Ecological and genetic differentiation of *Daphnia galeata* populations across Europe

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1. Introduction and outline of the thesis

1.1. General introduction

Studies on genetic structure in natural populations, including within and among population genetic diversity and genetic differentiation, have been a major topic in evolutionary ecology and genetics (Schmith 1998). This genetic architecture of a species depends on random processes such as the initial founder effect, genetic drift (the unpredictable change in gene frequency due to finite population size) and mutation and other non random events such as gene flow (the change of gene frequency due to movements of gametes, e.g. *via* individuals from one population to another), selection and reproduction mode (Avise 1994; Hartl and Clark 1989). Mutation, genetic drift and natural selection favour adaptations to local environmental conditions leading to the genetic differentiation of local populations, whereas effective gene flow will oppose that differentiation (Slatkin 1987). Since local adaptation may alter the genetic structure of a species, caused by natural selection, environmental characteristics mediate population structure (Turner *et al.* 2001). Currently there is growing interest in combining the tools of molecular genetics with the principles of ecological biogeography and landscape ecology. Although already recognized by Candolle (see in, Crisci 2001) and Wallace (Wallace 1860) in the end of the nineteenths century, only recent improvements in molecular genetic tools, combined with existing or new statistical tools (e.g. geostatistics, maximum likelihood and Bayesian approaches) and powerful computers have led to the emergence of the field of landscape genetics (Manel *et al.* 2003). This approach is an amalgamation of molecular population genetic and landscape ecology. Landscape genetics aims to provide information about the interaction between landscape features and evolutionary processes, such as gene flow, genetic drift and natural selection. Furthermore, it enables the spatial mapping of e.g. allele frequencies from one ore more species (or populations) and subsequently the correlation of such patterns with the current landscape. Thus, this approach can resolve population substructure across different geographical scales at fine taxonomic levels (see Manel *et al.* 2003) and will help us to understand the microevolutionary processes that generate genetic structure across space. Since it is an upcoming method in evolutionary genetics and ecology, only few studies have been conducted, including terrestrial (Piglucci and Barbujani 1991), marine (Riginos and Nachman 2001), and river systems (Castric *et al.* 2001) though not on large lakes (but see, Michels *et al.* 2001).

However, large scale demographic changes, which occur frequently in the evolutionary history of a species, also influence population genetic structure. Indeed, despite of evolutionary processes and landscape ecology, historic events, such as glaciation, have changed the genetic structure of many species (Hewitt 1999). The importance, however, of recent short-term and high-amplitude climatic oscillations during the late Pleistocene (130 000 to 10 000 years BP) on genetic structure and distribution of species was raised only recently (reviewed in, Taberlet *et al.* 1998). Molecular genetic data proved evidence for several ice age refugia, e.g. the Iberian Peninsula, the Balkan and the Caucasian region (**Fig. 1-1**). These refugia represent the source from which many species recolonized previously glaciated areas.

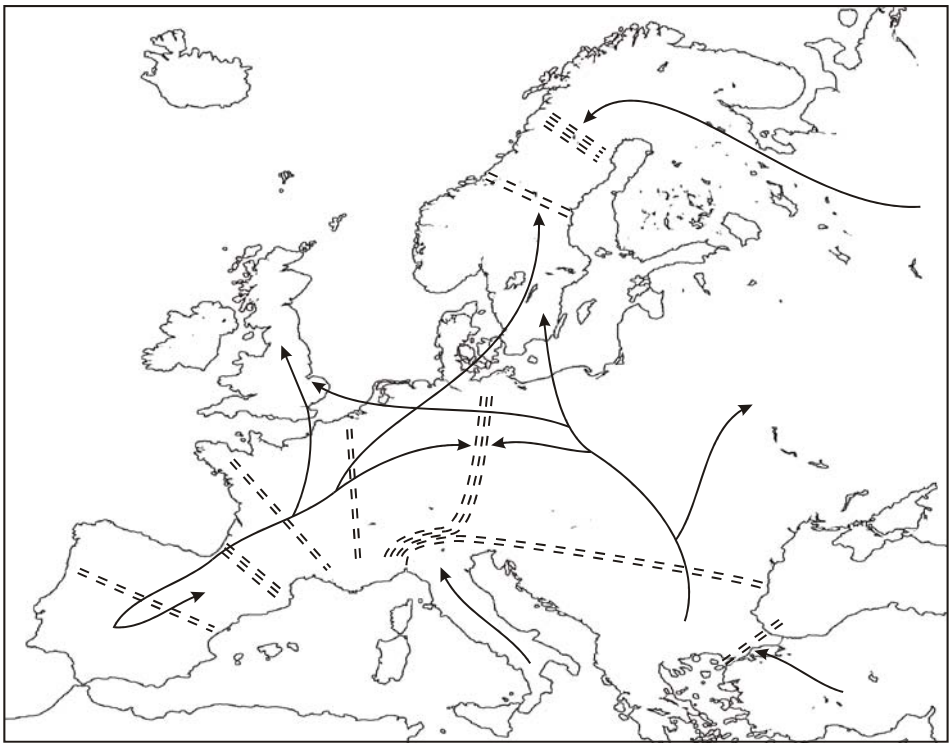

Fig. 1-1. Major postglacial colonization routes across Europe after last glaciation from the main refugial areas, as there are Iberia, Italy, the Balkan and Caucasus. Dashed lines represent general position of some well-known hybrid zones in Europe Figure modified from Hewitt (Hewitt 2000), primarily based on higher plants, insects and mammals.

During the Quaternary most species went through many range contractions/expansions, characterized by extinction of northern populations and a northward expansion from southern refugia. Such a colonization process implies severe bottlenecks that may lead to a loss of genetic diversity in northern populations (reviewed in, Hewitt 1999). In addition hybrid zones have been detected for several species across Europe (Hewitt 2000). These areas are

generated by secondary contact of two genetically diverged species or lineages as they expanded their ranges from separate glacial refugia (**Fig. 1-1**).

Biodiversity research investigates the interactions between evolutionary forces and landscape ecology influencing e.g. genetic composition of a species (Chapin *et al.* 2000; Weider and Hobaek 2000). Monitoring programs are used increasingly to assess spatial and temporal trends of biological diversity, with an emphasis on evaluating the efficiency of management policies (Yoccoz *et al.* 2001). Biodiversity studies on this level have mainly been applied to terrestrial organisms (e.g., Van der Heijden *et al.* 1998; Barnosky *et al.* 2001), but freshwater systems were rarely investigated (but see the EU-LAKES-project; http://www.cluwrr.ncl.ac.uk/projects/lakes/index.html). In order to investigate the complex biodiversity pattern in shallow lakes on a large geographic scale including ecological and genetic data the EU-project BIOMAN (http://www.kuleuven.ac.be/bio/eco/bioman/) was initiated. The topics of the BIOMAN project which are related to my theses entail:

1. The development of cost-effective biodiversity estimates for shallow lakes capable of predicting the biodiversity of the whole ecosystem and to develop a reliable method to evaluate the success of restoration measures.
2. The assessment of biodiversity across trophic levels in aquatic food webs to compile a database on the current state of biodiversity in a representative sample of European shallow bodies, covering the classical food web (fish, zooplankton, phytoplankton) as well as the microbial loop (bacterioplankton, heterotrophic nanoflagellates, ciliates), and also covering genetic diversity of zooplankton and diversity as measured through the egg bank.
3. The inclusion of both morphological and genetic criteria in the assessment of biodiversity at the taxon level (Roy and Foote 1997). Morphological and genetic criteria were applied at taxon level of three trophic levels (phytoplankton, ciliates, crustacean zooplankton). As genetic criteria, molecular tools such as DGGE (Denaturing gradient gel electrophoresis; phytoplankton, ciliates) and DNA polymorphisms (crustacean zooplankton) were used.
4. The joint assessment of genetic diversity at the within- and the between-taxon level. In addition to measuring taxon diversity, I also determined within-taxon genetic diversity at one trophic level (zooplankton). By incorporating genetic diversity estimates, the project encompasses the two main levels of biodiversity (intraspecific and taxon diversity), and my results allow us to determine whether the patterns for these two levels are similar.

With the emphasis to contribute in these topics I conducted the work presented in this thesis in the frame of the EU-project BIOMAN. As an example of within taxon level, the genetic

diversity of *D. galeata* populations across Europe was assessed. As a new approach in ecological genetics, I focussed amongst other aspects on the main issue of subunit 4, whether intraspecific biodiversity pattern (i.e. genetic diversity) resembles the one among taxa (species diversity).

In general, crustaceans have served as major target species in evolutionary ecology, since they provide an ideal model organism to study intra- and interpopulational genetic structure due to their island-like habitats (De Meester 1996). Furthermore freshwater zooplankton species, such as *Daphnia*, include uni- and biparental reproduction modes (Hebert 1978) and dispersal occurs only passively *via* dispersing vectors (Bilton *et al.* 2001). Thus, studies of population and community structure in freshwater zooplankton have traditionally emphasized on the role of local processes. Local factors known to influence the distribution and composition of species include lake area (Dodson 1992), chemical compositions (Tessier and Horwitz 1990), the supply of limiting nutrients (Dodson *et al.* 2000), competition (DeMott 1989), and predator abundance (Brooks and Dodson 1965). A vast amount of data concerning population genetics and ecological genetics as well as life history experiments in *Daphnia* species have been conducted (reviewed in, De Meester 1996; De Meester *et al.* 2004). However, the processes to alter genetic diversity and population genetic structure in cyclic parthenogenetic *Daphnia* species within and among habitat sites on a large European scale have hardly been tackled yet (De Gelas and De Meester 2005). Here I present the first comprehensive population genetic and ecological genetic study of a freshwater crustacean across a large geographic area. Using nuclear and mitochondrial DNA information of *Daphnia galeata* in combination with ecological data I describe population genetic structure, estimated gene flow and assessed the impact of ecological differentiation within and among populations on population genetic structure.

1.2. *Daphnia* as a model organism

Daphnia (Crustacea: Anomopoda) as a freshwater organism are present in a wide range of different water bodies, like temporary pools and large permanent lakes (Hebert 1978). The island-like nature of aquatic habitats facilitates the delineation of distinct populations. This is an important advantage in population genetic research of lake species. Indeed, *Daphnia* are a preferred model organism in a wide range of research fields such as (eco)toxicology, ecology, quantitative genetics and population genetics.

As a mainly cyclic parthenogenetically (alternating periods of parthenogenetic reproduction with sexual reproduction, **Fig. 1-2**) reproducing organism (Hebert 1978; Bell 1982; De Meester *et al.* 2004) it is easy to culture and clonal lineages can be maintained over a long time period. As long as environmental conditions remain favourable, the individuals reproduce by amictic parthenogenesis, forming all-female clonal lineages. When ecological conditions deteriorate (e.g. food shortage and quality deterioration, desiccation of the habitat, changing photoperiod, high population densities, Kleiven *et al.* 1992), the females of different clonal lineages (Fig. 1-2, De Meester and Vanoverbeke 1999) start to produce males and sexual females (Davison 1969; Hobæk and Larsson 1990). The sexual females produce sexual eggs that need to be fertilised and are released as dormant propagules, called ephippia. These resting egg stages are capable of surviving harsh conditions and remain viable for a long time, forming resting egg banks in the sediments of the habitat (De Stasio 1989; Cáceres 1999; Brendonck and De Meester 2003). As long as they float on the water surface, these sexual propagules are easily dispersed through migrating vectors such as birds since they either attached or survive gut passage (Proctor 1964; Figuerola and Green 2002). This high dispersal potential is reflected in the rapid colonization of new habitats (Hebert and Moran 1980; Louette and De Meester 2004) and may have a major ecological and evolutionary significance in the species composition (Bohonak and Jenkins 2003).

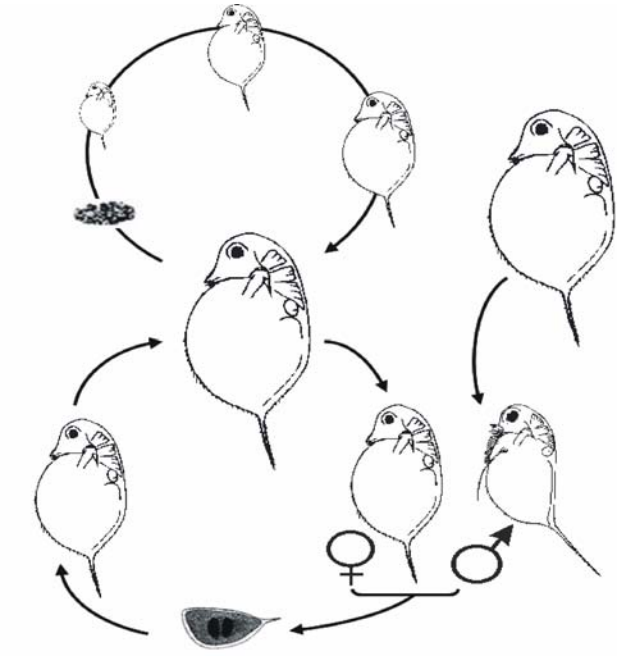

Fig. 1-2 Reproduction cycle of cyclic parthenogenetic *Daphnia* species

1.3. Population genetic structure and gene flow of a cyclic parthenogenetic organism

Daphnia has served in population genetic studies as a model organism already more than 30 years (Hebert and Ward 1972; Carvalho 1994; De Meester 1996). Most of the studies have used allozyme markers or DNA sequence data to determine the genetic structure within and among populations (De Meester 1996). However, recent studies have used also microsatellite

DNA data to reveal a more detailed analysis of the population genetic structure of several *Daphnia* species in North America or relatively narrow geographic ranges in Europe (e.g. Lynch *et al.* 1999; Pálsson 2000; Cousyn *et al.* 2001).

1.3.1. Genetic variation within Daphnia *populations*

Since the early seventies, habitat size and the length of the growing season have been recognised as some of the most important factors determining the genetic structure of cyclical parthenogenetic zooplankton populations. In addition, a distinction has been made between intermittent and permanent *Daphnia* populations based on several genetic studies initiated for instance by Hebert (1974a; 1974b). Intermittent populations are characterised by high levels of multi-locus genotype (MLG) diversity, stable allele frequencies and genotype frequencies generally being in Hardy-Weinberg equilibrium. In occasional deviations from H-W equilibrium intermittent pond populations show heterozygote deficiencies. In contrast permanent populations show a lower level of MLG diversity and severe shifts in allele frequencies within and between growing seasons. Furthermore permanent populations commonly deviate from H-W equilibrium exhibiting heterozygote excess. The differences in genetic structure are mainly explained by the time span clonal lineages are subjected to natural selection eroding genetic diversity (Hebert 1974a; Hebert 1974b; Young 1979). In contrast to these early findings, studies on *Daphnia* populations in permanent lakes revealed similar characteristics in population genetic structure than has been found for intermittent populations (Mort and Wolf 1986; Gießler 1987; Wolf 1988; Jacobs 1990). These results indicate that habitat permanency is not the ultimate factor influencing the genetic structure of cyclical parthenogenetic populations (see also, De Meester 1996).

1.3.2. Genetic differentiation among Daphnia *populations*

Despite the high dispersal capacity of most cyclic parthenogenetic zooplankton species, a high genetic differentiation among populations was generally observed and patterns of isolation by distance are often absent (Hebert 1987a; Carvalho 1994; Lynch and Spitze 1994; but see, De Gelas and De Meester 2005). Whereas for lake-dwelling *Daphnia* genetic differentiation between neighbouring populations is relatively low, and there is a clear pattern of increasing genetic differentiation with increasing geographic distance (Mort and Wolf 1986; Wolf 1988; Jacobs 1990). Founder effects have been shown to be important and

persistent in shaping the genetic structure among cyclical parthenogenetic zooplankton populations (Boileau *et al.* 1992). Recent studies have extended this persistent founder effect hypothesis by including the effects of large resting egg banks providing a vast amount of genetic diversity and the rapid local adaptation of resident populations to local conditions (De Meester 1996; Okamura and Freeland 2002). They integrated these components into one hypothesis termed "the Monopolization Hypothesis" (De Meester *et al.* 2002). It states that the genetic structure of cyclical parthenogens is largely determined by colonization events and that secondary gene flow is strongly impeded by the rapid monopolization of available recourses, i.e. the build up of a dormant egg bank and rapid genetic adaptation to local conditions.

1.4. The role of *Daphnia* sp. in freshwater trophic cascades

Although the population genetic structure in several *Daphnia* species has been well studied (De Meester 1996), less studies have focused on the environmental factors influencing the genetic structure in *Daphnia* species (Weider 1985; Hobæk *et al.* 1993; Boersma *et al.* 1999). Due to the above listed characteristics of widely distributed *Daphnia* such as their reproduction mode, the observed dispersal-gene flow paradox, the rapid local adaptation and their varying levels of genetic diversity, it is a highly suitable organism to investigate the environmental effects and processes influencing population genetic structure not only on the local scale but covering a large geographic range.

In terms of landscape, genetics ecological aspects in addition to e.g. population genetic are of major importance. Food webs in freshwater habitats are relative complex (Rothhaupt 2000), with a general pattern called "Trophic Cascades" (Carpenter *et al.* 1985). The basic idea of "Trophic Cascade" is that on the one hand the potential biomass and production of organisms at a given trophic level is determined by nutrient (food) availability (McQueen *et al.* 1986), whereas the realized biomass and dynamics of organisms at each trophic level on the other hand is regulated by the adjacent, higher trophic level *via* top-down control (Wickham 1998; Pace *et al.* 1999).

Daphnia, especially larger species, can exert a significant grazing pressure on the phytoplankton and the microbial loop in freshwater habitats (Lampert 1987a; Lampert 1987b). In turn, they serve as an important food source for a large number and variety of vertebrate (e.g. Boersma *et al.* 1991; Hoffman *et al.* 2001) and invertebrate predators (e.g. Riessen 1990; e.g. Kvam and Kleiven 1995; Branstrator 1998). Given their central position in

freshwater aquatic food webs, *Daphnia* can be considered a key-species in freshwater ecosystems. Therefore it is of major interest to infer the possible impact of diversity and biomass/density at adjacent trophic levels on genetic diversity of *Daphnia* populations, a relationship that has been discussed so far only in terrestrial systems (e.g. Agrawal 2003; Wade 2003).

1.5 Outline of the thesis

Several zooplankton species have been well studied concerning population genetic structure, life-history and ecological genetics (De Meester 1996). However, few studies have focused on a large scale population genetic structure across Europe (but see, De Gelas and De Meester 2005) and none on zooplankton species of large freshwater bodies such as *D. galeata* in lakes. In addition, fine-scale genetic markers like microsatellite DNA have only been used in one population genetic analyses of *Daphnia* on a relatively narrow geographic range in Europe (Daphnia pulex, Pálsson 2000). Furthermore additional information is needed about the interactions between environmental factors and genetic diversity. Only then one may understand how the genetic composition of cyclic parthenogenetic organisms is dependent on the local and regional biodiversity. The aim of my study is to reveal factors that do influence and mediate genetic diversity and population structure in a cyclic parthenogenetic organism at the heart of the crustacean species *D. galeata*. Therefore I focused in this thesis on four major points of interest in evolutionary ecology and landscape genetics (Population genetic structure, environmental impact, levels of gene flow, and historic events; **Fig. 1-3**).

In Chapter 2 differences in population genetic structure of lake-dwelling cyclic parthenogenetic species, *D. galeata*, were revealed. The general division of permanent and intermittent *Daphnia* populations has critically been discussed on the basis of population genetic estimates. Furthermore, I elucidated the paradox of genetic differentiation between populations on the background of dispersal capacity *via* ephippia (resting egg).

In Chapter 3 I concentrated on the historic and current population genetic structure and diversity in the species of interest revealed by mitochondrial DNA and nuclear marker systems. Microsatellite loci are rapidly evolving nuclear markers (Jarne and Lagoda 1996; Ellegren 2000) and are suitable to detect recent interactions between extant or recently separated populations. Mitochondrial DNA data evolves at a 10 fold lower level than microsatellite loci and reflects rather historic genetic processes (e.g. Hansen *et al.* 1999; Johnson *et al.* 2003). Since dispersal among lakes is an important evolutionary and ecological

process (Bohonak and Jenkins 2003), I intended to reconstruct the level and direction of gene flow for *D. galeata* after the last glaciations. Based on these results I discussed a possible impact of historic events on present genetic diversity and differentiation within and among *D. galeata* populations.

In Chapter 4 I focused on the possible interaction of intra- and interpopulation genetic diversity in *D. galeata* with different trophic levels in eutrophic lakes. Especially I analysed the importance of density and biomass of food and predator species and their diversity in adjacent trophic levels concerning *D. galeata*, as well as the influence of abiotic characters in eutrophic lakes.

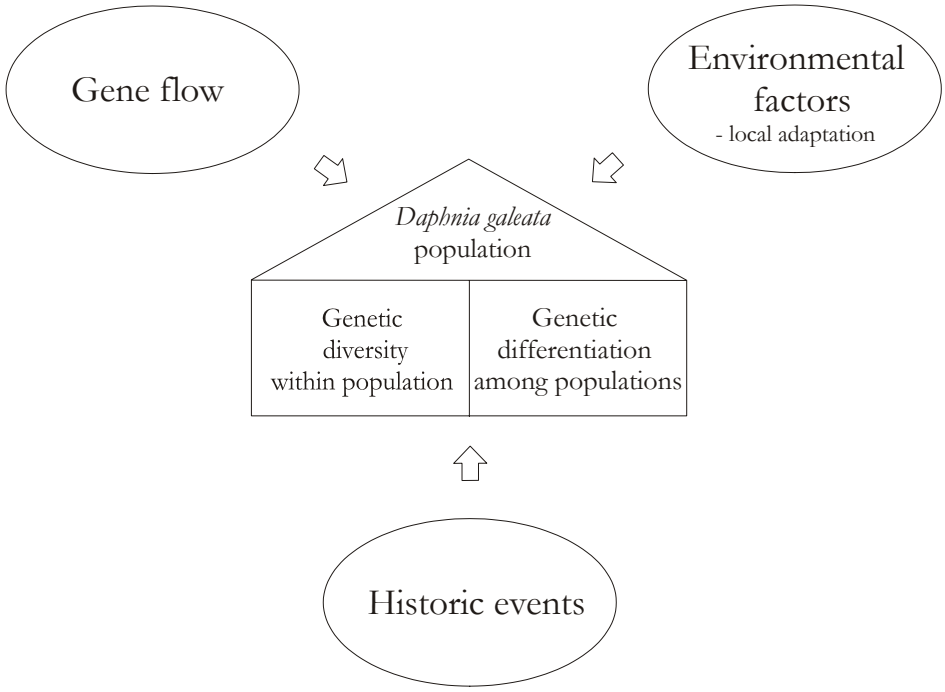

Fig. 1-3 Factors responsible for current population genetic structure in *Daphnia*

2. Clonal diversity and population structure of *Daphnia galeata*

2.1. Abstract

Microcrustaceans of the genus *Daphnia* became a favoured model organism in ecological genetics since species are widely distributed, reproduce mainly via cyclic parthenogenesis and occur in discrete habitats. So far, most studies have focussed on pond species and mainly on small geographic scales when studying genetic structure and among-population genetic differentiation. Here I analyse 23 populations of *D. galeata*, a large-lake cladoceran, distributed mainly across the Palaearctic. I detected high levels of clonal diversity and population differentiation using variation at six microsatellite loci across Europe. Most populations were characterised by deviations from H-W equilibrium and significant heterozygote deficiencies. Observed heterozygote deficiencies might be a consequence of simultaneous hatching of individuals produced during different times of the year or of the coexistence of ecologically and genetically differentiated subpopulations. A significant isolation by distance was only found over large geographic distances (> 700 km). This pattern is mainly due to the high genetic differentiation among neighbouring populations. My results suggest that historic populations of *Daphnia* were once interconnected by gene flow but current populations are now largely isolated. Thus local ecological conditions which determine the level of biparental sexual reproduction and local adaptation are the main factors mediating population structure of *D. galeata*.

2.2. Introduction

Islands and freshwater habitats, such as lakes and ponds, provide useful model systems for studying the origin and maintenance of genetic diversity and processes of genetic differentiation (e.g. Frankham 1997). These habitats of well defined boundaries offer a wide range of ecological niches, facilitate population differentiation within species and may even facilitate speciation processes (e.g. Schluter 2001). Spectacular illustrations of the outcome of these processes are the indigenous species flocks in cichlid fishes (e.g. Wilson *et al.* 2000) and the radiation of crustaceans in Lake Baikal (Fryer 1991). Many zooplankton taxa have long been considered to be cosmopolitan because of their morphological similarity across large geographic areas (e.g. Mayr 1963). Recent developments of genetic tools have,

however, made it possible to unambiguously delineate evolutionary lineages and to estimate effective gene flow among lenthic habitats (Colbourne and Hebert 1996; Schwenk *et al.* 1998; Freeland *et al.* 2001; Michels *et al.* 2001; Finston 2002; Gomez *et al.* 2002). The emergent pattern is one of provincialism and mosaic distributions (eg. Mitchell *et al.* 1998; Gomez *et al.* 2000; Witt and Hebert 2000).

For several aquatic taxa, like rotifers and bryozoans high levels of genetic differentiation among populations have been reported (Freeland *et al.* 2000a; Gomez and Carvalho 2000). Many earlier studies on population genetics in *Daphnia* have focussed on pond species like *D. pulex* (e.g. Lynch 1984; Hebert *et al.* 1988; Pálsson 2000) and *D. magna* (e.g. Korpelainen 1984; Vanoverbeke and De Meester 1997). Different levels of clonal diversity and genetic differentiation among *Daphnia* populations have mainly been related to habitat size and permanency (Hebert 1987b). Genetic differentiation among populations has been found to be more pronounced in pond than in lake habitats (Wolf 1988). High clonal diversity and genotype frequencies in agreement with H-W equilibrium are typical for intermittent ponds, whereas permanent pond populations exhibit lower levels of clonal diversity and often strong deviations from H-W equilibrium (Hebert 1987b; De Meester 1996). Studies on the population genetic structure and genetic diversity of lake-dwelling species (Mort and Wolf 1986; Jacobs 1990; Cerny and Hebert 1993; Gießler 1997; Limburg and Weider 2002) have not revealed consistent patterns with respect to deviations from H-W equilibrium (either excess or deficiencies), nor with respect to the level of genetic differentiation among populations. Recent empirical studies suggest that the amount of biparental reproduction and population size, rather than habitat characteristics, explain genetic diversity (De Meester 1996). Although many studies revealed high levels of genetic differentiation among *Daphnia* populations, ecological studies suggest high dispersal capacity via resting eggs, facilitating high gene flow rates (Bilton *et al.* 2001; Figuerola *et al.* 2005). In order to resolve this apparent paradox De Meester *et al.* (2002) proposed the "Monopolisation Hypothesis", which explains the lack of effective gene flow despite high dispersal capacity by a combination of stochastic effects, founder events, and fast local adaptation. Rapid population growth rate and the establishment of large resting egg pools provide a powerful buffer against newly invading genotypes (Hairston 1996) and thus further enhance priority effects.

Although many studies focused on the population genetic structure of zooplankton species (De Meester 1996), large-scale surveys using high resolution molecular markers are virtually absent for European *Daphnia* populations. With this study, I aim to describe the intra- and

interpopulational genetic variation of 23 *D. galeata* populations across Europe using six microsatellite loci.

2.3. Materials and methods

2.3.1. Sampling and identification of Daphnia galeata

Members of the *Daphnia longispina* species complex of eight Belgian lakes (lakes BNL1-8), six Danish lakes (DK1-6) and one Spanish lake (SP1; **Fig. 2-1**, **Table 2-1**) were sampled monthly during the growing season (April - October) of 2000 or 2001.
An additional six lakes were sampled once; one lake in Spain (SP2), one in France (FRA), one in Italy (ITA), one in Russia (RUS), one in Norway (NOR) and one in Hungary (HUN) were sampled in summer 1998, whereas two Finnish lakes (FIN1 and FIN2) were sampled in summer 2000 (**Fig. 2-1**, **Table 2-1**). Individuals were stored in ethanol and adult individuals were characterised both morphologically (Flößner 2000) and *via* recently developed nuclear DNA markers (Schwenk *et al.* 2000; Billiones *et al.* 2004). For the latter analysis, a random selection of 5-10 individuals per population was subjected to a Restriction Fragment Length Polymorphism (RFLP) analysis of an amplified Internal Transcribed Spacer (ITS) region (Billiones *et al.* 2004). If populations were composed of parental species and interspecific hybrids, all individuals (21 to 44) were subjected to PCR-RFLP analyses prior to microsatellite screening.

2.3.2. Microsatellite amplification and genotyping

Total DNA was extracted from an average of 35 (21 - 44) individuals per population using a modified procedure of Walsh *et al.* (1991). Each individual was incubated overnight in 50 - 100 µl 6% Chelex and 10 mg/ml Proteinase K (Boehringer Mannheim). I adjusted the volume of the incubation buffer to the size of the individuals.

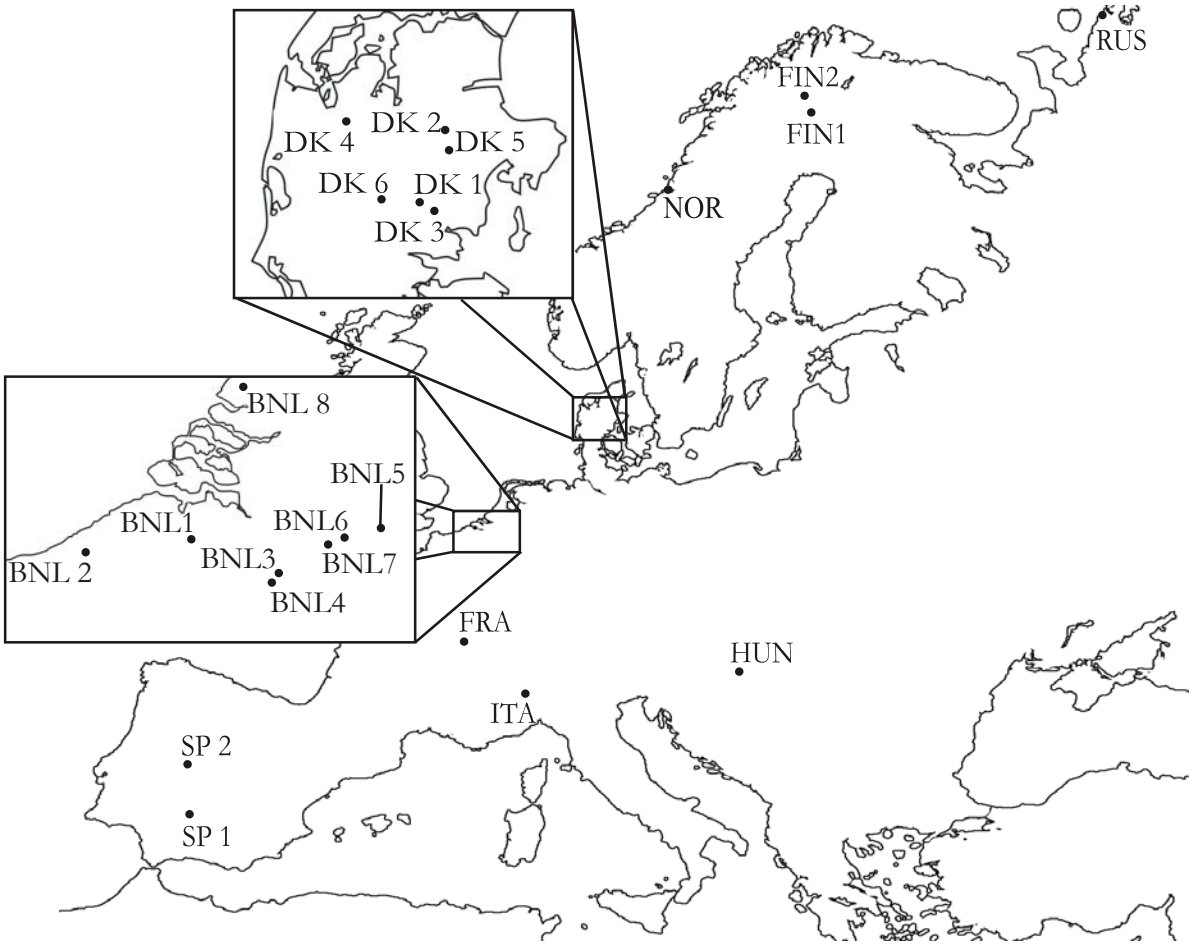

Fig. **2-1** Sampling sites of *Daphnia galeata* populations across Europe. Abbreviations correspond to lake names as listed in Table 2-1: SP 1+2 = Spain, ITA = Italy, HUN = Hungary, FRA = France, BNL = Belgium/The Netherlands, DK = Denmark, NOR = Norway, FIN 1+2 = Finland, RUS = Russia.

After brief vortexing, samples were boiled for 10 minutes. Homogenates (supernatant) were isolated from the Chelex resign and stored at 4 °C or -20°C. Polymerase chain reactions (PCR) were performed for six microsatellite loci per individual. Amplification conditions for the loci DaB10/14 (NCBI Database accession number: U41402), DaB10/15 (U41402), DaB17/16 (U41403; reverse primer was modified: 5'-TCT TTG GCA GGC CAC TGC CAA GG-3') and DaB17/17 (U41403) were modified based on Ender *et al.* (1996), i.e. 0.5 U of Taq (Invitrogen) in a total reaction volume of 10 µl. For two microsatellites, Dpu 06 (AY 057864) and Dpu 30 (AY 057865), the following PCR-temperature profiles were used: Dpu 06, denaturation at 95°C for 180s, 32 cycles at 95°C for 30s, 56°C for 30s and 72°C for 30s, followed by an elongation step of 20 minutes at 72°C; Dpu30, denaturation at 95°C for 3 minutes, 32 cycles of 95°C for 45s, 53°C for 45s, and 72°C for 45s, followed by an elongation step of 20 minutes at 72°C. Microsatellite fragments were visualised on an automatic DNA

Sequencer (ALF, Pharmacia) using 6% denaturing high-resolution polyacrylamide gels. To ensure consistency of allele sizing and comparability between different runs and lanes, gels were standardised using an external size ladder and internal implies in each lane four internal size markers. Internal markers and external size ladder were obtained by generating amplicons of defined length. In order to cover the entire size range of *D. galeata* alleles (77 bp to 234 bp), I applied an internal size ladder containing four fragments of 54 bp, 118 bp, 180 bp, and 252 bp in length. The external size ladder consisted of three additional fragments of 81 bp, 103 bp, and 228 bp. Alleles were scored and sized manually using an ALF-win^{TM} Fragment Analyser 1.00 (Pharmacia).

2.3.3. Genotypic diversity

Since *D. galeata* reproduces most of the growing season *via* parthenogenesis, identical multilocus genotypes (MLG's) are most likely the product of clonal propagation and represent a clonal group. Therefore, as suggested for cyclical parthenogens by Sunnucks (1997), I used only one individual per MLG for most population genetic analyses. Genetic diversity (*D*) was obtained by dividing the number of MLG's per population by the number of individuals (*N*). Linkage equilibria between loci and deviations from HW-equilibrium were tested for clonal lineages as well as for all individuals using the Markov chain method (1000 dememorisations, 100 batches, and 1000 iterations) following the algorithm of Guo and Thompson (1992). In cases of deviations from HW-equilibrium, alternative tests using GENEPOP (Rousset and Raymond 1995) were performed to test whether deviations are caused by heterozygote deficit or excess. Values for the observed and expected heterozygosity in populations (only MLG's) were obtained using GENETIX (Belkhir *et al.* 1996-97). To test HW-equilibrium and clonal structure, I compared simulated numbers of MLG's based on allele frequencies with the observed number of MLG's per population including all individuals (HWClon; Vanoverbeke, unpublished software).

To explore the impact of sample size on the number of detected MLG's, I calculated the number of MLG's for various random samples of individuals (*N* = 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, and 798). To obtain average MLG's and standard deviation, I selected 10 random subsamples for each sample size. A similar procedure was conducted for the number of loci (1-6); 10 random subsamples of each marker combination were used to calculate the average of MLG and standard deviation. To estimate the impact of hybridization on population structure of *D. galeata* I performed correlation

analysis (Statistica 1995) between the genetic diversity estimated of populations, in which hybrids or maternal lineages coexisted with *D. galeata* and "pure" *D. galeata* populations.

2.3.4. Genetic differentiation among populations

For the following analyses I used only one daphnid per genotype (MLG) and population. Proportions of shared alleles (D_{PS}, Bowcock *et al.* 1994) were calculated using MICROSATELLITE ANALYZER (MSA, Dieringer and Schlötterer 2003) and pairwise distances among populations were subjected to multidimensional scaling (Statistica 1995). Pairwise F_{ST} values for all population pairs were calculated based on Weir (1984) using GENETIX (Belkhir *et al.* 1996-97), and global F_{ST} estimates for each population were obtained using FSTAT (Goudet 1995). Significance levels for pairwise F_{ST} were obtained by permutation of individuals. Same analysis was performed for a subset of loci (DaB10/15, C, DaB17/17, G, Dpu 06, T, and Dpu 30, U), since results in Chapter 4 suggest that DaB17/16, F, and DaB10/14, B, are under natural selection. In addition to the estimation of molecular variances (AMOVA) among and within populations (N = 23), I also estimated the genetic variation among and within months within populations (ARLEQUIN ver. 2.0, Schneider *et al.* 2000). The latter analysis is based on only seven populations due to insufficient sample sizes in most monthly samples. This analysis allowed us to compare the impact of seasonal variation (analysis within populations) with that of spatial variation (analysis among populations).

Mantel test (GenAlEx v.5, Peakall and Smouse 2001) were performed to determine the association of geographic distances (log km) and pairwise genetic distances ($F_{ST}/(1 - F_{ST})$) among population. Each individual was assigned to a population based on MLG's, allele frequencies, and a Bayesian assignment method (GeneClass, Cornuet *et al.* 1999). I applied the following settings: 10.000 runs; rejection at P = 0.05 in combination with direct estimation of allelic frequencies, "Leave one out" and Nei's standard distance (D_S, Nei 1972).

2.4. Results

D. galeata densities in 23 populations varied from 0.53 (DK4) to 6.72 (BNL3) individuals per litre, with an average of 1.93 individuals per litre. I detected no correlation between the density of *D. galeata* and various diversity indices and estimates of genetic variation. Eleven (BNL1-3, BNL5-8, DK2, 3, 5, and SP1) out of 23 populations harboured *D. galeata* x

cucullata hybrids (10-15%). Estimates of linkage disequilibrium and HW-equilibrium showed similar results for both analysis approaches, either including all individuals (not shown) or only one individual per genotype.

2.4.1. Clonal diversity within Daphnia galeata *populations*

Overall, 497 MLG's were distinguished out of 798 individuals screened using six microsatellite loci (i.e. 62% of the individuals represented unique MLG's). The majority of MLG's (90.14%) occurred only in one population, 6.63% were found in two, 2.01% in three, 0.80% in four and 0.04% in five populations. Genetic diversity (*D*) was lowest in DK2 (0.375) and highest in BNL5 (0.865; **Table 2-1**). I detected no significant correlation between the amounts of MLG's per population and latitude (r^2 = 0.029; *P* = 0.435). The number of alleles varied from 6 to 15 (average = 9), and allelic richness (*A*/*N*) ranged from 0.342 (NOR and ITA) to 0.7 (BNL1), with an average of 0.484 (**Table 2-1**). Both the number of individuals and the number of loci were positively correlated with the number of observed MLG's (r^2 = 0.9958, *P* < 0.001 and r^2 = 0.9859, *P* < 0.001, respectively; **Fig. 2-2**). Genetic diversity was significantly correlated with *A*/*N* and polymorphism of loci (r^2 = 0.342, *P* = 0.003 and r^2 = 0.586, *P* = 0.003, respectively). The observed heterozygosity (H_{obs}) varied from 0.114 in Italy (ITA) to 0.405 in Denmark (DK5) with an average of 0.236.

In 18 of the 23 populations, the hypothesis of H-W equilibrium was rejected (**Table 2-1**). In all but three populations (SP1, FIN1, and FIN2), significant heterozygote deficits were observed (**Table 2-1**), even within months (data not shown). However, when testing for H-W equilibrium based on MLG's (HWClon) I found a larger amount of populations in H-W equilibrium (**Table 2-1**).

Table 2-1 Genetic diversity estimates among 23 *Daphnia galeata* populations across Europe.

Population	**Country**	***N***	**MLG**	**D**	**A/*N***	**PA**	***P***	**HwCl**	**H_{exp}**	**H_{obs}**	**HWE**	**HD**
Berlare Broek, BNL1	Belgium	30	19	0.633	0.721	1	1	**	0.418	0.229	**	**
Blankaartvijver, BNL2	Belgium	33	23	0.697	0.485	0	1	0.230	0.281	0.213	*	**
Oud Heverlee Troebel, BNL3	Belgium	38	32	0.842	0.605	1	1	**	0.424	0.261	**	**
Oud Heverlee Helder, BNL4	Belgium	37	25	0.730	0.622	0	1	**	0.582	0.371	**	**
Oude Maasmaender Maasveld, BNL5	Belgium	37	32	0.865	0.568	0	1	**	0.462	0.365	**	**
Plas Astrid, BNL6	Belgium	38	20	0.526	0.368	0	0.667	0.108	0.251	0.228	*	*
Voortmangelbeek, BNL7	Belgium	37	29	0.784	0.421	0	1	**	0.408	0.272	**	**
Delftse Houd, BNL8	Netherlands	36	22	0.611	0.417	0	0.833	**	0.359	0.288	**	*
Stigsholm sø, DK1	Denmark	38	19	0.5	0.414	0	0.667	**	0.335	0.251	**	**
Søbygaard sø, DK2	Denmark	32	12	0.375	0.406	0	0.833	**	0.294	0.218	**	**
Kvind sø, DK3	Denmark	38	21	0.553	0.368	0	0.833	0.270	0.262	0.223	0.117	**
Sunds sø, DK4	Denmark	36	30	0.833	0.472	1	0.833	0.082	0.357	0.242	**	**
Schousbye sø, DK5	Denmark	38	24	0.658	0.474	0	1	**	0.474	0.405	**	**
Ensø, DK6	Denmark	37	18	0.486	0.378	1	0.833	0.548	0.204	0.152	0.120	**
Cogollos, SP1	Spain	34	19	0.559	0.353	2	0.667	0.486	0.191	0.205	0.879	0.745
St. Paul-de-Varax, FRA	France	18	10	0.556	0.667	0	0.833	**	0.344	0.233	**	**
Demmingsvatn, NOR	Norway	38	20	0.526	0.342	0	0.833	**	0.2914	0.129	**	**
Pechora lake, RUS	Russia	37	22	0.595	0.486	0	1	*	0.3127	0.159	**	**
Embalse de Valdecano, SP2	Spain	44	30	0.682	0.591	4	1	**	0.558	0.131	**	**
Balaton, HUN	Hungary	39	26	0.667	0.358	3	1	**	0.464	0.161	**	**
Caluso/Turin, ITA	Italy	38	18	0.474	0.342	0	0.833	**	0.305	0.114	**	**
34, FIN1	Finnland	21	9	0.428	0.429	0	0.50	*	0.279	0.296	0.550	0.139
Toskaljärvi, FIN2	Finnland	22	16	0.727	0.591	2	0.667	0.114	0.296	0.292	0.290	0.611

For each population: *N*, number of individuals; MLG, number of multilocus genotypes; D, MLG/*N*; A/*N*, allelic richness; PA, number of private alleles; P, average polymorphism; HwCL, test of HW-expectations based on MLG (Vanoverbeke, unpublished software); H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; HWE, H-W-expectation; HD, heterozygote deficit. * = $P < 0.05$; ** = $P < 0.01$.

Only a small fraction of pairs of loci (27 out of 345 pairwise comparisons) were in significant linkage disequilibria (P = 0.0003 - 0.048). The amount of private alleles was generally higher in southern (SP1 = 2, SP2 = 4, and HUN = 3) than in northern populations. However, a few exceptions to this general trend were found, for instance, in Finland (FIN2 = 2; **Table 2-1**).

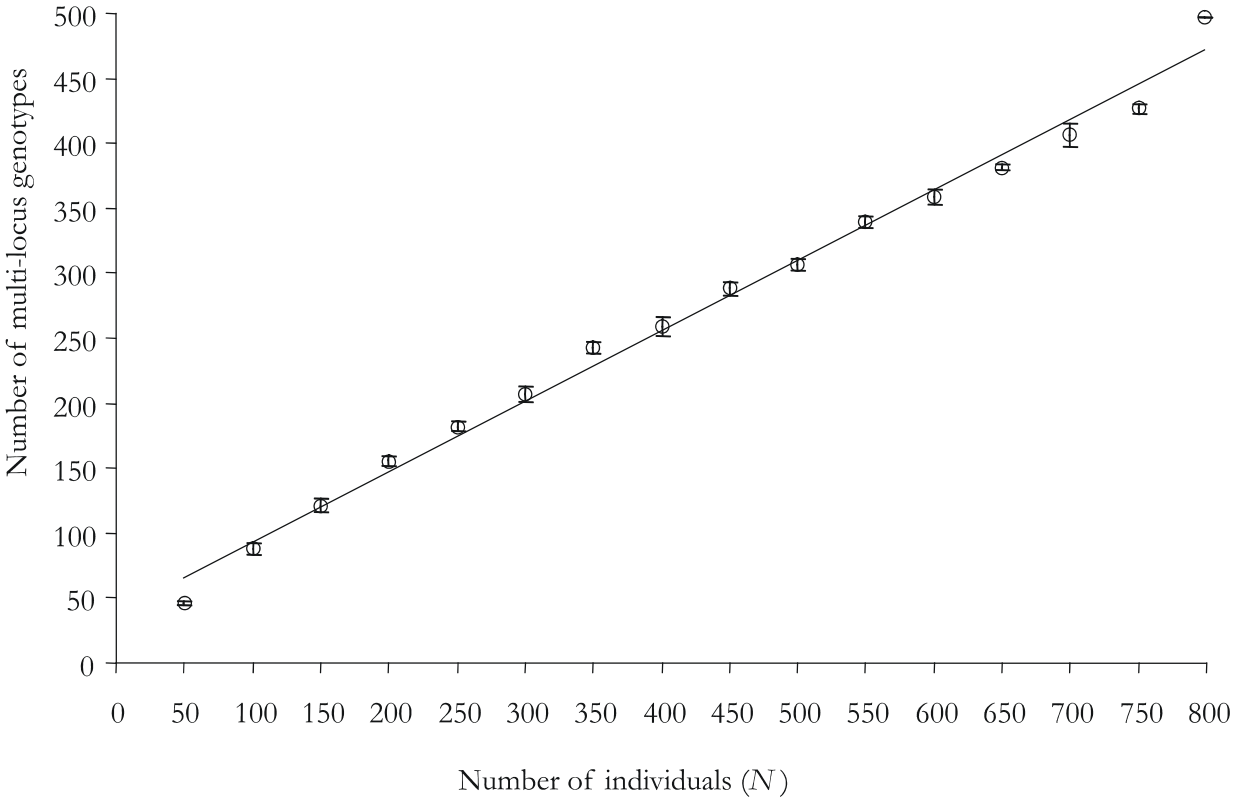

Fig. 2-2 Correlation and standard deviations between multiple randomised samples sizes of *Daphnia galeata* individuals and the revealed number of multilocus genotypes (MLG, $P < 0.001$, $r^2 = 0.9958$).

In order to test the potential impact of interspecific hybridization on population structure, I compared populations genetic parameters of *D. galeata* which co-occurred with hybrids (BNL1-3, BNL5-8, DK2, 3, 5, and SP1) with pure *D. galeata* populations. I observed a higher, but non significant, level of genetic variation (*D* and HET) in syntopic populations than in allopatric populations.

Table 2-2 Pairwise F_{ST} values (lower triangle) and corresponding significance levels (upper triangle) among *Daphnia galeata* populations. Abbreviations correspond to the populations listed in**Table 2-1**.

	BNL1	BNL2	BNL3	BNL4	BNL5	BNL6	BNL7	BNL8	DK1	DK2	DK3	DK4	DK5	DK6	SP1	FRA	NOR	RUS	SP2	HUN	ITA	FIN1	FIN2
BNL1		**	**	**	n.s.	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL2	0.26		**	**	**	**	**	**	**	**	*	**	**	**	**	**	**	**	**	**	**	**	**
BNL3	0.09	0.2		**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL4	0.26	0.33	0.26		**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL5	0.04	0.22	0.12	0.25		**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL6	0.11	0.48	0.21	0.42	0.23		**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL7	0.24	0.2	0.23	0.28	0.21	0.43		**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
BNL8	0.21	0.24	0.23	0.27	0.16	0.46	0.03		**	**	**	**	**	**	**	**	**	**	**	**	n.s.	**	**
DK1	0.24	0.29	0.21	0.26	0.12	0.51	0.25	0.18		**	**	**	**	**	**	**	**	**	**	**	**	*	**
DK2	0.16	0.35	0.31	0.31	0.1	0.44	0.31	0.25	0.25		**	**	**	**	**	**	**	**	**	**	**	**	**
DK3	0.3	0.05	0.21	0.35	0.26	0.49	0.24	0.3	0.35	0.42		**	**	**	**	**	**	**	**	**	**	**	**
DK4	0.1	0.24	0.06	0.28	0.09	0.33	0.24	0.19	0.13	0.27	0.3		**	**	**	**	**	**	**	**	**	**	**
DK5	0.21	0.28	0.25	0.1	0.17	0.44	0.21	0.16	0.17	0.21	0.33	0.22		**	**	**	**	**	**	**	**	**	**
DK6	0.35	0.41	0.32	0.36	0.2	0.61	0.38	0.32	0.09	0.37	0.47	0.23	0.29		**	**	**	**	**	**	**	**	**
SP1	0.44	0.55	0.47	0.4	0.32	0.63	0.48	0.47	0.47	0.41	0.58	0.44	0.39	0.55		**	**	**	**	**	**	**	**
FRA	0.43	0.44	0.42	0.39	0.37	0.61	0.37	0.41	0.43	0.51	0.52	0.44	0.41	0.52	0.65		**	**	**	**	**	**	**
NOR	0.42	0.42	0.4	0.4	0.34	0.62	0.42	0.39	0.36	0.43	0.5	0.39	0.38	0.46	0.59	0.18		**	**	**	**	**	**
RUS	0.25	0.08	0.19	0.32	0.21	0.47	0.16	0.16	0.23	0.34	0.19	0.19	0.27	0.31	0.53	0.36	0.37		**	**	**	**	**
SP2	0.22	0.2	0.24	0.19	0.2	0.39	0.17	0.17	0.24	0.24	0.28	0.24	0.17	0.33	0.39	0.14	0.16	0.17		**	**	**	**
HUN	0.27	0.25	0.27	0.28	0.25	0.44	0.06	0.09	0.27	0.33	0.33	0.27	0.24	0.37	0.47	0.2	0.31	0.15	0.09		*	**	**
ITA	0.27	0.28	0.26	0.31	0.25	0.51	0.06	0.02	0.25	0.35	0.36	0.24	0.22	0.39	0.57	0.41	0.42	0.13	0.16	0.06		**	**
FIN1	0.41	0.47	0.36	0.3	0.36	0.64	0.37	0.37	0.35	0.48	0.48	0.37	0.28	0.47	0.62	0.56	0.5	0.42	0.29	0.37	0.4		*
FIN2	0.39	0.47	0.35	0.3	0.34	0.61	0.37	0.35	0.31	0.44	0.47	0.33	0.25	0.37	0.57	0.55	0.51	0.4	0.32	0.38	0.38	0.09	

* : $0.05 > p > 0.01$; ** : $p > 0.01$; n.s. : non significant

2.4.2. Population structure and geographic patterns

Pairwise F_{ST} values varied from 0.02 to 0.65 and showed in general strong genetic differentiation (average among all populations, F_{ST} = 0.301) even among populations in close vicinity (**Table 2-2**). Only two pairs of populations showed no significant genetic differentiation (BNL1 - BNL5 and BNL8 - ITA). However, when excluding loci DaB17/16

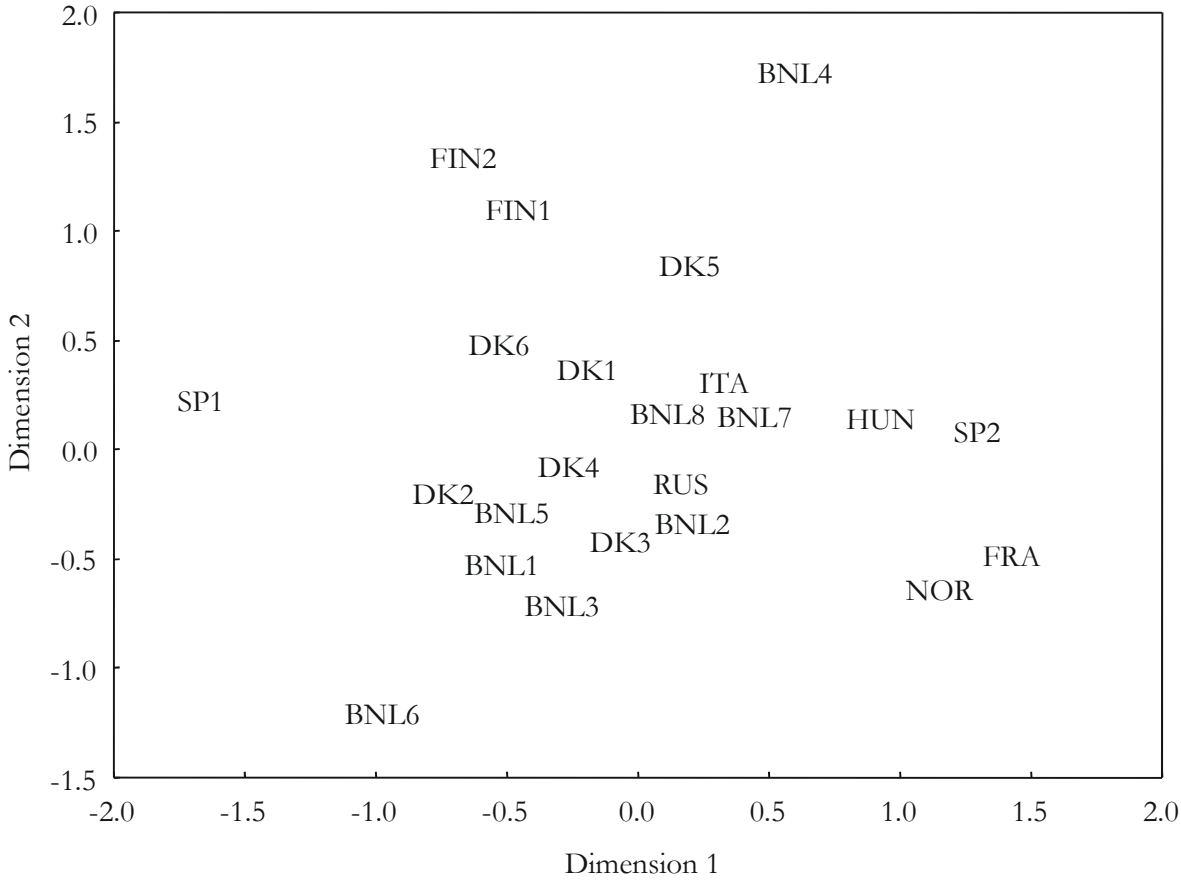

Fig. **2-3** Multidimensional scaling of European *Daphnia galeata* populations using genetic distances based on proportions of shared microsatellite alleles. Labels correspond to the populations as listed in Table 2-1: SP 1+2 = Spain, ITA = Italy, HUN = Hungary, FRA = France, BNL = Belgium/The Netherlands, DK = Denmark, NOR = Norway, FIN 1+2 = Finland, RUS = Russia.

and DaB10/14 from the differentiation analysis, in 10 % of all pairs of populations divergence was offset. This may be due to stochastic events, because only three loci were actually included for the analysis by the program. A similar picture emerged from a multidimensional scaling analysis of genetic distances based on shared alleles (**Fig**. **2-3**).

Table 2-3 Assignment of *Daphnia galeata* individuals (only one individual per MLG included) to populations (POP) based on Nei-distances (D_S, Nei 1972). Rows correspond to the population from which the individual was sampled and columns represent the population to which the individual was assigned (upper triangle) and *vice versa* for lower triangle. Values in parentheses represent the number of populations which contain misassigned individuals. Bold values show the number of individuals assigned to population of origin. Abbreviations correspond to the populations listed in Table 2-1.

POP	BNL1	BNL2	BNL3	BNL4	BNL5	BNL6	BNL7	BNL8	DK1	DK2	DK3	DK4	DK5	DK6	SP1	FRA	NOR	RUS	SP2	HUN	ITA	FIN1	FIN2
BNL1 (17)	**15**	4	14	6	15	8	6	6	5	6	4	8	8					6	7	5	6	1	
BNL2 (18)	9	**19**	19	3	17		12	10	9	2	17	10	5	1		1	1	19	21	12	6		
BNL3 (13)	19	5	**26**	2	21	8	1	1	4		5	18	1					6	6				
BNL4 (11)	1	1		**19**	3			1	2			1	4	1					2			1	1
BNL5 (17)	23	1	17	5	**27**	7	5	9	11	12	1	11	11	6				5	11	4	4		
BNL6 (5)	18		17		13	**19**						3							1				
BNL7 (14)	5	8	7		6		**24**	17	2	2	7	4	6					9	16	23	12		
BNL8 (18)	10	6	6	2	13		22	**21**	8	6	3	9	11	1				8	15	18	15	1	1
DK1 (16)	5	2	8	6	13		4	8	**17**	5		11	12	7				6	7	3	4	1	
DK2 (16)	9	1	4	1	11		2	4	5	**10**		4	5	3				3	5		2	1	1
DK3 (15)	5	17	15	1	11		10	4	3	1	**17**	4	1					15	13	3	2		
DK4 (18)	24	5	23	5	26	1	6	9	14	7	1	**27**	9	6				9	13	4	4	1	
DK5 (15)	4		1	19	9		1	6	6	6		5	**21**	4				1	8	1		1	1
DK6 (19)	3	1	7	2	15		2	4	16	5	1	14	11	**15**			1	7	8	3	2	5	3
SP1 (5)	1				6					2			2		**19**				1				
FRA (4)																**8**	4		7	3			
NOR (11)					3				2	1		1	1	1		8	**15**	2	17	3	1		
RUS (15)	4	14	14		12		11	10	7	1	6	9	3	1				**17**	19	16	10		
SP2 (16)	3	5	3	2	3		7	7	2	2		2	2			5	3	8	**18**	14	8		
HUN (13)	1	4	2		2		15	12				1	1			5	1	4	15	**19**	11		
ITA (14)	6	4	5		7		15	16	2	2	1	4	7					8	12	14	**10**		
FIN1 (7)			1		1				1		1		5						3			**9**	7
FIN2 (10)			1	2	2				2	1		2	7	1					2			15	**15**

A number of geographically close populations were genetically highly differentiated, (e.g. BNL4 and BNL2, SP1 and SP2), whereas others showed hardly any genetic differentiation (e.g. BNL1 and BNL5). A similar pattern of high and low genetic differentiation was observed among distant populations, e.g. a population from Russia clustered within the majority of central European populations.

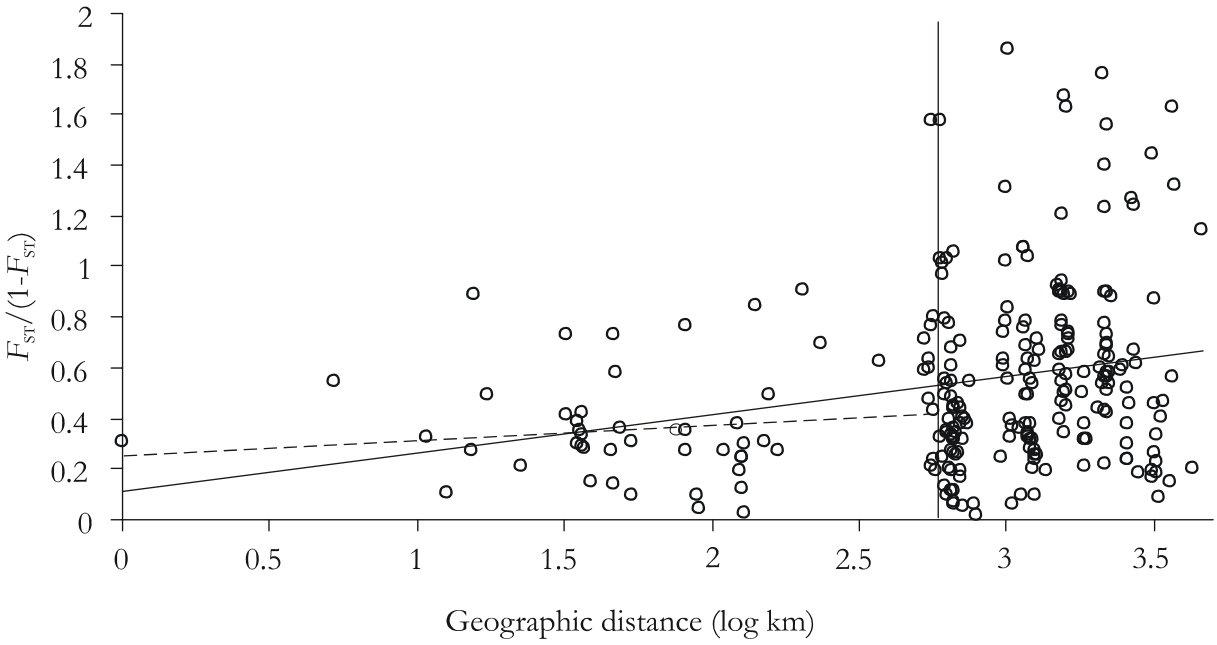

Fig. 2-4 Relationship between geographic distance (log km) and modified pairwise genetic differentiations (Rousset 1997) of 23 European *Daphnia galeata* populations based on microsatellite DNA data (solid line; $r^2 = 0.0643$, $P = 0.022$). Correlations among populations of less than 700 km geographic distance (vertical line) resulted in no significant association with genetic differentiation (dashed line, $r^2 = 0.01$, $P = 0.155$).

The results of the assignment test showed that 10 out of 23 populations appeared to be genetically very similar to other populations resulting in a high number of misassignments (**Table 2-3**). The geographic distances between the original population and the population to which the individuals were assigned ranged from 53 km (BNL1 - BNL3) to up to 4255 km (RUS - SP2).

Although many geographically distant populations showed low levels of genetic differentiation (e.g. ITA - BNL7 and ITA - BNL8) a Mantel test revealed a significant association ($P = 0.022$; **Fig. 2-4**) between geographic distance and standardised genetic differentiation ($F_{ST}/(1 - F_{ST})$). However, an analysis based on all population pairs with geographic distances below 700 km revealed no significant correlation ($P > 0.05$).

Table 2-4 Analysis of molecular variance of 23 *Daphnia galeata* populations across Europe. A: Analysis of molecular variance among and within regions and populations. B: Analysis of molecular variance among and within populations and months.

A.

Source of variation	d.f.	Percentage of variation	*P*
Among regions	8	8.51	*
Among populations within regions	14	17.56	*
Within populations	971	73.93	*
Total	993		

B.

Source of variation	d.f.	Percentage of variation	*P*
Among populations	16	22.7	*
Among months within populations	9	4.82	*
Within months	608	72.48	*
Total	633		

* $P < 0.01$

The largest proportion of the genetic variation was found within populations (73.93%), 8.51% of the variation was found among regions and 17.56% among populations within regions (**Table 2-4**A). The variation among months within populations was relatively low (4.82%) compared to the variation within months (72.48%; **Table 2-4**B). No major shifts in allelic composition were detected between months; however, the inter-monthly heterogeneity of allele frequencies was relatively high (data not shown). The number of MLG's did not change from month to month, but within a given population I detected complete substitution of MLG's among months (data not shown).

2.5. Discussion

2.5.1. Clonal diversity

In general, clonal diversity (*D*) appears to be relatively high in terrestrial as well as in freshwater cyclic parthenogenetic organisms (De Meester *et al.* 2004). For example, clonal diversity ranged from 0.46 to 0.92 in grain aphids (*Sitobion avenae*, Llewellyn *et al.* 2003), and Gomez (2000) detected 0.9 for the rotifer species *Brachionus plicatilis*. Clonal diversity in cyclical parthenogenetic freshwater zooplankton populations has been suggested to depend

on habitat characteristics such as permanency and lake or pond size (reviewed in De Meester 1996). Intermittent pond populations of *D. pulex* (Hebert *et al.* 1988; Pálsson 2000), *D. carinata* (Hebert and Moran 1980) and *D. magna* (Vanoverbeke and De Meester 1997; Mitchell *et al.* 1998) showed higher clonal diversity than permanent pond populations (Wolf 1988). By way of example, in several Finnish populations *D. pulex* has been revealed a mean diversity of $D = 0.697$ (range 0.04 – 1, Pálsson 2000). However, several studies on lake-dwelling species like *D. pulicaria* (Geedey *et al.* 1996; Weider *et al.* 1999a), *D. hyalina* and *D. cucullata* (Gießler 1997) found high levels of clonal diversity (D up to 0.5), which almost reach the average level of genetic diversity in intermittent pond populations. The population genetic data on large-lake populations revealed inconsistent patterns. For instance, Mort and Wolf (1986) detected a relatively low clonal diversity ($D = 0.139$) in large-lake *D. galeata* populations from northern Germany. Their study, however, relied on only three allozyme loci and an interconnected lake area. my data from 23 European *D. galeata* populations revealed a mean D of 0.62, which indicates high clonal diversity in lake species and a significant contribution of sexual reproduction to the genetic architecture of contemporary populations.

Resting eggs in most *Daphnia* species are produced sexually (except for a few arctic species). Therefore, the absolute number of resting eggs in a given lake represents the potential number of clones. Carvalho and Wolf (1989) estimated the number of resting eggs in the upper eight centimetres of Lake Kellersee sediments to range from 37,000 to 120,000 m^{-2}. Even if only one per cent of resting eggs hatch each year, then this lake would still contain 740-2400 clones m^{-2}. Thus enormous amount of resting eggs provides a potentially important source of genetic variation in large lakes. Observed high levels of genetic variation and establishment of populations suggests that sexual reproduction is more important in large lake populations than previously thought. Thus, my results support the notion that other factors than habitat permanency such as the amount of biparental reproduction, the level of recruitment from a large and genetically diverse resting egg pool and population size are important to explain variation in local diversity.

2.5.2. Genetic variation within populations

Daphnia populations of intermittent and permanent habitats are known to differ in levels of heterozygosity and with regard to deviations from HW-expectations. Overall, allele frequencies of intermittent pond populations are found to be in good agreement with HW-expectations (Korpelainen 1984; Hebert 1987b; Vanoverbeke and De Meester 1997). In

contrast, heterozygote excess is relatively common in permanent pond populations (De Meester 1996). These pattern have been explained by strong clonal selection and genetic erosion in permanent ponds, whereas occasional heterozygosity deficiencies of intermittent pond populations suggest Wahlund effects due to simultaneous hatching of sexual eggs produced in several growing seasons. Populations genetic studies of other cyclic parthenogens revealed similar results, e.g. the freshwater bryozoan *C. mucedo*, often shows heterozygote deficiencies and persistent deviations from HW-expectations in semi permanent habitats (Freeland *et al.* 2000b). The heterozygote deficiencies are probably due to inbreeding since released sperm is dispersed over only short distances which results in self-fertilisation or cross-fertilisation between closely related clonal lineages (Freeland *et al.* 2000b; Freeland *et al.* 2000a). A similar pattern of heterozygote deficiencies was observed in the aphid species *Rhopalosiphum padi* and attributed to allochronic isolation of differentiated lineages (Delmotte *et al.* 2002). Permanent lake populations of *Daphnia* are, in contrast to intermittent and permanent pond populations, not characterized by a consistent pattern of heterozygosity excess or deficiency (Mort and Wolf 1986; Gießler 1997). Although populations deviated from HW-equilibrium, they were found to exhibit either heterozygote excess or deficiencies. However, I observed strong deviations from HW-expectations in 18 of the 23 studied *D. galeata* populations. In 87% of the populations, I observed significant heterozygote deficiencies. Inbreeding as a cause of the observed heterozygote deficiencies is unlikely in *D. galeata*, since populations are diverse and inbreeding avoidance was reported (De Meester and Vanoverbeke 1999). In general, no major effects of genetic drift are expected in *Daphnia* since population sizes are large due to the establishment of resting egg banks. Some of the *D. galeata* populations that I monitored throughout the growing season ($N = 7$) showed complete clonal replacement between months suggesting strong allochronic isolation among clonal lineages. Therefore I argue that the observed heterozygote deficiencies are most likely the result of simultaneous hatching of individuals produced during different times of the year or the coexistence of ecologically or genetically differentiated subpopulations. These conclusions are consistent with field observations with regard to seasonal variation in clonal composition (Carvalho and Crisp 1987) and strong ecological differentiation of coexisting clonal groups (Lynch 1983; Hebert 1987b). If the amount of ephippia represents an essential factor determining clonal diversity, lake size is expected to be correlated with clonal diversity. I neither detected a significant positive correlation between lake size and clonal diversity, nor with any other measure of genetic variation. In part, this may be explained by the fact that the size range of the studied lakes was rather limited (3 - 124 ha, with a mean of 22 ha). Further

studies on the temporal and spatial dynamics of clonal diversity in lakes of a wide range in habitat sizes are needed. Another process, interspecific hybridisation, might contribute to the deviations from HW-equilibrium, since introgression substantially alters the genetic architecture of intercrossing species (Arnold 1992; Muller and Seitz 1995). Since *D. galeata* belongs to the most frequently hybridising taxa of the *D. longspina*-complex (Schwenk *et al.* 2000) observed population structures might represent a mix of pure and introgressed genotypes. Since I detected slightly higher clonal diversity and higher observed heterozygosity among *D. galeata* populations which co-occur with interspecific hybrids than among "pure" *D. galeata* populations, interspecific hybridisation might influence for elevated levels of genetic diversity within populations and increased levels among population differentiation.

In addition to the above-mentioned biological explanations for deviations from HW-expectations, stochastic effects may also have contributed to the observed heterozygote deficiencies. Due to the high observed genetic variation within populations I might have collected non-representative samples to detect HW-equilibrium. In order to test this hypothesis I are currently applying a larger set of molecular markers (15 microsatellites) to natural populations.

2.5.3. Population structure and geographic patterns

Zooplankton organisms dispersing passively via diapausing eggs have a high ability to colonise new habitats (Brendonck and De Meester 2003; Figuerola *et al.* 2003). Resting eggs withstand harsh conditions and are the means by which many aquatic organisms disperse (Bilton *et al.* 2001). Dispersal vectors include mammals, birds and, for short distances, even wind. Freshwater zooplankton taxa, although exhibiting good possibilities of dispersal, in most cases show high levels of genetic differentiation between populations (Hebert and Moran 1980; Crease *et al.* 1990; Vanoverbeke and De Meester 1997; Gomez and Carvalho 2000). Levels of genetic differentiation varying around 0.37 - 0.7 (F_{ST} or G_{ST}) were found in both intermittent or permanent ponds (Korpelainen 1984; Hebert *et al.* 1993; Vanoverbeke and De Meester 1997; Pálsson 2000). Pálsson (2000) observed a broad range of F_{ST} values among populations of *D. pulex* (0.05 - 0.9) and suggested that either strong gene flow or founding of nearby populations by the same genotype explains the instances of low genetic differentiation among geographically close populations. Much lower levels of differentiation than in pond species were found in the pelagic species *D. hyalina*, *D. cucullata*, (F_{ST} = 0.204,

0.165, respectively, Gießler 1997) and *D. galeata* (F_{ST} = 0.091, Mort and Wolf 1986). Significant levels of genetic differentiation despite high dispersal capabilities frequently observed in cladocerans might be due to the several processes summarised in the "monopolisation hypothesis" (De Meester *et al.* 2002). This hypothesis suggests that strong founder events in combination with rapid local adaptation result in a monopolization of resources by descendants of the first immigrants. Since resident genotypes are expected to be generally better adapted to the local environmental conditions than immigrant genotypes, gene flow among populations will be strongly constraint. My data do not reject predictions of the monopolisation hypothesis, since I found strong population differentiation even among neighbouring populations. A similar pattern was observed using estimates of differentiation derived from an assignment test. Most of the individuals were assigned to their source population (**Table 2-3**). Misclassified individuals were assigned to populations either in close vicinity (e.g. 53 km) or to populations distant from the source population (e.g. 4255 km). Since adult individuals are most unlikely transported between unconnected habitats, I attribute MLG-similarity among populations to stochastic effects. An analysis of allele frequencies across the study area did not reveal a consistent geographical pattern. Some alleles showed a decreasing south-north gradient, whereas others were mainly found in the north and decreased in frequency in southern populations. Populations previously covered by ice during the last glacial period (NOR, FIN1/2 and RUS, **Table 2-1**) do not show lower levels of genetic variation (*D*, H_{exp} and allelic richness) compared with populations of potential glacial refugia. One of the Finnish populations (FIN2), together with populations from Belgium, Denmark (e.g. BNL3, 4, 5, and 7, DK4 and 5; **Fig. 2-1**) and Spain (SP2), even exhibited the highest values of genetic diversity (*D* and relative number of alleles). This pattern is in sharp contrast to many empirical examples showing the highest genetic variation in Mediterranean populations versus populations of previously glaciated areas (Coope 1994; Hewitt 1996). Northern populations were either recolonized from central European areas (e.g. from pre-glacial lakes), or the high genetic variation in microsatellite markers reveals current levels of genetic differentiation, rather than patterns of initial colonisation after the retreat of glaciers. Further studies using less variable genetic markers, such as mtDNA, are required to unravel phylogeographic patterns and levels of "ancient" gene flow.

Previous population genetic studies on several freshwater zooplankton species covering large geographical areas revealed no isolation by distance (IBD, Hebert and Moran 1980; Hebert *et al.* 1989; Innes 1991; Freeland *et al.* 2000b). Similar patterns were found among neighbouring *D. galeata* populations and among mainly interconnected lakes (25 km, Mort

and Wolf 1986; 13 km, Gießler 1997). Vanoverbeke and De Meester (1997) instead found higher genetic differentiation with increasing geographic distance. In addition, the relationship between genetic differentiation (F_{ST}) and geographical distance was also significant at a larger geographical scale (*D. pulex* on both sides of the Baltic Sea, Pálsson 2000). The *D. galeata* populations studied here show a similar pattern. Within short distances (up to 700 km), no significant IBD was observed. The lack of any association between genetic differentiation and (short) geographic distance among populations is most likely based on the high genetic differentiation already observed among populations in very close vicinity (e.g. DK1-DK3 are only 5.2 km apart and show a F_{ST} value of 0.35). However, a significant positive correlation ($P = 0.019$) between geographic distance and genetic differentiation was observed if populations were included which are more than 1000 km apart.

2.6. Conclusions

In agreement with previous studies on lake-dwelling *Daphnia* species, I detected high clonal diversity within populations, but in contrast to earlier studies, I also observed consistent deviations from H-W equilibrium due to heterozygote deficiencies. Although *D. galeata* reproduces *via* cyclic parthenogenesis, population genetic data suggests a significant impact of sexual reproduction. Allochronic isolation of divergent clonal lineages or differential hatching of ecologically divergent sub-populations might explain the heterozygote deficiency in *D. galeata* populations. Patterns of genetic differentiation among populations on the one hand indicate no isolation by distance (only across large distances), and on the other hand suggest strong genetic differentiation of populations in close proximity.

3. Current and historic genetic differentiation among European *Daphnia galeata* populations

3.1. Abstract

The population genetic structure and diversity in *D. galeata* was investigated at a European scale using six microsatellite loci and 12S rDNA sequence data to infer and compare historical and contemporary patterns of gene flow. *D. galeata* has the potential for long-distance dispersal *via* ephippial resting eggs by wind and other dispersing vectors (waterfowl), but shows in general strong population differentiation even among neighbouring populations. A total of 427 individuals were analysed for microsatellite and 85 individuals for mitochondrial (mtDNA) sequence data from 12 populations across Europe. I detected genetic differentiation among populations across Europe and locations within sampling regions for both genetic marker systems (average values: mtDNA F_{ST} = 0.574; microsatellite F_{ST} = 0.389), resulting in a lack of isolation by distance. Furthermore, several microsatellite alleles and one haplotype were shared across populations. Partitioning of molecular variance was inconsistant for both marker systems. Microsatellite variation was higher within than among populations, whereas mtDNA data yielded an inverse pattern. Relative high levels of nuclear DNA diversity were found across Europe. The amount of mitochondrial diversity was low in Spain, Hungary and Denmark. Gene flow analysis at a European scale did not reveal typical pattern of population recolonization in the light of postglacial colonization hypotheses. Populations, which recently experienced an expansion or population-bottleneck were observed both in middle and northern Europe. Since these populations revealed high genetic diversity in both marker systems, I suggest these areas to represent postglacial zones of secondary contact among divergent lineages of *D. galeata*.

3.2. Introduction

The genetic architecture of natural populations depends on both, current factors related to the biology of the species (e.g. mating system, ecological differentiation) and historical (e.g. colonization process, isolation in geographical refugia). Therefore climatic fluctuations have drastically influenced the distribution of many plant and animal taxa. In particular, repeated glacial and interglacial cycles caused species to retreat into refugia or to expand from refugia during interglacial warming (Hewitt 1996). This process shaped significantly the populations

structure of current species across Europe (e.g., Taberlet *et al.* 1998). Based on the reconstruction of climate conditions and pollen data, three European refugial regions have been identified – the Iberian Peninsula, Italy, the Balkans and the Caucasus. Several studies indicate that recolonization of northern areas, occurred in successive steps; immigrants expanded to form new populations, which then served as new propagule pools for further northward colonization steps (e.g., Clark *et al.* 1998; Hewitt 1999; Hewitt 2000). Accompanied with colonization are repeated population bottlenecks which may lead to increased genetic differentiation among recently colonized populations and to a loss of genetic diversity (Taberlet *et al.* 1998; Hewitt 2000). In addition to historical events, the distribution of genetic variation is also affected by contemporary evolutionary forces related to for example local adaptation (e.g., Kawecki and Ebert 2004).

Freshwater habitats in most of northern Europe have been available to colonists for only 10 000 years, because much of this area was glaciated (Hewitt 1999). Widespread zooplankton species depend solely on the passive dispersal of their (mainly sexually) produced resting eggs by vectors, like waterfowl (Figuerola *et al.* 2005). Therefore, distribution of these passively dispersed clonal lineages is random (Bohonak and Jenkins 2003). In addition, multiple colonization events by several clonal lineages might be limited because of priority effects and local adaptation (De Meester *et al.* 2002). Hence, current phylogeographic pattern should represent original colonization (Boileau *et al.* 1992). Disentangling the extent to which population structure results from recurrent forces, such as gene flow, versus historical events, such as fragmentation and range expansion, will be crucial to understanding the frequency of dispersal in freshwater taxa. However the distributions of zooplankton species are often the result of recent glaciation as reported mainly for North American taxa (e.g. Dumont 1983; e.g. Weider 1989; Stemberger 1995). In Europe, however only a few species have been subjected to large scale population genetic studies using both nuclear and mitochondrial DNA (bryozoa, Freeland *et al.* 2000c; rotifers, Gomez *et al.* 2002) or phylogeographic studies, e.g. on *Daphnia magna* (De Gelas and De Meester 2005) using only mitochondrial DNA. All three studies reported population fragmentations during the late or early Pleistocene and some long-distance colonization. In contrast to the results of bryozoans populations, general findings of rotifers and *Daphnia* populations were consistent with regards to deep phylogeographic structure, and low levels of gene flow. Gomez et al (2000) and De Gelas and De Meester (2005) found isolation by distance, whereas Freeland et al (2000c) showed no relationship between population differentiation and their geographic

pattern. There are no data available yet on large scale phylogeographic pattern of lake-dwelling *Daphnia* species.

In order to fill this gap, I applied mitochondrial 12S rDNA sequences and six microsatellite loci in the cyclic parthenogenetic organism *D. galeata*. I assessed e.g. the modern-day distribution and survival of ancient clonal lineages. In addition, I compared the two marker systems in the light of evolutionary and population genetic properties, such as gene flow, genetic diversity, isolation by distance, and population differentiation. Based on these estimates I discuss the genetic assumptions associated with expanding populations and possible routes of recolonization pattern for *D. galeata* across Europe.

3.3. Materials and methods

3.3.1. Sampling and identification of Daphnia galeata

Members of the *D. longispina* species complex of four Belgian lakes (lakes BNL4-7) and two Danish lakes (DK1 and 2) were sampled monthly during the growing season (April - October) of 2000 or 2001, depending on the lake. An additional five lakes were sampled once in summer 1998, such as in Spain (SP2), in France (FRA), in Hungary (HUN), in Russia (RUS) and in Norway (NOR), whereas one lake in Finland (FIN2) was sampled once in summer 2000 (**Fig. 2-1** and **Table 2-1**). Individuals were stored in ethanol. Adult individuals were characterised both morphologically (Flößner 2000) and using recently developed nuclear DNA markers (Schwenk *et al.* 2000; Billiones *et al.* 2004) for identification as *D. galeata*. For the latter analysis, a random selection of 5-10 individuals per population was subjected to a RFLP (Restriction Fragment Length Polymorphism) analysis of an amplified ITS-region (Billiones *et al.* 2004). If populations were composed of parental species and interspecific hybrids, then all individuals (21 to 44) were subjected to PCR-RFLP analyses prior to microsatellite screening and sequencing.

3.3.2. Genotyping and sequencing

Total DNA was extracted from an average of 35 (21-44) individuals per population using a modified procedure of Walsh *et al.* (1991). Each individual was incubated overnight in 50-100 µl 6% Chelex and 10 mg/ml Proteinase K (Boehringer Mannheim). I adjusted the volume of the incubation buffer to the size of the individuals. After brief vortexing, samples were boiled for 10 minutes. Homogenates (supernatant) were isolated from the Chelex resign and

stored at 4 °C or -20 °C. Polymerase chain reactions (PCR) were performed for six microsatellite loci per individual carried out as described in Dove *et al.* (submitted).

Sequencing of the mitochondrial 12S rDNA gene was carried out for *D. galeata* specimens (varying N = 1-17) of a subsample of populations (N_P = 12) used for microsatellite analysis (Dove *et al.* submitted). The 12S rDNA fragment was amplified in a final volume of 35µL, containing template DNA, 0.1µL of each primer (12s6 forward: 5'-ATG CAC TTT CCA GTA CAT CTA C-3'; 12s7 reverse: 5'-AAA TCG TGC CAG CCG TCG C-3', (Colbourne and Hebert 1996)), 20mM dNTP, 1x PCR buffer (10 x, Invitrogen), 3mM $MgCl^{2+}$, 0.5 U Taq (Invitrogen), 0.1 µg/µL of BSA (100x). PCR was performed under the following conditions: 10 cycles of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, followed by 25 cycles of 45 sec at 92 °C, 1 min at 54 °C and 1 min at 72 °C. Direct sequencing was carried out with amplification primers in both direction from obtained PCR fragments using an ABI 377 automated sequencer (Perkin Elmer) using a Prism™ TaqFS dye terminator kit (Perkin-Elmer, Norwalk, CT, USA**)**.

3.3.3. Data analysis

Mean heterozygosity and mean numbers of alleles per locus (allelic diversity) were calculated using the program MSA 3.1 (Dieringer and Schlötterer 2003). Allelic richness was calculated as described by Petit *et al.* (Petit *et al.* 1998) using FSTAT version 2.9.3. (Goudet 1995). Values for the observed and expected heterozygosity (H_o and H_e) in populations (just clonal lineages) were obtained using GENETIX (Belkhir *et al.* 1996-97).

To be able to discuss e.g. the evolutionary effects on population structure, mitochondrial haplotype diversity, (*h,* the probability that two randomly chosen individuals have different haplotypes), nucleotide diversity (π, the average pairwise nucleotide difference between individuals within samples) (Nei 1987) and Tajima's *D* (Tajima 1989) were estimated for each population using ARLEQUIN vs 2.0 (Schneider *et al.* 2000). For the analysis of Tajima's *D* and several populations were combined (BNL: BNL4-7, DK: DK1 and 2, and NOR: NOR and FIN2). Tajima's *D* is expected to be zero when mating is random and populations have reached equilibrium; large significant values of *D* (negative or positive) indicate a deviation from neutrality which may be the result of (directional) selection, recovery from a population bottleneck or population expansion (Tajima 1989; Aris-Brosou and Excoffier 1996).

Populations in stable demographic equilibria have a multimodal frequency distribution of pairwise differences among haplotypes in a sample (Harpending 1994) whereas distribution appears unimodal in populations that have passed through recent demographic expansion (Rogers and Harpending 1992). Based on both nuclear and mitochondrial data (for mtDNA some populations pooled, BNL: BNL4-7, DK: DK1 and 2, and NOR: NOR and FIN2) the validity of the demographic expansion hypothesis was tested using a parametric bootstrap approach, in which the sum of square deviation (SSD) among the observed distribution was compared to the expected distribution (Mismatch distribution in ARLEQUIN vs 2.0, Schneider *et al.* 2000). The relationship $\tau = 2ut$ (Rogers and Harpending 1992) was used to estimate an appropriate time of expansion (t) for *D. galeata* populations, where τ represents the mode of the mismatch distribution, expressed in units of evolutionary time and u represents the mutation rate. The value u was calculated from the formula $u = 2\mu k$, where μ equals the mutation rate per nucleotide and k is the number of nucleotides assayed *via* sequencing (493 bp). Since no molecular clock calibration for *Daphnia* 12s DNA gene based on fossil records is available, I considered divergence rates of mitochondrial coding sequences, which have been reported for crustaceans to range from 1.66 to 2.6% per million years (Knowlton *et al.* 1993; Paternello *et al.* 1996; Schubart *et al.* 1998). Based on previous studies (Schwenk *et al.* 2000) I applied an average molecular clock rate of 2.3% sequence divergence per million. Additional hierarchical analysis of variation among populations were conducted using an analysis of molecular variance (AMOVA) as described by Excoffier *et al.* (1992) for microsatellite- and mtDNA sequence data. To investigate population genetic structure, both microsatellite and mitochondrial pairwise F_{ST} (Weir and Cockerham 1984) were calculated as implemented in ARLEQUIN vs 2.0 (Schneider *et al.* 2000). In the latter two analyses for mtDNA data only populations with $N \geq 3$ were applied and the same subset of populations was used for microsatellite data. Analysis of isolation by distance (IBD) and Mantel tests (Mantel 1967) of mitochondrial (only populations of $N \geq 3$) and nuclear (same subset of populations as for mtDNA) divergences (F_{ST}) were conducted using GenAlExV5_04PC (Peakall and Smouse 2001). BOTTLENECK (Cornuet and Luikart 1996) was applied to test for recent reductions in population sizes based on microsatellite data using a Wilcoxin sign-rank test (Luikart and Cornuet 1997) and a two phase model (TPM). This intermediate model combining a stepwise mutation model (SMM) and an infinite allele model (IAM), was suggested to fit microsatellite data the best (Di Rienzo *et al.* 1994). The Wilcoxon sign-rank test provides a high statistical power even if the number of loci and individual is low.

3.3.4. Gene flow anaylsis

Gene flow rates and directionality of gene flow were calculated for mtDNA sequence data using MIGRATE 1.7.6.1 (Beerli 2002), which employs Markov chain Monte Carlo (MCMC) sampling of gene trees to estimate θ and asymmetrical migration rates among populations. I pooled populations (BNL: BNL4-7; DK: DK1 and 2; NOR: NOR and FIN2) to circumvent the extremely long computational time required to analyse 12 populations. The MCMC searching strategy employed a series of 10 short chains (5000 gene trees) and three long chains (50,000 gene trees). Initial parameters were estimated using the default settings and the first 10.000 trees were ignored to ensure parameter stability. I repeated this analysis three times and the integrated estimates were used as input for a more exhaustive search using eight heated chains {static:1:1, 1.1, 1.3, 1.5, 2, 3, 5, 10}. The searching strategy was replicated 10 times, parameter estimates were integrated across the replicates and the analyses were repeated twice more to ensure stability. Results from all three analyses were similar and I report only one of them here. Effective population sizes are reported as θ, which translates to $N_e\mu$ for maternal haploid mtDNA, and migration rates as m/μ, calculated as $N_e m/\theta$.

A model-based clustering method implemented in STRUCTURE (Pritchard *et al.* 2000) was used to estimate the expected number of populations (*K* clusters) and the level of admixture among populations based on the number of expected populations. Estimated posterior probabilities for the simulated model fitting the data were calculated assuming a uniform prior for *K*, where *K* ranged from one to 19. To minimize the effect of the starting configuration during the Monte Carlo simulation, I discarded the first 30×10^3 iterations of the Markov chain before data for the parameter estimation were collected from another 10^3 iterations. Three independent runs of the Markov chain, each of 10^3 updates, were performed to assure convergence of the chain and homogeneity among runs for each prior of *K*. The posterior probabilities of *K* were then calculated using Bayes' rule. For the simulation the program was run without population identifiers (USEPOPINFO = 0) and allowing admixture mode (NOADMIX = 0). Alpha was allowed to vary for all simulations. In order to specifically assess the level of admixture between European *D. galeata* populations, I additionally calculated individual admixture coefficients for each population (USEPOPINFO = 1) assuming a model of $K = 18$ (burn-in period of 30 000 steps and 50 000 MCMC steps), as suggested by prior simulations and averaged the individual admixture coefficients per population.

3.4. Results

3.4.1. Estimates of genetic diversity

There was a negligible evidence of linkage disequilibrium in 12 out of 180 pairwise comparisons, whereas all estimates of heterozygosity were significantly lower than expected ($P < 0.05$).

Table 3-1. Comparison of nuclear and mitochondrial genetic variation in *Daphnia galeata* populations of Denmark and Belgium. *N*: number of individuals; MLG: number of multi-locus genotypes; H: Number of haplotypes; S_D: Shannon Wiener diversity of multi-locus genitypes; π: nucleotide diversity; *h*: gene diversity, Nei 1987.

	Nuclear DNA (six microsatellite loci)						Mitochondrial DNA (12S rRNA)			
Population	*N*	MLG	Allelic richness	S_D	H_O	H_E	*N*	*H*	π	*h*
BNL4	37	25	0.622	1.053	0.371	0.582	3	2	0.0027 ± 0.00278	0.67 ± 0.314
BNL5	37	32	0.568	0.829	0.365	0.462	1	1	0	1
BNL6	38	20	0.368	0.469	0.228	0.251	10	5	0.0031 ± 0.0023	0.822 ± 0.097
BNL7	37	29	0.421	0.663	0.272	0.408	3	1	0	0
DK1	38	19	0.414	0.579	0.335	0.251	3	1	0	0
DK2	32	12	0.406	0.488	0.218	0.294	4	1	0	0
FIN2	22	16	0.591	0.48	0.292	0.296	2	2	0.002 ± 0.00287	1 ± 0.5
SP2	44	30	0.591	0.981	0.131	0.558	12	2	0.0036 ± 0.0026	0.546 ± 0.061
RUS	37	22	0.486	0.559	0.159	0.313	8	3	0.00267 ± 0.00213	0.464 ± 0.2
NOR	38	20	0.342	0.477	0.129	0.292	14	1	0	0
FRA	18	10	0.667	0.524	0.233	0.344	8	2	0.0005 ± 0.0007	0.25 ± 0.18
HUN	39	26	0.358	0.811	0.161	0.464	17	2	0.0015 ± 0.0013	0.382 ± 0.11

Multi-locus genotype frequency was highest in BNL5 (0.865) and lowest in DK2 (0.375). These values are derived from results obtained in Chapter 2 (**Table 2-1**). Allelic richness varied between 0.342 (NOR) and 0.667 (FRA;**Table 3-1**) and up to two alleles of each of the six microsatellite loci was represented with varying frequencies across all populations (data

not shown). In addition, many microsatellite alleles were present from the Iberian Peninsula (SP2) up to the Pechora Lake (RUS). Private alleles were found only in the Spanish, Hungarian, and Finnish population. The Shannon Wiener diversity (**Table 3-1**) varied among sampling sites and was highest in BNL4 (1.053) and lowest in BNL6 (0.469).

Estimates of mtDNA variability varied strongly among populations (**Table 3-1**). In total 14 halotypes among 85 individuals were found. Haplotype frequencies varied from 1 (BNL5 and FIN2) to 0.07 (NOR), whereas most mitochondrial haplotypes were found in Belgium (BNL6).

Table 3-2 Demographic estimation from mismatch distribution and neutrality test results. *Daphnia galeata* populations of three lake regions were combined for these analysis (BNL: BNL4-7, DK: DK1 and 2, and NOR: NOR and FIN2) to obtain a proper number of sequences (*n*). *D* Tajima's D statistic; τ the expansion parameter expressed in units of mutational time; $P_{(Ssd)}$ the probability of observing by chance a higher values of the sum of squared deviations than the observed one under the hypothesis of population expansion; θ_0 and θ_1 are estimates for populations size before and after expansion; *t* time since expansion (in million years before present) based on τ and a divergence rate of 2.3% per million years.

	n	*D*	τ	$P_{(Ssd)}$	θ_0	θ_1	*t*
BNL	17	-0.216	2.360	0.562	0.001	12.114	0.052
DK	7	1.811	3.900*	0.013	0.000	3.496	0.066
NOR	16	-0.987	3.000	0.051	0.180	0.189	0.087
RUS	8	-1.595*	5.344	0.421	0.002	0.815	0.118
SP2	12	2.123	3.982*	0.000	0.000	2.783	0.088
HUN	17	0.731	3.086*	0.025	0.007	0.770	0.068
FRA	8	-1.055	0.324	0.328	0.000	4.580	0.007

* $P < 0.05$

Nucleotide diversity (π) was very low ranging from 0 (BNL5, BNL7, DK1, DK2, and NOR) to 0.0036 (SP2). Gene diversity (*h*) varied strongly among populations ranging from 0 (BNL7, DK1, DK2, and NOR) to 1 (BNL5 and FIN2). Except for BNL6 ($H = 5$), all populations consisted of one, two or three haplotypes (**Table 3-1**). Polymorphism within each population was consistent with neutral expectations (Tajima's $D = 0.731 - 2.123$; $P > 0.05$; **Table 3-2**) in populations of Denmark, Spain and Hungary, but non significant negative *D* - values were obtained for Belgian, French, Norwegian and Finish [$D = -1.055 – (-0.216)$; $P > 0.05$]. Only the Russian population differed significantly from neutrality ($D = -1.595$; $P = 0.035$). Two populations (FRA and FIN2; BOTTLENECK) yielded a deviation from the mutation-drift equilibrium. No significant association was detected when testing clonal and haplotype diversity against glaciated and non-glaciated areas.

3.4.2. Analysis of population structure

Microsatellite and mitochondrial DNA All *D. galeata* populations were significantly subdivided using microsatellite loci (**Table 3-3**).

Table 3-3 Pairwise F_{ST} values for mitochondrial sequence data (upper triangle) and microsatellite data (lower triangle) and corresponding significances levels among 10 European *Daphnia galeata* populations (for mtDNA only populations $N \geq 3$, same subset of populations for microsatellite data). Abbreviations correspond to populations listed in **Table 2-1.**

Population	BNL6	DK2	FRA	NOR	RUS	SP2	HUN
BNL6		0.573**	0.448**	0.584**	-0.025 n.s.	0.212*	0.361**
DK2	0.518**		0.940**	1.000**	0.686**	0.544*	0.798**
FRA	0.668**	0.599**		0.953**	0.500**	0.561**	0.689**
NOR	0.690**	0.532**	0.284**		0.703**	0.480**	0.793**
RUS	0.552**	0.441**	0.447**	0.443**		0.310**	0.396**
SP2	0.453**	0.305**	0.149**	0.173**	0.213**		0.540**
HUN	0.495**	0.384**	0.203**	0.325**	0.183**	0.102**	

* : $0.05 > P > 0.01$; ** : $P > 0.01$; n.s. : non significant

In fact, some of the genetic differences (F_{ST}) between populations separated by short geographic distance (BNL4-BNL7; 0.23) were larger than differences between populations between long distances (HUN-BNL7; 0.065).

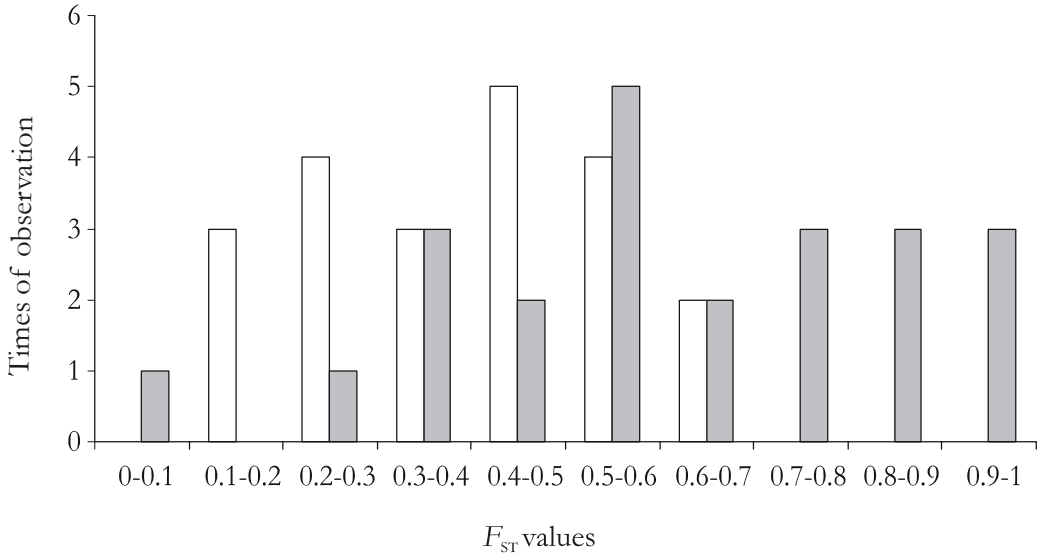

Fig. 3-1 Frequency distribution of F_{ST} values for mtDNA (filled bars) and microsatellite data (open bars) of *Daphnia galeata*. Only populations $N \geq 3$ for mtDNA data and same subset of populations used for microsatellite data.

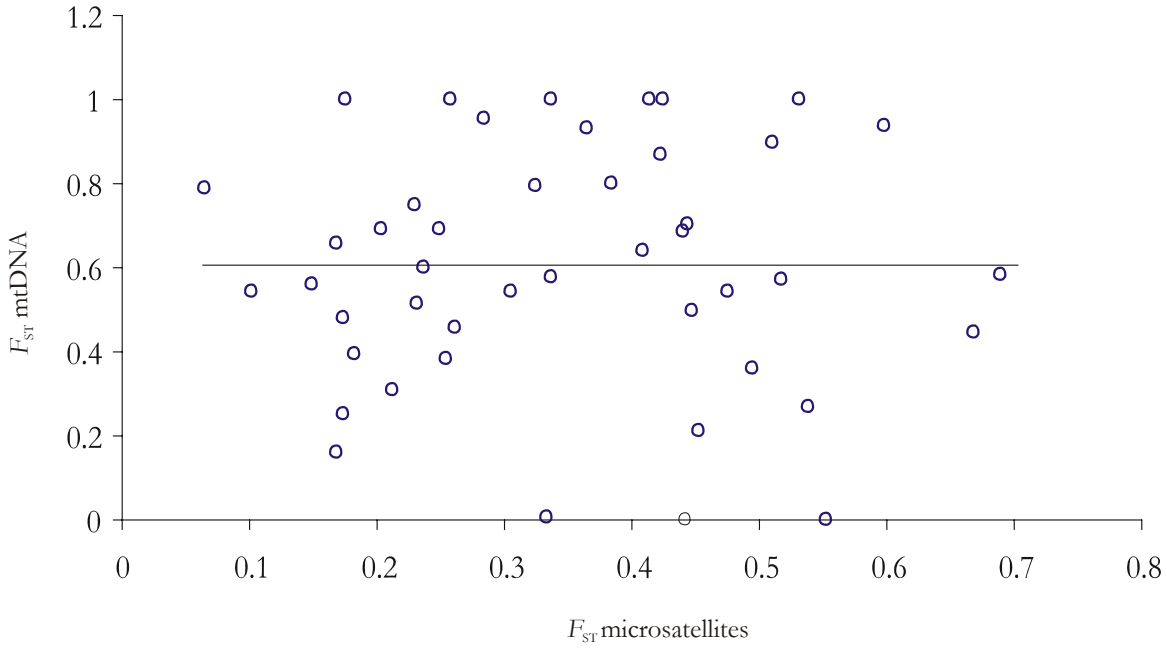

Fig. 3-2 Mantel test representing the relationship of both mitochondrial (mtDNA) and microsatellite (μsat) F_{ST} values; $R^2 = 0.00$, $P = 0.532$. Only populations $N \geq 3$ for mtDNA data and same subset of *Daphnia galeata* populations used for microsatellite data.

Pairwise F_{ST} analysis of mtDNA sequences revealed higher values of differentiation among populations than observed by microsatellite data (**Table 3-3**; e.g. see FRA-NOR, 0.953; **Fig. 3-1**).

Table 3-4 Analysis of molecular variance among and within 10 *Daphnia galeata* populations across Europe using mitochondrial (12S rDNA sequence; mtDNA, only populations $N \geq 3$) and nuclear genetic data (6 microsatellite loci; μ-sat, same subset of populations than for mtDNA data).

Source of variation	d.f.		Percentage of variation		Significance level	
	μ-sat	mtDNA	μ-sat	mtDNA	μ-sat	mtDNA
Among populations	9	9	32.84	59.8	*	*
Within populations	418	72	67.16	40.2	*	*
Total	427	81				

*: $P < 0.01$

However, populations which are geographically well separated revealed even non-significant genetic differences (BNL6-RUS; -0.025). One single mitochondrial haplotype was widely distributed (RUS, FRA, BNL4, and BNL6). The Finnish and Norwegian populations (FIN and NOR) did not share any haplotype neither with each other nor with other populations across Europe. No significant association between microsatellite and mtDNA divergence (F_{ST} values; **Fig. 3-2**) was detected (Mantel Test; $P = 0.532$). Results of analysis using F_{ST} values based on only three loci (Chapter 2) did not reveal notice worth differences.

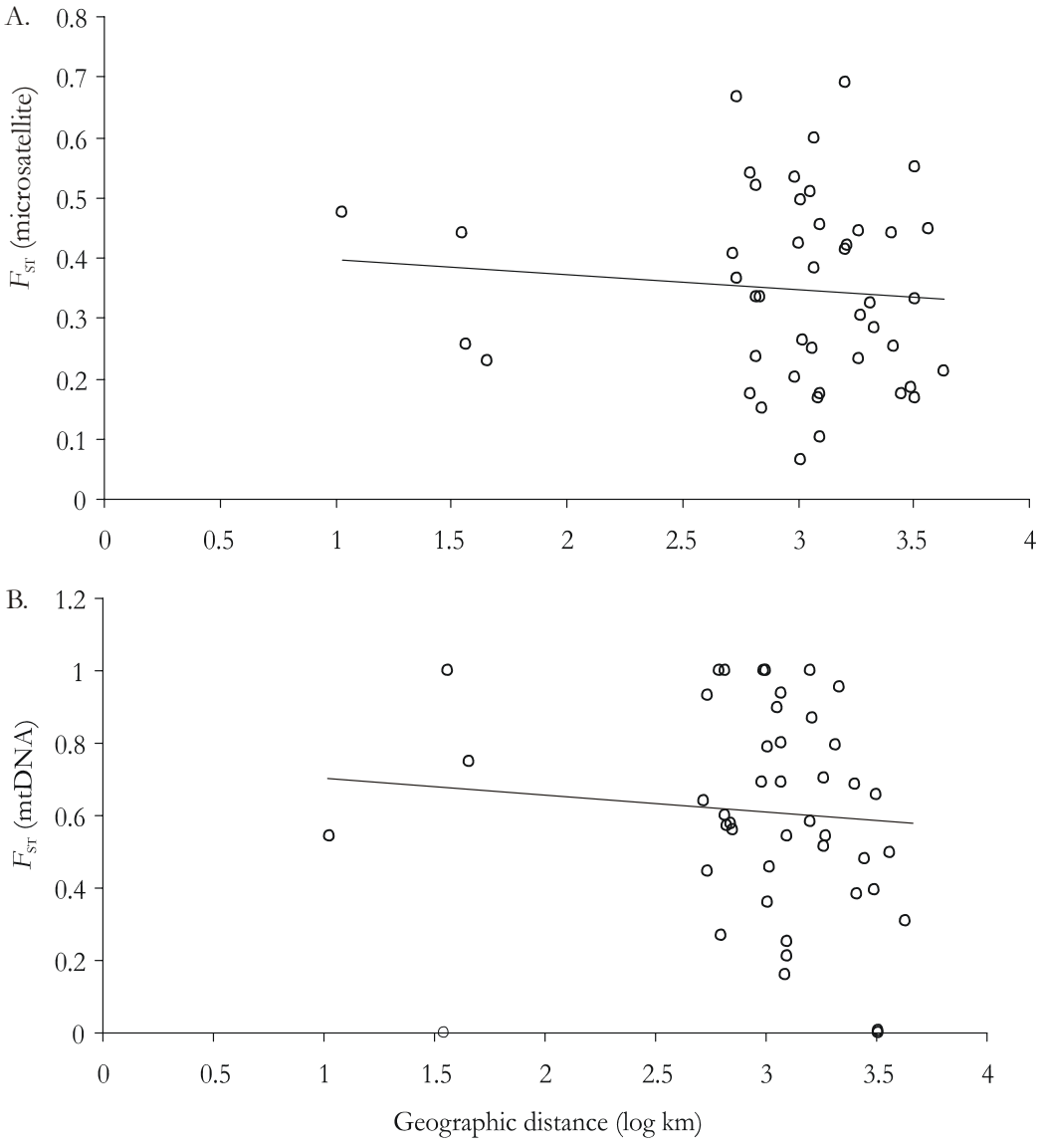

Fig. 3-3 The correlation of population differentiation (F_{ST}) with rising geographic distance (log km) represented for mitochondrial (mtDNA) and microsatellite (μsat) data sets. A. μsat $R^2 = 0.015$, $P = 0.66$; B. mtDNA $R^2 = 0.009$, $P = 0.629$. Only populations $N \geq 3$ for mtDNA data and same subset of *Daphnia galeata* populations used for microsatellite data.

Amova analysis Microsatellite and mtDNA markers revealed different pattern of population structure (AMOVA; **Table 3-4**). Using microsatellite data, 32.84 % of the variation was explained among groups and more than twice as much variation (67.16 %) was explained within populations. An inverse relationship for mtDNA was observed.

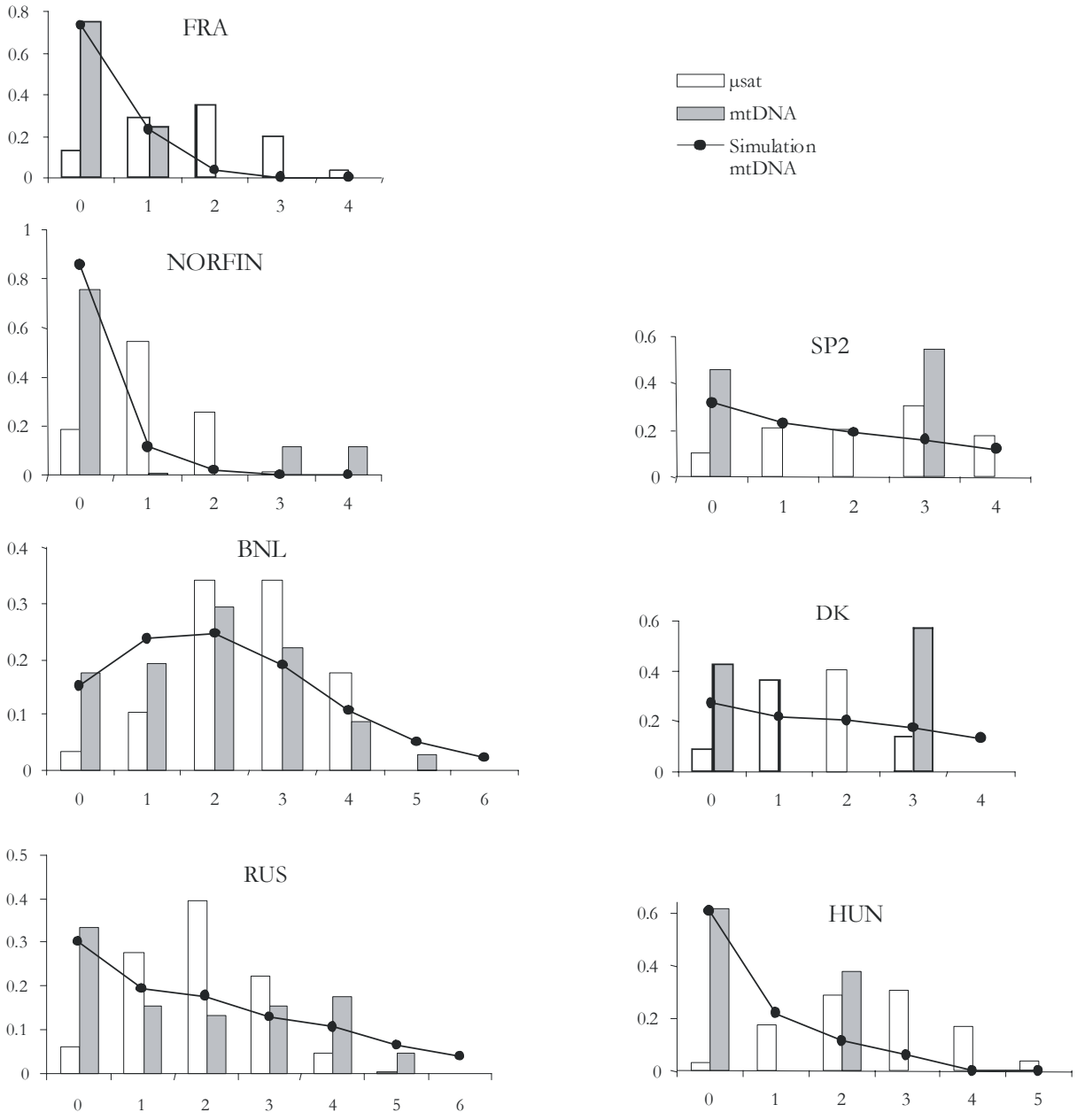

Fig. 3-4 'Mismatch distribution' established for the mitochondrial sequence data (partially pooled, with BEL: BEL4-7, DK: DK1 and 2, and NOR: NOR and FIN2) and microsatellite loci (NOR, BEL4, and DK1, as representatives of Fennoscandia, Belgium and Denmark) *Daphnia galeata* samples of lake areas. Bars represent observed frequency of pairwise differences among haplotypes within population groups (filled bars = mtDNA; open bars = microsatellite), while lines depicts the expected shape as predicted for a population that has undergone a demographic expansion in the past for mitochondrial data.

About 60 % of the variation was explained by variation among populations, whereas only about 40 % was explained within populations. Fixation indices for each proportions of variation were significantly different from zero ($P < 0.01$).

3.4.3. Gene flow

Isolation by distance The effect of geographical distance on genetic differentiation (F_{ST}) was more or less of similar magnitude among nuclear and mitochondrial markers. No significant relationship between F_{ST} and geographic distance (log transformed) neither with microsatellite nor with mtDNA sequence data (**Fig. 3-3**) was observed.

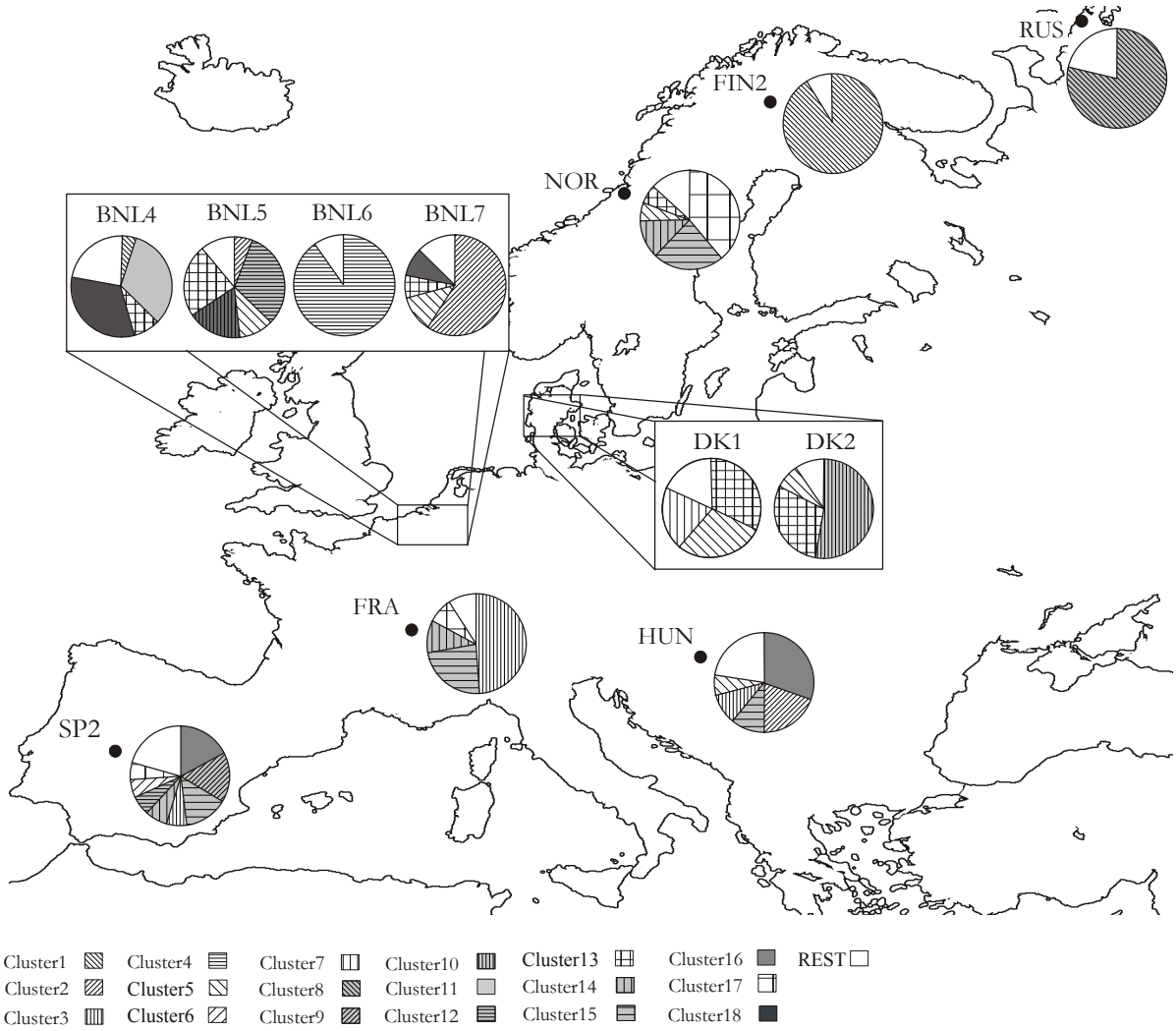

Fig. 3-5 Frequency distribution of admixed genotypes attributed to estimated clusters (K = 18) in STRUCTURE (Pritchard *et al.* 2000) distributed over 12 populations of *Daphnia galeata* across Europe. REST: sum of admixture coefficients lower than 0.05.

Mismatch distribution The mismatch distribution was unimodal for all populations based on microsatellite data (note: in **figure 4-4** DK1 and BNL4 represent DK and BNL, respectively). Among the mitochondrial data, three population-groups (SP2, DK, HUN) showed a bimodal distribution of mutation frequencies (**Fig**. **3-4**) and significant values for the corresponding τ (**Table 3-2**).

The other four lake areas (FRA, NOR, BNL, RUS) revealed low frequency of highly divergent haplotypes (i.e., smaller peaks in the right-hand part of the distribution; **Fig. 3-4**) with non-significant values for τ (**Table 3-2**). Using a divergence rate of 2.3% per million years my results suggest that the expansion of Belgian, Danish, and Hungarian populations date back about 52 000 - 68 000 years (**Table 3-2**). Norwegian, Finnish and Spanish populations expanded about 87 000 - 88 000 years B.P. and Russian population 118 000 B.P. years, whereas the population from France seems to be comparatively young (7 000 years B.P.).

Admixture estimation I derived estimates of admixture probabilistically using Bayesian statistics on individuals from all populations based on nuclear data (Pritchard *et al.* 2000). The majority of individuals showed high probabilities of ancestry in only one of the 12 populations (data not shown). The highest posterior probability was obtained for 18 clusters (K = 18, **Table 3-5**). The distribution of mean proportions of admixture per individual (K = 18) among the original 12 populations is shown in **Fig. 3-5**. Clusters, which occurred only once across Europe were detected in Spanish (SP2), Danish (DK1), Russian (RUS), and Belgian (BNL4) population. In Finnish (FIN2), Russian (RUS), and another Belgian (BNL 6) population only one cluster was observed at higher frequencies than 0.05, whereas the highest amount of genetic clusters were detected in Norway (NOR), Belgium (BNL5), and Spain (SP2).

Table 3-5 Estimated posterior probabilities of K assuming 19 genetic clusters of *Daphnia galeata*.

K	ln P (X | K)	P (X | K)
1	-1	~0
2	-3544.23	~0
3	-3160.00	~0
4	-2953.77	~0
5	-2762.10	~0
6	-2612.23	~0
7	-2480.37	~0
8	-2396.77	~0
9	-2329.40	~0
10	-2304.77	~0
11	-2255.03	~0
12	-2197.23	~0
13	-2169.40	~0
14	-2136.60	~0
15	-2126.93	~0
16	-2105.93	~0
17	-2089.73	0.02
18	-2086.50	0.49
19	-2116.07	~0

Migrate Gene flow based on mitochondrial data mainly occurred within central Europe and from central Europe to Norway and Russia (**Fig. 3-6**). Gene flow rates from central Europe to northern Russia and from Denmark to Belgium were about ten times higher than within central Europe (DK-FRA, FRA-HUN, BNL-DK, and FRA-NOR). Very low levels of gene flow was detected from southern to central Europe or northern sampling sites. Furthermore the Spanish population is characterized by a higher immigration than emigration rate.

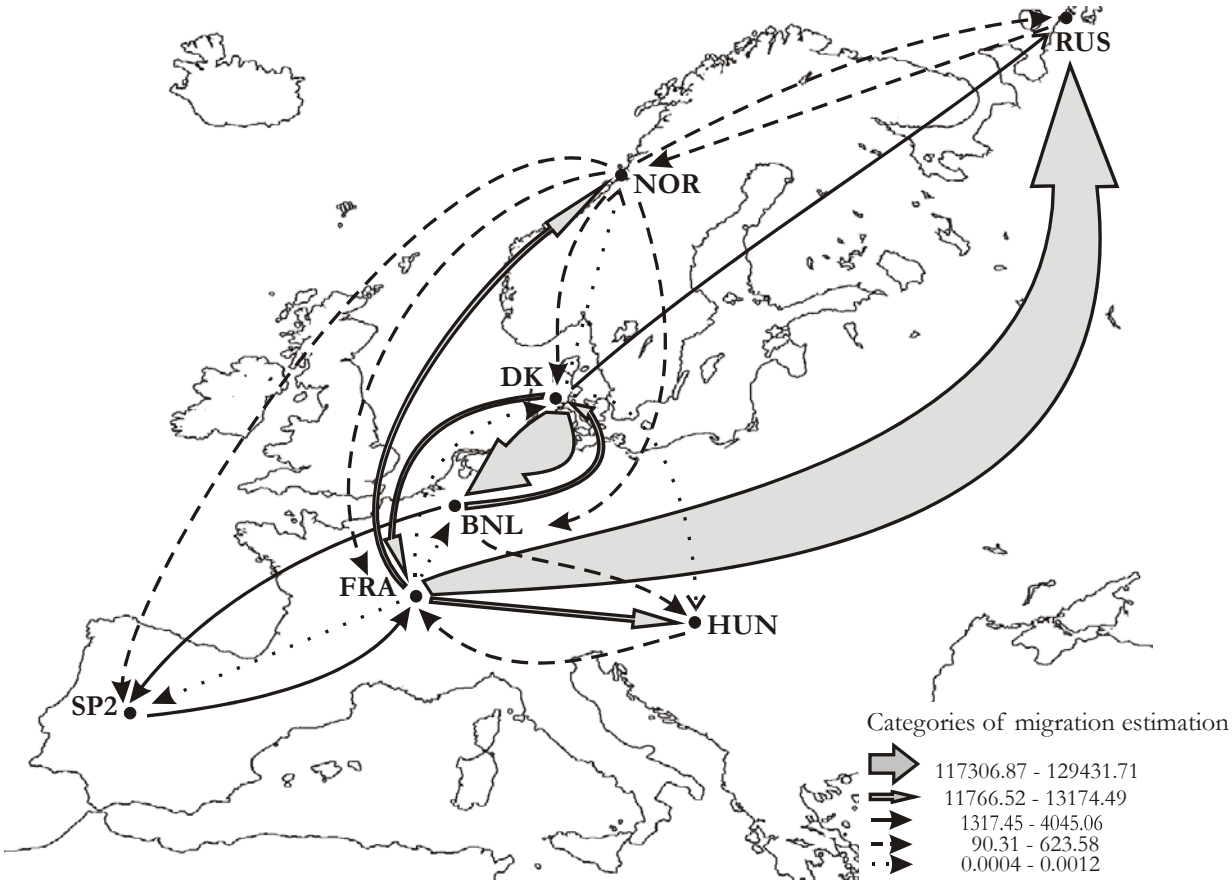

Fig. 3-6 Main probable gene flow rates of *Daphnia galeata* across Europe based on mitochondrial data and obtained by MIGRATE are depicted. Categories of migration should be seen as relative values, not as absolute N_m.

3.5. Discussion

The major result of my present study is the largely congruent pattern of highly differentiated and genetically depauparate populations of *D. galeata* across Europe based on both

mitochondrial and nuclear genetic data. Levels of population differentiation at either molecular marker system did not correlate with each other. Both marker systems showed no association with geographic population structure. In addition, I was not able to reject the general hypotheses of an association of genetic diversity pattern and expected recolonization routes after glaciation. Several areas were characterized as a possible hybrid zone.

3.5.1. Population genetic structure of Daphnia galeata *based on mitochondrial and microsatellite data*

A previous study (Dove et al submitted) revealed high levels of population structure based on microsatellite data. Results of mitochondrial analysis in here reinforce the pronounced population differentiation. The differentiation of individuals was even stronger (K = 18) after genetic cluster were simulated based on microsatellite data, suggesting the existence of subpopulation within lakes. Similar phylogeographic patterns, i.e. confirming high level of subdivision among populations has been generally found for several zooplankton species in the Holoarctic (Weider and Hobaek 1997; Gomez *et al.* 2000; Schwenk *et al.* 2000; Cox and Hebert 2001). The strong population structure for both marker systems in the absence of physical barriers may be a result of past subdivision events and can be explained by a rapid colonization of newly available habitat (Hewitt 2000). In addition, the higher estimates of population subdivision found for mitochondrial data are probably due to its higher susceptibility to effects of genetic drift and bottleneck during historical population foundation (Birky *et al.* 1989; Hedrick 2000). A phenomenon, that has also been reported in other studies on *Daphnia* (Lynch *et al.* 1999) and rotifer (Gomez *et al.* 2002). Furthermore, population divergence occurred among populations in close vicinity and over large geographic distance, which explains the lack of isolation by distance for either of both marker systems. Nuclear data of European *D. galeata* (Dove et al submitted) and *D. magna* populations using mitochondrial DNA (De Gelas and De Meester 2005) each work including twice as many sampling sites than presented in the current work, did reveal isolation by distance over large geographic areas. Thus, the lack of isolation by distance may be due to the low number of samples used. However, I argue that restricted gene flow caused by priority effects and strong local adaptation maintained pronounced population genetic differentiation. As a consequence, the phylogeographic pattern reflects historical colonization rather than contemporary gene flow.

In spite of the high level of population structure found in both marker systems, direction of gene flow pattern slightly differs between the two marker systems. Mitochondrial data revealed substantial gene flow e.g. from France to Russia (**Fig. 3-6**), whereas the Russian and Finnish populations are, based on nuclear data, well separated from the other populations. In addition, no correlation was detected between the degrees of differentiation estimated for nuclear and mitochondrial genomes. This dissimilarity in the patterns of gene flow and population differentiation in the two marker systems may be due to chance events like genetic drift (influencing stronger mtDNA) or mutation (faster in microsatellite loci). Another explanation may be differences in male and female mediated gene flow, since evidences for male-only producing clones in *D. magna* have been observed (Ferrari and Hebert 1982; Hobæk and Larsson 1990). Similar results have been found in rotifers by Gomez *et al*. (2002). However, results of hatching experiments among lake-*Daphnia* did not reveal resting eggs to release males (e.g., Carvalho and Wolf 1989). In addition, hierarchical analysis of population structure revealed different results for the genetic variation among and between populations. Genetic variation among populations was pronounced in mitochondrial data, whereas microsatellite data varies stronger within populations. These patterns of dissimilarities in the level of population differentiation and inverse genetic variation among and within populations may be caused by the occurrence of distinct haplotypes among populations, because recolonization of different sites may have occurred from different refuges (Hewitt 2000). Instead, the higher variability of microsatellite loci within populations may be the result of high mutation rates in combination with local adaptation (Dove in preparation). Thus chance events such as genetic drift and mutation rate are rather the reason than indifferences in male or female mediated gene flow.

3.5.2. Population history and genetic pattern of recolonization

In general a south to north range expansion of most species has been reported across the Holarctic (Hewitt 2000). Several examples of zooplankton species from Northern America, and Russia/Siberia reflect geographically segregated lineages with the general recolonization routes after Ice Ages representing derivation of same and different Pleistocene refugia (Weider and Hobaek 1997; Freeland *et al.* 2000b; Cox and Hebert 2001). As an example for Cladocera in Eurasia, representatives of the *D. pulex* species complex, closely allied taxa (two sister species, *D. tenebrosa* and *pulicaria*) showed different postglacial histories, therefore responded independently to the allopatric effects of glacial cycles (Weider *et al.* 1999a;

Weider *et al.* 1999b). Further phylogeographic analyses in zooplankton taxa showed a wide spread mtDNA haplotype lineages from glacial refugia to northern habitats after the glacial retreat (Gomez *et al.* 2000; Cox and Hebert 2001; Weider and Hobæk 2003; De Gelas and De Meester 2005). As an evidence for rapid recolonization after glacial period of *D. galeata* across Europe I observed a wide range distribution of one haplotype and several microsatellite alleles (FRA-BNL-RUS), which may represent ancestral lineages as a result of long distance dispersal followed by successful colonization in the past (Avise 2000; De Meester *et al.* 2002). However, little support was given for a unidirectional south to north range expansion of clonal lineages neither by mitochondrial nor by nuclear data (**Fig**. **3-5** and **3-6**). Furthermore is postglacial colonization expected to decrease genetic diversity within expanding populations (Hewitt 1996; Avise 2000; Hewitt 2000). In Russia/Siberia and arctic Canada indeed higher clonal variation for *D. pulex* in unglaciated sites, close to the Beringian glacial refuge, than recently deglaciated regions have been reported (Weider and Hobaek 1997; Weider and Hobæk 2003). The Iberian Peninsula has been depicting for a wide range of species repository for the northern latitudes of Europe after the Pleistocene ice ages (references in, Gomez and Lunt 2005). However, among *D. galeata* populations neither haplotype nor clonal diversity was correlated with any latitudinal gradient. Despite of relative high allelic richness across Europe e.g. the Spanish population (SP2) was characterized by low haplotype diversity. In addition gene flow analysis based on mtDNA sequences did indicate relatively little migration from the Spanish population towards the central and northern Europe (**Fig**. **3-6**). Differential distribution and fragmented nature of suitable habitats favour the occurrence of multiple glacial refugia in the Iberian Peninsula (Gomez and Lunt 2005). Thus by chance SP2 has not served as southern refugia for northern populations. Again, a noticeable amount of gene flow from France to Russia and, though less pronounced, to Norway has been indicated. However, equal levels of migration have been suggested within central Europe (**Fig**. **3-5** and **3-6**). Based on these results I cannot reject the general hypothesis concerning distribution of genetic diversity after postglacial colonization European species. However, most probably I have not analysed sufficient number of samples in the Mediterranean region. Though another reason for these contrasting results may be that *D. galeata* has recolonized Europe from other refugia, than previously thought. Geological events during the last glaciations are likely to have resulted in repeated isolations and connections of lakes, thus creating additional pre-glacial lakes south of the ice cover in Northern Europe. Stewart and Lister (2001) for instance suggest the existence of refugia in Belgium. A colonization route from Eastern Europe has been proposed by Bilton *et al.* (1998).

The Baltic had two freshwater phases (the Baltic ice lake and later the Ancylus Lake) during deglaciation after the Weichsel. These huge freshwater lakes have played a major role in recolonization of freshwater habitats in the Scandinavian Peninsula (Økland and Økland 1999). Väinölä *et al.* (1994) concluded that post-glacial colonization of Fennoscandian lakes occurred primarily from proglacial lakes east of the Scandinavian dome. Mitochondrial data of Russian and Finnish populations represent haplotypes not observed in any of the other populations present in this study. As above-mentioned, the Beringia was the largest and is believed to have been the most important Holarctic glacial refugia (Sanmartin *et al.* 2001). In Europe eastern Russia and northern Baltic areas may have served as a refuge for the northern population. In any case, the limited number of sampling sites makes it difficult to assign certain refugia for European *D. galeata*. Further sampling, especially in the Mediterranean area as well as Eastern Europe and Russia, of *D. galeata* is needed to distinguish glacial refugia and recolonization routes of this zooplankton species.

Empirical studies published on *Daphnia*-phylogeography across Europe are scarce (but see, De Gelas and De Meester 2005). In their study they investigated the historic gene flow, thus colonization pattern of extant *D. magna* populations, and revealed an important geographical subdivision based on mitochondrial data. In addition they observed recent population expansion with population growth after glacial termination II (130 000-90 000 BP) from different, but not attributable, refugia. The expansion times estimated for *D. galeata* here suggest population expansion and growth in the glacial cycles during late Pleistocene (118 000-52 000 BP), and after last glaciation (FRA, 7 000 BP). The Russian population seems unexpectedly to be the oldest one (RUS, 118 000 BP) and the French population the youngest (7 000 BP). However, the consequences of oscillation in the Pleistocene ice ages makes dating of expansion time even more complex, since not all refugia or recolonized areas were equally suitable habitats during and between glaciations (Gomez and Lunt 2005). Furthermore I must take into account the large standard error, which appears, when few sequence data of little gene variation are analysed. Since population genetic analysis across a large geographic area of two freshwater cladoceran species, *D. magna and D. galeata*, revealed similar population geographic pattern, I suggest that the same historic processes (e.g. glaciation) and evolutionary forces (e.g. local adaptation and genetic drift) have most probably influenced their population genetic structure.

Besides Belgian *D. galeata* populations surprisingly Finnish and Russian populations revealed as well high allelic richness and relative high mitochondrial diversity. At the same time results of mismatch distribution, Tajimas' *D* and BOTTLENECK predicted a recent

population expansion for the given populations. This incongruence may occur at hybrid zones where individuals or populations of the same species from two or more source areas experience a secondary contact after recolonization events (Hewitt 1993; Hewitt 1999). Populations of *D. galeata* have most probably been isolated for long time periods at different yet unknown refugia, where they may have undergone frequent hybridisation with other members of the *Hyalodaphnia* species complex. This is a common phenomenon (Schwenk 1993) and leads to genetic introgression in *D. galeata* (N. Brede, personnel communication, Schwenk and Spaak 1995). Therefore, depending on the *Hyalodaphnia* community structure in the refugia, more or less genetically differentiated clonal lineages of *D. galeata* recolonized the same recently available sites (e.g. Finnish, Russian and Belgian lake areas) from different refugia. Furthermore, extreme divergence of populations within sampling areas (e.g. Belgium) indicates colonization events from different sites and subsequent strong local adaptation leading to minimal or non existing effective gene flow among sites (De Meester *et al.* 2002). However, the relative high mitochondrial diversity in Belgian populations may support the idea of refugia in central Europe (Pfenninger *et al.* 2003).

3.6. Conclusion

In conclusion, both mitochondrial and nuclear marker revealed similar patterns of allele and haplotype distribution as well as similar pattern of genetic differentiation in *D. galeata* populations across Europe. This congruence indicates the strong influence of evolutionary forces and local adaptation in the population genetic structure in *D. galeata* populations. No isolation by distance was detected for either of the two marker systems. The general pattern of south to north genetic expansion and declining gradient of genetic diversity, in the light of postglacial colonization was only partially supported; instead, refugia in central Europe may have existed. Several lakes have probably been recolonized after glaciation by allopatric differentiated clonal lineages of *D. galeata* populations from different refugia, which results in a pattern of hybrid zones. However, further sampling efforts in the Mediterranean and Eastern Europe have to be conducted to reveal possible refugia and colonization pattern in *D. galeata*.

4. The role of environmental factors for inter- and intrapopulational genetic diversity of *Daphnia galeata* populations

4.1 Abstract

Understanding the patterns and processes of local adaptation requires a detailed knowledge of how environmental factors structure populations. Although the intra- and interpopulational genetic diversity and ecological differentiation are well studied, few investigations have considered both components in concert. I determined the intra- and interpopulational genetic diversity and differentiation of 15 *D. galeata* (subgenus *Hyalodaphnia*: Cladocera: Anomopoda) populations in Belgium/The Netherlands and Denmark using six polymorphic microsatellite loci. In addition, I characterized all freshwater systems included in my study with regard to abiotic factors, diversity and biomass at adjacent trophic levels. In order to reveal the relationship between population genetic structure of *D. galeata* and the relative contribution of environmental factors, I used a statistical framework based on canonical correspondence analysis. Although I detected no single ecological gradient mediating the genetic differentiation in either lake regions, it is noteworthy that the same ecological factors were significantly correlated with intra- and interspecific genetic variation of *D. galeata*. For example, I found a relationship between genetic variation of *D. galeata* and differentiation with higher and lower trophic levels (phytoplankton, submerged macrophytes and fish) and a relationship between clonal variation and species diversity within Cladocera. Variance partitioning had only a minor contribution of each environmental category (abiotic, biomass/density and diversity) to genetic diversity of *D. galeata*, while the largest proportion of variation was explained by shared components. My work illustrates the important role of ecological differentiation and adaptation in structuring genetic variation, and it highlights the need for approaches incorporating a landscape context for population divergence.

4.2. Introduction

Many evolutionary studies have focussed on genetic structures within and among populations as well as on evolutionary processes responsible for population differentiation, including restricted gene flow, stochastic demographic processes (e.g., bottlenecks and founder effects), or selection (Boileau and Taylor 1994; Lowe *et al.* 2004). These

evolutionary processes determine biodiversity patterns and community structure influenced by extant ecological factors like productivity, climate, and extreme environmental conditions (e.g. Mittelbach *et al.* 2001) (Grady-Steed *et al.* 1997; Barnosky *et al.* 2001). Variation in biodiversity patterns with regard to interactions between trophic levels and local and regional effects has been well studied, both theoretically and empirically (Persson *et al.* 1992; Carpenter and Kitchell 1993; Siemann *et al.* 1998; Shurin *et al.* 2002; Jeppesen *et al.* 2003; Krivan and Schmitz 2003; Hawkins and Pausas 2004). However, only a few studies have addressed the effects of local landscape and environmental factors on the population genetic structure of a given species (Shaw 1994; Kudoh and Whigham 1997; Keyghobadi *et al.* 1999; Turpeinen *et al.* 2001; Pfenninger 2002; Costello *et al.* 2003). The combination of ecological and population genetic data has revealed the impact of spatial structure on genetic differentiation and the role of historical events in explaining patterns of contemporary genetic diversity (Angers *et al.* 1999; Brouat *et al.* 2004). However, the relative contribution of community structures, biodiversity patterns and landscape characteristics to the population structure of a given species has only rarely been studied (Manel *et al.* 2003).

Lakes provide an ideal model habitat to study population differentiation in relation to environmental gradients, since they represent well defined entities (Dodson *et al.* 2000; Jeppesen *et al.* 2000; Hessen *et al.* 2003). Local and regional processes jointly regulate local species diversity and composition in many aquatic systems (Shurin 2000). In addition, several studies have shown that, for instance, predation levels and abiotic factors have a major impact on freshwater communities and taxon diversity within a particular trophic level (Gliwicz and Pijanowska 1989; Beaver and Havens 1996; Hobaek *et al.* 2002; Matveev 2003; Mitchell *et al.* 2004). Similar patterns have also been found between environmental conditions and life history traits of species or clonal groups (Laurén-Määttä *et al.* 1997; Walls *et al.* 1997; Boersma *et al.* 1999). Ecological genetics studies of zooplankton have revealed a differential response of *Daphnia* genotypes to variation in biotic (Pijanowska *et al.* 1993; Epp 1996; Hietala *et al.* 1997) and abiotic factors (Weider 1985; Weider 1989; Plath and Boersma 2001). This “horizontal” impact (environmental factors per se) on intraspecific genetic variation might have an direct or indirect effect on the genetic or taxon diversity at other trophic levels (“vertical“ interaction), or vice versa, since trophic cascades hinge on strong interactions (McQueen *et al.* 1986; Carpenter and Kitchell 1993; Pace *et al.* 1999). Although recent studies have considered the relationship of species diversity among multi-trophic levels in lakes (Dyer and Letourneau 2003; Declerck *et al.* 2005), a detailed analysis of the impact of environmental factors on intraspecific genetic diversity is lacking.

To assess both the horizontal and vertical interactions within aquatic habitats, I determined the relative impact of environmental factors and diversity across trophic levels on extant genetic variation within and among populations of the microcrustacean species *D. galeata*. I aim to reveal the relationship between taxon diversity and clonal diversity across trophic levels. Further, I describe the impact of abiotic factors, diversity and biomass/density of food species (ciliates, phytoplankton), competitors (copepods, cladocerans) and predators (fish) on the genetic diversity within and differentiation among 15 European *D. galeata* populations. Finally, I determine the relative contribution of biotic and abiotic factors on the genetic diversity and variation within and between *D. galeata* populations.

4.3. Materials and methods

4.3.1. *Study sites and sampling*

I analysed 15 lakes (**Table 4-1**; a subset of a total of 98 lakes sampled for a European project/the EU BIOMAN (for details see Declerck *et al.* 2005) within two European lake regions: Denmark and Belgium/The Netherlands.

For all lakes bacterio-, protisto- and zooplankton, fish and macrophytes, as well as chemical, physical and landscape variables, were sampled (see appendix **Table 4-2**). Plankton was sampled monthly in each lake during the growing season (May to October) during either 2000 or 2001. Depending on the surface area of the lakes, equal volumes of depth-integrated samples were taken at eight (<5 ha; 6 L) or 16 (>5 ha; 3 L) locations and subsequently pooled. This pooled sample was then thoroughly stirred and quantitatively subsampled for bacteria, ciliates, flagellates, phytoplankton, and zooplankton. Crustacean zooplankton was collected by filtration of 15 L through a 64 µm mesh and stored in 70-80 % ethanol for subsequent DNA analysis. For detailed information on sampling techniques and storing processes see Declerck et al. (2005).

Table 4-1. Geographic location of sampling sites; abbreviations of sampling sites (Code); average observed heterozygosity (HET) and genetic diversity (MLG/*N*) are provided for each *Daphnia galeata* population (Lake).

Lake	Code	Country	Latitude	Longitude	HET	MLG/*N*
Berlare Broek	BNL1	Belgium	51.02	3.95	0.23	0.83
Blankaartvijver	BNL2	Belgium	50.97	2.86	0.21	0.70
Oud Heverlee Troebel	BNL3	Belgium	50.84	4.66	0.26	0.84
Oud Heverlee Helder	BNL4	Belgium	50.85	4.66	0.37	0.68
Oude Maasmaender Maasveld	BNL5	Belgium	51.06	5.77	0.36	0.87
Plas Astrid	BNL6	Belgium	50.99	5.11	0.23	0.53
Voortmangelbeek	BNL7	Belgium	51.02	5.26	0.27	0.78
Delftse Houd	BNL8	Netherlands	51.98	4.35	0.29	0.61
Stigsholm Sø	DK1	Denmark	55.98	9.49	0.25	0.50
Søbygård Sø	DK2	Denmark	56.26	9.81	0.22	0.39
Kvind Sø	DK3	Denmark	56.03	9.50	0.22	0.55
Sunds Sø	DK4	Denmark	56.22	9.02	0.24	0.83
Schousbye Sø	DK5	Denmark	56.17	9.62	0.39	0.66
Ensø	DK6	Denmark	55.94	9.30	0.15	0.47

4.3.2. Determination of taxon richness and clonal diversity

Ciliates, adult copepods, cladocerans, fish and macrophytes were identified with standard taxonomic methods to species level, and rotifers and phytoplankton to genus level. Bacterial and protist communities in the two particle size fractions were analysed with Denaturing Gradient Gel Electrophoresis (DGGE, Muyzer 1999, G. Zwart pers. comm.).

Species specific markers for the *D. longispina* complex were used to identify species and interspecific hybrids (Schwenk *et al.* 2000; Billiones *et al.* 2004). All individuals were morphologically identified and a subsample was subjected to a Restriction Fragment Length Polymorphism (RFLP) analysis of an amplified ITS-region (Billiones *et al.* 2004). All individuals (21 to 44) of mixed populations containing *D. galeata* and interspecific hybrids were tested by PCR-RFLP analysis prior to microsatellite screening. Genetic diversity was determined using six variable microsatellite loci (Chapter 2; Dove et al submitted).

Environmental data - A total of 38 independent variables (**Table 4-2**) were recorded from 15 sampling sites and grouped into three categories: abiotic factors (SECCHI DEPTH, CON,

TEMP, PH, TP, SI, TN, ORTHOP, AREA, and DEPTH), diversity (FISHSHA, NDPSHA, DPSHA, SUBMSHA, PHYTSHA, MPASHAS, DGBASHAS, CLADSHAS, CLADSHAL, and CILSHAS) and biomass/density (PHYTTOT, ZOOPTOT, BACTDENS, HNFDENS, %COVSUBM, SUSORM, TA, NTA, EA, NEA, DPFN, DPFYN, DPFMN, DPFKG, DPFYKG, and DPFMKG). Methods for estimating diversity indices of species and organismal groups are described in Declerck et al. (2005).

Genetic data - Genetic differentiation among populations was inferred from the frequency of alleles occurring at a given locus (allele frequencies per population), resulting in six matrices, i.e. one per locus: B (DaB10/14), C (DaB10/15), F (DaB16/17), G (DaB17/17), T (Dpu06), and U (Dpu30; see also Chapter 2). Intrapopulational genetic diversity was inferred from the number of alleles (NALL), the single-locus genotype frequencies (SLG/N) and observed heterozygosity per locus (HET), obtained using the program Arlequin vs 2.0 (Schneider *et al.* 2000). For each of these estimates a separate matrix was constructed. Thus, I analysed a total of nine separate matrices of genetic data for each geographic region (BNL and DK).

4.3.3. Statistical analysis

Prior to canonical correspondence analysis a multiple regression was performed to determine those environmental variables which revealed a significant association with genetic variation (number of multi-locus genotypes per population, MLG/N, and observed average heterozygosity). In addition, variables were tested for normality (Kolmogorov-Smirnov Test), and correlations (Pearson Product-Moment Correlation) between single environmental and genetic variables were performed (Statistica 1995). A canonical correspondence analysis (CCA) was carried out using the program CANOCO for Windows 4.5 (ter Braak 1988) to assess the relative impact of environmental factors (grouped into three categories: abiotic factors, biomass/density, and diversity) on the genetic variation within and among populations. CCA incorporates both ordination and multiple regression techniques for analysis of the relationships between tables of multivariate data, assuming an unimodal relationship between dependent and independent variables.

The variables contributing most to the explanation of genetic variation were selected using a forward selection procedure available in CANOCO, with a cut-off point of lambda (P) = 0.1 and a significance level $P = 0.05$, based on 1000 Monte Carlo permutations. The contribution

of each set of variables (i.e. model) explaining genetic diversity was estimated independently using the sum of eigenvalues. The statistical significance of the selected model was assessed by Monte Carlo permutation tests of the sum of all eigenvalues, using 1000 permutations as implemented in CANOCO. In order to determine the relative contribution of each set of environmental factors (abiotics, biomass/density, and diversity) to genetic diversity and differentiation, I used the method of variance partitioning proposed by Bocard et al. (1992), which allows revealing the relative contribution of environmental components to explain patterns of population structure. I calculated the pure component of variation explained by the three environmental categories (abiotic, biomass/density and diversity), the variation shared between the three environmental categories, and the residual (unexplained) variance. Partial CCA was based on variation at loci B, C, F and HET since these were significantly associated with environmental factors of at least two environmental categories. Significance levels of the five components (pure biotics, pure abiotics, pure biomass, shared and residuals) were assessed by permuting the sum of all eigenvalues (N_P = 1000) using CANOCO.

4.4. Results

Canonical correspondence analysis revealed a differential pattern of associations among environmental factors and genetic population structure of *D. galeata*. Environmental factors explaining intra- and interpopulational variation differed between the Belgian/Dutch and Danish lake areas (**Table 4-3** and **4-4**). Nearly the same set of environmental factors was associated with intrapopulational diversity and interpopulational differentiation; however, about twice as many significant associations were found for genetic variation among populations. Among all significant factors, the density and diversity of fish preying on *Daphnia*, the diversity and percentage coverage by submerged macrophytes and pH appeared to be the five main factors mediating genetic diversity in Belgian/Dutch and Danish *D. galeata* populations.

4.4.1. Environmental factors and interpopulation genetic differentiation

The CCA revealed a strong influence of several environmental factors in Belgium/The Netherlands on the interpopulational component of genetic variation in *D. galeata* (**Table 4-3**). Fourteen factors out of 33 were retained by a forward selection procedure as significant predictors of the variation in allele frequencies. These accounted for 29.92% (diversity; locus

U) to 92.08% (abiotics; locus B) of total variation in allele frequencies, depending on the locus. Factors related to submerged macrophytes (SUBMCOV and SUBMSHA) and fish preying on *Daphnia* (DPFKG, DPFYKG, and DPSHA) were associated with several loci (**Table 4-3**). The relationship between interpopulation genetic diversity and selected environmental variables was statistically significant at all loci ($0.023 < P > 0.001$;**Table 4-3**).

Table 4-3. Summary statistics for canonical correspondence analysis of genetic diversity in *Daphnia galeata* and environmental variables among lakes in Belgium/The Netherlands (BNL). For each category of environmental factors (abiotics, biomass, and diversity) at the loci (B, F, G, and U) and measures of genetic variation (observed heterozygosity, HET), eigenvalues are given in parentheses. For most of the environmental variables correlation coefficients are available for at least one axis; asterix represents P < 0.05.

Locus		Canonical coefficients			
Genetic diversity measure	Predictor	Axis 1	Axis 2	*P model*	Explained variation in %
Interpopulational genetic diversity					
B	Abiotics	(0.158)	(0.025)	0.001	92.08
	TEMP	-0.243*	0.115*		
	PH	0.153*	0.033		
	TP	0.261*	0.082*		
	Biomass	(0.156)	(0.017)	0.003	85.64
	SUBMCOV	0.427*	-0.02		
	DPFKG	-0.115*	0.138*		
F	Abiotics	(0.606)	(0.468)	0.009	76.56
	PH	0.797*	0.051		
	AREA	0.393*	-0.2		
	SECCHI	-0.118	0.645*		
	Biomass	(0.661)	(0.541)	0.001	81.92
	BACTDENS	-0.044	0.261		
	SUBMCOV	1.585*	1.862*		
	DPFYKG	-0.894*	-2.152*		
G	Diversity	(0.461)	(0.064)	0.012	77.57
	PHYTSHA	-0.622*	0.034		
	DPSHA	0.366	0.064		
	SUBMSHA	0.182*	0.221*		

Table 4-3 (*continued)*

Locus Genetic diversity measure	Predictor	Canonical coefficients Axis 1	Axis 2	*P model*	Explained variation in %
U	Biomass	(0.246)	(0.180)	0.001	70.41
	PHYTTOT	0.183*	0.430*		
	SUBMCOV	0.534*	0.016		
	Diversity	(0.181)	(0.251)	0.023	29.92
	CILSHAS	-0.425*			
Intrapopulational genetic diversity					
HET	Abiotics	(-0.363)	(-0.083)	0.001	76.33
	TEMP	-0.648*	0.380*		
	PH	-0.058*	0.490*		
	Biomass	(0.349)	(0.104)	0.015	59.86
	DPFMKG	-0.591*			
	Diversity	(0.364)	(0.059)	0.004	72.38
	SUBMSHA	-0.402*	0.199		
	MPASHAS	-0.337*	-0.218*		

Abiotics (abiotic factors): TEMP: temperature (°C); TP: total phosphorus (µg l^{-1}); SECCHI: Secchi depth; SUBMCOV: percentage coverage of submerged macrophytes; biomass: DPFKG: fish preying on *Daphnia* (fry and adult, kg); DPFYKG: fish preying on *Daphnia* (fry, number of fish net^{-1} $night^{-1}$: CPUE in kg); DPFMKG: fish preying mainly on *Daphnia* (adult, CPUE in kg); BACTDENS: total bacterial density (10^6 cells ml^{-1}); PHYTTOT: total phytoplankton biomass (µg C l^{-1}); diversity: DPSHA: Shannon Wiener (SHA) of fish preying on *Daphnia*; PHYTSHA: SHA of phytoplankton species level; SUBMSHA: SHA of submerged macrophytes; CILSHAS: SHA of small ciliates; MPASHAS: SHA of multiprobe array of small bacteria

In addition, a significant relationship between interpopulational genetic diversity and environmental factors was detected in Danish populations. The environmental variables selected accounted for 44.39 % (Abiotics; locus C) to 88.48 % (Diversity; locus C) of the total variation depending on locus (**Table 4-3**). Nine out of 38 environmental variables were retained as significant predictive variables; three being related to fish biomass and diversity (DPFN, DPSHA, and FISHSHA). The relationships between allele frequencies and environmental variables were statistically significant at all loci ($0.05 < P > 0.014$; **Table 4-4**), but revealed no consistent pattern across different loci.

Table 4-4. Summary statistics for canonical correspondence analysis of genetic diversity and environmental variables among lakes in Denmark (DK). For each category of environmental factors (abiotics, biomass, and diversity) at the loci (B, C, and F) and measures of genetic variation (observed heterozygosity, HET and single-locus genotypes, SLG), eigenvalues are given in parentheses. For most of the environmental variables correlation coefficients are available for at least one axis; asterix represents $P < 0.05$.

Locus Genetic diversity measure	Predictor	Canonical coefficients		*P* model	Explained variation in %
		Axis 1	Axis 2		
Interpopulational genetic diversity					
B	Abiotics	(0.066)	(0.039)	0.014	56.41
	SI	0.256*			
C	Abiotics	(0.289)	(0.213)	0.05	44.39
	PH	-0.537			
	Biomass	(0.468)	(0.097)	0.015	86.94
	HNFDENS	0.965*	0.191		
	DPFN	0.461	0.431		
	Diversity	(0.478)	(0.097)	0.012	88.48
	PHYTSHA	-0.267	-0.598		
	MPASHAS	-0.907*	-0.453		
F	Biomass	(0.952)	(0.559)	0.037	47.79
	NTA	0.976*			
	Diversity	(0.894)	(0.552)	0.04	72.59
	DPSHA	0.634*	0.5736*		
	FISHSHA	-0.846*	0.368*		
Intrapopulational genetic diversity					
HET	Abiotics	(0.164)	(0.061)	0.004	73.53
	TEMP	-0.301*	0.18*		
	PH	0.203*	0.225*		
	Biomass	(0.161)	(0.077)	0.009	52.61
	HNFDENS	0.401*			
	Diversity	(0.133)	(0.074)	0.019	43.46
	DGBASHAS	0.364*			

Table 4-4 (continued)

Locus Genetic diversity measure	Predictor	Canonical coefficients Axis 1	 Axis 2	*P* model	Explained variation in %
SLG	Biomass	(0.068)	(0.049)	0.011	45.33
	DPFKG	-0.26*			

Abiotics (abiotic factors): TEMP: temperature (°C); TP: total phosphorus (µg l-1); SI: silicate (mg l-1); SUBMCOV: percentage coverage of submerged macrophytes; biomass: DPFN: fish preying on *Daphnia* (number of fish net-1 night-1, CPUE); DPFKG: fish preying on *Daphnia* (fry and adult, CPUE in kg); DPFMKG: fish preying mainly on *Daphnia* (adult, CPUE in kg); HNFDENS: total density of heterotrophic nanoflagellates (cells ml-1); NTA: non toxic algae. Diversity: DPSHA: Shannon Wiener (SHA) of fish preying on *Daphnia*; PHYTSHA: SHA of phytoplankton species level; SUBMSHA: SHA of submerged macrophytes; CILSHAS: SHA of small ciliates; MPASHAS: SHA of multiprobe array of small bacteria

4.4.2. Environmental factors and intrapopulation genetic diversity

In Belgian/Dutch populations five environmental variables were significantly associated with intrapopulational genetic diversity (HET; P = 0.001 - 0.015; **Table 4-3**). The first and second canonical axes were significant for all environmental factors, except for SUBMSHA (Diversity). The explained variation was highest for abiotic factors (76.33 %) and lowest for biomass (59.86 %). No relationship between environmental factors and NALL was found.

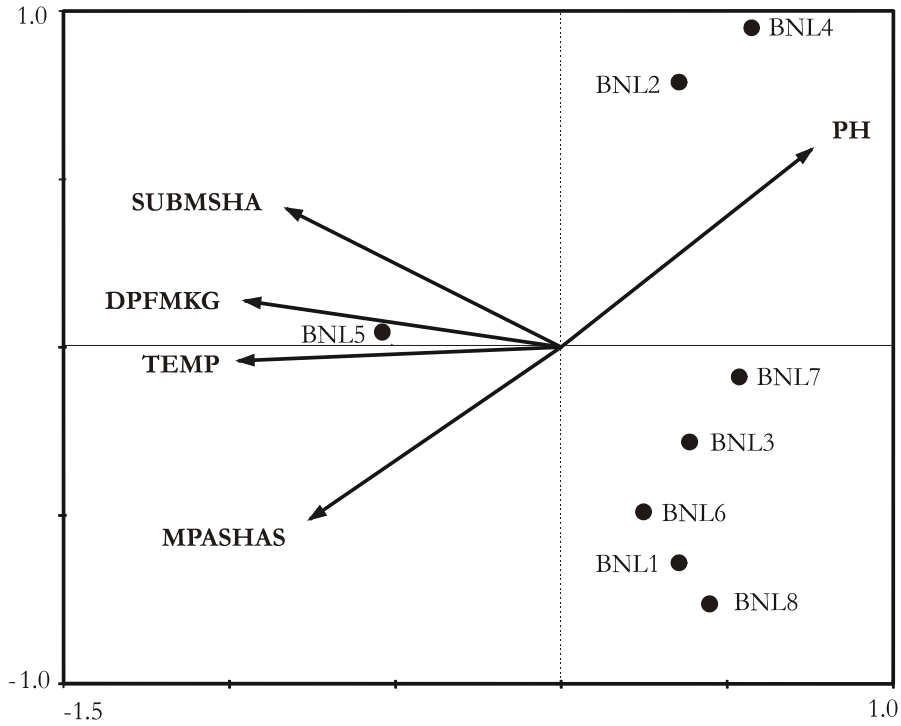

Fig. 4-1. Bi-plot opf a canonical correspondence analysis (CCA) comparing observed heterozygosity with environmental factors among eight Belgian/Dutch (BNL1-8) lakes. Significant effects (P<0.05) are shown as vectors; TEMP: temperature (°C); DPFMKG: fish (net^{-1} night^{-1} CPUE in kg) preying mainly on *Daphnia*; SUBMSHA: Shannon Wiener (SHA) of submerged macrophytes; MPSHAS: SHA of multiprobe array of small bacteria.

In Danish population HET was significantly associated with four out of 38 environmental variables, whereas SLG correlated only with DPFKG (P = 0.004 – 0.019; **Table 4-4**). The first and second canonical axes were significantly correlated with HET. In general, the explained variation was lower than in Belgian/Dutch populations, varying between 43.46 % and 73.53 %. No relationship between environmental factors and NALL was found.

Ordination scores were used to create a bi-plot that simultaneously represents an ordination diagram of environmental factors and observed heterozygosity of Belgian/Dutch populations (**Fig**. **4-1**). The figure shows that most populations are located along the first axis which is associated with temperature, fish mainly preying on *Daphnia*, diversity of submerged macrophytes, diversity of multiprobe array of bacteria, and pH. Heterozygosity of two populations (BNL2 and BNL4) was associated with pH and MPASHAS.

A correlation analysis of environmental variables with genetic variation revealed significant associations between the catch of fish per night per net preying on *Daphnia* (DPFN) with frequency of multi locus genotypes (MLG/N, **Fig 4-2**A) and Shannon Wiener of phytoplankton (PHYTSHA), and between large (not shown) and small cladoceran genera (CLADSHAS) with observed average heterozygosity (HET, **Fig. 4-2**B and C, respectively).

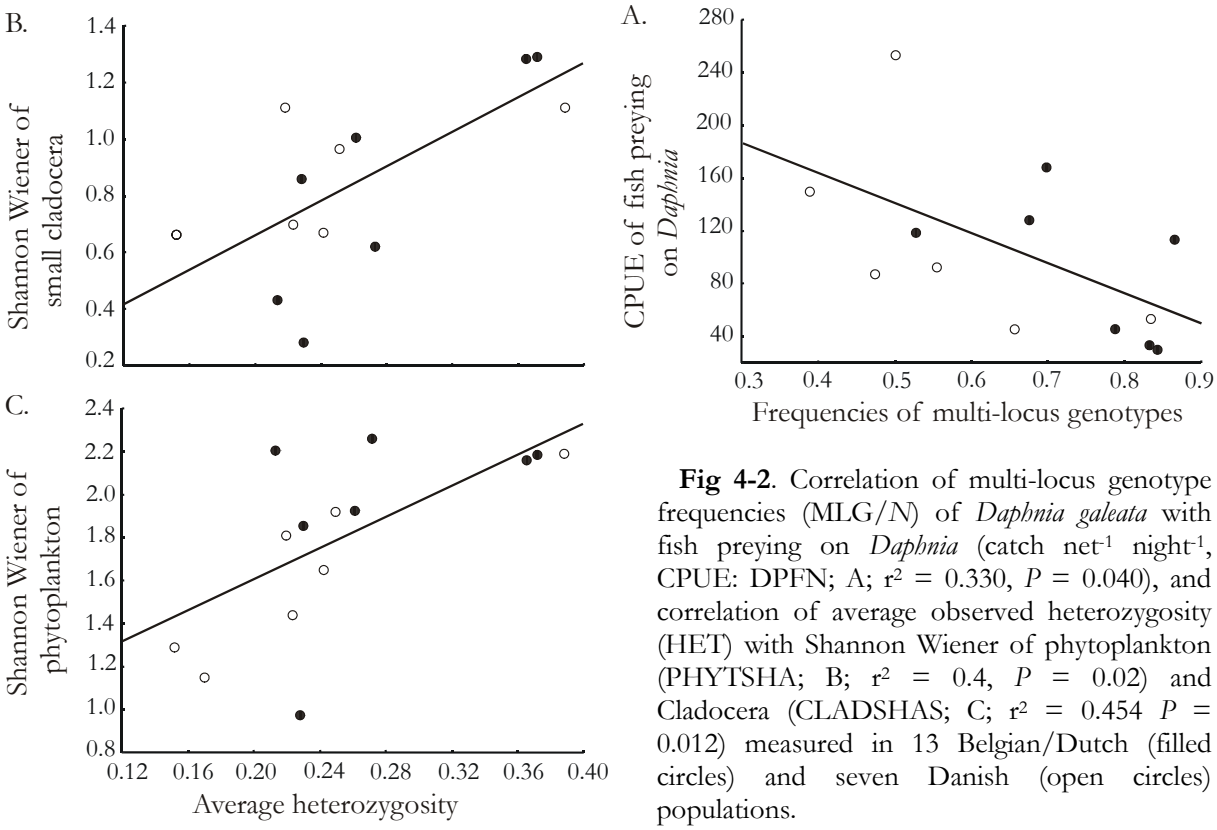

Fig 4-2. Correlation of multi-locus genotype frequencies (MLG/*N*) of *Daphnia galeata* with fish preying on *Daphnia* (catch net^{-1} night^{-1}, CPUE: DPFN; A; r^2 = 0.330, P = 0.040), and correlation of average observed heterozygosity (HET) with Shannon Wiener of phytoplankton (PHYTSHA; B; r^2 = 0.4, P = 0.02) and Cladocera (CLADSHAS; C; r^2 = 0.454 P = 0.012) measured in 13 Belgian/Dutch (filled circles) and seven Danish (open circles) populations.

4.4.3. Partition of explained variation within and differentiation among populations

Results of the partial CCA (**Table 4-5**) revealed in two out of 15 cases significant variance components (BNL, locus B, "pure abiotics", $P = 0.018$ and "pure biomass", $P = 0.034$) and a large amount of shared variation between environmental categories. No variation was left unexplained.

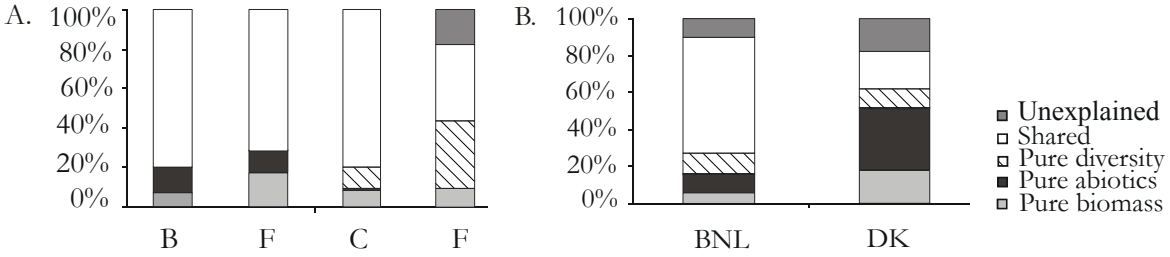

Fig 4-3. Partition of total variation into five independent components: pure abiotics, pure biomass, pure diversity, shared (among all three environmental categories), and unexplained. **A** and **B** represent the explained variation of allele frequencies of locus B, F and observed heterozygosity (HET) for Belgian/Dutch (BNL) and C, F and HET for Danish (DK) *D. galeata* populations.

In Danish populations, although non–significant, it is noteworthy that "pure diversity" contributed with 10.75 % (Locus C) and 34.29 % (Locus F) to the variance.

Table 4-5. Partitioning of total variation into five independent components: pure abiotics, pure biomass, pure diversity, shared (among all three environmental categories), and unexplained. Variation is presented in percentages. NA: data not available. Bold numbers refer to *P*-values and NS indicates non-significant results after 1000 Monte Carlo permutations.

	Pure abiotics	Pure biomass	Pure diversity	Shared	Unexplained
Interpopulation genetic diversity					
Belgium/The Netherlands					
B	13.37 (**0.018**)	6.93 (**0.034**)	NA	79.70	0
F	11.43 (NS)	16.78 (NS)	NA	67.85	0
Denmark					
C	1.54 (NS)	8.14 (NS)	10.75 (NS)	79.57	0
F	NA	9.54 (NS)	34.29 (NS)	38.25	17.92
Intrapopulation genetic diversity					
Belgium/The Netherlands					
HET	9.95 (NS)	6.00 (NS)	10.98 (NS)	62.95	10.12
Denmark					
HET	33.66 (NS)	17.65 (NS)	10.46 (NS)	20.59	17.65

Intrapopulational genetic variation was not explained by a significant effect of any variance component, neither in Belgium/The Netherlands nor in Denmark (**Table 4-5**). In Belgium/The Netherlands I observed a high proportion of shared variance (62.95 %), whereas in Denmark most variation was explained by the independent component "pure abiotics" (33.66 %).
In both regions variation was left unexplained. In general, the amount of explained variance differed qualitatively and quantitatively among components, loci and regions (**Fig 4-3**).

4.5. Discussion

The canonical correspondence analysis provided no evidence for the existence of one major ecological gradient explaining genetic variation of *D. galeata* populations. I found strong regional differences in the associations of genetic diversity and differentiation with environmental variables. Although environmental factors of all categories contributed to genetic variation and differentiation among populations I found only a small amount of variation explained by pure categories, but most of the genetic variation was explained by shared components. However, intra- and interpopulational variation is explained by nearly the same set of environmental variables (**Table 4-3** and **4-4**). Since *Daphnia* clones are ecologically differentiated with regard to food preferences, predation resistance and behaviour (e.g., Spitze 1993; De Meester *et al.* 1995; Boersma *et al.* 1998), I consider clones as ecological units, similar to species. Based on this assumption I compare the association of clonal diversity of *D. galeata* with abiotic factors, diversity and biomass at other trophic levels with studies dealing with the association of taxon diversity and environmental factors. First, I discuss the impact of environmental variables on intrapopulational genetic variation and, secondly, I focus on associations of environmental gradients and interpopulational genetic differentiation.

4.5.1. Variation within populations

The genetic diversity within *D. galeata* populations (clonal richness and genetic variation) was explained by at least two factors of each environmental category, but this pattern differed quantitatively and qualitatively among regions. For example, I found a negative correlation between the diversity of submerged macrophytes and genetic diversity in Belgian *D. galeata* populations, but not among Danish lakes. However, since a relationship of submerged macrophytes and zooplankton biodiversity is well documented my data suggest that similar

functional relationships mediate species and clonal variation (Jeppesen *et al.* 2000; Declerck *et al.* 2005). Aquatic macrophytes form a physical structure that creates multiple environmental gradients supplying a unique substrate and shelter to many types of organisms (e.g. macro-invertebrates) and serving as a refuge for predation (Lauridsen and Lodge 1996; Moss *et al.* 1998; Lauridsen *et al.* 1999). Stansfield (1997) found that the abundance and distribution of large bodied *Daphnia* covaries with macrophyte growth and abundance. If macrophyte stands develop in the middle of the growing season, then *Daphnia* are found throughout the summer in the littoral. my results revealed a higher genetic diversity of *Daphnia* in lakes with lower macrophyte diversity, but lower genetic diversity (**Table 4-3** and **4-4**, **Fig**. **4-3**A) in lakes with high biomass of fish preying on *Daphnia*. This pattern fits very well the results from population dynamic studies on fish, aquatic macrophytes and *Daphnia*, suggesting that increased macrophyte coverage supports a higher number of invertebrates and planktivorous fish, which results in higher predation levels for *Daphnia* (Irvine *et al.* 1989; Stansfield *et al.* 1997; Van de Meutter *et al.* 2005). Increased positive size selective predation might be responsible for the loss of intraspecific genetic variation. Due to directional selection previous studies have revealed that *Daphnia* clones vary as to a number of life history traits (such as size at first reproduction and diel vertical or horizontal migration) related to fish predation. Life history studies have revealed a differential response of *Daphnia* clones to variation in predation levels (Cladocera: Spitze 1993; Boersma *et al.* 1998) (*D.galeata*, De Meester *et al.* 1995; Weber and Declerck 1997). Thus, increased predation levels result in reduced body sizes (e.g. Hessen *et al.* 1995; Declerck and Weber 2003) and reduced sizes at first reproduction (Declerck and De Meester 2003). Field studies on comparing predation levels (by juvenile fish and invertebrates) indicate strong positive-size selective predation on zooplankton communities (Brooks and Dodson 1965). Furthermore, fish predation causes significant changes in life characteristics among *Daphnia* clones (Boersma *et al.* 1998; Boersma *et al.* 1999). My field observations of reduced genetic variability among *D. galeata* populations under high predation levels are in concordance with laboratory and field observations.

In addition to the relationship of *D. galeata* variation with a higher trophic level, I found also an association with lower trophic levels. For example, correlation analyses revealed a positive association between phytoplankton diversity and heterozygosity of *D. galeata*. Repka (1997) and Vanni (1992) found that *Daphnia* clones respond differentially to variation in food sources (different phytoplankton species) in various life history traits. Increased algal diversity may facilitate the adaptation of *Daphnia* lineages to different ecological niches and

thus the maintenance of elevated levels of genetic variation. Furthermore, species diversity of small (**Fig**. **4-3**C) and large cladocerans is correlating with average heterozygosity of *D. galeata*, suggesting similar processes acting on diversity at clonal and species level. Further experimental studies are necessary to test the generality of this pattern (comparing several species) and to verify the functional relationship between habitat heterogeneity and intraspecific genetic variation.

Previous studies described the relationship between several physico-chemical factors, such as lake size and pH (Heino *et al.* 2003) and zooplankton and macroinvertebrate diversity. In Belgian/Dutch and Danish lake regions temperature and pH were both associated with genetic variation within populations. Although variation in pH is linked to several other ecological factors, it remains noteworthy that species richness in zooplankton and clonal richness of *Daphnia* are both associated with variation in pH. Compared with the study of Dodson (1992) and Allen (1999), I covered only a limited range of lake sizes, which might explain the lack of correlation between genetic variation and lake area.

4.5.2. Variation among populations

In general, genetic differentiation of zooplankton populations has been attributed to founder effects (Boileau *et al.* 1992), regional dispersal (Shurin and Allen 2001) and long distance dispersal by birds (Figuerola *et al.* 2005). Furthermore, chance events, habitat age and structure are considered to be important for population differentiation (Boileau and Taylor 1994; De Meester 1996), and empirical studies of neighbouring populations have revealed high levels of differentiation in neutral genetic markers and ecological relevant traits (Spitze 1993; Vanoverbeke and De Meester 1997; Declerck *et al.* 2001; Dove *et al.*). In contrast, many aquatic invertebrates are capable of dispersing via resting stages and recent studies document that migrating birds effectively transport propagules even across large distances (Figuerola *et al.* 2003; Figuerola *et al.* 2005). In order to provide a solution to this paradox, De Meester et al. (2002) proposed the monopolization hypothesis, explaining the lack of effective dispersal by strong local adaptation, chance and priority effects. Chance effects are only important in populations with low clonal diversity, which is not the case for *D. galeata* (Dove *et al.*).

Although different environmental factors were associated with genetic differentiation among *D. galeata* populations, several factors were related to the levels of predation and submerged macrophytes (**Table 4-3** and **4-4**). For example, I found a positive relationship of

increasing submerged macrophyte cover (biomass and diversity) with genetic differentiation among Belgian/Dutch *D. galeata* populations. In addition, CCA analysis showed that diversity and biomass of fish preying on *Daphnia* and phytoplankton diversity were associated with genetic differentiation in both regions. These patterns suggest that diverse habitats, with regard to macrophyte and fish, provide a higher potential for clonal differentiation among populations than less diverse freshwater systems. Thus, besides the documented impact of physical isolation among populations (e.g. Vanoverbeke and De Meester 1997) and historic effects (e.g. Weider and Hobaek 1997), ecological differentiation contributes to genetic population differentiation. These field observations are largely consistent with experimental studies of local adaptation in *Daphnia* (Declerck *et al.* 2001) and predictions of the monopolisation hypothesis (De Meester *et al.* 2002). My results of the partitioning of the environmental variation into five components showed a small contribution of pure biomass, diversity and abiotics, but relative large amounts of shared variance. Thus, population differentiation represents a multifactorial phenomenon being mediated by strong interactions among environmental factors with regard to abiotic factors, competition, predation and richness in adjacent trophic levels. Similar observations have been reported at the species level, where a variety of evidence suggests a lack of richness associations between all trophic levels (Declerck *et al.* 2005). The correlation of microsatellite loci and environmental factors indicates these markers not to be neutral. However, in Chapter 3 a simulation analysis without the loci, which seem to be under natural selection, revealed an equal number of populations than has been introduced to the analysis and population genetic estimates did not differ note worthy from each other.

4.6. Conclusion

In general, I found large regional differences with regard to the impact of environmental factors on the genetic population structure of *D. galeata*. Furthermore, no single environmental gradient emerged at the one major responsible factor for population differentiation within a region. Since my study revealed a significant contribution of ecological factors (such as predation levels) to the population genetic structure of *D. galeata*, I expect that future studies on the population structure, phylogeography and ecology of freshwater organisms will benefit from interdisciplinary approaches combining genetic information, historic processes and ecological differentiation.

5. General discussion

The genetic structure of natural populations is formed by evolutionary processes such as founder effects and genetic drift within populations on the one hand, and selection and effective gene flow among populations on the other hand (Avise 1994). Furthermore may landscape characteristics influence genetic variation within and between populations (Manel *et al.* 2003). In addition has the genetic structure of most species been mediated by historic processes such as glacial oscillations (Hewitt 2000). The recent improvements in molecular techniques have made the application of molecular markers available for evolutionary, ecological, and population genetic questions especially in cyclic parthenogenetic organisms (e.g., Halkett *et al.* 2005). The assessment of genetic diversity within and between populations enables us to infer the processes, that shape the inter and intrapopulation genetic structure. In general neutral genetic markers as used in the current study are useful to investigate the impact of historical and stochastic processes, reveal the influence of environmental factors on extant population genetic structure.

In the present work I describe the first study on a European scale to investigate processes and factors, which may influence the intra- and interpopulation genetic structure of a lake-dwelling cyclic parthenogenetic species, *D. galeata* (subgenus *Hyalodaphnia*: Cladocera: Anomopoda). First I assessed the population genetic structure of European *D. galeata* in 23 lakes (Chapter 2). In addition, I discussed the relationship of historic events and current population genetic pattern of European *D. galeata* (Chapter 3). Further, several population genetic aspects (using mitochondrial and microsatellite data), such as diversity indices and level of population differentiation, have been applied to reveal the impact of environmental characteristics (Chapter 4) on these aspects.

5.1 Genetic structure within *Daphnia galeata* populations

In general, clonal diversity (*D*) appears to be relatively high in freshwater cyclic parthenogenetic organisms (De Meester *et al.* 2004) and has been suggested to depend on habitat characteristics such as permanency and lake or pond size (reviewed in De Meester 1996). *D. galeata* populations described in this thesis revealed high genetic diversity based on nuclear data (Chapter 2). I did not observe a relationship between habitat size and clonal diversity or any other diversity index (Chapter 4), which may be due to the narrow size range of the lakes presented in this study. Lake sediments contain a vast amount of ephippia (resting egg bank) from several generations with each egg representing a different clone. It has been

shown that clonal lineages of the intermittent pond-dwelling species hatch at different times of the growing season (Carvalho and Crisp 1987), which suggests that ephippia are produced as well throughout the summer. When *Daphnia* clones hatch from ephhippia produced the last decade (Hairston 1996) subpopulations of a species exist in the extant water column. This phenomenon may be present in *D. galeata* populations (Chapter 2). The population genetic analysis of coexisting subpopulations, caused by the admixture of resting eggs produced in different years with different allelic composition, results in a Wahlund effect and can be observed as heterozygote deficiencies (Chapter 2). However, heterozygote deficiencies, allele frequency shifts and total replacement of multi locus genotypes (MLG) were detected in several months during one growing season, whereas high levels of genetic diversity throughout the growing season have been observed (Chapter 2). Therefore I suggest the changes in allele frequency and replacement of MLGs' to be the result of hatching of different genotypes within a month (Chapter 2). These coexisting lineages of *D. galeata* may be due to ecologically differentiated clones, as has been shown for *Daphnia* species of intermittent habitats (Carvalho 1987; Weider and Hebert 1987; Tessier and Leibold 1997). Generally it has been thought that lake dwelling *Daphnia* produce resting eggs only at the end of the growing season, although resting production can be initiated as well for example by predation pressure (Spaak *et al* 2004; Pijanowska and Stolpe 1996). I revealed a correlation of genetic diversity in *Daphnia galeata* populations with abundance of fish (Chapter 4). Thus, *Daphnia galeata* may turn into the sexual phase also during the growing sesaon. The significant correlation, which I revealed In Chapter 4, between clonal variation within populations and biotic and abiotic variables indicating that some loci used in this study may not be neutral as such. Indeed, due to linkage of the entire genome during clonal reproduction, neutral markers will hitch-hike with successful clones and reflect locally adapted genotypes (background selection, Maynard Smith and Haigh 1974). However, this finding does not have an impact on the genetic diversity within *D. galeata* populations (Chapter 2).

The results discussed above support the suggestion, that the genetic structure of permanent populations, such as *D. galeata*, rather resemble intermittent pond populations (De Gelas in prep)(reviewed in De Meester 1996). Thus habitat permanency does not have an impact on genetic diversity within a population; whereas local adaptation has a large influence on genetic diversity within *D. galeata* populations In addition I suggest that lake-dwelling *Daphnia* populations, such as *D. galeata* undergo frequent sexual reproduction.

5.2. Genetic structure among *Daphnia galeata* populations

Freshwater zooplankton taxa, although exhibiting good possibilities of dispersal, in most cases show high levels of genetic differentiation between populations (Hebert and Moran 1980; Crease *et al.* 1990; Vanoverbeke and De Meester 1997; Gomez and Carvalho 2000; Dove et al subm). *D. galeata* populations showed also strong population differentiation even among neighbouring populations (Chapter 2), which probably led to lack of isolation by distance, and persistent genetic differentiation on historical scale (mtDNA, Chapter 3). These significant levels of genetic differentiation despite high dispersal capabilities frequently observed in cladocerans are recently explained by several processes summarised in the "monopolisation hypothesis" (De Meester *et al.* 2002). Based on the results in Chapter 4 genetic population differentiations represent a multifactorial phenomenon which is mediated by strong interactions among environmental factors with regard to abiotic factors, competition, predation and richness at adjacent trophic levels. Since this implies that some microsatellite loci are not neutral, I analysed the data for population differentiation under the exclusion of the given loci. However, the results did not yielded in noteworthy changes compared with previous results (Chapter 2) and therefore there is only an indication for effective gene flow between *D. galeata* populations. However if gene flow would exist shared alleles and shared haplotypes among *D. galeata* populations should be observed (Chapter 2 and 3). Yet, most of the populations are genetically highly differentiated based on nuclear and mitochondrial data (Chapter 2 and 3). Therefore, I suppose, that the offset of genetic differentiation among some populations is rather an artefact, because only three loci have been included in the second analysis. Thus besides geographic distance (Chapter 2) and historic events (Chapter 3), local adaptation in combination with monopolization of local recourses has a major influence on genetic structure among *D. galeata* population (Chapter 4). As a consequence of priority effects in combination with local adaptation, the phylogeographic pattern in passively dispersed zooplankton might reflect historical colonization rather than contemporary gene flow (De Meester *et al.* 2002). In *D. galeata* populations, mitochondrial and nuclear data revealed the same phylogeographic pattern, i.e. confirming high level of subdivision among populations (Chapter 2 and 3), which has been reported for several zooplankton species in the Holoarctic (Weider and Hobaek 1997; Gomez *et al.* 2000; Schwenk *et al.* 2000; Cox and Hebert 2001; De Gelas and De Meester 2005). Furthermore *D. galeata* populations, contained endemic mitochondrial haplotype, a pattern which is congruent with the distribution of microsatellite alleles and nuclear clusters across

Europe (Chapter 2 and 4). However, the level of population divergence based on mtDNA and nuclear levels were not associated (Chapter 3). This phenomenon is due to a combination of two different chance events (mutation and genetic drift). New microsatellite alleles have emerged probably rapidly in *D. galeata* populations across Europe (Chapter 2 and 3) due to the fast mutation rate in microsatellite loci (Ellegren 2000). Mitochondrial genome however evolves at a slower rate and is more susceptible to chance events like genetic drift, since it is inherited solely by females (Birky *et al.* 1989). These different characteristics of the two marker system lead on the one hand to high genetic diversity in genetically differentiated population revealed by microsatellite loci (Chapter 2 and 3) and on the other hand to low gene diversity in strongly diverged populations based on mitochondrial data, even shortly after range expansion events (Chapter 3).

Decreased genetic diversity within populations at formerly glaciated areas and a south to north range expansion of most species are expected after postglacial colonization (Hewitt 1996; Hewitt 2000). These patterns were not totally supported by the genetic data of *D. galeata* (Chapter 2 and 3). Level of haplotype diversity was not declining towards northern Europe. However, mitochondrial gene flow has occurred mainly within central Europe and from central Europe to Russia and Norway and a widely distributed mitochondrial haplotype among *D. galeata* populations (Chapter 3) may be a relict of ancestral lineage as a result of long distance dispersal at a south-north route followed by successful colonization in the past (Avise 2000). Thus a south to north range expansion has probably occurred, but the pattern of genetic diversity distribution may be mediated by other processes, like genetic drift and mutation rate (Chapter 2 and 3) on the one hand and local adaptation (Chapter 4) on the other hand.

The elevated level of endemic haplotypes in *D. galeata* populations of northern Europe indicates, that there may have occurred gene flow from other than refugia in southern Europe as generally suspected (Hewitt 1999). The genetic cluster distribution based on nuclear marker (Chapter 3) revealed in central and northern *D. galeata* populations to consist of only one genetic cluster, which does not occur in the other European populations. This may be a result of high mutation rate at microsatellite loci, but also it might support mitochondrial data and represent immigration of alleles from other populations than described here.

5.3. Concluding remarks

In summary, this study provides the first deep insight into the intra- and interpopulation genetic structure of *D. galeata* across Europe both on current and historic scale. The results yielded population genetic structure of *D. galeata* to be mediated by several factors such as pronounced sexual reproduction, local adaptation, mutation rates and historic events. To test the outcome of this work additional studies have to be conducted. Further sampling in geographic regions such as Northern and Eastern Europe as well as the Mediterranean and Russia would probably elucidate the way of colonization of European *D. galeata* populations. The sampling design should focus on lakes along several gradients of the environmental factors which I found to influence genetic diversity and differentiation within and among *D. galeata*. Life history analysis, such as replacement experiments of clonal lineages from different habitat can be used in addition to prove whether the biotic and abiotic factors are actually important in natural populations. This would be even more effective, if the same environmental factors would be tested for *Daphnia* species of different kinds of habitats (intermittent-permanent ponds and lakes), in addition to lakes, on both a temporal scale during the growing season and a geographic scale.

6. Zusammenfassung

Die genetische Populationsstruktur, d.h. die Vielfalt innerhalb und Differenzierung zwischen natürlichen Populationen, ist auf der einen Seite durch stochastische Prozesse wie zum Beispiel Gründungsereignisse, genetische Drift und Mutation bedingt, auf der anderen Seite durch deterministische Prozesse wie natürliche Selektion und Genfluss (Hartl and Clark 1989; Avise 1994). Die inselartige Beschaffenheit von Süsswasserhabitaten, setzt im Allgemeinen Grenzen für den Genfluss zwischen Populationen (Slatkin 1985) und ermöglicht somit die Untersuchung genetischer Differenzierung zwischen Populationen und ihre lokale Anpassung. Zooplanktonarten, wie z.B. Cladocera, können diese Grenzen jedoch durch die passive Verbreitung von Dauereirstadien (Ephippien) überschreiten. Somit bieten Organismen dieser Gruppe ein geeignetes Modell, um die populationsgenetische Struktur zu analysieren und Faktoren oder Prozesse zu ermitteln, die diese Struktur beeinflussen könnten. Aufgrund dessen und weil die genetische Populationsstruktur lakustriner zyklisch parthenogenetischer Cladocera noch unklar ist, habe ich *Daphnia galeata*, eine europaweit verbreitete Art des *Hyalodaphnia*-Komplex (Crustacea: Anomopoda) untersucht. Anhand von sechs nukleären Markern (Microsatelliten Loci) habe ich von 23 Seen aus ganz Europa die genetische Populationsstruktur von *D. galeata* untersucht (Kapitel 2). Um einen möglichen Zusammenhang zwischen historischen Prozessen und der rezenten genetischen Struktur von *D. galeata*-Populationen herzustellen, wurden anhand einer weiteren vergleichenden Analyse basierend auf Sequenz- (mitochondriale 12S rDNA) und Microsatelliten-Daten 12 Populationen auf phylogeographische Muster untersucht (Kapitel 3). Anhand von Korrelationsanalysen wurde weiterhin getestet, ob und wie diese genetische Struktur von Umweltfaktoren beeinflusst wird (Kapitel 4). Ziel der Arbeit war es Faktoren und Prozesse herauszuarbeiten, die die genetische Struktur innerhalb und zwischen Populationen einer zyklisch parthenogenetischen Art beinflussen und formen.

Die klonale Diversität von zyklisch parthenogenetischen Organismen (u.a. Rotatorien und Cladoceren) im Süßwasser ist im Allgemeinen relativ hoch, jedoch unterscheiden sich *Daphnien*-Arten stark z.B im Bezug auf Heterozygotie-Werte und klonaler Stabilität über eine Wachstumsperiode hinweg (De Meester *et al.* 2004). Diese Unterschiede wurden bisher mit Eigenschaften des Habitats wie z.B. der Stabilität der Gewässer in Verbindung gebracht (De Meester 1996). Unter einem permanenten Gewässer, wie einem See, versteht man z.B., dass die saisonalen Unterschiede, wie die Klarwasserstadien und Veränderungen der Wassertiefe, nicht ausgepgrägt sind. Nicht permanente Gewässer, wie Teiche, hingegen können im

Sommer austrocken, was im Falle von *Daphnia* in der Regel im Vorfeld zur sexuellen Reproduktion von Dauereiern (Ephippien) führt. In permanenten Gewässern wurden höhere Heterozygtie-Werte als unter Hardy-Weinberg Gleichgewichts zu erwarten wäre durch starke klonale Selektion und somit genetischer Erosion erklärt (Mort and Wolf 1986; Wolf 1988). Im Gegensatz dazu wurden für *Daphnia*-Arten in nicht permanenten Süsswasserhabitaten eher niedrige Heterozygotie-Werte gemessen (De Meester 1996). Da *D. galeata* in permanenten Habitaten wie Seen vorkommt würde man annehmen, eine hohe genetische Diversität, aber auch bezüglich der Erwartungen des H-W Gleichgewichts signifikant hohe Heterozygotie-Werte in populationsgenetischen Studien zu finden.

Im Einklang mit zuvor gefundenen Ergebnissen habe ich in *D. galeata*-Populationen europäischer Seen eine hohe genetische Variabilität gefunden (Kapitel 2), jedoch zeigten alle Populationen niedrige Heterozygotie-Werte auf, sowohl über die Wachstumsperiode hinweg als auch innerhalb eines Monats. Zudem wiesen einige der *D. galeata* Populationen ($N = 7$) eine völlig andere genetische Zusammensetzung in den einzelnen Monaten über die Wachstumsperiode hindurch auf. Ein Grund dafür kann allochronische Isolation sein, d.h. zeitlich versetzte Isolation von genetischen Linien. Diese Linien sind z.B. dadurch getrennt, dass sie aus verschiedenen Generationen der Dauereirstadienbank eines Sees stammen. Das gleichzeitige Untersuchen dieser klonalen Linien könnte niedrigere Heterozygotie-Werten als erwartet, in den von mir untersuchten Populationen erklären (Kapitel 2). Da in anderen empirischen Studien die Koexistenz von sich ökologisch unterscheidenden Klonen geschildert wurde (Carvalho 1987; Weider and Hebert 1987; Tessier and Leibold 1997) können die hier gefundenen unterschiedlichen klonalen *D. galeata* Linien ebenfalls ökologisch differenzierte klonale Linien darstellen. Studien von andere *Daphnia*-Arten, die allerdings nicht permanente Habitate bewohnen, haben ähnliche Ergebnisse gefunden. In *D. magna* z.B. traten über das Jahr hinweg verschiedene Klone auf (Carvalho and Crisp 1987), was ein Anzeichen dafür sein kann, dass Dauereier das ganze Jahr über produziert werden. Für *D. galeata* wäre es nicht unwahrscheinlich, dass diese Hypothese zutrifft, denn auch *Daphnia* von permanenten Habitaten können zu anderen Zeitpunkten als nur zum Ende der Wachstumsperiode zur sexuellen Reproduktion übergehen. Diese Art der Fortpflanzung kann nämlich auch durch andere Effekte, wie Prädation und der Reduzierung der Futterressourcen iniziiert werden (Spaak *et al.* 2004). Demnach führen in nicht permanenten Gewässern die verschlechterten externen Bedingungen zur Produktion von Ephippien, in permanenten Gewässern sind zudem innerhalb der Wachstumsperiode z.B. der Prädationsdruck durch Fische dafür verantwortlich (Pijanowska and Stolpe 1996). Da die Seen, die ich untersucht habe unter anderem auch

Fische enthalten, weisen die von mir für *D. galeata* erhobenen Ergebnisse darauf hin, dass sexuelle Reproduktion in diesen Habitaten regelmäßiger vorkommt, als zuvor angenommen.

Im Allgemeinen führt man die genetische Differenzierung zwischen Populationen auf sowohl regionale (Shurin 2001) wie auch Verbreitung über weite Distanzen (Figuerola *et al.* 2005), Gründungseffekte (Boileau *et al.* 1992) und lokale Adaptation (De Meester 1996) zurück. Zooplankton-Organismen, die sich passiv durch Dauereier verbreiten, die widrige Umweltbedingungen überstehen, können mit erhöhter Wahrscheinlichkeit neue Habitate erreichen und kolonisieren (Bilton *et al.* 2001; Brendonck and De Meester 2003). Trotzdem weisen mehrere Studien eine starke Differenzierung zwischen Zooplankton-Populationen in diskreten Süßwasserhabitaten auf (Hebert and Moran 1980; Crease *et al.* 1990; Vanoverbeke and De Meester 1997; Gomez and Carvalho 2000; Schwenk *et al.* 2004; Dove *et al.* submitted). Populationsgenetische Studien an *Daphnia* in nicht permanenten Gewässern haben gezeigt, dass sowohl nah gelegene als auch weit entfernte Populationen genetisch stark differenziert sind (z.B., Vanoverbeke and De Meester 1997). Populationen permanenter Gewässer hingegen weisen nur über weite Entfernungen genetische Differenzierung auf (z.B., Mort and Wolf 1986). Für eine signifikante Populationsdifferenzierung trotz der hohen Verbreitungsmöglichkeiten sind wahrscheinlich Prozesse verantwortlich, die unter der *Monopolization Hypothesis* zusammengefasst wurden (De Meester *et al.* 2002). Die Hauptaussage der Hypothese ist, dass beständige Gründungseffekte in Kombination mit rascher lokaler Adaptation zur Monopolisierung der Ressourcen durch die Nachkommen der ersten Immigranten führen. Meine Ergebnisse unterstützen diese Hypothese, denn ich habe unter Anderem starke genetische Differenzierung auch über kurze Distanzen gefunden, obwohl ich aufgrund der bisherigen Ergebnisse anderer lakustriner Arten genetische Ähnlichkeiten erwartet hätte. Die Populationsdifferenzierung, ermittelt anhand von Mikrosatelliten-Daten, zeigt, dass es nur über weite Distanzen (> 700 km) zur genetischen Isolation in *D. galeata* kommt. Meine Ergebnisse in Kapitel 2 ähneln somit eher der Populationsstruktur von Arten, die in nicht permanenten Gewässern vorkommen, in denen sexuelle Reproduktion häufig stattfindet (z.B., De Meester 1996; z.B., Vanoverbeke and De Meester 1997). Somit vermute ich, dass, obwohl für *D. galeata* während der Wachstumsphase eine hauptsächlich asexuelle Reproduktionsweise angenommen wurde, eine sexuelle Reproduktion weitaus häufiger stattfindet als erwartet (De Meester 1996). Aufgrund dessen komme ich zu dem Schluss, dass die ökologische Stabilität des Habitats wahrscheinlich nicht für die Populationsstruktur von *D. galeata* verantwortlich ist, sondern z.B. der Grad der sexuellen Reproduktion und möglicherweise die lokale Anpassung von klonalen Linien.

Ein weiterer Aspekt meiner Arbeit (Kapitel 3) beschäftigt sich mit den Auswirkungen historischer Prozesse auf die genetische Struktur der untersuchten *Daphnia*-Populationen. Als allgemein bekannt gilt, dass die Eiszeiten die genetische Struktur und geographische Verteilung von Europäischen Tierarten geprägt haben (diskutiert in, Hewitt 1996; Hewitt 2000), jedoch wurden Süßwasserarten, speziell *Daphnien*, in diesen Studien selten berücksichtigt (siehe aber, De Gelas and De Meester 2005). Wie in meiner Untersuchung dargestellt, wurde hier ein Ansatz gewählt, der sowohl nukleäre Mikrosatelliten wie auch ein mitochondriales Markersystem kombiniert. Beide Markersysteme zeigten ähnliche populationsgenetische Strukturen von *D. galeata* in Europa auf.

Als Konsequenz des Prioritätseffektes der ersten Immigranten laut der *Monopolization Hypothesis* wird das phylogeographische Bild bei sich passiv verbreitenden Zooplanktonarten die ursprüngliche Kolonisierung darstellen (De Meester *et al.* 2002). Somit können genetische Marker, auch wenn sie in verschiedenen Zeitfenstern evolvieren (z.B. mtDNA und Microsatelliten Loci), das gleiche Bild genetischer Differenzierung diskreter Populationen widerspiegeln. Zwischen *D. galeata* Populationen habe ich starke genetische Abgrenzungen anhand von mitochondrialen Sequenzen (mtDNA) und Microsatelliten-Daten gefunden (Kapitel 3). Diese Ergebnisse sind vergleichbar mit denen anderer Studien von Zooplankton-Arten der Holarktis (Weider and Hobaek 1997; Gomez *et al.* 2000; Schwenk *et al.* 2000; Cox and Hebert 2001; De Gelas and De Meester 2005). Weder anhand mitochondrialer Sequenzdaten noch der Microsatelliten-Daten, konnte in den von mir untersuchten 12 Populationen eine genetische Isolation über die Distanz gefunden werden. Das unterstützt, im Gegensatz zu dem vorherigen Kapitel, die bisher für lakustrine Arten gefundenen Ergebnisse (Mort and Wolf 1986; Wolf 1988; Jacobs 1990), ist aber wahrscheinlich auf die reduzierte Anzahl an Populationen zurückzuführen. Trotz der ähnlichen Differenzierungsmuster konnte ich keine Korrelation zwischen mtDNA und Mikrosatelliten-Daten ermitteln werden. Der Grund liegt wahrscheinlich darin, dass zum einen mtDNA stärker von genetischer Drift beeinflusst wird als nukleäre DNA (Birky *et al.* 1989) und zum anderen Microsatelliten Loci schneller evolvieren, als Sequvenzen des mitochondrialen Genom (Ellegren 2000).

Die genetischen Daten in Kapitel 3 weisen nur teilweise die typischen postglazialen Muster auf, die für viele europäische Tierarten gefunden wurden (Hewitt 1999). Die weite Verbreitung von mitochondrialen Haplotypen in *D. galeata*-Populationen (Kapitel 3) könnte anzestrale Linien darstellen, die nach der Ausbreitung in Süd nach Nord Richtung in der Vergangenheit erfolgreich neue Habitate kolonisiert haben (Avise 2000). Meine Mikrosatelliten-Daten unterstützen diese Aussage, was wahrscheinlich darauf zurückzuführen

ist, dass eine weitere Kolonisierung durch neue klonale Linien aufgrund der *Monopolisation Hypothesis* begrenzt ist (De Meester *et al.* 2002). In *D. galeata*-Populationen hat ein mitochondrialer Genfluss zwar in einer Süd nach Nord Richtung stattgefunden, aber nicht von der iberischen Halbinsel sondern von Mitteleuropa aus. Ausserdem war der Genfluss innerhalb Mitteleuropas zwischen Dänemark und Belgien am höchsten. Weiterhin habe ich kein typisches Bild der Verarmung genetischer Diversität in einer Süd nach Nord Richtung aufgrund der postglazialen Rekolonisierung (diskutiert in, Hewitt 1996; Hewitt 2000) für *D. galeata*-Populationen zeigen können. Die Mikrosatelliten-Variation war in ganz Europa hoch, die Diversität der mitochondrialen Haplotypen allerdings war nur in den mittel- und nordeuropäischen Populationen (BEL, FIN und RUS) erhöht. Zusätzlich haben Analysen meiner Arbeit ergeben, dass diese Populationen eine starke Reduzierung ihrer effektiven Populationsgröße erfahren haben oder sich rezent ausgebreitet haben. Diese gegensätzlichen Interpretationen können z.B. in Hybridzonen gefunden werden, wo die Kolonisierung aus verschiedenen glazialen Refugien stattgefunden haben muss (Hewitt 1993; Hewitt 1999). Meine Ergebnisse stellen höchstwahrscheinlich das syntope Auftreten von *D. galeata*-Linien dar, die sich allopatrisch auf der genetischen Ebene verändert (erklären) haben. Diese Veränderung kann durch Introgression aufgrund von erfolgreicher Hybridisierung in den Refugien bei *D. galeata* (Schwenk 1993; Schwenk and Spaak 1995; N. Brede persönliche Kommunikation) noch verstärkt worden sein. Die Präsenz von einmalig auftretenden Haplotypen in nordeuropäischen Populationen und privaten Allelen in über Europa verteilten Populationen lässt annehmen, dass diese Seen auch aus anderen Gebieten rekolonisiert worden sein können, wie z.B. Mittel- oder Osteuropa (Väinölä *et al.* 1994; Bilton *et al.* 1998; Økland and Økland 1999; Stewart and Lister 2001; Pfenninger *et al.* 2003) als aus den generell bekannten südeuropäischen Refugien wie z.B. die iberische Halbinsel oder der Balkan (diskutiert in, Hewitt 1999).

Um den ökologisch-genetischen Aspekt in meine Arbeit mit einzubeziehen, habe ich in Kapitel 4 die Zusammenhänge von Umweltfaktoren und populationsgenetischer Struktur von *D. galeata* untersucht. Es ist bekannt, dass die Eigenschaften eines Habitats einen Einfluss auf die Artenzusammensetzung haben (z.B., Shurin 2000; z.B., Matveev 2003). Allerdings ist noch nicht klar, ob es auch einen Zusammenhang zwischen Habitat-Charakteristika mehrerer trophischer Ebenen und genetischer Vielfalt innerhalb einer Art z.B. *Daphnia* gibt. Da sich *Daphnia*-Klone parthenogenetisch reproduzieren und sie ökologisch differenziert sind z.B. im Bezug auf Nahrung, Prädationsresistenz und Verhalten (i.e., Brooks and Dodson 1965; i.e., Spitze 1993; De Meester *et al.* 1995; Boersma *et al.* 1998), setze ich klonale Linien

ökologischen Einheiten wie z.B. Arten gleich. Ich konnte anhand mehrerer signifikanter Korrelationen zeigen, dass die genetische Diversität von *D. galeata* Populationen signifikant mit abiotischen und biotischen Faktoren höherer und tieferer trophischer Ebenen (z.B. pH-Wert, Fisch-Abundanzen und -Artenvielfalt, Unterwasserflora, Phytoplankton-Artenvielfalt und Bakterien-Vielfalt und -Dichte) korreliert. Jedoch kam es zu einer regionalen Differenzierung in der Quantität und Qualität der Umweltfaktoren auf die intra-populationsgenetische Struktur von *D. galeata.* Somit konnte keine Kategorie der Umweltfaktoren (Abiotik, Biomasse oder Artenvielfalt verschiedener trophischer Ebenen) für sich als erklärende Variable der genetischen Populationsstruktur in *D. galeata* ermittelt werden. Weiterhin habe ich Evidenzen dafür gefunden, dass die genetische Differenzierung zwischen *D. galeata*-Populationen ebenfalls ein multifaktorielles Phänomen ist, welches durch gleiche Interaktionen von Umweltfaktoren, z.B. abiotischen Faktoren, Konkurrenz, Prädation und Artenreichtum in angrenzenden trophischen Ebenen gekennzeichnet ist wie das der genetischen Diversität. Vergleichbare Ergebnisse auf der Taxon Ebene (Brooks and Dodson 1965; Dodson 1992; Jeppesen *et al.* 2000; Heino *et al.* 2003; Declerck *et al.* 2005) führen zu dem Schluss, dass die Artenzusammensetzung innerhalb eines Habitats und die klonale Variabilität innerhalb einer Art durch ähnliche funktionelle Zusammenhänge geformt werden. Hier möchte ich anmerken, dass signifikante Korrelationen zwischen genetischen Markern und bestimmten Faktoren die Neutralität dieser Marker anzweifeln lässt. Allerdings haben weitere Analysen anhand eines reduzierten Datensatzes (ohne die möglicherweise unter natürlicher Selektion stehenden Loci) keine nennenswerten Unterschiede in der Populationsstruktur von *D. galeata* ergeben. Meine Ergebnisse weisen weiterhin darauf hin, dass diverse Habitate in Bezug auf Makrophyten-Zusammensetzung und Fisch-Abundanzen ein höheres Potential für eine genetische Differenzierung zwischen Populationen haben als weniger diverse Süßwasser-Systeme. Demnach kann neben physikalischer Isolation (Kapitel 2) und historischen Prozessen (Kaptiel 3) ökologische Differenzierung (Kapitel 4) im Falle von *D. galeata* ebenfalls zu genetischer Differenzierung führen und eine Auswirkung auf die genetische Diversität haben.

Die in dieser Promotion erarbeiteten Ergebnisse geben Aufschluss über die verschiedensten Faktoren, die die genetische Struktur von *D. galeata* beeinflussen. Diese Arbeit bildet somit die Grundlage für experimentell motivierte Studien, um die hier erarbeiteten Hypothesen zu testen. Es konnte anhand von *D. galeata* gezeigt werden, dass eine detaillierte Aufnahme sowohl abiotischer als auch biotischer Faktoren mehrerer trophischer Ebenen notwendig ist, um die microevolutiven Vorgänge innerhalb eines Habitates wie auch zwischen Habitaten zu

verstehen. Somit können nun basierend auf dieser empirischen Arbeit z.B. durch *Life-History* Experimente überprüft werden, ob bestimmte Umweltfaktoren artspezifisch wirken, oder generell für Cladoceren eine Rolle in Bezug auf ihre Populationsstruktur in Abhängigkeit ihres Habitattyps aufweisen.

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Appendix

Table 4-2. Description of the environmental variables used for canonical correspondence analysis. DGGE: denaturing gradient gel electrophoresis, GALD: greatest axial linear dimension, CPUE: catch night^{-1} multi-mesh gillnet^{-1}.

Category	Abbreviation	Description	Mean (Range)	Unit
Abiotic	SECCHI	Secchi depth	107 (35.5-268.9)	cm
	DEPTH			
	CON	Conductivity	417.4 (165.95-898.65)	micro S.cm^{-1}
	TEMP	Temperature	16.87(13.79-20.09)	°C
	PH	PH	8.18 (7.62-9.06)	
	TP	Total phosphorus	166.973 (8-519.75)	microg l^{-1}
	SI	Silicate	3.433 (0.05-9.99)	mg l^{-1}
	AREA	Lake surface	22.637 (1.942-124)	Ha
	DEPTH	Lake average depth	1.26 (0.58-3.5)	m
Biomass-Density	PHYTTOT	Total phytoplankton biomass	1196.3 (0.6-55.68.85)	µg C l^{-1}
	ZOOPTOT	Total zooplankton biomass	794.5 (77.09-2801.8)	µg dry weight l^{-1}
	BACTDENS	Bacterial density	5.489 (1.497-10.63)	10^6 cells ml^{-1}
	HNFDENS	Density of heterotrophic nanoflagellates	1994.58 (357-596917)	cells ml^{-1}
	SUBMCOV	Coverage by submerged macrophytes	19.457 (0-73)	%
	SUSORM	Suspended organic matter	9.86 (1-31)	mg l^{-1}
	TA	Toxic algae	213.23 (0-2016.37)	mg C l^{-1}
	NTA	Nontoxic algae	1.767 (0-15.15)	mg C l^{-1}
	EA	Edible algae 0 < 20 > 40 microm large GALD phytoplankton and grazable ciliates	517.786 (19-3452)	mg C l^{-1}
	NEA	Non edible algae > microm GALD	1.99 (0.1-3.7)	mg C l^{-1}
	DPFN	Fish preying on *Daphnia* (sum of DPFYN and DPFMN)	101.98 (30.5-251.83)	CPUE
	DPFYN	Fry preying on *Daphnia*	49.516 (4-215.167)	CPUE
	DPFMN	Fish preying mainly on *Daphnia*	38.115 (0-161.17)	CPUE

Table 4-2 (*continued*)

Category	Abbreviation	Description	Mean (Range)	Unit
	DPFKG	Fish preying on *Daphnia* (Sum of DPFYKG and DPFMKG)	5054.22 (649.5-10660.64)	CPUE in kg
	DPFYKG	Fry preying on *Daphnia*	1803.88 (82.33-3955.73)	CPUE in kg
	DPFMKG	Fish preying mainly on *Daphnia*	3251.33 (0-8565.85)	CPUE in kg
Diversity	FISHSHA	Fish Shannon Wiener on species level	1.055 (0.156-1.614)	
	NDPSHA	Fish not preying on *Daphnia* Shannon Wiener on species level	0.167 (0-0.68)	
	DPSHA	Fish preying on *Daphnia* Shannon Wiener on species level	0.843 (0.005-1.34)	
	SUBMSHA	Submerged macrophytes Shannon Wiener on species level	0.448 (0-1.968)	
	PHYTSHA	Total phytoplankton Shannon Wiener on genus level	1.826 (0.986-2.266)	
	MPASHAS/L	Multiprobe array hybridisation data on bacteria taxa (S: small; L: large)	1.736 (0.973-1.968); 0.374 (0-1.036)	
	DGEUSHAS/L	DGGE Eukariotes (S: small; L: large) Shannon Wiener	1.946 (0.99-2.598); 1.912 (1.22-2.675)	
	DGBASHAS/L	DGGE Bacteria (S: small; L: large) Shannon Wiener	3.015 (2.491-3.298); 2.571 (1.874-3.031)	
	CLADSHAS/L	Cladoceran (S: small; L: large) Shannon Wiener on genus level	0.806 (0.262-1.29); 0.707 (0.262-1.178)	
	CILSHAS	Ciliates (small) Shannon Wiener	2.152 (1.009-2.637)	

Fish preying (DPFxx, fry and adult) on *Daphnia*: *Rutilus rutilus, Perca fluviatilis, Abramis brama, Blicca bjoerkna, Gobio gobio, Leucaspius deliniatus, Carrassius carrassius, Pseudorasboa parvu, Acerina cernua, Leppomis gibbosus, Coregonus lavaretus, Tinca tinca,*
Fish not preying (NDPxx) on *Daphnia*: *Rhodeus sericeus amarus, Scardinius sp., Esox lucius (?), Lucioperca lucioperca* (?)

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Publikationen

Nilsson, J., R. Gross, T. Asplund, **O. Dove**, H. Jansson, J. Kelloniemi, K. Kohlmann, A. Löytynoja, E. E. Nielsen, T. Paaver, C. R. Primmer, S. Titov, A. VasemÄgi, A. Veselov, T. Öst and J. Lumme. 2001. Matrilinear phylogeography of Atlantic salmon (Salmo salar L.) in Europe and postglacial colonization of the Baltic Sea area. **Molecular Ecology** 10 (1) 89-102.

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Dove, O., K. Schwenk, S. Declerck, E. Jeppesen, J. M. Conde Porcuna, & L. de Meester. 2002. Are biotic and abiotic factors of lakes associated with intraspecific genetic variation in *Daphnia galeata*? The VIth International Symposium on Cladocera, Wiercba, Poland; **Vortrag**

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