1995).

The species *Chironomus riparius* is a model organism in aquatic ecotoxicology and is frequently used in sediment and fresh water toxicity tests (Vogt et al., 2007a). The species is easy to culture in the laboratory and has a short generation time of only three to four weeks under laboratory conditions.

In order to measure genetic variation in *C. riparius* populations in the laboratory and the field, microsatellite markers were developed for this species (**Chapter 2**). The markers were also tested on the closely related species *C. piger* in order to allow for species discrimination in field samples.

The third chapter (**Chapter 3**) addresses the question, whether environmentally relevant concentrations of the highly toxic biocide tributyltin (TBT) affect genetic variation in *C. riparius* populations. To this end, strains of the species were kept for 12 generations either exposed to TBT or under control conditions, and both genetic variation and several life-history traits were monitored over time.

The finding that genetic variation is negatively impacted by chemical stress leads to the question, if genetic erosion, which has been addressed in the previous chapter, influences extinction risk of *C. riparius* populations in polluted environments. In **Chapter 4**, a study is described in which *C. riparius* strains with different levels of inbreeding and reduced genetic variation were exposed to various cadmium concentrations.

If genetic variation affects susceptibility towards chemical stress, then ecotoxicological exposure tests might be biased due to high levels of genetic impoverishment in caged laboratory cultures. **Chapter 5** documents the extent and rate of genetic impoverishment in ten laboratory strains of *C. riparius*.

The next chapter (**Chapter 6**) addresses the question, if decreased genetic variation indeed affects laboratory exposure tests. Six genetically characterized strains from different laboratories were tested for variation in life-history response to cadmium exposure.

All previously described studies were performed under laboratory conditions. **Chapter 7** describes patterns of genetic variation and species composition of *Chironomus* in the highly human-impacted Rhein-Neckar region in southwestern Germany. This study

documents the usefulness of molecular genetic tools, e.g., DNA-barcoding and microsatellite analyses, for the discrimination of morphologically cryptic invertebrates, like *Chironomus* larvae. In addition, the results document the role of population dynamic processes in field investigations on genetic erosion.

In the last chapter (**Chapter 8**) the results of all previously described studies are shortly summarized in order to present a general discussion on the consequences of environmental pollution on genetic diversity in laboratory and natural populations of *Chironomus riparius*.

2 Development and Localization of Microsatellite Markers for the Sibling Species *Chironomus riparius* and *Chironomus piger*

Abstract

Five variable microsatellite loci are reported for the non-biting midge species Chironomus riparius *and* Chironomus piger. *All loci show considerable intraspecific variation and species-specific alleles, which allow to discriminate among the two closely related species and their interspecific hybrids, and to estimate genetic diversity within and between populations. Additionally, the loci were localized on* C. riparius *polytene chromosomes to verify their single copy status and investigate possible chromosomal linkage. The described markers are used in different studies with regard to population and ecological genetics and evolutionary ecotoxicology of* Chironomus.

2.1 Introduction

Chironomids are a worldwide distributed family of nematocerans which occupy nearly all kinds of fresh water environments (Armitage et al., 1995). They play a key role in many lake and river systems because of their high abundance and their detritus consumption, and they represent an important food resource for many bird and fish species (Armitage et al., 1995). Although several studies investigated population dynamics within the Chironomidae, no suitable DNA based population genetic markers for species of this family have been developed so far. One of the most widely distributed and frequent species, *Chironomus riparius* Meigen is used as a model organism in ecotoxicological sediment biotests (OECD 2004). In order to answer the question if anthropogenic pollution stress has consequences on the genetic diversity of *Chironomus* populations in the laboratory and the field, we developed five microsatellite markers for this species. These markers were tested for their applicability to its sister taxon *Chironomus piger* Strenzke, which is morphologically highly similar to *C. riparius* and has been shown to form interspecific hybrids with the latter species (Hägele, 1999). Furthermore, the loci were located on the salivary gland chromosomes of the species via in situ hybridization in order to test for single copy status and physical independence.

2.2 Methods

For the construction of a genomic DNA library we extracted DNA from 100 individuals of a *C. riparius* culture sampled in Bochum (Germany). After shearing of the genomic DNA by nebulization, 0.5-1.5 kb sized fragments were separated electrophoretically, electroeluted and purified. The fragments were ligated into pUC 19 vector plasmids cut with *SmaI* and then transformed into DA10B *E. coli* host cells via electroporation. To identify fragments containing typical microsatellite DNA sequence motifs, we picked 2700 insert-containing clones on gridded nitrocellulose filters (Schleicher & Schuell, BA85) for colony filter hybridization (Grunstein & Hogness, 1975). Clones were screened for microsatellites using six different radioactive labelled synthetic oligonucleotide probes (((CA_{15}, $(GA)_{15}$, $(AAT)_{10}$, $(AAG)_{10}$, $(ATG)_{10}$, $(GATA)_6$). Fifty-five hybridizing clones were picked at random, and their inserts were amplified by PCR using standard forward and reverse vector primers. Amplification products were column-purified (Qiagen), sequenced by dye terminator cycle sequencing using the ABI BigDye Version 3.1 kit (Applied Biosystems) and separated on an ABI 3730 capillary sequencer. For genotyping microsatellite loci, fluorescent labeled primer pairs were designed manually or using FASTPCR (Kalendar 2003) for 27 loci containing microsatellite motifs. After optimization of PCR conditions, reactions were performed in a total volume of 10 µl containing 0.25 mM dNTPs, 2.4 mM $MgCl_2$, 1 x reaction buffer (20 mM Tris-HCl, 50 mM KCl; Invitrogen), 0.2 µM of each primer and 0.5 U *Taq* DNA polymerase (Invitrogen). In total, 24 microsatellite regions were successfully amplified and tested for variability using an ALF DNA sequencer (Pharmacia). All loci were analysed using at least 12 individuals from *C. riparius* field samples. Only five loci showed variability within or between the investigated populations (Table 2.1). Those loci were then amplified for 24 individuals each of *C. riparius* and *C. piger* sampled at different localities within the Rhein-Neckar region in Germany. Standard population genetic parameters were calculated using GENEPOP online version 3.4 (Raymond & Rousset 1995).

To localize the microsatellite loci on the chromosomes, in situ hybridization was performed following published procedures (Hankeln et al. 1988; Schmidt et al. 1988). Briefly, salivary gland polytene chromosomes were prepared from 4th instar larvae of *C. riparius*. Plasmid DNA from microsatellite-containing clones was labelled using biotin-labelled dUTP and nick-translation (Gibco-BRL). Biotinylated probes were hybridized overnight to the denatured chromosomes in 5 x SSC (0.75 M NaCl, 0.075 M Na citrate)/0.5% SDS, and

detected after washing in 2 x SSC by rabbit anti-biotin IgG primary antibody and a FITC-conjugated goat anti-rabbit IgG secondary antibody. Positions of hybridization signals were determined on the published chromosomal map of Hägele (1970).

2.3 Results and Discussion

The observed number of alleles ranged from three to 10 for *C. riparius* and two to 13 for *C. piger*, with expected heterozygosity values ranging from 0.621 to 0.864 (*C. riparius*) and 0.041 to 0.859 (*C. piger,* Table 2.1). All markers showed variation within both species. Significant linkage disequilibrium (Fisher´s exact test, $p < 0.05$) was found for *C. riparius* between the loci MSC2 and MSC5 after Bonferroni correction. A significant heterozygote deficit was observed for three loci within *C. riparius* and two loci in the *C. piger* samples (Exact HW test; Guo & Thompson 1992) which is most likely caused by population substructuring.

Table 2.1. Repeat motifs, primer sequences, size range, number of alleles (A), observed (H_O) and expected (H_E) heterozygosity, and GenBank accession numbers of five microsatellite loci for *C. riparius* and *C. piger* (in parenthesis). Population genetic parameters are based on 24 individuals per species sampled in the Rhein-Neckar region (Germany). * indicates significant ($p < 0.05$) heterozygote deficiencies.

Locus	motif	primer sequence	size [bp]	A	H_O/H_E	access. No.
MSC1	CA_9	for: CAT CAT CCT TAA CAA CCC AC rev: CTA GCT TTG CAG GCG AGT GC	95-103 (96-100)	8 (4)	0.500/0.860* (0.291/0.328)	DQ408105
MSC2	$(TAA)_9$, T_{10}	for : AGA CTA ATG ACC AGA CTT GC rev : CTT GTG ATG CGA AAA GCC TG	114-141 (112-141)	8 (10)	0.750/0.864 (0.333/0.780*)	DQ408106
MSC3	$(GT)_{14}$, T_9, T_6	for : ACT ACG CGT GCC TCA ACA GC rev : AGC TAA TTC TCA TGT TGG TC	168-176 (159-189)	7 (13)	0.583/0.758* (0.541/0.857*)	DQ408107
MSC4	$(TCA)_6$	for : TGA CTG AAC TTC CGC AAT GGG rev : CCG AGA ATG CTG CGA TCC AG	211-216 (215-216)	3 (2)	0.458/0.621 (0.041/0.041)	DQ408108
MSC5	$(CA)_{11}$, A_9	for : AAC ATT TGA ACG CAC ATC G rev : ATT TGA TTG TAT GTC CTG	264-278 (269-290)	10 (13)	0.750/0.830* (0.666/0.859*)	DQ408109

The in-situ hybridization resulted in a single hybridization signal for each of the 5 clones, which is most likely due to presence of unique DNA sequences flanking the microsatellites in the clones (Fig. 2.1). The hybridization thus verified the 'single copy' status of the microsatellite loci. Two of the identified loci reside on the small telocentric chromosome IV which contains the nucleolus (MSC2 and MSC4). However, genetic data did not reveal linkage for these loci. Possibly, high recombination rates due to the presence of the internally repetitive Balbiani Ring genes on chromosome 4 obliterate linkage of MSC2 and MSC4 alleles. The other three loci reside on the large metacentric chromosomes I (MSC1) and III (MSC3 and MSC5). Thus linkage disequilibrium found between the loci MSC2 and MSC5 is not due to close physical location on the same chromosome.

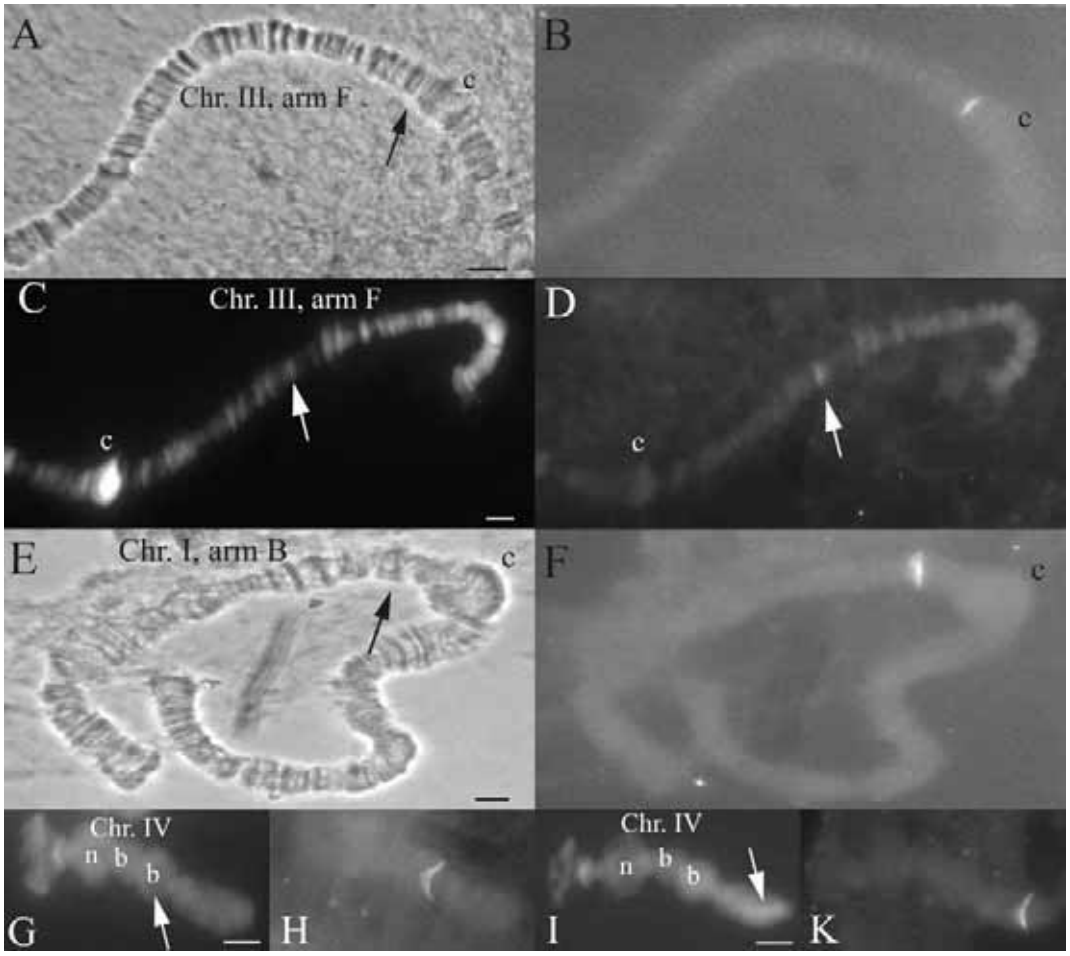

Figure 2.1. Localization of microsatellite loci by in situ hybridization to salivary gland polytene chromosomes of *C. riparius* (MSC5, A and B; MSC3, C and D; MSC1, E and F; MSC2, G and H; MSC4, I and K). Micrographs B, D, F, H and K show the signals of hybridized DNA probes, A and E represent phase contrast pictures of the respective chromosomes and C, G and I show chromosomal banding patterns after staining with the DNA dye DAPI. Arrows indicate positions of hybridization signals. Centromeres ©, nucleoli (n) and Balbiani Ring puffs (b) are indicated. Bars represent 10 µm.

The presented markers are useful tools for the assessment of the genetic population structure of *C. riparius* and *C. piger*. They can be applied for species discrimination due to the occurrence of species-specific alleles at all five loci (data not shown) as well as for the investigation of differentiation and hybridization between both species. The markers are currently used in several projects including studies on the regional population structure of *C. riparius* and *C. piger* and the genetic consequences of pollution stress for *C. riparius* populations.