Population genetic structure in European *Hyalodaphnia* species:

Monopolization versus gene flow

Dissertation

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Carmen Dietrich, 2006

Contents

Abstract

The principles of population genetics are the basis for an understanding of evolutionary processes, and are further essential for other biological fields, e.g. genomics, ecology, and conservation biology. The reproductive mode is, amongst others, an important determinant of population genetic structure of species. Cyclic parthenogens display an alternation of asexual and sexual reproduction which has consequences for the genetic structure of these organisms. In zooplankton cyclic parthenogens (Cladocera and Monogononta) amictic females reproduce asexually during favorable environmental conditions. If these conditions deteriorate, male production and sexual females are induced. This results in the formation of dormant eggs and thus a dormant egg bank in the freshwater sediments. Dormant eggs not only facilitate dispersal through time but also through space as they are carried away by waterfowl, wind or water currents. The clonal diversity of cyclic parthenogenetic zooplankton populations is dependent on the size of the dormant egg bank, which contributes new genetic variants to the populations, and clonal erosion over time, which reduces the number of different clones through stochastic and selective processes. Although freshwater invertebrates are good dispersers through their dormant stages, the influence of gene flow is assumed to be negligible, as the local populations successfully monopolize the available resources. Several processes are combined under the term monopolization to explain the high genetic differentiation, which is often observed among cyclic parthenogenetic zooplankton populations. As these populations reach carrying capacity fast due to the asexual reproduction, the first colonizing individuals are able to successfully establish in the habitat, resulting in a priority effect which hinders the invasion of new genotypes. Due to clonal selection and sexual reproduction a population will locally adapt over time and will establish a dormant egg bank which facilitates the fast re-colonization after a hostile period.

The aim of my thesis was to evaluate the processes altering the population genetic structure of cyclic parthenogenetic zooplankton with a special focus on the concepts of monopolization as well as the counteracting effects of gene flow, using the lake species *Daphnia cucullata*, *D. galeata*, and *D. longispina*.

I developed thirty-two variable microsatellite DNA markers which enable the fine-scale genetic study of populations of several species belonging to the *D. longispina* group, as cross-species amplification was successfully tested for several loci. In addition, I evaluated a subset of twelve markers regarding their suitability for species assignment and hybrid class detection, and was able to successfully delineate among the species *D. cucullata*, *D. galeata* and *D. longispina*. Hybrid detection in the species pair *D. galeata-cucullata* was limited due to three loci exhibiting *D. cucullata* specific null alleles. However, successful assignment to hybrid classes was possible using only nine of the twelve markers.

With this marker set and an additional mitochondrial DNA marker I studied forty-four natural European populations of the species *D. cucullata*, *D. galeata* and *D. longispina*. In *D. galeata*, most populations were characterized by low clonal diversities which suggest high influence from clonal erosion over the growing season and a low contribution from the dormant egg bank. Further, recent expansions as well as gene flow were detected, probably caused by the anthropogenic alteration of freshwater habitats, in particular eutrophication of many European lakes or the introduction of fish in erstwhile fishless water bodies. This facilitated the invasion of *D. galeata* in habitats formerly occupied by *D. longispina*, which resulted either in the replacement of the residing species or in massive interspecific hybridization events. *D. longispina* and *D. cucullata* revealed a different genetic structure compared to *D. galeata*, with high genetic differentiation among populations. This indicates low levels of effective gene flow which is in line with the predictions of the monopolization hypothesis. Further, high clonal diversities were found in populations of the two taxa, suggesting a high contribution from the dormant egg bank while clonal erosion was often not detectable. In *D. longispina*, mitochondrial data revealed an ancient expansion which was probably initiated by the formation of glacial lakes after the last ice age. In addition, in *D. longispina* not only clonal diversity but also genetic diversity was high, indicating that during the build-up of the studied populations the influence from gene flow was probably high. To better understand the processes that act on early populations I experimentally studied the population build-up with regard to the temporal advantage of clones during invasion succession and discovered that priority effects shape population structure of *Daphnia* species. However, in certain cases highly superior clones resulted in the extinction of inferior clones independent of the temporal advantage the single clones had. This clearly shows that not only the time of succession is important in population assembly but also the competitive strength.

In conclusion, the results obtained during my Ph. D. studies show that the population genetic structure in cyclic parthenogenetic zooplankton species is impacted by various processes. In addition to earlier studies, which mainly focus on local adaptation, clonal erosion and the size of the dormant egg bank to understand population genetic structure, I could show that gene flow may be effective as well. During population build-up the advantage of early arriving individuals does not necessarily predicts the outcome of population assembly, as additional genotypes may contribute to the population. Finally, the genetic structure of established populations may be severely impacted by effective gene flow, if severe environmental changes alter the habitat of the locally adapted population.

General introduction

Population genetics: Objectives and approaches

The often quoted proposition "Nothing in biology makes sense except in the light of evolution", going back to an inspiring paper written by Theodosius Dobzhansky (1973), was recently reformulated by Michael Lynch (2007) to emphasize the importance of population genetic principles for an understanding of evolutionary processes:

"Nothing in evolution makes sense except in light of population genetics"

But these principles are not just the basics of a broad evolutionary perspective, they are also essential for many other areas of modern biology, like genetics, genomics, animal breeding, ecology, natural history, forestry, horticulture, conservation, and wildlife management (Hartl & Clark 2007).

Population genetics is probably the only biological science that was first elaborately developed as a theoretical discipline before empirical and experimental research had a significant impact (Veuille & Slatkin 2002). During the 1920s and 1930s the classical core principles were established by Ronald A. Fisher, John B. S. Haldane, and Sewall Wright (Provine 2001) and included theoretical expectations to fuse Darwin's concept of natural selection with the principles of Mendelian inheritance. These expectations integrated that natural selection is able to alter allele frequencies rapidly within populations, that mutation and recombination supply genetic variation, that mating patterns and gene flow shape genetic differentiation, and that the effective population size regulates the process of genetic drift (Hamilton 2009).

With the advent of molecular methods in the late 1960s, first allozymes and amino acid sequences, and later DNA sequence data (in the 1980s) unraveled the genetic structure of many natural populations and laid out the empirical basis which revived the discipline of population genetics and resulted in a new set of questions (Hedrick 2011). Today, advanced methods are available for fine-screening and characterizing population genetic structure (e.g.

microsatellite DNA and single nucleotide polymorphisms), or which aid the collection of massive amounts of genomic data from almost any organism. Further, not only the technical abilities to collect large amounts of genetic information have been developed, but also analysis techniques are becoming more sophisticated by the minute as computation capacity increases (Hamilton 2009).

Population genetic structure in cyclic parthenogenetic zooplankton

In addition to genetic drift, gene flow and natural selection the mode of reproduction influences the genetic structure of populations. In comparison to obligate sexual organisms, cyclic parthenogens interrupt the bisexual phase more or less regularly by several cycles of parthenogenetic reproduction (Hebert 1987). Among animals this reproduction mode has evolved in several thousand species of only seven taxonomic groups (Monogononta, Cladocera, Digenea, Homoptera, Hymenoptera, Diptera, and Coleoptera; Hebert 1987). The rarity of this reproduction in animals is attributed to the difficulty of evolving mechanisms permitting the coordinated occurrence of both asexual and sexual reproduction (Hebert 1987). In cyclic parthenogenetic zooplankton organisms (Cladocera and Monogononta) individuals develop from amictic eggs during the asexual phase of the life cycle which is maintained as long as environmental conditions are favorable (Zaffagnini 1987). When these conditions tend to deteriorate, the population turns to sexual reproduction and males appear that fertilize sexual eggs produced by mictic females (see **Figure I-1**; Zaffagnini 1987).

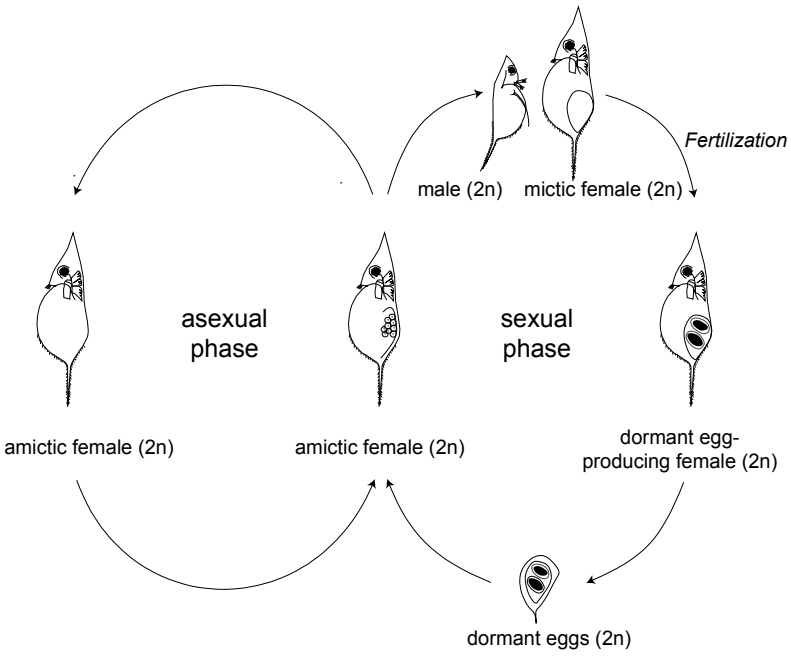

Figure I-1 Cyclic parthenogenetic reproduction in *Daphnia*. Drawings were modified from Flößner (2000).

The sexual products (dormant eggs), in cladocerans encased in a protective structure (ephippium), remain viable for a long time (Hairston *et al.* 1995). These dormant eggs facilitate dispersal in time and space to survive harsh conditions like freezing or drying or to colonize new habitats, respectively (Brendonck & De Meester 2003).

The occurrence of parthenogenetic and sexual generations has consequences for the maintenance of genetic diversity and for population genetic structure. At the beginning of the growing season populations are characterized by a high clonal diversity as resurrection from the dormant egg bank supplies genetic variation to the population, because each hatchling represents a unique clone produced through the segregation and recombination of genes. During the parthenogenetic phase, chance extinctions and selection of clones are expected to erode clonal diversity, which may strongly impact the genetic structure of populations (Vanoverbeke & De Meester 2010; Young 1979). Given that most natural populations are expected to start the growing season with millions of hatchlings from dormant eggs (Lynch 1987), the observation of clonal erosion, by studying multi-locus genotypes, indicates that the change in clonal structure must be very pronounced. De Meester *et al.* (2006) suggest that the degree to which clonal erosion affects the genetic structure of cyclic parthenogens is depending on three main factors: (1) size of the population (size of the active dormant egg bank), (2) permanency of the population (length of the growing season), and (3) strength of clonal selection (**Figure I-2**).

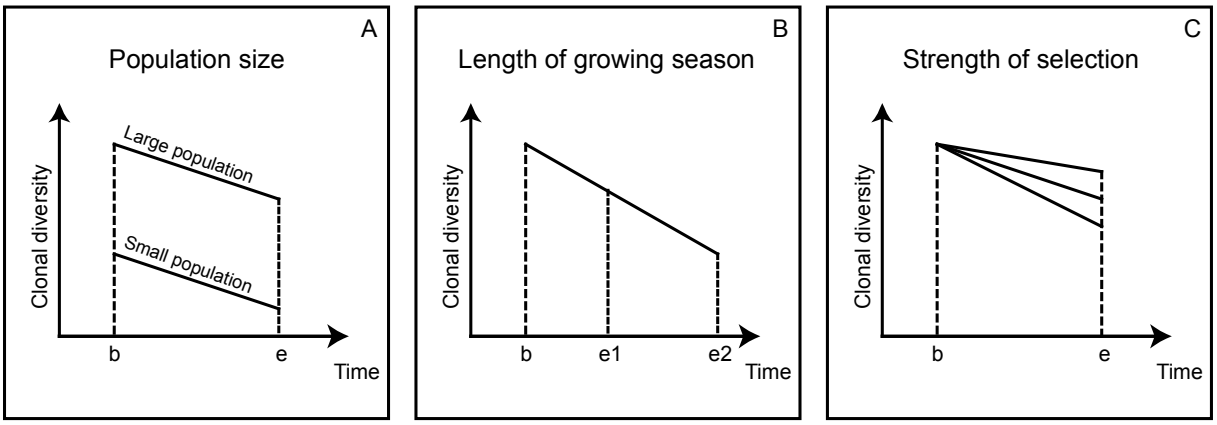

Figure I-2 Schematic representation of the three main factors determining clonal diversity of cyclic parthenogenetic populations. A: influence of the size of the dormant egg bank; B: influence of the length of the growing season; C: influence of the strength of clonal selection. The beginning (b) and the end (e) of the growing season are indicated. Modified from De Meester *et al.* (2006).

Although parthenogenetically reproducing populations of freshwater cladocerans and monogonont rotifers usually rapidly attain carrying capacity, these populations may be

characterized by only a limited clonal diversity which results in a low effective population size (Vanoverbeke & De Meester 2010; Young 1979). Further, not all clones in a population reproduce sexually with the same probability (Keller & Spaak 2004) and also interpopulational differences in sexual investment are recorded (Allen & Lynch 2011). This results only in the partial contribution of genetic variation to the dormant egg bank. At the beginning of a growing season, populations are re-established or additionally supplied by genotypes from these dormant eggs, hence, the number of different clones at the beginning of the growing season depends on the size and characteristics of the dormant egg bank. In addition to variation in hatching percentage among populations, there is also variation in hatching percentage among growing seasons within one population, because hatching cues may vary among years (Cáceres & Tessier 2003). To sum up, populations occupying large habitats are expected to start the growing season with a higher number of hatchlings, and thus a higher number of clones, than populations inhabiting small habitats. Also the age of the population is essential as younger populations are supported by a less variable dormant egg bank than older ones.

The longer the growing season, the longer chance extinctions and selection may erode genetic diversity in the population. The best unit to express the length of the growing season in relation to clonal erosion is the number of parthenogenetic generations the population has gone through since hatching from the egg bank, as some populations are sustained over several years (Hamrová *et al.* 2011; Zeis *et al.* 2010). If the strength of clonal selection is variable among populations, this may strongly influence differences in clonal structure among natural populations of cyclical parthenogens. Selection pressures may also vary within a population over time. As clonal diversity is mainly established at the beginning of the growing season, it tends to decline afterwards at a pace depending on the selective conditions in the habitat, e.g. parasitism (Yin *et al.* 2012a), predation regime (Lampert 2011), or food quality (Seidendorf *et al.* 2007).

Dispersal versus monopolization

Effective gene flow resulting through the dispersal of individuals among populations is not considered in the above mentioned concept by De Meester *et al.* (2006) although it is an important force in the population genetics concept. Under effective gene flow, population diversity should increase, while differentiation should decrease with time since founding (Boileau *et al.* 1992; Haag *et al.* 2006). Together with recombination it should reduce allelic

sequence divergence and the proportion of private alleles. In contrast, if little to no gene flow is occurring, frequency differences of haplo- and genotypes, the proportion of private alleles, and allelic sequence divergences should increase among populations with time. Cyclic parthenogenetic zooplankton organisms have a high dispersal capacity mediated by their dormant stages. Several vectors for dispersal are known, in particular animals like waterfowl, but also wind and water currents are considered (reviewed by Havel & Shurin 2004). Experimental evidence of waterfowl as dispersal vector is numerous and suggests that dormant stages survive gut passages (Figuerola *et al.* 2003) and also adhere to the feather coat (Figuerola & Green 2002).

The first population genetic studies using cyclic parthenogenetic zooplankton organisms started in the 1970s using allozyme markers and revealed varying levels of genetic diversity within populations and often very high genetic differentiation among populations (Hebert 1975; Hebert & Moran 1980; Jacobs 1990; Korpelainen 1984; Vanoverbeke & De Meester 1997; Wolf 1988). Later the analysis of sequence and high-resolution data from microsatellite markers supported those first allozyme studies (Gómez *et al.* 2002; Gómez & Carvalho 2000; Hamrová 2011; Hamrová *et al.* 2011; Ishida & Taylor 2007a, b; Petrusek *et al.* 2007; Xu *et al.* 2009; Yin *et al.* 2010). In 2002 Luc De Meester and colleagues published the *monopolization hypothesis* to explain this paradox of high dispersal capacity and low effective gene flow. This hypothesis combines stochastic and selective forces and states that the successful colonization and the explosive population growth lead to an advantage as the new population reaches carrying capacity very fast which makes it difficult for other genotypes to invade, i.e. a priority effect is observed. During the sexual phase, dormant eggs are produced that are incorporated in a dormant egg bank. This egg bank provides an powerful buffer, as new genotypes hatch as soon as conditions are favorable, which was observed as early as one week after filling of an intermittent pond by Cáceres and Tessier (2004). This advantage increases the priority effect. Furthermore, clonal selection and sexual reproduction may lead to better adapted genotypes than later invading ones. In short, through strong population growth, genetic drift and local adaptation, the residing population is protected against invading genotypes thus reducing gene flow to a minimum (De Meester *et al.* 2002).

Historic environmental changes

To describe and interpret the genetic structure of a taxon, further aspects must be considered, e.g. the evolutionary history of a given species. One important factor that influenced the population genetic structure of many species is the last ice age. During the last glacial maximum (17000-25000 BP; Petit *et al.* 2003) northern parts of Europe were covered by a massive ice sheet. Animals and plants that could not actively migrate southwards became eventually extinct. In widely distributed species the survival was assured in unglaciated refuge regions, like Mediterranean Europe (Hewitt 1996) or eastern Siberia (Weider & Hobæk 1997, 2003). This was probably also the fate of freshwater inhabitants, like cladocerans and monogonont rotifers. As the ice sheet slowly retreated and left numerous new oligotrophic lakes and ponds behind, range expansions from such refuge regions occurred, in cyclic parthenogenetic zooplankton most probably by means of waterfowl, but also wind and water streams, as well as other animal vectors like fish and mammals are conceivable (Havel & Shurin 2004). While refugial populations are assumed to be characterized by a high genetic diversity, colonizers of new populations would dominate the populations gene pool as later migrants would contribute little since they would be entering established populations at carrying capacity with only replacement dynamics (Hewitt 1996).

Contemporary environmental changes

Thomas Smith and Louis Bernatchez (2008) appositely formulated in the preface of the special issue "Evolutionary change in human-altered environments" that we are witnessing a global, but unintended, evolutionary experiment concerning the biotic diversity of the planet as a consequence of human impact on all ecosystems. Already with the beginning of agriculture (starting 14000-10000 years BC) *Homo sapiens* became an increasing force in environmental systems (Diamond 2002). Early influences, e.g. farming and deforestation, impacted the aquatic ecosystems for example as run-off and therefore nutrient inflow were not retained, often even increased (Bradshaw *et al.* 2005). This enriched many freshwater systems leading to a change in productivity. Especially in some standing water bodies this resulted in eutrophication already as early as 6000 years BP (Fritz 1989; Korponai *et al.* 2010). The transportation and breeding of fish, for example through clergy and noble men, is recorded for at least thousand years, and is assumed to have occurred even earlier (Balon 1995). This led to the establishment of several thousand fish ponds and the introduction of new species (Van Damme *et al.* 2007). Hitchhiking of other species in the course of fish stocking was and still is

probably common (Van Damme *et al.* 2007). Starting with industrialization around 200 years ago effects on freshwater habitats became even more severe. The main consequences are increased acidification and pollution of habitats (Cammarano & Manca 1997; Nevalainen *et al.* 2011), the increased intentional introduction of alien species (Hesthagen & Sandlund 2004; Knapp *et al.* 2001), the repeated invasion of hitchhiking species, for example in ballast water of ships (Hebert & Cristescu 2002; Taylor & Hebert 1993a), the damming of rivers, changing lotic to rather lentic habitats with steep ecological gradients promoting co-existence of species, massive eutrophication of water bodies (Correll 1998) as well as increased fish stocking for aquaculture since the middle of the 20th century (e.g., Musil *et al.* 2010).

Daphnia longispina **species complex**

Genus Daphnia

A well-studied representative cyclic parthenogenetic organism living in freshwater habitats is *Daphnia* (Arthropoda, Branchiopoda, Cladocera). This ecologically plastic group is widely distributed and dominates the planktonic community in a variety of pool, pond and lake ecosystems, where it occupies a key position in aquatic food webs (Lampert 2011). Above that, members of this genus are used as models in teaching and evolutionary biology, ecology, toxicology, and only recently were nominated as model organism for biomedical research of the National Institutes of Health (Bethesda, Maryland, USA). *Daphnia* received this special status as many aspects of its biology, ecology, and physiology are known (Lampert 2011; Peters & de Bernardi 1987). Additionally, the *Daphnia* Genomics Consortium is showing high effort in sequencing several species belonging to this genus (*D. pulex*: Colbourne *et al.* 2011; *D. magna* and *D. galeata*: https://daphnia.cgb.indiana.edu).

Therefore, it is unexpected that despite this knowledge and progress, the systematic status of the most common taxa is still subject of debate (Adamowicz *et al.* 2009; Benzie 2005; e.g. Frey 1982a; Petrusek *et al.* 2008). There are several reasons accounting for this problem: First, phenotypic plasticity is often observed within the genus which makes body shape and other aspects of morphology highly variable, depending on environmental factors (Juračka *et al.* 2011; Laforsch *et al.* 2006; Laforsch & Tollrian 2004; Petrusek *et al.* 2009). Second, local races develop, as populations are often founded by a small number of individuals (Petrusek *et al.* 2008). Third, interspecific hybridization is often observed within this genus, which may

further complicate morphological assignment (Benzie 2005) and fourth, cryptic species are discovered by molecular analyses (Adamowicz *et al.* 2009; Penton *et al.* 2004).

Phylogenetic relationships of the D. longispina *complex*

The eponym of the *D. longispina* species complex was first described by O. F. Müller in 1776. Since then several new species and subspecies were characterized often including the morphological description of hybrids, and some of them were merged again (Benzie 2005; Flößner 2000; Frey 1982a). A recent taxonomic reappraisal by Petrusek *et al.* (2008) straightened this complex by using morphological and genetic evidence collected. The phylogenetic relations of the *D. longispina* complex are reasonably robust through evidence from morphology, 12S rDNA, 16S rDNA, COXI, CytB, ITS-2 rDNA, and allozyme loci (Petrusek *et al.* 2008; Schwenk 1993; Schwenk *et al.* 2000; Schwenk *et al.* 1998; Taylor & Hebert 1994; Taylor *et al.* 1996; Taylor *et al.* 2005). The most basal clade contains *D. lacustris* and *D. umbra*, while the major clade contains currently five described species: *D. longispina* and *D. cucullata* in Eurasia; *D. dentifera* and *D. mendotae* distributed throughout the Nearctic, but are also found in Japan (Ishida *et al.* 2011; Ishida & Taylor 2007a, b); and *D. galeata*, which is found across the whole Holarctic range (Ishida & Taylor 2007b). Although, this complex is highly studied in regard to their morphology, physiology, ecology and phylogenetic relationships, new genetic lineages are recovered, even in well-studied habitats (**Figure I-3**; Ishida *et al.* 2011; Petrusek *et al.* 2008; Petrusek *et al.* 2012).

Biology, ecology, and distribution of the species D. cucullata*,* D. galeata*, and* D. longispina

The three species used for this study are all distributed throughout Europe and regularly occur syntopically whereas they occupy different ecological niches. *D. cucullata* is distributed throughout the Palaearctic where it is most abundant in cool temperate and montane, but not arctic areas (Benzie 2005; Flößner 2000). In general, it is found in small and large shallow lakes rarely in small ponds; sometimes it occurs in deep stratified lakes or reservoirs due to recent eutrophication, where it is found either in the upper layers of the epilimnion (Flößner 2000) or in the upstream regions (Vaníčková *et al.* 2010). *D. cucullata* is known to be well adapted to fish predation, especially due to its small body size. However, it is competitively inferior to larger *Daphnia* species under somewhat relaxed predation conditions. Consequently, localities with high fish densities, especially eutrophic ones, are often inhabited by *D. cucullata*.

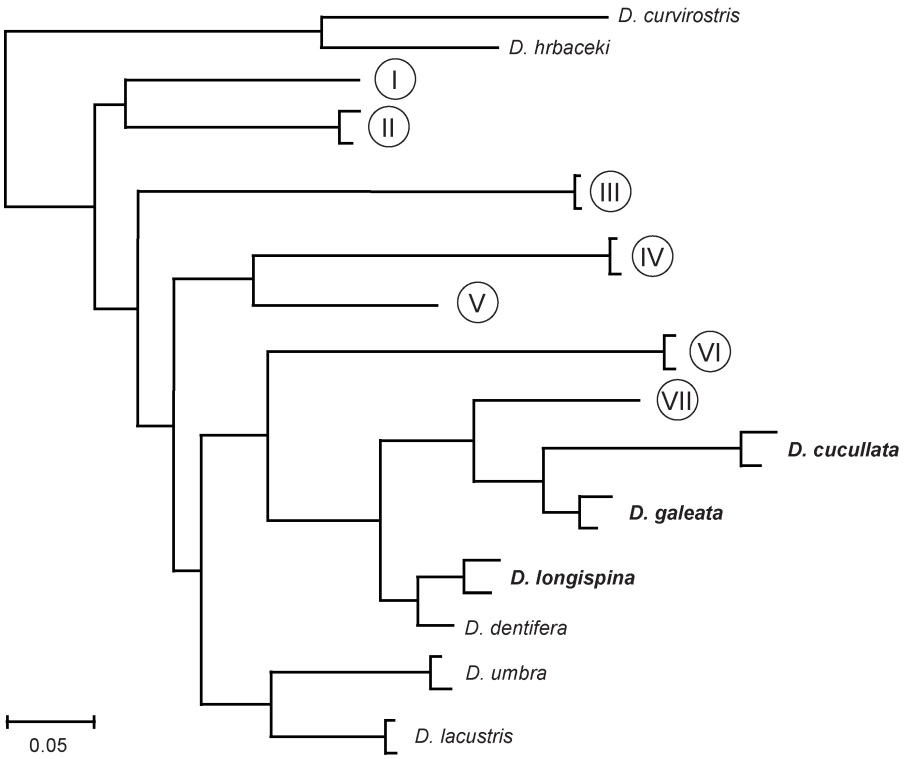

Figure I-3 Bayesian tree using the 12S rRNA gene representing genetic variation among and within lineages of the *D. longispina* complex, including recently reported new lineages indicated by Roman numerals. The species relevant for my thesis are indicated by bold letters. Modified from Petrusek *et al.* (2012).

D. galeata has probably the widest distribution among species of the *D. longispina* complex; it occurs throughout the Holarctic and may have even invaded the Neotropic (Benzie 2005). Usually this taxon is found in small to very large, permanent ponds and lakes; rarely in temporary waters. Further, the immigration into eutrophicated deep stratified lakes and reservoirs is observed (Flößner 2000). *D. galeata* feeds efficiently on algae and tolerates increased fish predation, outcompeting *D. longispina* and other larger *Daphnia* species (Nilssen *et al.* 2007).

D. longispina is a morphologically highly plastic taxon, which includes morphs formerly describes as four separate species (*D. hyalina*, *D. longispina*, *D. rosea*, and *D. zschokkei*), and now merged under the oldest description (Petrusek *et al.* 2008). This taxon is distributed throughout Eurasia and Africa, although, a relatively recent study by Ishida and Taylor (2007a) indicates that the distributional range may be limited to the western Palaearctic and maybe Afrotropic. In general, *D. longispina* favors deep, oligotrophic freshwater lakes with no or relaxed fish predation (Gliwicz 2003), but is also detected in large pools, sometimes smaller ponds, and in slow flowing water.

Interspecific hybridization processes within the D. longispina *species complex*

A reason why this species complex draws the attention of many researchers is the high level of ongoing interspecific hybridization. In Europe the species belonging to this complex are not clearly separated by habitat, although different preferences exist as explained above. The mix of taxa depends mostly on food (Seidendorf *et al.* 2007) and predator regimes (Declerck & De Meester 2003), which fluctuate throughout the year (Schwenk *et al.* 1995). However, other factors such as parasites (Wolinska *et al.* 2006) may also strongly affect the patterns of species coexistence.

The parental species are distinct (**Figure I-3**) and display high mitochondrial sequence divergence of often more than 10 percent (Schwenk 1993; Schwenk *et al.* 1995). Genetic evidence has been collected suggesting that especially *D. galeata* is able to produce viable F_1 offspring, prevalent also backcross individuals and introgressants, with several species: *D. longispina* (Brede *et al.* 2009; Hebert *et al.* 1989; Reid *et al.* 2000; Rellstab *et al.* 2011; Ruthová 2008; Wolf 1987; Wolf & Mort 1986; Yin *et al.* 2012a; Yin *et al.* 2010), *D. cucullata* (Hebert *et al.* 1989; Müller & Seitz 1993, 1995; Ruthová 2008; Schwenk *et al.* 1998; Spaak 1996; Wolf 1987; Wolf & Mort 1986), *D. dentifera* (Ishida *et al.* 2011), *D. mendotae* (Taylor & Hebert 1993a; Taylor *et al.* 2005), and *D. lacustris* (Hobæk *et al.* 2004). Also *D. cucullata* and *D. longispina* are able to hybridize, although hybrids are recorded rarely, often in low numbers, and genetic evidence revealed only F_1 hybrids (Gießler 1997a, b; Gießler *et al.* 1999; Keller *et al.* 2008; Müller & Seitz 1995; Spaak *et al.* 2004; Wolf 1987; Wolf & Mort 1986). In addition, the species *D. mendotae* is assumed to be one of few examples among animals that arose due to the interspecific hybrid reproduction of individuals belonging to *D. galeata* and *D. dentifera* and recent interspecific hybridization events among *D. dentifera* and *D. mendotae* are still observed (Taylor & Hebert 1993b).

The little mitochondrial DNA sequence divergence between maternal species and interspecific hybrids indicates a recent origin of hybrids. Further, the detection of several genotypes among hybrid groups provides evidence for the multiple and continuing production of hybrids (Schwenk *et al.* 1995). Nuclear genes (study of allozymes and RAPDs) show a closer relationship between *D. galeata* and *D. longispina* (Gießler *et al.* 1999; Schierwater *et al.* 1994), and a more distant relationship of this pair to *D. cucullata*, while mtDNA shows a closer relationship between *D. galeata* and *D. cucullata*, and a more distant relationship of this pair to *D. longispina* (Schwenk *et al.* 1995) which indicates that interspecific

hybridization processes may have had important impact on genetic structure of species for a long time.

Thesis outline

Aim of this study

The general aim of my thesis is the evaluation of the processes altering the population genetic structure in cyclic parthenogens with a special focus on the concept of monopolization (according to De Meester *et al.* 2002) as well as the counteracting effects of gene flow. In order to achieve this goal I pursuit three different approaches: Starting with a methodological project I developed variable microsatellite markers and evaluated a subset of these markers for species delineation and hybrid class detection. I used this subset of nuclear markers together with a mitochondrial DNA marker to empirically study the detailed population genetic structure of three syntopically occurring *Daphnia* species that reproduce via cyclic parthenogenesis. The resulting patterns of high genetic diversity within *D. longispina* populations (**Chapter 3**) are rather unexpected in the light of monopolization as only very few colonizers are assumed to effectively monopolize the resources which would result in a low genetic diversity, only slightly increasing through recombination and mutation over time. This suggests that monopolization is probably impaired by further successful invaders in the early stages of population build-up. This may be explained by various factors: first, if the succession of invasions occurs rapidly, a priority effect of the first colonizers might be prevented; secondly, as long as local adaptation has not occurred, better pre-adapted genotypes may successfully invade at some later time; and thirdly, low clonal diversity may result in inbreeding depression during sexual reproduction, also favoring invading genotypes. This inspired the last part of my thesis where I experimentally analyzed these early stages of population build-up in regard to the temporal advantage of genetic lineages in different succession treatments and in combination with differential competitive strength of genotypes.

In particular, in my thesis I focus on the following objectives:

1. Development and evaluation of a set of molecular markers for population genetic studies (methodological approach), in particular:
    - Establishment of variable microsatellite markers and assessment of successful cross-species amplification (**Chapter 1**)
    - Delineation of the three species *D. cucullata*, *D. galeata*, and *D. longispina* using a subset of the established genetic markers (**Chapter 2**)
    - Detection of interspecific hybrids and evaluation of null alleles for hybrid class assignments (**Chapter 2**)

2. Application of molecular markers (microsatellite and mitochondrial DNA) to study the fine-scale genetic diversity within and to assess the genetic differentiation among populations of the species *D. cucullata*, *D. galeata* and *D. longispina* (empirical approach) exploring in particular the following aims:
    - Estimation of clonal diversity in each species; evaluation of clonal erosion and the role of the dormant egg bank for the level of intrapopulational genetic variation (**Chapter 3** and **4**)
    - Evaluation of the genetic structure among populations in due consideration of monopolization on the one hand and effective gene flow on the other hand (**Chapter 3** and **4**)
    - Discussion of contemporary and historical environmental changes as drivers of effective gene flow among populations and expansion of the distributional ranges (**Chapter 4**)

3. Assessment of the impact of priority effects on the genetic structure during population build-up (experimental approach) with a focus on the:
    - Influence of a temporal advantage of genotypes during invasion succession on the population genetic structure (**Chapter 5**)
    - Assessment of differential clonal strength on the priority effects and therefore on the population genetic structure (**Chapter 5**)

Chapter overview

In the first chapter (**Microsatellite markers for European *Daphnia***) I present 32 polymorphic microsatellite markers for species of the European *Daphnia longispina* group which allow intra- and interspecific genetic studies on, i.e. population genetic structure, interspecific hybridization events and introgression.

In the second chapter (**Discrimination of hybrid classes using cross-species amplification of microsatellite loci: methodological challenges and solutions in *Daphnia***) I focus on the suitability of a subset of the markers described in **Chapter 1** for species identification and hybrid detection. Therefore, laboratory lineages as well as natural populations were studied. Using multi-locus genotypes and model-based assignment testing, the identification of all three taxa (*D. galeata*, *D. longispina*, and *D. cucullata*) produced reliable results. By using laboratory bred F_1 hybrids of *D. galeata* and *D. cucullata* species specific null alleles were detected that hamper hybrid detection. Better results in detecting hybrid classes were obtained when using a subset of nine loci that amplified equally well in both species.

In the third chapter (**Contribution of cyclic parthenogenesis and colonization history to population structure in *Daphnia***) I assess the patterns of genetic variation within and among populations in the eurytopic and morphologically variable species *Daphnia longispina*, using data from both nuclear and mitochondrial markers from a large set of populations sampled across Europe. Most populations are characterized by very high clonal diversity, reflecting an important impact of sexual reproduction and low levels of clonal selection. Among-population genetic differentiation is very high for both nuclear and mitochondrial markers, and no patterns of isolation-by-distance are observed. The findings of high levels of within-population genetic variation combined with high among-population genetic differentiation are in line with predictions of the *monopolization hypothesis*, which suggests that in species with rapid population growth and potential for local adaptation, strong priority effects due to monopolization of resources lead to reduced levels of gene flow.

In the fourth chapter (**The impact of historical and contemporary environmental changes on the population genetic structure of large lake *Daphnia* species**) I use a comparative approach to study the genetic population structure of the three hybridizing species *D. galeata*, *D. longispina*, and *D. cucullata* using nuclear and mitochondrial DNA markers. While *D. longispina* shows high population diversity and differentiation in line with monopolization

(presented in more detail in **Chapter 3**), *D. galeata* reveals almost opposite patterns (low population diversity; few mtDNA haplotypes shared between many populations; low genetic differentiation at nuclear loci) suggesting either recent gene flow or expansion. The above described patterns indicate that *D. longispina* probably expanded very early with the deglaciation and the formation of glacial lakes, while *D. galeata* benefits from recent human-mediated environmental changes (eutrophication and fish introduction). The third studied taxon *D. cucullata* does exhibit mtDNA patterns similar to *D. longispina* and also high clonal diversity within populations at the nuclear level, however, no in- or decrease in effective population size over time is detected which may be a sign of a taxon at equilibrium.

In the fifth chapter (**Priority effects and fitness differences determine genetic structure during population build-up in the waterflea *Daphnia***) I present the results of an outdoor mesocosm experiment that was conducted to test whether the sequence of arrival determines the relative contribution of genetic lineages (clones) to populations. Clones with an initial time advantage had in general higher relative abundances compared to treatments with simultaneous inoculation of genetic lineages. However, abundances of clones varied in some cases strongly when inoculated simultaneously, revealing evident differential fitness among clones. This study highlights the importance of two different processes that determine the fate of clonal lineages and ultimately population genetic structuring. First, the sequence of arrival is crucial, with an advantage of five days being already sufficient to dominate the population in specific cases. Secondly, fitness differences among clones, reflecting the degree to which they are pre-adapted to the environment, may overrule priority effects.

Chapter 1

Microsatellite markers for European *Daphnia*

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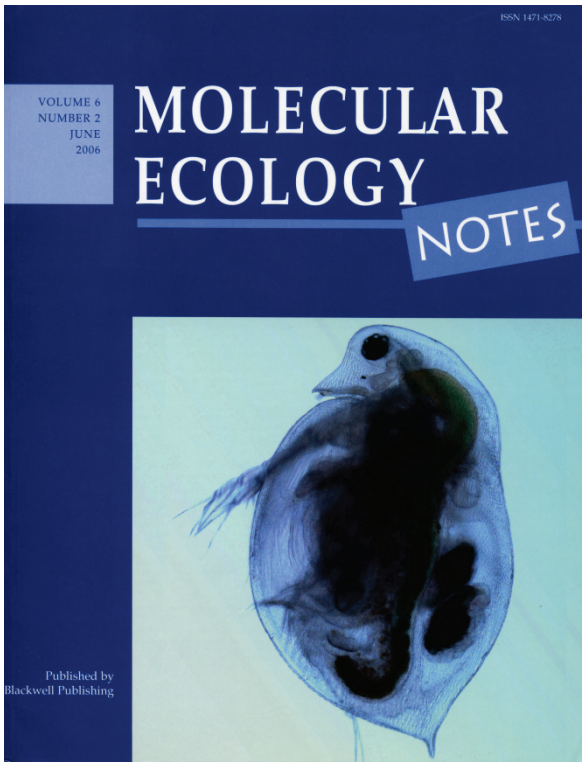
Abstract

We present 32 polymorphic microsatellite markers for species of the European *Daphnia longispina* group: *D. galeata*, *D. hyalina*, *D. rosea*, *D. cucullata* and *D. curvirostris*. Microsatellite markers were either isolated from genomic libraries or optimized based on previously published sequence information of sister taxa. Cross-species tests revealed that all but one of the polymorphic markers are applicable to more than one species, which allows intra- and interspecific genetic studies on, i.e. population structure, hybridization events and introgression.



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Cladoceran species of the genus *Daphnia* have become an important model organism in ecotoxicology, limnology, ecological genetics and, recently, in genomics (Lynch & Spitze 1994; The *Daphnia* Genomics Consortium: http://daphnia.cgb.indiana.edu/; Stark & Banks 2003). This prominence of *Daphnia* is based on several characteristics of this genus: species are widely distributed, representatives are key species in trophic cascades and occur across all kinds of freshwater habitats, they are easy to rear in the laboratory, and because of their reproductive mode (cyclic parthenogenesis) they represent ideal experimental animals. In addition, *Daphnia* diapausing eggs were successfully used to reconstruct changes of taxon composition over evolutionary relevant time periods (e.g. Hairston *et al.* 1999).

The frequent occurrence of interspecific hybridization among species of the *Daphnia longi-spina* complex resulted in several taxonomic problems, but motivated many studies with regard to the origin, maintenance and fate of hybrid lineages (e.g., Jankowski & Straile 2004; Schwenk & Spaak 1995). Previous studies were mainly limited by the small number of fixed loci among species (allozymes: Schwenk & Spaak 1997) and the limited discriminatory power of other molecular markers (e.g., Billiones *et al.* 2004; Schwenk *et al.* 2000).

Although several microsatellite markers have been developed for American species (Colbourne *et al.* 2004; Fox 2004), only a small number of markers has been published for European taxa (Ender *et al.* 1996; Fox 2004).

Here we present 32 microsatellite markers for the European *D. longispina* group, which were partly optimized using the *Daphnia pulicaria* markers by Colbourne *et al*. (2004; Dp). Furthermore, we tested previously published primer sequences developed either for European *Hyalodaphnia* or for North American *Daphnia galeata mendotae* (DaB: Ender *et al.* 1996; Dgm: Fox 2004). An additional set of microsatellite markers (SwiD) were developed using an enriched library from size-selected genomic *D. galeata* DNA ligated into SAULA/SAULB linker (Armour *et al.* 1994) and enriched by magnetic bead selection with biotin-labeled $(CA)_{14}$ and $(ACAG)_7$ oligonucleotide repeats (Gautschi *et al.* 2000). Out of 570 recombinant colonies screened, 98 gave a positive signal after hybridization. Plasmids from 72 positive clones were sequenced, and primers were designed for 15 microsatellite inserts (ECO-GENICS GmbH). Of these, 13 were tested for polymorphism.

In order to cover a representative array of species of the *D. longispina* group, we selected laboratory clones originating from a broad geographical range. *Daphnia galeata* (one from each country: Germany, Switzerland, the Netherlands, North Ireland), *D. hyalina* (one from Northern, Middle and Southern Germany and one from Switzerland), *D. rosea* (one from Northern and two from Central Germany), *D. cucullata* (one from each country: Switzerland

and the Netherlands) and *D. curvirostris* (two from Northern Germany, one from each of the following countries: Central Germany, Czech Republic) were tested as well as one interspecific hybrid of the following species: *D. galeata* x *hyalina* (the Netherlands), *D. galeata* x *rosea* (Israel) and *D. galeata* x *cucullata* (the Netherlands). Six of the 32 loci (DaB10/15, DaB17/17, DaB17/16, DaB10/14, Dp512 and Dp519) have been tested on 23 populations of *D. galeata* across Europe. The allelic richness (alleles/*N*) ranged from 0.34 to 0.72 (Ø = 0.484) and an average observed heterozygosity of 0.236 (range: 0.114–0.405; Dove 2005). Since species of the *D. longispina* group form clonal assemblages, generations overlap due to diapause, and introgressive hybridization occurs frequently, thus heterozygosity deficiencies and linkage disequilibria are often observed (Schwenk & Spaak 1995).

DNA preparation of *Daphnia* individuals was carried out in 70 µL H3 buffer [1×: 10 mM Tris-HCl (pH 8.3 at 25 °C), 0.05 M potassium chloride, 0.005% Tween 20 and 0.005% NP-40]. After adding 2 µL of proteinase K (Sigma, 10 mg/mL), samples were vortexed and centrifuged. Incubation varied between 4 and 16 h at 56 °C. Samples were then boiled at 96 °C for 12 min, centrifuged shortly and stored at 4 °C. Polymerase chain reactions (PCR) were performed in 0.2 mL tubes using either a Biometra T3 or a DYAD thermal cycler. All reactions were first performed with a 10 µL reaction volume containing 2.4 mM $MgCl_2$, 1× PCR buffer, 0.25 mM dNTPs, 0.2 µM of each primer and 0.5 U *Taq* DNA polymerase (chemicals and primers by Invitrogen). Cycling conditions started with a 3 min denaturing step at 95 °C followed by 35 cycles of 1 min steps at 95 °C, 55 °C and 72 °C. A final 7 min synthesis step at 72 °C completed the program. Depending on the specificity of each primer set, PCR conditions varied mainly in annealing temperature (see **Table 1-1**). When pure PCR products were obtained, the PCR was repeated with labeled forward primers (Invitrogen, MWG). Amplicons were diluted and electrophoresed on a CEQ 2000 (Beckman Coulter) or on an ALF sequencer (Amersham) with self-designed size standards based on Lambda virus DNA (Symonds & Lloyd 2004).

From 32 loci, 25 were variable for at least three species. Sixteen of the markers showed a large shift of fragment lengths; indicating potentially diagnostic insertions or deletions among species (see **Table 1-2**). Several primer combinations were tested in multiplex PCRs to allow faster and efficient screening of populations. Primer concentrations were adjusted in order to amplify similar amounts of PCR products. For example, primers DaB17/17 (0.1 µM), DaB10/14 (0.075 µM), Dp512 (0.3 µM) and Dgm109 (0.075 µM) were subjected to a multiplex PCR with 3 mM $MgCl_2$, 1× PCR buffer, 2% BSA (NEB), 2% DMSO (Sigma), 1 U *Taq* (Invitrogen) in a reaction volume of 10 µL. Cycling conditions are identical to those presented

Table 1-1 Characteristics of the 32 microsatellites: locus name, repeat motif, primer sequences, annealing temperature (T_a), fragment size range, cross-species test with number of alleles.

Locus	Repeats	Primer sequences (5'–3')	*Ta* (°C)	Size range (bp)	GenBank Accession no.	Cross-species test*				
						D. galeata	*D. hyalina*	*D. rosea*	*D. cucullata*	*D. curvirostris*
DaB10/15†	$(TC)_6$	F: AGAGAAGTGTTTGCGTTTC R: TGTTTCCTATATCCCTCGG	55	75-89	U41402	5	2	NT	4	2
DaB17/17†	$(T)_9$	F: GAGAACCTTTTATCAGCTTCG R: ACTCATCTGGTGAGATGGATC	55	100-109	U41403	4	4	NT	4	1
DaB17/16‡	$(GA)_{10}$	F: AGGGAACGAGCGGCGATAAG R: TCTTTGGCAGGCCACTGCCAAGG	55	189-195	U41403	3	2	NT	2	NO
DaB10/14†	$(CAA)_8$	F: CTCTTATAACCAGCACCTCG R: CTATTATTCCATCGTCCGTC	55	222-234	U41402	4	4	NT	1	NO
Dp512§	$(TG)_4\ldots(GT)_8$	F: TTTCGTTCTACCCAGGGAAG R: TTTGCTCGTCTGTGATAGGC	56	125-141	AY057864	5	4	NT	3	NT
Dp519§	$(TG)_6(GA)_7$	F: AGTCGCGACGACATAAAGC R: GTGGTAGTTGTGGAATCCG	50	144-160	AY057865	5	5	NT	6	NO
Dgm101††	$(GA)_{10}AGA$	F: TCTTGCTCGAATTCTCTCC R: CCTGTCTCACACGGAGC	54	162-177	AY542275	3	2	3	3	4
Dgm102††	$(TTG)_{10}\ldots(TTG)_4$	F: AGCGTATTCGATTTCAGG R: ACGGATTCGATGATGAACCC	57	118-131	AY542276	1	1	3	1	2
Dgm105††	$(CAG)_8AG$	F: ATGTGAGCGCGCGAGCATTT R: GTCCAGCCGGCCCATTTCAGTT	55	172-197	AY542269	2	4	3	2	1
Dgm106††	$(CAA)_8CCAA$	F: ACCACCACCTCCTCCGCCACAT R: TTCGTCGATTTCCTCACCCATTTC	50	124-155	AY542270	2	2	2	NT	NT
Dgm107††	$(TGC)_7$	F: CCTTTGGCATCGTTTCTTATTCTT R: CCTGCCAACCTCCCAGTCCT	51	117-132	AY542271	2	3	NO	1	NO
Dgm109††	$(ACC)_7AC$	F: CCAGCTGTTGACCACCTG R: TGCGCGAGGATTTCCAACAC	61	247-266	AY542272	2	4	3	5	NO
Dgm112††	$(TGC)_6TGG$	F: GGAAATAGGCCTAGATGCTGTGT R: TTATTGATCTTCCGGCTGACTTTA	55	109-130	AY542274	3	2	1	NO	3
Dgm113††	$(GTC)_7$	F: TGCCACGAATCGTCTATAATGGTG R: AAGCCCACATGTAGGCACAAGTCA	55	133-155	AY542279	3	3	4	NO	2
Dp196NB¶	$(AC)_5$	F: ATTTTCCGCCCTTATTCTGC R: TCTTGGTCGCGTTCCAGC	50	115-130	WFms0000201	3	3	2	NT	1
Dp238NB¶	$(AG)_8$	F: ACAAGCAACTCACCAAAAGG R: CTAGATGTACACTGGGC	52	61-77	WFms0000246	4	1	3	1	2
Dp281NB¶	$(T)_{10}$	F: AATAACACTCGTAGCACG R: AGCGGACCGGAAGTGGTAGG	55	69-78	WFms0000290	2	2	2	1	1

SwiD1	$(TG)_{18}$	F: GCCGTGTTCGAAAGCTAGTC R: AGCCGAACGAAAAACATGC	61	116-142	3	3	2	2	NO	
SwiD2	$(TG)_{18}$	F: GTCAAGTTGTTCTTGTTATTGTGC R: TTTTGTAGGTCCGCGTAAATG	55	164-194	3	2	2	1	NO	
SwiD4	$(CA)_{17}$	F: GACCCAAAGTCTCTCTCCATC R: TGGAGATGTATCACATCCATACG	56	159-204	2	3	2	1	3	
SwiD5	$(GA)_{13}$	F: ACTATGCATAACACAGACACACG R: GAAGTACGGCAAGGAGCAAC	61	145-165?	3	1	2	NO	NO	
SwiD6	$(CA)_{13}$	F: GATCAGCAAGATGAAATACAC R: ATTTGAAGGCATTTCCTGTACG	55	123-142	4	2	3	1	NO	
SwiD7	$(CTG)_6$	F: TCAGACTGGTGATTACGACTGC R: TCTGATAAAGCGGATGAGAGAAC	58	159-171	2	2	2	1	2	
SwiD8	$(TG)_4A(TG)_{13}$	F: GATATTCTCTTGGACTGCGTTTG R: GATATGACAAGCCGACGTCAT	56	117-159	3	1	1	NO	NO	
SwiD10	$(TG)_{19}$	F: TGTAGATATCAGCCAGCAGCTC R: AAGGTTATTCTCTCCGCTCGTC	60	182-204	2	2	1	2	2	
SwiD11	$(GT)_{20}$	F: ACTCGACAAACTTGGAGAGGTC R: GGGGTGGCTATAGATAGACTGG	57	155-177	3	NO	2	1	3	
SwiD12	$(TC)_{14}TTA(TG)_{12}$	F: ATTCTTATTGCCCCAAATAACC R: GCCGCTTTTTCTATCTGCATAC	55	105-127	4	4	3	NO	1	
SwiD14	$(GT)_{12}...(GT)_7$	F: AGACGATCGTTGGTTCATCC R: CCGGATAGTTGCTGGAAAAG	59	173-191	2	1	3	1	1	
SwiD15	$(GT)_{14}$	F: TCTTCTTTTCCCATACAGACTCTC R: CTCCCTCTGATTTGGCGTAACT	54	79-99	2	3	1	NO	NO	
SwiD16	$(CA)_{15}$	F: CATCGACAATGTACGGTGGGAG R: GGCTGGTGGTGGTCCAGTGGTT	57	168-187	2	1	2	1	3	
SwiD17	$(GA)_9$	F: CGTATGGATGTGATACATCTCCA R: GGAATGAGTTGGAAAGAGGGA	53	76-98	1	1	1	2	3	
SwiD18	$(CA)_9$	F: GGATGCCAACTCTCTCCCCTA R: CGTGTGTCTGTGTTATGCATAGT	55	85-97	3	2	3	2	NO	

*Cross-species testing results: > 1: variable microsatellite — number of alleles, 1: amplicon obtained but tested as invariable, NO: no amplicon obtained, NT: not tested for this species; †Ender *et al.* (1996), ‡primer sequence differs from Ender *et al.* (1996), §Colbourne *et al.* (2004), ¶primer sequence differs from Colbourne *et al.* (2004), ††Fox (2004).

Table 1-2 Selection of 16 potentially species specific microsatellite markers: locus name, detected alleles for tested species and interspecific hybrids.

	Cross-species and interspecific hybrid test*							
Locus	***D. galeata***	***D. hyalina***	***D. rosea***	***D. cucullata***	***D. curvirostris***	***D. galeata* x *hyalina***	***D. galeata* x *rosea***	***D. galeata* x *cucullata***
Dgm102‡	131	118	118/124/131	118	**140**/**144**	131	128/131	115
Dgm105‡	186/193	184/187/190/195	183/185/187	177/**179**	177	193	186/193	193
Dp196NB†	115/117/121	121/127	121/**135**	NT	130	119/121	119/121	NT
Dp238NB†	61/65/71/**77**	65	61/65/69	59	59/61	61/65	**77**	59
SwiD1	125/130/134	**116**/121/134	**116**/125	125/130	NO	134	130	125
SwiD2	164/**182**/**184**	168/172	168/172	164	NO	172	172	172
SwiD5	158/162/164	**127**	153/158	NO	NO	160	162	NO
SwiD7	158/161	161/164	161/164	158	**144**/**152**	158/161	155/161	158
SwiD8	122/**141**/**155**	122	122	127	NO	122	117	**155**
SwiD10	186/**202**	186/192	186	184/196	192/196	186	184	180
SwiD11	158/163/175	NT	166/175	150	**139**/**141**/**145**	NO	163	NT
SwiD12	111/119/124/**127**	111/113/115/119	113/115/117	NO	**98**	115/122	105/113	124
SwiD15	**91**/**97**	79/84/86	79	NO	NO	79/**97**	**95**	**97**
SwiD16	168/172	172	170/172	**182**	**180**/**185**/**187**	168/172	168/172	168
SwiD17	80	80	80	80/83	76/78/**98**	80	80	80
SwiD18	85/87/93	93/97	91/93/97	81/91	NO	NO	85	**77**

*Cross-species testing results: bold numbers, alleles potentially species-specific; NO, no amplicon obtained; NT, not tested for this species or hybrid; †primer sequence differs from Colbourne *et al.* (2004) and ‡Fox (2004).

above. For the primer set SwiD1, SwiD10 and SwiD14 (each 0.1 µM), an annealing temperature of 60 °C was appropriate. Generally, many primer combinations were successfully tested in multiplex PCR; however, sufficient amplification was only achieved if all primers were labeled with the same dye.

A total of 110 primer pairs that positively amplified microsatellite markers in American *Daphnia dentifera* (Colbourne *et al.* 2004) were tested and resulted in eight polymorphic loci. Only 65 primer pairs successfully amplified a fragment, and only 34 amplicons exhibited the expected fragment size (±50 bp; after testing amplification conditions of Colbourne *et al*. (2004) and alternative conditions using a *D. pulicaria* clone from the Netherlands as a positive control). DNA sequencing revealed that only 18 fragments corresponded with the reference sequence, of which eight loci (7.3% of the tested microsatellites) contained repetitive units found in *D. pulicaria* (Colbourne *et al.* 2004). Due to inefficient amplification yield for the *D. longispina* group, three primer pairs (Dp196NB, Dp238NB and Dp281NB) containing a variable microsatellite were newly designed. Our study shows that the high sequence divergence of microsatellite flanking regions hampers the application of primer sets originating from sister species. However, considering the large genetic differentiation among *D. pulicaria* and members of other subgenera (i.e. *Ctenodaphnia* and *D. longispina* group), we expect that the application of the described markers will be most efficient for closer related species.

These markers together with the newly developed markers provide a powerful 'toolbox' of 32 microsatellite loci for *Daphnia* taxonomy, ecology and evolutionary biology. These markers were already applied to determine natural population structure in *D. galeata* (Dove 2005) and for clonal identification of experimental animals (Seidendorf *et al.* 2007). In addition, due to the discriminatory power of microsatellite loci among species and hybrid classes, we were able to reconstruct evolutionary changes from *Daphnia* dormant egg banks (Brede *et al.* 2009).

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Chapter 2

Discrimination of hybrid classes using cross-species amplification of microsatellite loci: Methodological challenges and solutions in *Daphnia*

A. Thielsch, E. Völker, R.H.S. Kraus, K. Schwenk

Abstract

Microsatellite markers are important tools in population, conservation and forensic studies and are frequently used for species delineation, the detection of hybridization and introgression. Therefore, marker sets that amplify variable DNA regions in two species are required; however, cross-species amplification is often difficult, as genotyping errors such as null alleles may occur. In order to estimate the level of potential misidentifications based on genotyping errors, we compared the occurrence of parental alleles in laboratory and natural *Daphnia* hybrids (*Daphnia longispina* group). We tested a set of twelve microsatellite loci with regard to their suitability for unambiguous species and hybrid class identification using F_1 hybrids bred in the laboratory. Further, a large set of 44 natural populations of *D. cucullata*, *D. galeata* and *D. longispina* (1715 individuals) as well as their interspecific hybrids were genotyped to validate the discriminatory power of different marker combinations. Species delineation using microsatellite multi-locus genotypes produced reliable results for all three studied species using assignment tests. *D. galeata* x *cucullata* hybrid detection was limited due to three loci exhibiting *D. cucullata* specific null alleles which most likely are caused by differences in primer binding sites of parental species. Overall discriminatory power in hybrid detection was improved when a subset of markers was identified that amplifies equally well in both species.

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Introduction

Microsatellites, or short tandem repeats (STRs), are repetitive stretches of DNA of one to six nucleotides scattered throughout the genome. They are typically inherited co-dominantly and are used as powerful molecular markers with a wide range of applications, e.g., in population genetic analyses, conservation biology and forensic genetics including kinship studies (Goldstein & Schlötterer 1999). Rare occurrences of mutations in the repeat structure as well as in the primer binding site (which might lead to null alleles), chromosomal mutations, homoplasy, and amplification bias against larger alleles (large allele dropout) can lead to spurious results, such as anomalous number of peaks that do not follow Mendelian inheritance (Pompanon *et al.* 2005). Further, human and methodological errors, for instance cross-contamination or the presence of 'stutter bands', generated by slippage of *Taq* polymerase during PCR, resulting in scoring errors, may complicate the interpretation of results.

Recently, microsatellite markers became a popular tool for the detection of interspecific hybrids, because microsatellite loci usually exhibit high levels of polymorphism even across species borders (Selkoe & Toonen 2006). The detection of hybrid and introgressed individuals is crucial in conservation biology since many studies showed that interspecific hybridization might be caused or maintained by human activities such as habitat modification and fragmentation, species eradication or species introduction (reviewed by Crispo *et al.* 2011). In addition, hybridization may lead to extinction of rare or threatened species (Adams *et al.* 2007; Rhymer & Simberloff 1996). However, hybridization is an ecological and evolutionary process which naturally occurs in a wide range of organisms. It is very common in plants (Wissemann 2007) but also well described for a number of animal taxa (Schwenk *et al.* 2008). During the past decade many hybrid studies were based on microsatellite analyses for hybrid detection (e.g., Adams *et al.* 2007; Dubut *et al.* 2010). Usually, variable markers were developed for one of the parental species and subsequently used to identify both hybridizing taxa and their interspecific hybrids (cross-species amplification). Although this approach has been routinely employed in many studies, the question remains whether genetic markers reveal parental variation equally well, as, for example the variation in primer binding sites among species may result in largely different quantities and qualities of PCR products and thus alter the outcome of hybrid class assignments.

Our main aim was to determine the discriminatory power of *Daphnia* microsatellite markers using cross-species amplification and to discuss this approach for animal hybridization studies in general. Specifically, we tested microsatellite loci developed for *Daphnia galeata* to discriminate among species and hybrids of the *D. longispina* species complex. Members of

this complex reproduce via cyclic parthenogenesis (Hebert 1987), an alternation of asexual and sexual reproduction, in which the asexual phase is maintained under favorable conditions. *D. galeata* is known to hybridize frequently (including occasional later generation backcrossing) with other members of this species complex (for instance, *D. longispina* and *D. cucullata*) if sexual reproduction is induced (e.g. Schwenk 1993; Wolf 1987). Therefore, species assignment as well as detection of hybrids and introgressed individuals is difficult, especially as morphology is highly variable and dependent on environmental factors (e.g., Laforsch & Tollrian 2004). Several molecular methods have been established to facilitate this task in the *D. longispina* complex, but all of these methods have some limitations (Dlouhá *et al.* 2010). Microsatellite markers established for this species complex (Brede *et al.* 2006) show an excellent suitability for *D. longispina* and *D. galeata* and proved to be very useful in the study of population structure and clonal identification (Brede *et al.* 2009; Hamrová *et al.* 2011; Thielsch *et al.* 2009; Yin *et al.* 2012a; Yin *et al.* 2012b; Yin *et al.* 2010).

In particular, we wanted to assess the discriminatory power of different loci for the detection of hybrid classes among hybridizing species of *Daphnia*. For convenience, we labelled this marker set as *CGL µsat kit* (DaB10/14, DaB17/17, Dgm105, Dgm109, Dgm112, Dp196NB, Dp281NB, Dp519, SwiD6, SwiD12, SwiD14 and SwiD18; Brede *et al.* 2006). The *CGL µsat kit* was used on seven laboratory lineages, belonging to the *Daphnia longispina* species complex: five of these seven clones represent artificially bred F_1 hybrids between a *D. galeata* and a *D. cucullata* clone; hence, allowing a qualitative evaluation of the studied loci. The clones were studied at two points in time: shortly after the artificial crosses in 1996 and 14 years later in 2010. Further, we used extensive genotypic data of natural populations of all three hybridizing species, *D. galeata*, *D. longispina*, and *D. cucullata*, to quantitatively study the same set of markers. This approach allows a detailed examination of the technical properties of those markers for species identification and hybrid detection.

Material and Methods

Samples

Laboratory lineages from 2010 of *D. galeata* (G1) and *D. cucullata* (C2) originating from Tjeukemeer in the Netherlands (Schwenk *et al.* 2001) were studied. Interspecific hybrids (CG1, CG4, CG5, CG6, and CG8) of these two clones were bred in the laboratory and also cultured clonally. Four of those F_1 hybrids (CG1, CG4, CG5, and CG6) are a cross of a female *D. cucullata* and a male *D. galeata*, while clone CG8 results from the reciprocal cross

(Schwenk *et al.* 2001). After hatching of interspecific hybrids (in 1996) some individuals were stored in ethanol (70%) and were also analyzed for this study.
In addition, natural *Daphnia* populations spanning a wide geographic range across Europe (ranging from northern Norway to South Italy and from Great Britain to Russia; for more detailed information see supplement **Table 2-S1**) and three species (*D. galeata*, *D. longispina* and *D. cucullata*) were sampled and stored in ethanol (70% or above). In total 1715 individuals from 44 populations were investigated.

DNA extraction and microsatellite amplification

DNA extraction of all individuals was conducted either using the hotshot protocol according to Montero-Pau *et al.* (2008) or by proteinase K digestion described by Schwenk *et al.* (1998). The clonal laboratory lineages (C2, G1, CG1, CG4, CG5, CG6, and CG8) were genotyped using the Type-it® Microsatellite PCR Kit (Qiagen) as described below. Further, each locus was genotyped according to Brede *et al.* (2006) in at least three replicates.
Amplification of twelve microsatellite loci (CGL µsat kit; Brede *et al.* 2006) for natural populations was performed as described by Thielsch *et al.* (2009) or using the Type-it® Microsatellite PCR Kit (Qiagen) for eleven loci and a single PCR for locus Dp196NB which was conducted according to Brede *et al.* (2006). In case no amplicons were obtained, these loci were amplified individually according to Brede *et al.* (2006). Multiplex PCR using the Type-it® Kit contained in a total of 5 µL: 1 µL prepared DNA, 1x Type-it® Multiplex PCR Master Mix, 1x Q-solution (or lower concentration up to 0.5x), and a variable amount of each primer depending on the locus (0.06 µM Dp281NB, 0.25 µM SwiD18, 0.07 µM DaB17/17, 0.2 µM SwiD12, 0.6 µM Dgm112, 0.15 µM SwiD6, 0.4 µM Dp519, 0.09 µM SwiD14, 0.35 µM Dgm105, 0.08 µM DaB10/14, 0.6 µM Dgm109 for each forward and reverse primer). Thermal cycling for Type-it® multiplex PCR started with a 15 min denaturation step at 95 °C, followed by 30 cycles of 30 sec at 94 °C, 90 sec at 54 °C and 60 sec at 72 °C. A final 30 min synthesis step at 60 °C completed the program. Forward primers were fluorescently labeled using IRD700 (SwiD18, SwiD12, SwiD6, Dgm105; Metabion), Alexa647 (Dp281NB, DaB17/17, Dp519, SwiD14, DaB10/14, Dp196NB; Invitrogen) or Alexa750 (Dgm105, Dgm109 and Dgm112; Invitrogen).
Amplicons were electrophoresed on a CEQ 2000 (Beckman Coulter; denaturation at 90 °C for 2 min; injection at 2.0 kV for 30 sec; separation at 6.0 kV for 45 min) using a self-designed size standard (Symonds & Lloyd 2004) or DNA Size Standard Kit - 400 (Beckman Coulter).

Analysis of clonal laboratory lineages and sequencing of primer binding sites

We compared allele composition of parental and hybrid clones to check if genotypes of artificially bred hybrids (CG1, CG4, CG5, CG6, and CG8) indeed represent the outcome of a cross between individuals of clones G1 and C2 assuming Mendelian inheritance.

As amplification of some microsatellite loci was especially difficult for *D. cucullata* clone C2, the primer binding sites of several loci were sequenced. Therefore, we designed new primer pairs flanking the original primer binding site. For seven of twelve loci (either to amplify the forward or reverse primer binding site) sufficient sequence information was available for primer design (**Table 2-S2**; for more information see Brede *et al.* 2006). Individuals of the clone C2 were used for amplification (PCR conditions are available on request from the authors). The amplicons were purified using either PureLink^{TM} PCR Purification Kit (Invitrogen) or AMPure (Agencourt) and were subsequently sequenced in both directions on a capillary sequencer (Beckman Coulter CEQ 2000) using Beckman Coulter standard protocols (CEQ^{TM} DTCS Quick Start Kit and Agencourt CleanSEQ). Sequences were manually edited using GENEIOUS PRO 5.4.4 (Drummond *et al.* 2011) and aligned with reference sequences obtained from Brede *et al.* (2006). Based on the sequence results, new primers for amplifying some microsatellite loci (DaB10/14, Dp519, Dgm109, and Dgm112) were designed (according to the sequence information given in **Table 2-1**) and tested for different results compared to the original primers using clones C2, CG1, CG4, CG5, CG6 and CG8 (PCR conditions identical to those used with original primers).

Table 2-1 Differences in the sequences of primer binding sites in the *D. cucullata* reference clone C2. Reference sequences were retrieved from Brede *et al.* (2006). Nucleotide sites that deviated from the reference sequence are marked in boldface letters. IUPAC codes (M and K) indicate heterozygous loci.

Locus	Primer binding site	Source	Sequence 5'–3'
DaB10/14	forward	reference	CTCTTATAACCAGCAC**C**TCG
		C2	CTCTTATAACCAGCAC**M**TCG
	reverse	reference	CTATTATTCCATCGTCC**G**TC
		C2	CTATTATTCCATCGTCC**K**TC
Dgm109	reverse	reference	TGCGC**G**AGGATTTCC-AACAC
		C2	TGCGC**C**AGGATTTCC**C**AACAC
Dgm112	forward	reference	G**G**AAATAGGCCTAGATG**CT**GTG**T**
		C2	G**A**AAATAGGCCTAGATG**TC**GTG**G**
Dp519	reverse	reference	GTGGTAGTTGTGGAATCC – G
		C2	GTGGTAGTTGTGGAATCC**AT**G

Analyses of natural populations

To identify the species *D. galeata*, *D. cucullata* and *D. longispina* using the *CGL µsat kit*, a model-based assignment test as implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000) was applied. Since previous genetic studies showed high levels of population differentiation in species of the *D. longispina* complex (Thielsch *et al.* 2009) we limited the range of *K* (1-6) and therefore the number of assumed groups, as we were only interested in detecting differentiation at the species level rather than at the population level. A total of ten independent runs with 50,000 burn-in iterations and 150,000 MCMC (Markov chain Monte Carlo) steps were conducted at each value of *K* using the admixture model and independent allele frequencies among populations. Individual assignment probabilities, Ln P(D), and convergence between runs were used to assess the most likely value of *K*. Furthermore, the method described by Evanno *et al.* (2005) implemented in STRUCTURE HARVESTER (Earl & von Holdt 2011) which employs an ad hoc statistic ΔK based on the rate of change in Ln P(D) between successive *K* values was used to detect the uppermost hierarchical level of population structure.

Individuals with a probability above 95% belonging to one of the proposed clusters were considered as pure species. Admixed individuals, defined as those with less than 95% assignment probability, were regarded as individuals with a certain hybrid status. Subsequently, the dataset was analyzed using a factorial correspondence analysis implemented in GENETIX 4.05 (Belkhir *et al.* 1996-2004). The values of the first two factors were plotted and species assignments based on the posterior probabilities obtained from Structure ($K = 3$) were used as labels.

MICRO-CHECKER 2.3.3 (Van Oosterhout *et al.* 2004) was used to detect possible genotyping errors (errors due to stuttering, large allele dropout or presence of null alleles) in each single taxon population with a population size of at least 15 individuals (*D. longispina*: 16 populations; *D. galeata*: 18 populations, *D. cucullata*: 8 populations; see supplement **Table 2-S1**).

For the detection of hybrid classes within the natural populations, all individuals detected as *D. cucullata* and *D. galeata* (> 0.95 according to STRUCTURE analysis with $K = 3$; see above for more details) as well as all admixed individuals that belonged to one of the two taxa with an assignment probability < 0.95 and with assignment probability < 0.01 belonging to *D. longispina* were selected. NEWHYBRIDS 1.1 beta (Anderson & Thompson 2002) was used to identify the posterior probability of each genotype belonging to one of six classes (parental taxon 1, parental taxon 2, F_1 hybrid, F_2 hybrid, backcross with taxon 1, backcross with taxon

2). A total of five independent runs with at least 200,000 iterations and default settings were conducted with each locus combination (**Table 2-2**). We chose eleven different locus combinations, considering the obtained genotype results of this study: one including all twelve loci from the *CGL µsat kit*, one including nine loci that amplified equally well in both parental species and in the hybrids, and nine combinations including a total of nine loci but with variable contribution of loci that amplified well in all taxa and loci that revealed *D. cucullata* specific null alleles either in the hybrid genotypes or in pure *D. cucullata*. If an individual had a posterior probability of belonging to a specific genotype frequency class of 0.95 or higher, then it was scored as belonging to that particular class.

Table 2-2 Microsatellite locus combinations used for hybrid assignment testing. 6+3 a-c: 6 loci performing well in both species, randomly chosen of the nine well performing loci, together with three loci not amplifying properly in *D. cucullata* or *D. cucullata* x *galeata* hybrids. For example, in the set 6+3a, loci Dp196NB, DaB17/17 and Dgm109 were randomly excluded. 7+2 a-c: 7 loci performing well in both species, randomly chosen as above, together with two of three loci not performing properly in *D. cucullata* or *D. cucullata* x *galeata* hybrids. 8+1 a-c: 8 loci performing well in both species, randomly chosen as above, together with one of three loci not performing properly in *D. cucullata* or *D. cucullata* x *galeata* hybrids. 9: all nine loci amplifying well in both species and hybrids. 12: all twelve loci used. Loci not properly amplifying in *D. cucullata* or *D. cucullata* x *galeata* hybrids either due amplification failure (Dgm112) or to insufficient amplification in F_1 hybrids (SwiD12, DaB10/14) are marked in bold.

Locus	**6+3a**	**6+3b**	**6+3c**	**7+2a**	**7+2b**	**7+2c**	**8+1a**	**8+1b**	**8+1c**	**9**	**12**
Dp196NB			x	x	x	x	x		x	x	x
Dp281NB	x	x		x	x	x	x	x	x	x	x
Dp519	x	x	x	x		x		x	x	x	x
DaB10/14	**x**	**x**	**x**	**x**	**x**		**x**				**x**
DaB17/17		x		x	x	x	x	x	x	x	x
Dgm105	x	x	x				x	x	x	x	x
Dgm109		x		x	x	x	x	x	x	x	x
Dgm112	**x**	**x**	**x**		**x**	**x**			**x**		**x**
SwiD06	x		x	x	x		x	x	x	x	x
SwiD12	**x**	**x**	**x**	**x**		**x**		**x**			**x**
SwiD14	x		x	x	x	x	x	x	x	x	x
SwiD18	x	x	x		x	x	x	x		x	x

Results

Assignment of artificial F_1 hybrids using genotyping data of clonal lineages

Genotyping of individuals belonging to seven clonal laboratory lineages was successful for all twelve loci (**Table 2-3**). Contemporary individuals as well as individuals collected 14 years ago of these lineages resulted in the same multi-locus genotypes. F_1 hybrid genotypes (CG1, CG4, CG5, CG6, and CG8) are considered to contain one *D. cucullata* allele and one

D. galeata allele at each locus when assuming Mendelian inheritance. As the multi-locus genotypes (MLG) of the parental clones (G1, C2) are known, we detected two loci which deviated from expectations. For locus SwiD12 only the *D. galeata* allele was detected in hybrid clones. Further, in four of those hybrids (CG1, CG4, CG5, and CG6) only the *D. galeata* allele was found at locus DaB10/14, also after applying newly designed forward and reverse primers (performing equally well in parental clones of both species).

Differences in flanking regions among species

In five out of eight cases we detected substitutions or indels (**Table 2-1**) between primer site sequences of clone C2 and originally published sequences for the *D. longispina* complex (Brede *et al.* 2006). The other three primer binding site sequences, showing no differences to the reference sequences, were detected for loci Dp196NB (forward binding site), Dgm112 (reverse), and SwiD14 (reverse). In both primer binding sites of the locus DaB10/14 we detected single nucleotide polymorphisms. In locus Dgm109 (reverse primer binding site) we detected a G to C substitution and one nucleotide insertion. Another insertion (two nucleotides) occurred in the reverse primer binding site of Dp519. The biggest difference was detected in locus Dgm112 (forward primer binding site) where four substitutions occurred.

Species identification of individuals in natural populations using microsatellite DNA

In total, 1715 individuals (44 localities) were analyzed using the *CGL µsat kit*. All loci amplified well in all studied individuals except locus Dgm112, which showed low performance (no amplification) in most populations that morphologically belong to *D. cucullata*. The inspection of Ln P(D) values as well as Evanno's Δ*K* (Evanno *et al.* 2005; Figure 2-S1) suggest that $K = 3$ is adequate to describe the structure within the dataset. When $K = 3$, the three clusters were distinct with 85.5% of individuals (1466/1715) assigned with > 95% probability to one of the clusters and all ten independent analyses for $K = 3$ revealed the same result. 521 individuals were assigned to *D. longispina*, 666 to *D. galeata*, 279 to *D. cucullata* and 249 individuals could not be affiliated to one of the groups when applying the 95% criterion and are hence considered admixed genotypes (**Figure 2-1**). The populations belonging to *D. cucullata* revealed two loci that were monomorphic (Dp196NB and Dp519); the allele that was obtained from all *D. cucullata* individuals at locus Dp519 is most likely species specific (allele size: 141 nucleotides in our studies) as it was found neither in *D. galeata* nor in *D. longispina* individuals. MICRO-CHECKER 2.3.3 did not detect evidence

for large allele dropout in any of the pure taxon populations, however, possible genotyping errors due to stuttering or null alleles were detected rarely in single populations at varying loci (for further information see supplement **Table 2-S1**). We had to remove locus Dgm112 from the analysis of *D. cucullata* populations in the program MICRO-CHECKER as we were not able to amplify this locus in most populations.

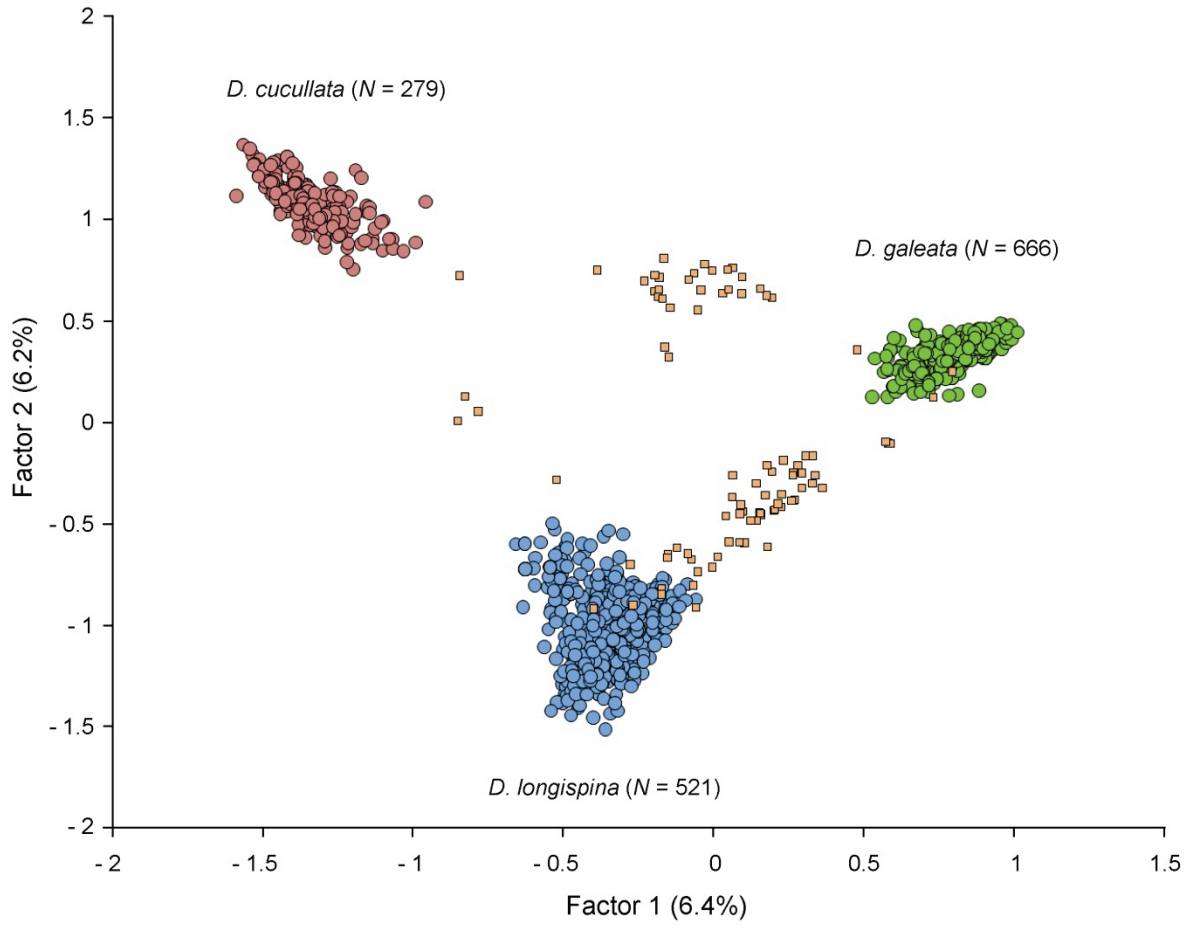

Figure 2-1 Genetic relationship of 1715 individuals of the *Daphnia longispina* complex using the first two factors of a factorial correspondence analysis based on microsatellite multi-locus genotypes calculated in GENETIX 4.05. Individuals are labeled as *D. longispina* (blue circles), *D. galeata* (green circles), *D. cucullata* (red circles) or admixed individuals (orange squares) depending on their assignment probabilities calculated in STRUCTURE 2.3.3.

Assignment of D. galeata *x* cucullata *hybrids in natural populations*

As we detected two loci deviating from expected allele frequencies in F_1 hybrids (SwiD12 and DaB10/14) as well as one locus not amplifying in most genotyped *D. cucullata* individuals (Dgm112) we tested suitability of several locus combinations for the identification of *D. cucullata* x *galeata* hybrids. In the dataset using the genotype information of the natural populations (666 *D. galeata* individuals, 279 *D. cucullata* individuals and 93 admixed

Table 2-3 Allele sizes for twelve microsatellite loci of individuals of seven laboratory clonal lineages. For each locus the allele size is given as three integers for each allele (e.g., 072 072 means homozygous for allele 72). Allele compositions that deviate from patterns expected under Mendelian inheritance are highlighted in bold.

Clone	Dp281NB	SwiD18	DaB17/17	SwiD12	Dgm112	Dp196NB	SwiD06	Dp519	SwiD14	Dgm105	DaB10/14	Dgm109
C2	072 072	091 091	100 100	114 114	120 120	120 120	122 122	141 141	187 187	177 180	232 232	256 259
G1	072 072	085 087	101 101	108 120	120 120	120 120	129 133	147 147	178 178	194 194	227 227	264 264
CG1	072 072	085 091	100 101	**120 120**	120 120	120 120	122 129	141 147	178 187	180 194	**227 227**	259 264
CG4	072 072	087 091	100 101	**108 108**	120 120	120 120	122 129	141 147	178 187	177 194	**227 227**	259 264
CG5	072 072	087 091	100 101	**120 120**	120 120	120 120	122 133	141 147	178 187	180 194	**227 227**	256 264
CG6	072 072	085 091	100 101	**120 120**	120 120	120 120	122 133	141 147	178 187	180 194	**227 227**	256 264
CG8	072 072	085 091	100 101	**108 108**	120 120	120 120	122 133	141 147	178 187	180 194	227 232	259 264

individuals as defined by STRUCTURE 2.3.3) the parental genotypes were assigned correctly in all locus combinations (except for 6+3b where three individuals of *D. galeata* had a posterior probability of less than 0.95 belonging to this class). The 93 admixed individuals were assigned to different classes depending on the locus combination (**Figure 2-2**). We found that the most representative result is given by the nine loci working equally well for both species, where all admixed individuals were sorted as F_1 hybrids (except for one individual which was assigned as *D. galeata*). In all other combinations we detected individuals that could not be assigned to any of the six groups when applying a posterior probability threshold of > 0.95. When using a subset of the *CGL µsat kit* which includes all three loci not properly performing in *D. cucullata* the number of individuals that could not be assigned increased drastically and assigned individuals were partly identified as backcross individuals with *D. galeata* instead as F_1 hybrids.

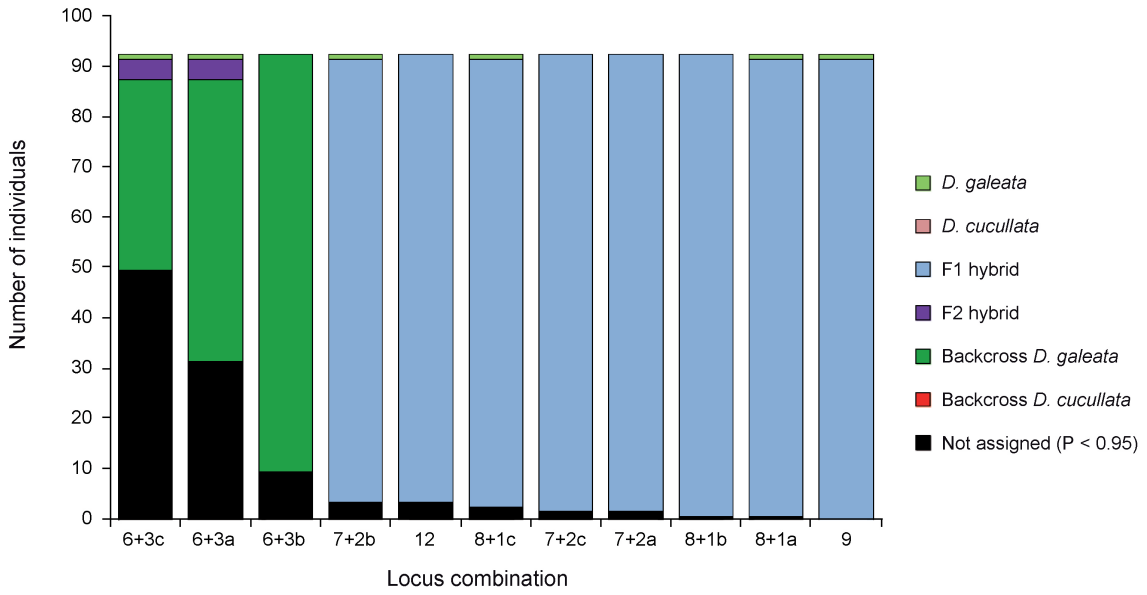

Figure 2-2 Assignment tests of *D. galeata* x *cucullata* hybrids based on a simulation of various combinations of microsatellite loci using software NEWHYBRIDS. Shown are the detailed classifications of 93 individuals with hybrid status as previously detected by STRUCTURE 2.3.3 (see *Material and methods* for more details). Individuals were sorted to one of the six hybrid classes, if posterior probability of individual assignments to a class was 0.95 or higher or if no assignment was possible ($p < 0.95$), to the unspecified admixed group. Locus combinations (categories on x-axis) are explained in **Table 2-2**.

Discussion

Cross-species amplification of microsatellite loci and consequences for hybrid detection

Molecular ecologists studying for example comparative population genetic structure and divergence, speciation processes, and interspecific hybridization require universal markers that can readily be transferred between taxa. Therefore, cross-species amplification of microsatellite markers is frequently used. The success of this approach is often measured by two variables, positive amplification of parental markers and their polymorphism.

The main reason for hindered cross-species amplification is variation within primer binding sites. Microsatellite flanking regions together with their repeat motifs (if located in non-coding regions) are thought to mutate unconstrained. This was indicated by Brohede & Ellegren (1999) who detected similar substitution rates in microsatellite flanking regions and introns (both considered as selectively neutral) within bovine and ovine animals. The consequence of differences in primer binding sites is the occurrence of null alleles. In parental assignment testing, no matter if applied within or among species, null alleles result in the problem of parental exclusion. For example, Eggleston-Stott *et al.* (1997) could show one case in which already two substitutions lead to a null allele and subsequently resulted in parental exclusion in horses.

We detected decreased performance of some loci (DaB10/14, Dgm112, and SwiD12) for *D. cucullata*, compared to *D. longispina* and *D. galeata*. The F_1 hybrids of clones G1 and C2 revealed two loci (SwiD12 and DaB10/14) at which we detected only one parental allele (from *D. galeata*), which would exclude clone C2 as potential parent. Hence, we sequenced eight primer binding sites of six microsatellite loci using parental clone C2, of which five deviated from reference sequences (DaB10/14 forward and reverse, Dp519 reverse, Dgm109 reverse, and Dgm112 forward; Brede *et al.* 2006). Still, newly designed primers for those loci did not change or improve amplification efficiency for the seven clonal lineages studied. As we were not able to sequence primer binding sites of locus SwiD12 we cannot confirm differences in primer sequences, but the presence of mutations or indels and therefore non-optimal binding of primers is a likely scenario. This locus was tested negative for *D. cucullata* by Brede *et al.* (2006) but could be successfully amplified in most natural *D. cucullata* populations (although single locus amplifications were necessary in most cases). This would explain the occurrence of null alleles within all artificial hybrids, as the *D. galeata* allele will be preferred and amplified more frequently during PCR.

Since daphnids reproduce via cyclic parthenogenesis, variation in nuclear loci in clonal cultures may arise through: (1) intraclonal sexual reproduction; (2) ameiotic recombination (cross-over and gene conversion; Omilian *et al.* 2006); (3) hemizygous deletions (Xu *et al.* 2011); or (4) mutation events within the microsatellite structure itself (Seyfert *et al.* 2008). As we genotyped individuals from 14 years ago, from a point in time shortly after the artificial crosses of clones G1 and C2 occurred, and detected the same multi-locus genotypes, we conclude that the observed homozygotes at locus SwiD12 and DaB10/14 are not explained by mechanisms that occurred over time. None of the above mentioned alternative explanations are applicable, and the *D. cucullata* null allele at locus DaB10/14 in hybrid individuals cannot be explained by inefficient primer binding, as redesigned primers did not result in correct multi-locus genotypes. One explanation for the *D. cucullata* specific null allele at locus DaB10/14 may be large allele dropout, although there were no indications for this when tested with MICRO-CHECKER. In locus SwiD12 it is more probable that the specific null alleles are observed due to inefficient primer binding.

Amplification of natural *D. cucullata* populations showed that the performance of Dgm112 is very low in general. This is in concordance with the cross-species results of Brede *et al.* (2006) where no amplicons were obtained for this locus in *D. cucullata* (however only two clones were tested). These results are most likely caused by multiple substitutions in the forward primer binding site. The recent study by Yin *et al.* (2012a) revealed an additional locus (SwiD2) that often failed to amplify, but in *D. longispina* and not in *D. cucullata*, although this locus was tested polymorphic by Brede *et al.* (2006) for this taxon.

Carlsson (2008) found that the number of loci and the level of genetic differentiation seem to have greater effects on the accuracy of assignment testing than does the presence or absence of null alleles. Also, Adams *et al.* (2007) tested the assignment of parental and hybrid individuals with two different sets of microsatellite loci and found that the assignment with 18 loci is more successful than the assignment with just 8 of those loci. However, our assignment tests using NEWHYBRIDS and 29 natural *Daphnia* populations revealed a reduced discriminatory power if analyses are based on twelve loci. The classification improved if only nine loci were used that worked equally well in both species, *D. galeata* and *D. cucullata*. If choosing an inappropriate set of loci misidentification of hybrid classes might be the result, and since a microsatellite set with less than ten markers is often used (Koskinen *et al.* 2004), the right choice of loci seems important. Therefore, for hybrid detection within the species pair *D. galeata* and *D. cucullata* we recommend the usage of a subset of the *CGL µsat kit*, as three loci revealed *D. cucullata* specific null alleles (Dgm112, SwiD12 and DaB10/14).

As addressed above, the positive amplification of parental markers as well as the polymorphism of those markers is crucial for cross-species amplification. The main reason for monomorphic loci during cross-species amplification may be either absence of the repetitive sequence or fixation for one allele. Within *D. galeata* and *D. longispina* all tested microsatellite loci were polymorphic. In contrast, we discovered two loci in *D. cucullata* that were monomorphic for all individuals tested (Dp196NB and Dp519), due to a fixed allele at each locus (unpublished results), and we detected two more (Dp281NB and DaB17/17) with one dominant allele (abundance > 90%).

The results discussed above are remarkable and unexpected as *D. cucullata* and *D. galeata* are assumed to represent sister species and to share a recent common ancestor (Petrusek *et al.* 2008; Taylor *et al.* 1996). However, it was reasoned for example by Schwenk *et al.* (1995) that *D. galeata* shares a more recent common ancestor with *D. longispina* than with *D. cucullata*, as discordant phylogenetic trees for mitochondrial and nuclear DNA were observed. Our results support the latter hypothesis, but the high degree of hybridization within this complex requires more detailed studies to unravel their true phylogenetic relationships.

Consequently, the obtained results show that even among closely related taxa cross-species amplification success might be negatively affected, resulting in ambiguous classification of species and hybrids.

Delineation of species in the D. longispina *species complex*

High phenotypic plasticity induced by environmental cues is often recorded in *Daphnia* species (for instance Laforsch & Tollrian 2004), causing ambiguous or wrong species assignments. Furthermore, local races within species are known and interspecific hybridization leads to intermediate phenotypes which complicates morphological classification (Dlouhá *et al.* 2010; Schwenk *et al.* 2001). Several molecular marker systems, e.g., allozymes, ITS-RFLP, RAPD, sequencing of selected genes, and microsatellites, were used in the past to facilitate the assignment of individuals to certain species or hybrid classes (Dlouhá *et al.* 2010). Still, each method exhibits certain disadvantages and limitations. Dlouhá *et al.* (2010) showed that allozyme as well as microsatellite loci offer a similar reliability and are best suited for species identification among other methods. As fixed (species specific) alleles are common in allozyme but not in microsatellite markers for the *D. longispina* complex, species identification of each individual using only microsatellite loci requires more elaborate statistical support. Following Noble *et al.* (2010) who suggest using microsatellite DNA information for species delineation we were able to assign most of the genotyped

individuals to one of the three studied species (*D. longispina*, *D. galeata*, and *D. cucullata*) by employing a model-based clustering method implemented in STRUCTURE (Pritchard *et al.* 2000). Other recent studies used a similar approach (Dlouhá *et al.* 2010; Yin *et al.* 2010), but combined factorial correspondence analysis and model-based assignment tests as implemented in NEWHYBRIDS (Anderson & Thompson 2002). Therefore, we recommend these approaches, if it is certain that all pure species occur in the studied geographic region or if a reference dataset is available.

Conclusions

Studies on interspecific hybridization using cross-species amplification of microsatellite loci require not only the amplification of polymorphic loci for each parental species, but also equally well amplifying loci, to reduce the risk of species specific null alleles among hybrid individuals. Either laboratory hybrids or artificial hybrids (using DNA of both parental taxa) provide a valid test for the efficiency of microsatellite markers. Alternatively, our data show that a sufficiently large number of microsatellite loci are capable of diluting the effect of unequal amplification.

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Chapter 3

Contribution of cyclic parthenogenesis and colonization history to population structure in *Daphnia*

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Abstract

Cyclic parthenogenesis, the alternation of parthenogenetic and sexual reproduction, can lead to a wide scope of population structures, ranging from almost monoclonal to genetically highly diverse populations. In addition, sexual reproduction in aquatic cyclic parthenogens is associated with the production of dormant stages, which both enhance potential gene flow among populations as well as impact local evolutionary rates through the formation of dormant egg banks. Members of the cladoceran genus *Daphnia* are widely distributed key organisms in freshwater habitats, which mostly exhibit this reproduction mode. We assessed patterns of genetic variation within and among populations in the eurytopic and morphologically variable species *Daphnia longispina*, using data from both nuclear (13 microsatellite loci) and mitochondrial (partial sequencing of the 12S rRNA gene) markers from a set of populations sampled across Europe. Most populations were characterized by very high clonal diversity, reflecting an important impact of sexual reproduction and low levels of clonal selection. Among-population genetic differentiation was very high for both nuclear and mitochondrial markers, and no strong pattern of isolation-by-distance was observed. We also did not observe any substantial genetic differentiation among traditionally recognized morphotypes of *D. longispina*. Our findings of high levels of within-population genetic variation combined with high among-population genetic differentiation are in line with predictions of the *monopolization hypothesis*, which suggests that in species with rapid population growth and potential for local adaptation, strong priority effects due to monopolization of resources lead to reduced levels of gene flow.

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Introduction

Cyclic parthenogenesis occurs in over 15 000 animal species, spread over seven taxonomic groups (Monogononta, Cladocera, Digenea, Homoptera, Hymenoptera, Diptera, Coleoptera; Hebert 1987). As this mode of reproduction combines the advantages of sexuality with the high demographic potential of asexuality, it has been intensely studied by evolutionary biologists and ecologists (e.g., de Meeûs *et al.* 2007; Decaestecker *et al.* 2007; Gómez & Carvalho 2000; Sunnucks *et al.* 1997; Taylor *et al.* 1999). However, the impact of cyclic parthenogenesis on population genetic structure depends on many factors that determine the relative importance of sexual and parthenogenetic phases (De Meester *et al.* 2006; Hughes 1989). In addition, especially in aquatic taxa, sexual reproduction is associated with the production of dormant stages. This may at the same time alter local rates of micro-evolutionary processes and facilitate dispersal among populations (Figuerola *et al.* 2005; Hairston & De Stasio 1988). For example, dormant eggs of cladocerans and monogonont rotifers are usually produced in high numbers and, as they are produced by sexual recombination in most species, they represent a source of new recombinant genotypes. Thus, hatching from egg banks has a strong impact on the level of clonal diversity within populations as well as significant effects on the population structure and potential for local adaptation (Boersma *et al.* 1999; Brendonck & De Meester 2003; Cousyn *et al.* 2001; De Meester 1996; Declerck *et al.* 2001). On the other hand, the parthenogenetic part of the life cycle may result in the persistence of clonal lineages over long time periods and a reduction of local diversity through clonal erosion (De Meester *et al.* 2006).

Daphnids (genus *Daphnia*; Cladocera) are cyclic parthenogenetic crustaceans constituting key components of freshwater systems. Their parthenogenetic reproduction takes place during favorable growth conditions, whereas sexual reproduction usually occurs when deteriorating environmental conditions are indicated by environmental cues such as shortening photoperiod, food shortage, crowding, or predator infochemicals (Hobæk & Larsson 1990). Sexual reproduction results in the production of long-lasting dormant eggs, encased in a protective chitinous structure called ephippium. Depending on the relative importance of re-combination and parthenogenetic reproduction in *Daphnia*, populations will vary in local diversity and genetic population structure. Recently, De Meester *et al*. (2006) developed a conceptual framework for cyclic parthenogenetic freshwater zooplankton, describing three main factors that determine population structure: the size of the dormant egg bank, the length of the growing season, and the strength of clonal selection.

Genetic drift and differential natural selection are thought to be key forces enhancing among-

population genetic variation in freshwater zooplankton (De Meester *et al.* 2006). In addition, phylogeographical studies on cladocerans suggest that historical factors (such as glaciation and past fragmentation) are also responsible for their population differentiation (Cox & Hebert 2001; Ishida & Taylor 2007a; Weider & Hobæk 2003). For example, populations of the *Daphnia longispina* complex from older unglaciated habitats (i.e. Japan) showed higher DNA sequence divergences than populations from presumed younger habitats (i.e., non-Beringian North America; Ishida & Taylor 2007a). The same study also found evidence for rapid population expansion, which suggests that most of the observed population differentiation is unlikely to be due to dispersal limitation. These patterns are consistent with earlier studies on continental zooplankton which revealed high levels of among-population differentiation (Boileau *et al.* 1992; De Meester 1996; Gómez *et al.* 2002), despite empirical evidence for effective dispersal of dormant eggs (mainly by waterfowl; Figuerola *et al.* 2005). Potential factors explaining this paradox are summarized in the *monopolization hypothesis*, which states that rapid population growth and rapid local adaptation upon colonization of a new habitat result in the effective monopolization of resources, yielding strong priority effects (De Meester *et al.* 2002). Potentially, local adaptation and the presence of a large dormant egg bank provide a powerful buffer against newly invading genotypes. Phylogeographical patterns in *Daphnia* seem to be in accordance with the *monopolization hypothesis*, as they indicate that historical processes (e.g. initial colonization from glacial refugia) rather than recent processes (such as gene flow) contributed significantly to the current distribution of evolutionary lineages (Cox & Hebert 2001; Ishida & Taylor 2007a; Weider & Hobæk 2003).

Here we present data on the population structure of the widely distributed and ecologically plastic Old World water flea species *D. longispina*. This lineage occurs in small ponds as well as large lakes, and has a particularly problematic taxonomy. Recently, Petrusek *et al*. (2008) showed that *D. longispina* encompasses morphotypes traditionally identified as separate species (*Daphnia hyalina* and *Daphnia rosea*, as well as a pigmented alpine form, originally described as *Daphnia zschokkei*). Reliable identification of this as well as other taxa of the *D. longispina* complex has been hampered due to common interspecific hybridization with two other related *Daphnia* species (*D. galeata* and *D. cucullata*; Schwenk & Spaak 1995). Hybridization in the *D. longispina* complex occurs throughout Central Europe, and although a number of populations exhibit backcrossed genotypes and cytonuclear recombinants, the levels of introgression remain relatively low (Schwenk 1993; Schwenk & Spaak 1995; Spaak 1996). Interspecific gene flow is apparently not strong enough to merge species gene pools. Yet, partly permeable species boundaries and temporal hybrid superiority enabled the

establishment of evolutionary stable recombinants within the complex, in particular in North America (Taylor *et al.* 2005).

The present study had two aims. First, using nuclear as well as mitochondrial DNA markers, we examined the level of within-population genetic variation as well as among population differentiation of 14 *D. longispina* populations (including various morphs of this taxon), collected from a wide range of habitats across Europe. Second, we examined whether the observed patterns are consistent with the predictions of the *monopolization hypothesis* which assumes a high level of population differentiation despite high dispersal rates.

Materials and methods

Sampling and morphological characterization

Daphnia individuals were sampled from 20 localities across Europe between 1995 and 2006 (**Table 3-1**). Fourteen of these populations represented all main morphotypes (previously recognized as separate taxa) pooled under *Daphnia longispina* by Petrusek *et al*. (2008). The remaining six populations were represented by *Daphnia galeata* and *Daphnia cucullata* (each by three populations). The sampling sites of *D. longispina* populations represent a range of habitats from temporary ponds to large lakes, covering a substantial latitudinal gradient (Spain to Norway). In most cases, sampling was carried out at the end of the growing season, so most *Daphnia* populations were likely present as parthenogenetic populations for at least several months. Exceptions from this sampling scheme were the populations from Göteborg (Sweden), Stechlinsee (Germany) and Delftse Houd (Netherlands), which were sampled at the beginning of the growing season. Samples were collected using plankton nets with mesh sizes of 100-200 μm, in case of ponds and small lakes by oblique tows from the shore, in large lakes by vertical or oblique tows from a boat. After collection, the zooplankton was preserved in 96% ethanol.

Our aim was to analyze population structure of one polymorphic *Daphnia* species with its various morphotypes, not to characterize the whole *D. longispina* species complex. We therefore analyzed only populations which contained only a single morphotype corresponding to one of the described forms of *D. longispina*, to avoid as much as possible the presence of interspecific hybrids that commonly occur in this complex (Schwenk & Spaak 1995). When selecting individuals from the samples, we assessed various morphological characters, including the body shape, position of antennular setae, morphology of antennular mound, pigmentation of ocellus and antennae, etc. However, despite the effort, we could not

completely rule out the possibility that some individuals morphologically similar to the parental *D. longispina* represent recombinant genotypes.

To test whether *D. longispina* morphotypes (previously treated as separate species) show any pattern of genetic differentiation, we compared molecular variation with classical morphological-based groupings (e.g. *D. rosea*, *D. zschokkei* and *D. hyalina*). *D. galeata* Sars and *D. cucullata* Sars, two closely related species, which are known to form interspecific hybrids among each other and with *D. longispina* (all three combinations, see Schwenk *et al.* 2001), were used as reference taxa (three populations per species). Apart from morphological characters, their identification was confirmed by genetic markers, i.e. internal transcribed spacer–restriction fragment length polymorphism (ITS–RFLP, Billiones *et al.* 2004), microsatellite DNA (Brede *et al.* 2006) and 12S rDNA sequences (Schwenk *et al.* 2000).

DNA preparation

In total, 663 individuals isolated from 20 localities were genetically characterized using on average 33 individuals (20-42) per sampling site (**Table 3-1**). *Daphnia* individuals were incubated in 70-150 µL of H3 buffer with proteinase K, containing 10 mM Tris-HCl (pH 8.3 at 25 °C), 0.05 M potassium chloride, 0.005% Tween 20, 0.005% NP-40 and 1.5 µg/mL proteinase K (Sigma). Samples were vortexed, briefly centrifuged and incubated for 4-16 h at 56 °C. Finally, samples were heated at 96 °C for 12 min, centrifuged briefly and stored at 4 °C or frozen.

Microsatellite analysis

We amplified 13 microsatellite loci (Brede *et al.* 2006) for each individual. Eleven loci were combined to four multiplex polymerase chain reactions (PCR; MP1: Dp281NB + SwiD14 + DaB10/14; MP2: DaB17/17 + Dp196NB; MP3: SwiD6 + SwiD12 + SwiD18; MP4: Dgm105 + Dgm109 + Dgm112) and the remaining two loci (Dp519 and SwiD1) were amplified in single PCRs. The forward primers were labeled with the fluorescent dyes Alexa 647 (Invitrogen; Dp281NB, SwiD14, DaB10/14, DaB17/17, Dp196NB and Dp519), Alexa 750 (Invitrogen; Dgm105, Dgm109 and Dgm112) and IRD700 (MWG; SwiD1, SwiD6, SwiD12 and SwiD18). Multiplex PCRs were performed in 0.2 mL tubes with 10 µL reaction volume containing 3.0 mM $MgCl_2$, 1x PCR buffer, 0.2 mM dNTPs, 1 U *Taq* polymerase (Invitrogen), 0.2 mg/mL BSA (New England Biolabs), 1% DMSO (Roth) and 2 µL prepared DNA. Except for locus Dp281NB (0.1 µM), we used a primer concentration of 0.2 µM. Each single PCR for

the loci SwiD1 and Dp519 contained 1x PCR buffer, 2.4 mM (SwiD1) or 2.5 mM (Dp519) MgCl2, 0.25 mM dNTPs, 0.2 µM of each primer, 0.5 U *Taq* polymerase (Invitrogen), 0.1 mg/mL BSA (New England Biolabs) and 2 µL prepared DNA. Cycling conditions for the multiplex PCRs and the locus SwiD1 started with a 3 min denaturing step at 95 °C followed by 35 cycles of 1 min steps at 95 °C, 55 °C (MP 1, 3 and 4) or 53 °C (MP 2) or 63 °C (SwiD1) and 72 °C. A final 7 min synthesis step at 72 °C completed the programs. Cycling conditions for the locus Dp519 started with a 3 min denaturing step at 95 °C followed by 32 cycles of 45 sec steps at 95 °C, 53 °C and 72 °C. A final 20 min synthesis step at 72 °C completed the program. Amplicons were diluted and electrophoresed on a CEQ 2000 (Beckman Coulter; denaturation at 90 °C for 2 min; injection at 2.0 kV for 30 sec; separation at 6.0 kV for 35 min) with a self-designed size standard based on Lambda phage DNA (Symonds & Lloyd 2004).

Amplification and sequencing of the 12S rDNA fragment

We sequenced the 580 bp long fragment of the mitochondrial gene for 12S rRNA from a range from 2 to 13 randomly selected individuals (except *D. galeata* from Cogollos, Spain, and *D. cucullata* from Drabužis, Lithuania). Each 20 µL PCR consisted of 2 µL DNA template, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer (5′-ATGCACTTTCCAGTACATCTAC-3′ and 5′-AAATCGTGCCAGCCGTCGC-3′) and 0.5 U *Taq* polymerase (Invitrogen). The cycling conditions followed Schwenk *et al*. (2000). PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen) or the GFX PCR DNA Purification Kit (Amersham Biosciences), and sequenced directly on capillary sequencers (ABI PRISM 3130 or Beckman Coulter 2000) using the forward primers.

Data analysis

To visualize the distribution of genetic variation at microsatellite markers across individuals, we performed a factorial correspondence analysis (FCA; calculated in GENETIX version 4.01) of multi-locus genotypes; this allowed us to distinguish genetically most differentiated groups and to compare patterns of genetic differentiation with prior information on morphotypes and populations (Belkhir *et al.* 1996-2004). To verify the potential occurrence of hybrids in our samples, we subsequently used the program NEWHYBRIDS 1.1 (Anderson & Thompson 2002). This software implements a Bayesian assignment approach dedicated to the quantification of hybridization events from individual multi-locus genotypes to estimate posterior probability

that an individual belongs to a certain hybrid class. We performed five runs of 10^6 iterations with a burn-in of 10^5 iterations using a Jeffrey's prior. Inspired by the observed population structure revealed from factorial correspondence analyses, we subjected all *D. longispina* and *D. galeata* (but not *D. cucullata*) populations to assignment tests. We conducted three different analyses, as NEWHYBRIDS is not able to differentiate more than two potential parental taxa and their hybrid classes. In the first analysis, we tested all *D. galeata* populations and all *D. longispina* populations except deviating populations from Zahillo (ZH) and Badajoz (BA); in the second, we tested all *D. galeata* populations and those from ZH and BA. In the third analysis, we tested several *D. longispina* populations, which suggested the potential presence of two partially separated but hybridizing lineages represented by clusters of Goksjø (GO), Göteborg (GB), Ismaning (IS), Mondsee (MS), Nižné Jamnícke (NJ), St. Bernard (SB), Stechlinsee (SS), Storveavatn (SV) and ZH and BA.

Mitochondrial 12S rDNA sequences were initially aligned using ClustalW (Higgins *et al.* 1996) and manually adjusted using BIOEDIT (Hall 1999). Haplotypes were identified using COLLAPSE 1.2 (by D. Posada, University of Vigo, Spain; http:// darwin.uvigo.es/software/ collapse.html). To visualize the pattern of divergence among and within the three species (*D. longispina*, *D. galeata* and *D. cucullata*), we constructed a neighbor-joining tree with the program MEGA 4.1 (Tamura *et al.* 2007) using pairwise deletion of gaps, Kimura 2-parameter model and 10^5 bootstrap replicates.

We estimated molecular differentiation among *D. longispina* populations based on microsatellite (F_{ST}) and mtDNA markers (Φ_{ST}) and conducted also two analyses of molecular variance (AMOVA) for both markers using ARLEQUIN 2.0 (Schneider *et al.* 2000). The first AMOVA was based on two hierarchical levels [populations ($N = 14$) and individuals ($N = 463$ for microsatellites and 119 for mtDNA, respectively)]. The second analysis was performed to evaluate the potential contribution of genetic differentiation between *D. longispina* morphotypes. It used three hierarchical levels [morphotype (*hyalina* and *rosea*), populations ($N = 14$) and individuals ($N = 463$ for microsatellites and 119 for mtDNA, respectively)].

To estimate the most likely number of clusters in the data set, which minimize Hardy-Weinberg and interlocus disequilibrium, we performed the Bayesian assignment method using STRUCTURE version 2.1 (Falush *et al.* 2003; Pritchard *et al.* 2000). Five independent runs were carried out for each value of a number K (K from 1 to 23) of clusters. For each run, 10^6 iterations were carried out after a burn-in period of 10^5 iterations. Analyses were performed without prior assumptions concerning the populations (morphotype or geographical location) using the following settings: admixture model, α inferred with an

initial value of 1, a maximum value of 10, a uniform prior, and the same value for all populations; different values of F_{ST} for different subpopulations; prior mean F_{ST} of 0.01; prior SD of 0.05; and constant λ with a value of 1.

To analyze intraspecific relationships among haplotypes of *D. longispina*, we created a haplotype network based on statistical parsimony using TCS version 1.21 (Clement *et al.* 2000). In addition, we tested for associations of genetic differentiation either at nuclear or mitochondrial markers with geographical distances, and among nuclear and mitochondrial genotypes, using the Mantel test (Isolation-by-Distance Web Service, version 3.15; Jensen *et al.* 2005).

Based on the lack of any significant genetic differentiation among *D. longispina* morphotypes, we subjected all forms to one population genetic analysis (see Results). Estimation of clonal diversity and genetic differentiation was based on several population genetic parameters, such as genetic variance partitioning, calculation of linkage disequilibrium and the proportion of distinct multi-locus genotypes (MLGs). Since individuals of the same MLG represent either members of the same clone or members of different clonal groups, we applied several approaches to assess the level of clonal selection. We calculated diversity measures based on the assignment of individuals to clones; in addition, we accounted for scoring errors, PCR artifacts and somatic mutations which may cause small differences among individuals from the same clonal lineage (Meirmans & Van Tienderen 2004). Pairwise distances among all genotypes were determined to select a threshold that defines the maximum distance between two individuals at which they are still assigned to the same clonal lineage using GENOTYPE (Meirmans & Van Tienderen 2004). We used two different data sets to calculate MLG/*N* (where *N* indicates the number of individuals) and diversity (Nei's genetic diversity; Nei 1987) for each population. The first data set was based on the assumption that all genetic differences are real (= no scoring errors, PCR artifacts or mutations; t0). Second, we defined a threshold at the third distance class (t3) to account for an overestimation of genetic variation due to methodological errors.

Following recommendations of Halkett *et al*. (2005), additional population genetic parameters such as deviations from Hardy-Weinberg and linkage equilibria, inbreeding coefficient and number of private alleles were calculated using the program GENEPOP version 3.4 (Raymond & Rousset 1995) or GENALEX (Peakall & Smouse 2006). These parameters were estimated without repeated identical multi-locus genotypes. A sequential inference of these parameters allows the assessment of the level of clonal selection. For example, negative F_{IS} values indicate an excess of heterozygotes relative to random mating, suggesting clonal selection;

similarly, linkage disequilibrium, which is detected in a population only when repeated identical MLGs are considered, suggests a recent burst of asexual reproduction (Halkett *et al.* 2005).

Results

Genetic differentiation among species of the Daphnia longispina *complex*

Among 663 analyzed daphnids using 13 microsatellite loci, we distinguished 567 different MLGs. Among 102 individuals of *D. galeata,* we found 63 MLGs, among 98 *D. cucullata* we found 98 MLGs and among 463 *D. longispina* individuals, we found 406 MLGs (**Table 3-1**). In the factorial correspondence analysis, most multi-locus genotypes of *D. longispina* formed a mixed cluster, clearly separated from the reference populations of *D. galeata* and *D. cucullata* (**Figure 3-1**). Two populations (from the pool Zahillo, Spain, and Lake Nordfjordvatn, Norway) were nevertheless found well isolated from the main *D. longispina* cluster (**Figure 3-2**A). Whether these geographically separated populations are genetically isolated, or whether they appear isolated because we failed to sample intermediate populations, remains open for further studies. Some individuals from the localities Piano (Italy) and Vranov (Czech Republic) were positioned closer to the *D. galeata* cluster, and individuals from the Villar del Rey reservoir in Badajoz (Spain) showed an intermediate position between Zahillo and the main *D. longispina* cluster. NEWHYBRIDS analyses revealed several recombinant genotypes. In the first test, which included all *D. galeata* and all *D. longispina* populations (except ZH and BA), recombinants were found in three populations (Piano = 71%, Vranov = 25% and Storveavatn = 3%). In the second test, which included all *D. galeata* populations and populations ZH and BA, recombinant genotypes were only found in Badajoz (40%). In the third test, which included selected *D. longispina* populations, recombinant genotypes were not detected. Even though all five runs of the third test assigned all populations only to potential parental groups and did not assign them to hybrid classes, the actual assignments of the fifth run (group 1 = GO, SV, BA, ZH; group 2 = GB, IS, MS, NJ, SB, SS) differed from that of the other four runs (group 1 = GO, GB, IS, MS, NJ, SS, SV; group 2 = SB, BA, ZH). These differing results are probably due to the high population differentiation in *D. longispina* which is supported by the STRUCTURE analysis (see below).
Mitochondrial 12S rDNA fragments were sequenced for two *D. galeata* populations (altogether 13 sequenced individuals resulting in five haplotypes from Stanovice, Czech Republic and Delftse Houd, Netherlands; GenBank Accession nos. FJ178301–FJ178305), for

two *D. cucullata* populations (11 sequenced individuals resulting in four haplotypes from Usingen, Germany and Brno, Czech Republic; GenBank Accession nos. FJ178306–FJ178309) and for 14 *D. longispina* populations (119 sequenced individuals, 36 haplotypes; Gen-Bank Accession nos. FJ178310–FJ178345) using 3 to 13 individuals per population. The number of observed haplotypes per population ranged from one to six (**Table 3-1**). The sequence divergence (Kimura 2-parameter distance) between *D. longispina* and *D. galeata* averaged 8.4%, between *D. longispina* and *D. cucullata* 10.8%, and between *D. galeata* and *D. cucullata* 8.4%. The sequence divergence within species was significantly lower (0.5% in *D. galeata;* 0.6% in *D. cucullata* and 1.0% in *D. longispina*). Both mitochondrial and microsatellite DNA analysis supported the three main species clusters (**Figure 3-1**).

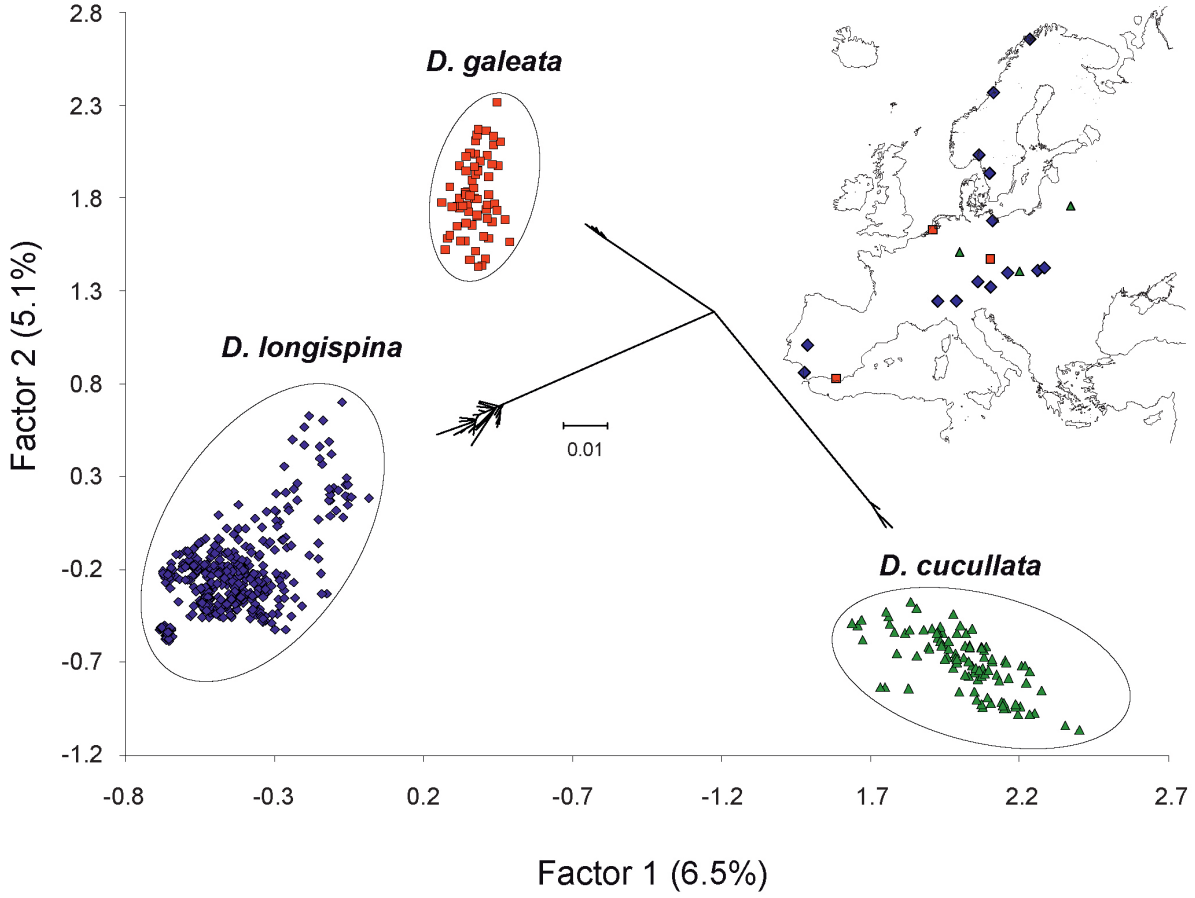

Figure 3-1 The first two factors of a factorial correspondence analysis (FCA) using microsatellite DNA information of individuals belonging to the three most common European species of the *Daphnia longispina* complex. *D. galeata* (red) and *D. cucullata* (green) are both represented by three populations, *D. longispina* (blue) is represented by 14 populations (**Table 3-1**). The small map in the right upper part of the graph shows the sampled locations for the three species. In the middle of the plot, a Neighbour-Joining tree is shown, based on 45 different haplotypes of the 531 bp long 12S rDNA fragment of the three species, constructed in MEGA 4.1 (Tamura *et al.* 2007) using the Kimura-2-Parameter model. All three species are supported by 100% of 10^5 bootstrap replicates.

Genetic differentiation among populations of D. longispina

The 14 geographically distant locations included in this study were strongly differentiated with both types of molecular markers (mtDNA: average Φ_{ST} = 0.79; microsatellites: average F_{ST} = 0.41). Nearly all pairwise *F*-statistic comparisons (89% of mtDNA and 100% of ncDNA comparisons) were highly significant ($P < 0.001$). To exclude the probability that the populations containing recombinant genotypes [BA, PI, SV and Vranov (VR)] and the one from Zahillo, which represents a genetically highly differentiated cluster, falsely increase the differentiation among populations, we calculated the mean Φ_{ST} for mtDNA and F_{ST} for microsatellites again, excluding all of them. The resulting values were very similar (mtDNA: average Φ_{ST} = 0.76; microsatellites: average F_{ST} = 0.46), confirming the strong population differentiation. An AMOVA analysis based on two hierarchical levels [populations (N = 14) and individuals (N = 463 for microsatellites and 119 for mtDNA, respectively)] revealed the distribution of variation within (21.2% mtDNA and 55.5% microsatellites) and among populations (78.8% mtDNA and 44.5% microsatellites). If an additional hierarchical level representing morphotypes corresponding to previously recognized taxa (*Daphnia hyalina* and *D. rosea*) was added to the AMOVA, the results did not differ substantially, which reflects that differences among populations representing these traditionally recognized forms explained only a negligible proportion of variation (1.6% mtDNA and 0.4% microsatellites).

We estimated the number of distinct populations in our sample applying a Bayesian model-based clustering algorithm to the microsatellite data (Pritchard *et al.* 2000). Among replicated runs (K = 1–23), the highest probabilities were found for 14 populations, and probabilities were very low for all other runs [$P(K|X) < 6.93 \times 10^{-53}$]. Posterior probabilities indicate that the number of sampled localities is identical with the number of populations represented in our microsatellite data (**Figure 3-2**B).

Mitochondrial DNA variation showed a similar pronounced geographical structure as obtained from the nuclear DNA (Φ_{ST} = 0.79). We found only two haplotypes which occurred in more than one studied population (**Figure 3-2**C). In general, mitochondrial and nuclear DNA divergences were highly similar. With only a few exceptions, we found a consistent cytonuclear differentiation among populations. Mantel tests revealed a significant correlation between genetic differentiation of populations based on mtDNA and on microsatellite data (R = 0.502; p = 0.022 using 1000 randomizations; **Figure 3-3**A). However, no significant associations between genetic differentiation and geographical distances were detected, irrespective whether nuclear (p = 0.156 from 1000 randomizations; **Figure 3-3**B) or mitochondrial (p = 0.32 from 1000 randomizations; **Figure 3-3**C) markers were used; this

was despite the fact that the highest genetic divergence was observed between the two populations found at the periphery of the sampled geographical area, Zahillo (Spain) and Nordfjordvatn (Norway).

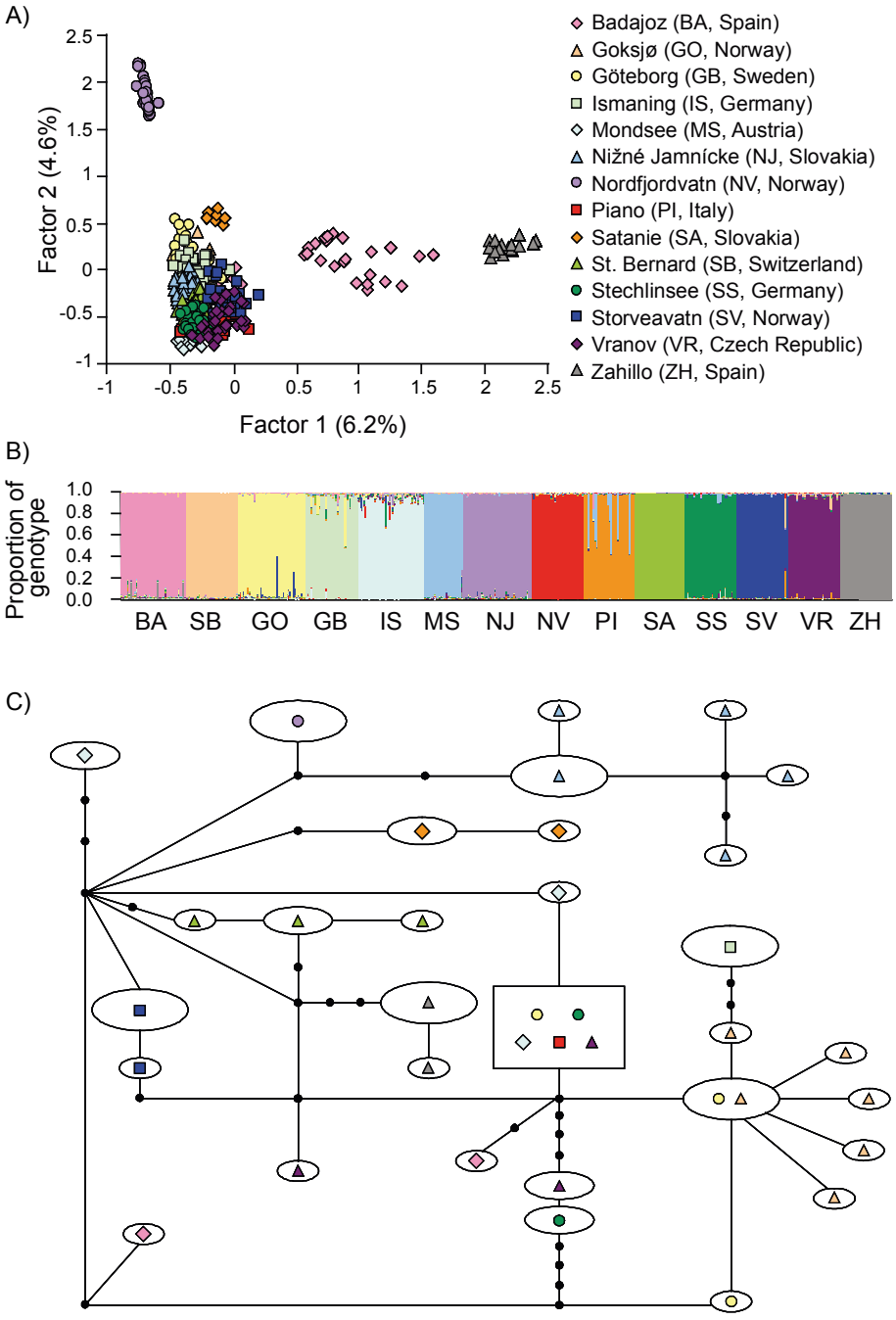

Figure 3-2 A) The first two factors of a factorial correspondence analysis (FCA) using microsatellite DNA information of individuals belonging to the taxon *Daphnia longispina*. B) Admixture analyses of all *Daphnia longispina* populations performed using STRUCTURE with $K = 14$. Each individual is represented as a vertical bar partitioned into K segments, whose length is proportional to the estimated membership in the K clusters. The number and composition of estimated clusters is identical with the number and population composition of the 14 sampled sites. C) Haplotype network of 119 sequences of the 531 bp long 12S rDNA fragment from the taxon *D. longispina*.

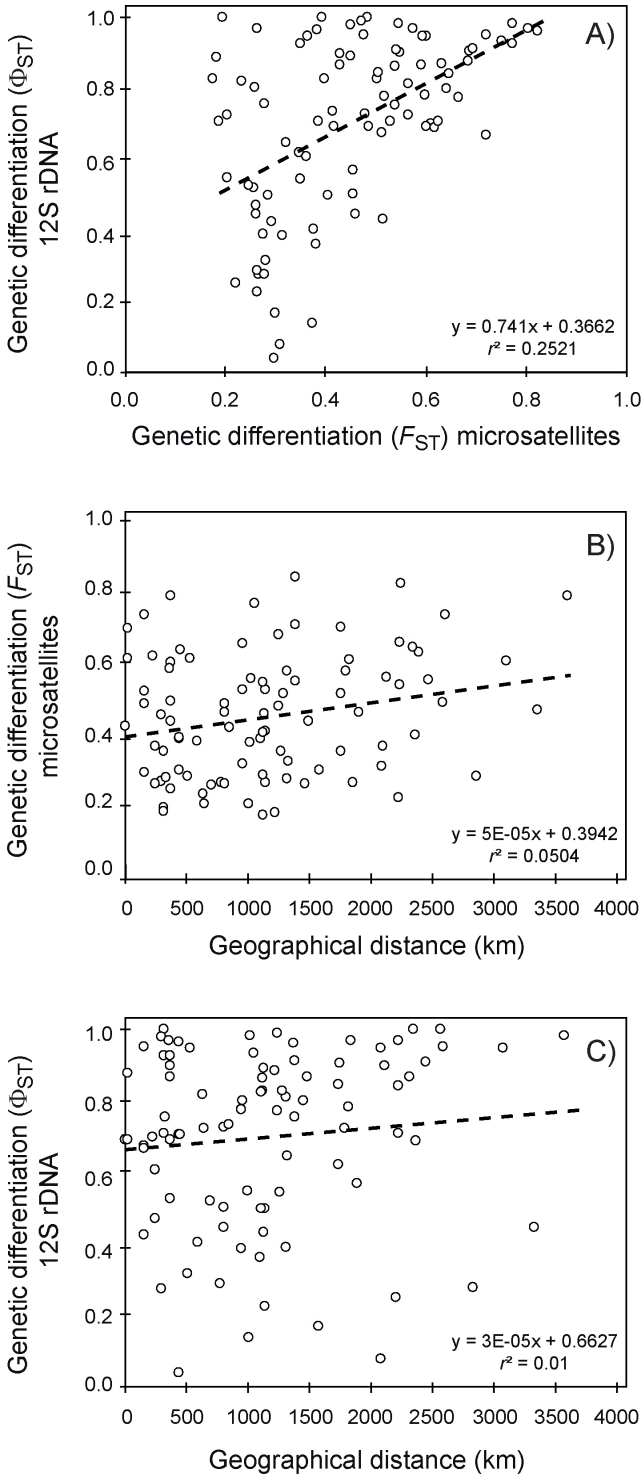

Figure 3-3 Linear regression of A) genetic differentiation (F_{ST}) based on microsatellites versus genetic differentiation (Φ_{ST}) based on 12S rDNA, B) pairwise geographic distances between populations (km) versus genetic differentiation (F_{ST}) based on microsatellites, and C) geographic distances versus genetic differentiation (Φ_{ST}) based on 12S rDNA. The adjusted regression equations and r^2 are presented inside the plot. Only the regression shown in 3A was significant (p = 0.028).

Table 3-1 Estimates of genetic diversity among 20 *Daphnia* populations at 13 microsatellite loci across Europe. Two-letter abbreviations of localities (in parentheses) are used throughout the manuscript. The first three lines provide data on *D. galeata* (gal), the next three lines provide data on *D. cucullata* (cuc) and the remaining populations represent *D. longispina* (ls).

Locality	Country	Taxon	Latitude	Longitude	Area (km^2)	Sampling date	*N*	MLG_{t0}	MLG_{t3}	MLG_{t0}/N	MLG_{t3}/N	Div_{t0}	Div_{t3}	PA	H_{exp}	H_{obs}	HWE	HD	$F_{IS(all)}$	$F_{IS(woc)}$	LD_{prop}	LD_{prop*}	N_{mtNDA}	N_{hap}
Cogollos (CO)	Spain	gal	37.210 N	2.835 W	0.119	05.09.2001	32	31	n.c.	0.97	n.c.	n.c.	n.c.	4	0.33	0.31	*	0.1	n.c.	n.c.	n.c.	n.c.	-	-
Delftse Houd (DH)	Netherlands	gal	51.980 N	4.350 E	23	16.06.2001	31	9	n.c.	0.29	n.c.	n.c.	n.c.	4	0.41	0.50	0.10	0.6	n.c.	n.c.	n.c.	n.c.	7	3
Stanovice (ST)	Czech Rep.	gal	50.175 N	12.880 E	1.4	09.07.2004	39	23	n.c.	0.59	n.c.	n.c.	n.c.	3	0.34	0.38	0.74	0.9	n.c.	n.c.	n.c.	n.c.	6	2
Brno (BR)	Czech Rep.	cuc	49.260 N	16.455 E	2.3	20.07.2004	33	33	n.c.	1.00	n.c.	n.c.	n.c.	2	0.31	0.28	*	*	n.c.	n.c.	n.c.	n.c.	5	1
Drabužis (DR)	Lithuania	cuc	54.567 N	24.650 E	0.514	24.09.1995	29	29	n.c.	1.00	n.c.	n.c.	n.c.	5	0.30	0.20	*	*	n.c.	n.c.	n.c.	n.c.	-	-
Usingen (US)	Germany	cuc	50.344 N	8.509 E	0.016	15.08.2006	36	36	n.c.	1.00	n.c.	n.c.	n.c.	0	0.32	0.30	*	*	n.c.	n.c.	n.c.	n.c.	6	3
Badajoz - Villar del Rey (BA)	Spain	ls (r)	39.178 N	6.846 W	12.74	16.05.1997	40	28	20	0.70	0.50	0.97	0.94	5	0.67	0.66	*	*	0.028	0.069	0.987	0.872	2	2
Goksjø (GO)	Norway	ls (h)	59.173 N	10.165 E	3.32	01.09.1999	42	42	42	1.00	1.00	1.00	1.00	7	0.59	0.43	*	*	0.280	0.280	0.064	0.064	10	6
Göteborg (GB)	Sweden	ls (r)	57.700 N	12.000 E	<0.005	02.05.2004	20	20	20	1.00	1.00	1.00	1.00	1	0.48	0.39	*	*	0.222	0.222	0.046	0.030	10	3
Ismaning (IS)	Germany	ls (r)	48.221 N	11.772 E	<0.01	22.09.2004	41	41	41	1.00	1.00	1.00	1.00	5	0.69	0.53	*	*	0.245	0.245	0.051	0.051	10	1
Mondsee (MS)	Austria	ls (h)	47.850 N	13.390 E	13.8	28.10.2004	24	23	21	0.96	0.88	1.00	0.99	0	0.41	0.34	*	*	0.178	0.191	0.135	0.068	10	3
Nižné Jamnícke (NJ)	Slovakia	ls (r)	49.203 N	19.772 E	0.01	24.09.2000	42	42	28	1.00	0.67	1.00	0.96	2	0.35	0.33	*	*	0.088	0.088	0.051	0.051	13	5
Nordfjordvatn (NV)	Norway	ls (r)	69.273 N	19.021 E	0.27	25.09.2001	32	32	6	1.00	0.19	1.00	0.65	1	0.21	0.19	*	*	0.078	0.078	0.022	0.022	10	1
Piano (PI)	Italy	ls (h)	45.780 N	10.230 E	0.63	03.07.1999	32	26	16	0.81	0.50	0.97	0.91	1	0.59	0.67	*	0.9	-0.128	-0.090	0.782	0.692	3	1
Satanie (SA)	Slovakia	ls (r)	49.170 N	20.063 E	<0.005	27.09.2003	31	7	1	0.23	0.03	0.72	0.00	0	0.07	0.09	1	0.4	-0.234	0.182	1.000	0.000	7	2
St. Bernard (SB)	Switzerland	ls (z)	45.871 N	7.170 E	<0.005	06.09.2005	32	30	7	0.94	0.22	1.00	0.44	1	0.25	0.21	*	*	0.153	0.158	0.000	0.000	8	3
Stechlinsee (SS)	Germany	ls (h)	53.150 N	13.033 E	4.25	10.05.2004	31	31	30	1.00	0.97	1.00	1.00	1	0.53	0.42	*	*	0.207	0.207	0.064	0.064	10	2
Storveavatn (SV)	Norway	ls (r)	64.844 N	11.376 E	0.578	08.09.1998	32	32	32	1.00	1.00	1.00	1.00	0	0.47	0.42	*	*	0.138	0.138	0.051	0.051	10	2
Vranov (VR)	Czech Rep.	ls (h)	48.908 N	15.817 E	7.7	11.10.2004	32	32	30	1.00	0.94	1.00	0.99	0	0.58	0.56	*	0.1	0.047	0.047	0.218	0.231	7	3
Zahillo (ZH)	Spain	ls (r)	36.990 N	6.510 W	<0.01	12.05.1997	32	20	4	0.63	0.13	0.96	0.66	1	0.21	0.22	*	0.1	-0.036	0.006	0.429	0.191	9	2
Total							463	406	298	0.88	0.64	0.97	0.82	29	0.44	0.39			0.090	0.130	0.280	0.170	119	36

Morphotypes of *D. longispina* populations are indicated by a letter in parentheses: ls (h) = *hyalina*, ls (r) = *rosea*, ls (z) = melanised population from the type locality of *D. zschokkei* Stingelin (see methods and Petrusek *et al.* 2008 for details). Total = total and average values for all *D. longispina* populations (excluding *D. galeata* and *D. cucullata*). Abbreviations: *N* = number of individuals; MLG_{t0}, MLG_{t3} = number of multi-locus genotypes (without threshold and with threshold of 3, respectively); Div_{t0}, Div_{t3} = genetic diversity (without threshold/with threshold of 3); PA = number of private alleles, H_{exp}, H_{obs} = expected and observed heterozygosity; HWE = Hardy-Weinberg-Equilibrium; HD = heterozygote deficiency; $F_{IS(all)}$, $F_{IS(woc)}$ = inbreeding coefficients (using all individuals and excluding identical multi-locus genotypes, respectively), LD_{prop}, LD_{prop*} = proportion of loci which deviate significantly from linkage equilibrium (using all individuals and excluding repeated identical multi-locus genotypes, respectively); N_{mtDNA}, N_{hap} = number of individuals sequenced and number of different haplotypes, respectively; n.c. = not calculated; asterisks indicate $p < 0.05$; Rep. = Republic.

Genetic differentiation within populations and among clonal lineages

In general, we found high F_{ST} values combined with high clonal diversity (**Table 3-1**). Out of 463 *D. longispina* individuals screened using 13 microsatellite loci, we found 406 different MLGs. Populations varied in their clonal diversity, which apparently reflects different levels of clonal selection, and identical MLGs occurred only within, but never among populations. Even when taking scoring errors, PCR artifacts and (somatic) mutations into account, we detected at least 298 different MLGs (**Table 3-1**; **Figure 3-4**A, B). The average clonal diversity of all *D. longispina* populations was 0.97 (Div_{t3} = 0.82) and ranged from 0.72 (Div_{t3} = 0.00) in Satanie to 1.00 (Div_{t3} = 0.44–1.00) in Göteborg, Goksjø, Ismaning, Mondsee, Nižné Jamnícke, Nordfjordvatn, St. Bernard, Stechlinsee, Storveavatn and Vranov (**Figure 3-3**C). Removing all populations that contained recombinant genotypes did not change the pattern, their average genetic diversity was 0.97 (Div_{t3} = 0.77).

Based on the resolution power of the 13 selected microsatellite markers, we simulated diversity estimates (MLG/*N*, where *N* = number of individuals) using various subsets and combinations of markers (1–13). For most of the populations, we found that five to six markers were sufficient to uncover around 90% of all MLGs (identified using 13 loci). However, for populations with a lower level of genetic variation, MLG/*N* curves did not reach saturation, indicating that a larger number of nuclear markers (> 10) would be needed to reveal the majority of genotypes, and using a lower number of less variable markers would substantially underestimate real clonal diversity (**Figure 3-4**C).

The observed level of heterozygosity per population ranged from 0.09 in Satanie to 0.67 in Piano (**Table 3-1**). Exact tests (Guo & Thompson 1992) revealed strong deviations from Hardy–Weinberg equilibrium for almost all populations and at most of the loci (**Table 3-1**). Ten out of 14 cases showed significant deficiencies of heterozygotes. Only three populations (Piano, Satanie and Zahillo) showed negative $F_{IS(all)}$ values. If repeated with identical MLGs removed, no large negative values of $F_{IS(woc)}$ were obtained (**Table 3-1**). Most populations exhibited a low proportion (less than 10%) of loci which significantly deviated from linkage equilibrium (LD_{prop}; **Table 3-1**). Only three populations (Mondsee, Satanie and Zahillo) exhibited a higher proportion of such loci. However, this proportion substantially decreased if repeated identical multi-locus genotypes were removed from the analysis.

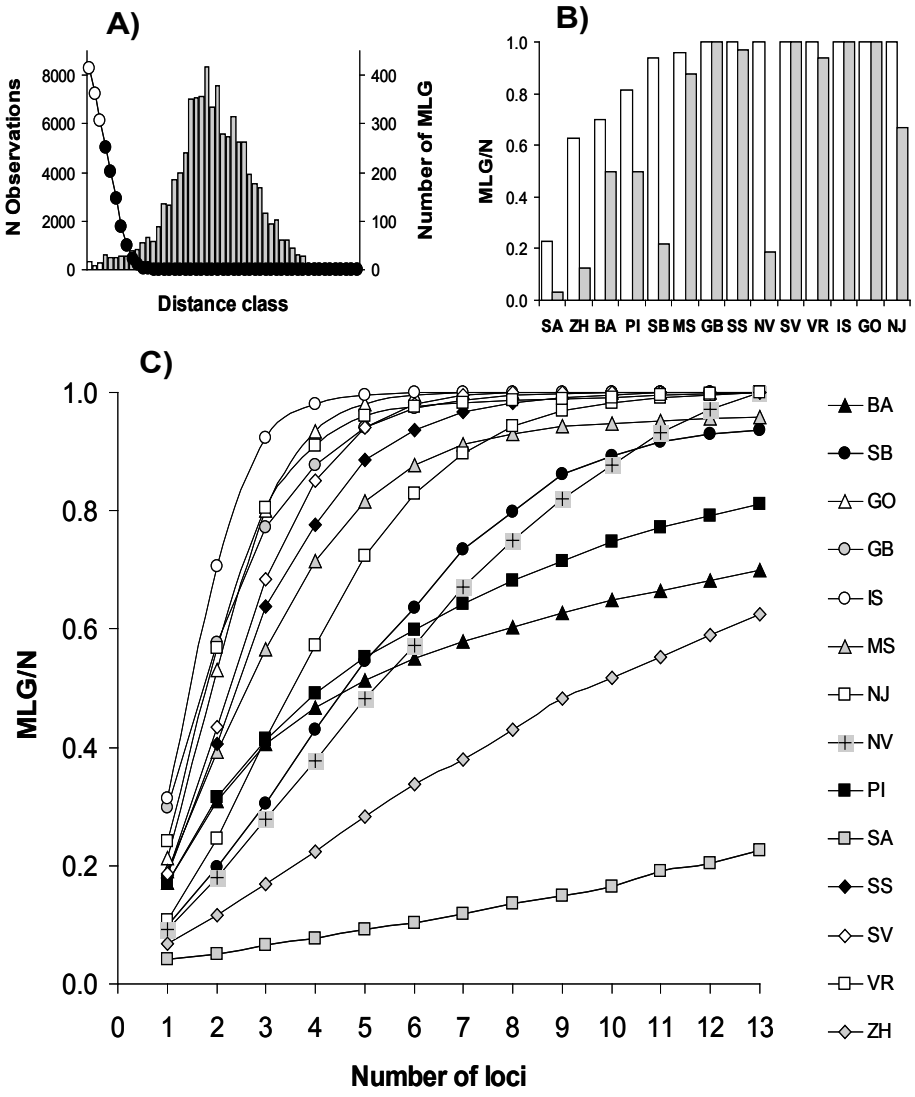

Figure 3-4 A) Frequency distribution of pairwise distances and the number of multi-locus genotypes detected (MLG), assuming either no threshold (N = 406 MLG; circles) or discarding the first three distance classes (N = 298 MLG; black circles only); B) MLG/N per population, either using all distance classes (white columns) or the reduced number of distance classes (grey columns). C) Relative number of multi-locus genotypes (MLG/N) per population as detected by different numbers of loci (one to 13 loci). Abbreviations of populations as in **Table 3-1**.

Discussion

Previous studies on large lake inhabiting *Daphnia* populations (*D. galeata, D. hyalina* – i.e. lake *D. longispina* form*, D. cucullata, D. pulicaria*), relying on allozyme markers, showed high MLG diversity, genotype frequencies in good agreement with Hardy-Weinberg expectations, and rather low levels of among-population genetic differentiation (Černý & Hebert 1993; Mort & Wolf 1985, 1986). More recent studies reported relatively low genotypic diversity and prominent shifts in genotype composition and allele frequencies within a growing season (Jankowski & Straile 2004; King *et al.* 1995; Weider & Stich 1992).

The *D. longispina* populations studied by us showed a very high average genetic diversity (mean Div_{t0} = 0.97; **Table 3-1**), suggesting a sexual phase in most populations and little effects of clonal selection on the genetic diversity, which is in concert with several previous studies. However, the level of genetic variation within our studied populations was much higher than expected from studies based on allozyme electrophoresis. This discrepancy most likely, at least in part, reflects different analytical methods. It is probable that unique MLGs detected by allozyme markers represent in most cases mixtures of a substantial number of different clones. Our data strongly suggest that even within large lake species, recruitment from dormant egg banks is an important factor for build-up of new populations at the beginning of the growing season.

Strong deviations from Hardy-Weinberg equilibrium were detected for almost all populations (13 out of 14 populations), and for a majority of loci. For most (10 out of 13) of these deviating populations, we observed significant heterozygote deficiencies (**Table 3-1**). This pattern indicates either inbreeding or the co-occurrence of at least two reproductively isolated subpopulations (Wahlund effect), although other factors such as recent immigration, recent selection at linked loci, and chance effects may also play a role. We suggest that recruitment from the dormant egg pools causes a very dynamic and heterogeneous assembly of short-lived clones. The combination of overlapping generations and chance effects are most likely responsible for deviations from Hardy-Weinberg equilibrium. In contrast, evolutionary young populations which lack a sufficient dormant egg pool, and which had been possibly founded by a few individuals carrying a limited number of alleles, are expected to exhibit only lower number of multi-locus genotypes. An example for this pattern is the population of Satanie; this lake was re-colonized only recently after an acidification period (Petrusek *et al.* 2007).

Following the recommendations of Halkett *et al*. (2005) for the analysis of partially clonal organisms, we found convincing evidence for strong effects of sexual reproduction among most of the studied *Daphnia* populations. The number of repeated multi-locus genotypes (even taking scoring and interpretation errors into account), the proportion of linked pairs of loci, and mean F_{IS} values among loci suggest a relatively low contribution of parthenogenetic reproduction to the genetic structure of most of the analyzed populations (**Table 3-1** and **Figure 3-4**B).

Although we tried to select samples which harbored only one morphotype in order to avoid interspecific hybrids, three populations in our data set exhibited individuals which probably have originated from interspecific hybridization. The factorial correspondence analysis suggested recombinant genotypes generated by the interbreeding of *D. longispina* and

D. galeata (Lake Piano, Italy; reservoir Vranov, Czech Republic) and interbreeding between *D. longispina* and the lineage found in Zahillo (Spain). However, both the Bayesian assignment method (STRUCTURE; **Figure 3-2**B) and a test for interspecific hybrids (using NEWHYBRIDS; Anderson & Thompson 2002) identified only few populations containing individuals that exhibit recombinant genotypes. In addition, we found in 10 out of 14 populations significant heterozygote deficiencies, which is in stark contrast to the expectation of multiple hybridization events which would lead to heterozygote excess. Thus, we exclude the possibility that interspecific hybridization or introgression explains the high genotypic diversity we found in the majority of populations.

Although many zooplankton species are passively dispersed by dormant stages via wind or birds (Bilton *et al.* 2001; Figuerola *et al.* 2005), many studies have reported high levels of genetic differentiation among populations (e.g., Gómez & Carvalho 2000; Vanoverbeke & De Meester 1997). High F_{ST} and G_{ST} values up to 0.7 were detected in neighboring *Daphnia* populations (Hebert *et al.* 1993; Ishida & Taylor 2007a; Pálsson 2000; Vanoverbeke & De Meester 1997). In general, we found highly similar patterns of genetic differentiation in 12S rDNA sequences and microsatellites (**Figure 3-3**A), despite the large difference in mutation rates, dispersal probabilities and modes of inheritance among mitochondrial and nuclear markers. Nearly all populations were characterized by private MLGs and private haplotypes. Other invertebrates from continental waters, including other *Daphnia* taxa, show either moderate (De Gelas & De Meester 2005) or strong overall genetic divergence (Ketmaier *et al.* 2005; Muñoz *et al.* 2008; Zierold *et al.* 2007) or a differential pattern of nuclear or mitochondrial DNA (Freeland *et al.* 2000). Our data, however, show strong genetic divergence in both nuclear and mitochondrial loci, suggesting that population differentiation is not a recent phenomenon, but persisted probably since the initial colonizations and the build-up of large propagule banks.

The close association of mitochondrial and nuclear divergence among populations confirms the prediction that dispersal in *D. longispina* is egg-mediated, where dormant eggs are produced sexually and hatch into parthenogenetic females, thus offspring among immigrant and local genotypes are able to exchange mitochondrial and nuclear genes. Although empirical studies on other *Daphnia* species described phenomena such as male-only producing clones or allochronic differentiation (Ferrari & Hebert 1982; Hobæk & Larsson 1990), which may lead to reduced likelihood of transmission of mitochondrial genes and therefore to other patterns in population differentiation, our data suggest that such processes did not have strong influence on *D. longispina* population structure.

Genetic differentiation among populations of *D. longispina* suggests low levels of gene flow among populations and persistent founder effects, not only on the continental scale but also among neighboring populations (see also Petrusek *et al.* 2007). This observation is consistent with the *monopolization hypothesis* for cyclic parthenogens (De Meester *et al.* 2002) which predicts high population differentiation due to rapid population growth, potential for local adaptation and the monopolization of resources (priority effects). The alternative explanation that the pattern of strong genetic differentiation among populations is caused solely by high levels of genetic drift is unlikely given the high levels of genetic variation observed within populations.

We detected no evidence for isolation-by-distance in mtDNA and ncDNA. Some populations, which were separated by great geographical distances, showed only relatively low levels of differentiation (e.g. Göteborg and Ismaning; F_{ST} = 0.17 based on microsatellite data) whereas some populations relatively close to each other (e.g. Satanie and Nižné Jamnícke) were highly genetically differentiated (F_{ST} = 0.62). This is despite their ecological similarity – both localities of the latter pair are alpine lakes – suggesting that the large genetic differentiation of the local *Daphnia* populations results from independent colonization of the lakes (Petrusek *et al.* 2007). Overall, the highest genetic differentiation was detected between the Central European populations and Zahillo and Nordfjordvatn in the periphery of the sampled range. Although the mitochondrial markers confirmed the close relationship to other *D. longispina* populations, the Zahillo population was substantially divergent at the microsatellite markers. Individuals of Zahillo might possibly represent independent evolutionary units. They share, however, alleles with the second Spanish population (Badajoz, Villar del Rey; **Figure 3-2**) and group well in the overall *D. longispina* cluster (see **Figure 3-1**). Whether these elevated levels of intraspecific divergence are caused by ecological processes (Zahillo was the only representative of a temporary dune lake in our data set), incomplete sampling, or the geographical isolation remains open for further studies. Based on the large morphological and ecological divergence within *D. longispina,* we predict that the populations from Zahillo and probably also Nordfjordvatn represent lineages on the dawn of speciation. They offer the unique opportunity to study the origin and the maintenance of genetic divergence in a cyclical parthenogenetic freshwater zooplankter under natural conditions.

Our findings of a lack of differentiation among various *Daphnia* morphotypes (previously named *D. rosea*, *D. hyalina* and *D. zschokkei*) based on microsatellite and mitochondrial data sets are consistent with results of previous genetic studies (Gießler *et al.* 1999; Petrusek *et al.* 2008; Schwenk *et al.* 2000; Taylor *et al.* 1996) and support the interpretation that phenotypic

differences between these morphs represent only intraspecific variation (Petrusek *et al.* 2008). Hierarchical analysis of genetic variation confirmed that the differentiation among populations is much higher than differentiation among morphotypes (see also **Figure 3-2**). Neither phylogenetic nor factorial correspondence analysis identified an association of formerly recognized species and genetic clusters, suggesting that previous classifications were based on polymorphic traits which do not reflect the phylogenetic history.

Conclusions

Population structure in *Daphnia longispina* could not be explained solely by gene flow and genetic drift, as the patterns of mtDNA and ncDNA diversity are significantly associated. Populations show strong genetic differentiation without isolation-by-distance, and highly structured populations are found in geographical proximity. The observed patterns are in concordance with the *monopolization hypothesis*, and most likely reflect priority effects mediated by rapid population growth and potential for local adaptation, limiting effective gene flow due to a reduced establishment success of immigrants. Most populations of *D. longispina* are characterized by high clonal diversity, low levels of clonal selection, predominance of sexual reproduction and heterozygote deficiencies. The latter is most likely caused by multiple events of hatching from the dormant egg banks, the co-existence of clones of different temporal origin leading to a Wahlund effect. In general, the diversity within *D. longispina* populations seems to be closer to sexual than obligate asexual organisms despite extended phases of clonal reproduction during the growing season and an opportunity for substantial clonal erosion.

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Chapter 4

The impact of historical and contemporary environmental changes on the population genetic structure of large lake *Daphnia* species

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Abstract

The population genetic structure of cyclic parthenogenetic freshwater zooplankton is characterized by clonal diversity that is found within populations and the genetic differentiation detected among populations. Although the factors influencing within population clonal diversity are often studied and discussed, the mechanisms regulating effective gene flow among populations are less understood. Especially the impact of severe environmental changes, either natural or human-mediated, might be important for the population genetic structure of species. We used three syntopically occurring taxa of the *Daphnia longispina* complex that may have responded differently to ancient and contemporary environmental changes and thus may exhibit different population genetic structures. We studied the clonal diversity using twelve microsatellite loci and further applied a mitochondrial DNA marker to detect potential expansion and bottleneck events. For two species we detected an expansion in effective population size, *Daphnia longispina* most probably expanded due to the formation of oligotrophic glacial lakes after the last ice age, while *D. galeata* successfully distributed recently due to the severe changes in many water bodies, shifting to a more eutrophic status. Although we expected a recent expansion of *D. cucullata*, due to reduced vulnerability to fish predation, we found no significant changes in effective population size. This pattern might be explained by the frequent interspecific hybridization with *D. galeata*. Overall, ancient processes such as glaciation as well as current, mainly man-made environmental changes explain local genetic diversity as well as patterns of dispersal and gene flow among large lake *Daphnia* populations.

Unpublished manuscript

Introduction

The more or less regular alternation of asexual and sexual reproduction is called cyclic parthenogenesis and evolved amongst others in the zooplankton groups: Monogononta (monogonont Rotifera) and Cladocera (Hebert 1987). Individuals develop from amictic eggs during the asexual phase of the life cycle as long as environmental conditions seem favorable. Cues for changing environmental deterioration induce sexual reproduction (Hebert 1987) and the resulting dormant eggs, which are encased in a protective structure (ephippium) in cladocerans, enable the survival of harsh conditions, like freezing and drying (Brendonck & De Meester 2003), as well as the dispersal with different vectors, like animals, wind, and water currents (Havel & Shurin 2004). This special reproduction has consequences for the population genetic structure of those animals. Recently, De Meester *et al.* (2006) summarized the major factors impacting the clonal diversity within populations: the length of the growing season, the strength of clonal selection and the size of the dormant egg bank. The longer the growing season is the longer is the asexual phase in that clonal erosion, due to selection and drift processes, will reduce the variability within the population (Vanoverbeke & De Meester 2010). Additionally, strong clonal selection will act negatively on the genetic diversity as it increases clonal erosion (e.g. due to parasitism; Yin *et al.* 2012a). More competitive clones will limit the growth of inferior clones, which eventually go extinct over time (**Chapter 5**). The last factor, the size of the active dormant egg bank, has a positive impact on genetic diversity. Larger egg banks store more genetic variation, as dormant eggs are produced sexually, than smaller ones.

Clonal diversity in local populations is not only explained by processes within populations (e.g. clonal erosion and selection), but also by processes among populations. For example, the level of dispersal, the age of populations as well as environmental change (in contemporary and historical times). In general, among populations of freshwater invertebrates, including cyclic parthenogens, high levels of genetic differentiation were found (Boileau *et al.* 1992; Gómez & Carvalho 2000; Hebert 1974a, b; Hebert & Wilson 1994; Pálsson 2000; Thielsch *et al.* 2009; Vanoverbeke & De Meester 1997; Xu *et al.* 2009) indicating low impact of effective gene flow on the population structure, although many evidence for highly efficient dispersal was recovered (e.g., Figuerola & Green 2002; Figuerola *et al.* 2003). This obvious paradox of high dispersal capacity but low gene flow is supposed to occur due to stochastic and adaptive processes summarized as the *monopolization hypothesis* (De Meester *et al.* 2002). Through the efficient colonization and the explosive population growth encountered in partly asexual freshwater animals an advantage arises as this population reaches carrying capacity which

makes it difficult for other genotypes to invade (priority effect). During the sexual phase, dormant eggs are produced that are incorporated in the dormant egg bank which provides an extremely powerful buffer, as new genotypes hatch as soon as conditions are favorable (e.g. hatchlings observed one week after refilling of a pond by Cáceres & Tessier 2004). Furthermore, clonal selection and sexual reproduction may lead to locally adapted genotypes compared to those invading the habitat afterwards (De Meester *et al.* 2002) further enhancing the efficient prevention of invading genotypes. In principle, this hypothesis assumes a rather stable habitat, however, recent studies indicate that a severe change in environmental conditions may overrule monopolization because pre-adapted genotypes become inferior to invading clones (Brede *et al.* 2009; Jankowski & Straile 2003; Rellstab *et al.* 2011). These studies focused on rapid shifts in species abundances associated with environmental change (such as eutrophication) and thus raise the question whether environmental changes may also alter the local clonal structure of populations. In particular, as man-made ecological changes have been significantly altered water bodies probably as early as 6000 years BP (Fritz 1989; Korponai *et al.* 2010). Starting with industrialization around 200 years ago effects on freshwater habitats became even more severe. The main consequences are increased acidification and pollution of habitats (Cammarano & Manca 1997; Nevalainen *et al.* 2011), the increased intentional introduction of alien species (Hesthagen & Sandlund 2004; Knapp *et al.* 2001), the repeated invasion of hitchhiking species, for example in ballast water of ships (Hebert & Cristescu 2002; Taylor & Hebert 1993a), the damming of rivers changing lotic to rather lentic habitats creating ecological gradients, massive eutrophication of water bodies (Correll 1998) as well as increased fish stocking for aquaculture since the middle of the 20th century (Musil *et al.* 2010). Besides human-induced environmental changes, natural events such as the last ice age played an important role in shaping current animal population genetic structures across Europe (Hewitt 2000). During the last glacial maximum (17000-25000 BP; Petit *et al.* 2003) the ice sheet covered North and parts of Central Europe and animals that did not migrate actively become eventually extinct. In widely distributed species the survival was assured in unglaciated refuge regions, like Mediterranean Europe (Hewitt 1996) or eastern Siberia (Weider & Hobæk 1997, 2003). This was probably also the fate of many freshwater inhabitants, like cladocerans and rotifers. As the ice sheet retreated slowly leaving numerous new oligotrophic glacial lakes and ponds behind, range expansion from refuge areas occurred. The colonizers of these new populations would dominate the populations gene pool as later migrants would contribute little as they would be entering established populations at carrying capacity with only replacement dynamics (Hewitt 1996). Without any significant level of

effective gene flow those populations had the opportunity to differentiate over time and to establish patterns of strong local adaptation (monopolization).

The main aim of our study was to assess the impact of historical and contemporary environmental changes on the population genetic structure of cyclic parthenogenetic zooplankton animals. Therefore, we studied three ecologically differentiated species belonging to the genus *Daphnia* (*D. cucullata*, *D. galeata*, and *D. longispina*) which are widespread and inhabit a large range of different pond and lake environments in the Palaearctic. Hybridization among these species is common whereas *D. galeata* is the most prominent hybridization partner (Schwenk 1993; Schwenk 1997; Schwenk *et al.* 2000; Schwenk & Spaak 1995) and also produces hybrids with other than the above mentioned species (Hobæk *et al.* 2004; Ishida *et al.* 2011; Taylor & Hebert 1993a; Taylor *et al.* 2005). Although syntopically occurring, the three species occupy different ecological niches. Taxon *D. longispina* favors deep, oligotrophic freshwater lakes with no or relaxed fish predation (Gliwicz 2003), is also found in alpine regions above the tree line as well as in large pools, sometimes smaller ponds, and in slow flowing water (Benzie 2005; Flößner 2000). After the retreat of the ice sheet glacial lakes formed that represented ideal habitats for *D. longispina* which very likely resulted in an expansion of this taxon and in the massive founding of new populations. However, recent eutrophication events and increased fish stocking probably caused the replacement by other taxa, like *D. galeata* (Brede *et al.* 2009; Nilssen *et al.* 2007) which is found in small to very large, permanent ponds and lakes; rarely in temporary waters (Benzie 2005; Flößner 2000). The successful immigration of *D. galeata* into eutrophicated deep stratified lakes and reservoirs is observed (Flößner 2000) which entailed probably a recent population expansion. *D. cucullata* is most abundant in cool temperate and montane, but not arctic areas (Benzie 2005; Flößner 2000). In general, it is found in small and large shallow lakes rarely in small ponds; sometimes it occurs in deep stratified lakes or reservoirs due to recent eutrophication, where it is found either in the upper layers of the epilimnion (Flößner 2000) or in the upstream regions (Vaníčková *et al.* 2010). *D. cucullata* is known to be well adapted to fish predation, especially due to its small body size. However, it is competitively inferior to larger *Daphnia* species under somewhat relaxed predation conditions. Consequently, localities with high fish densities, especially eutrophic ones, are often inhabited by *D. cucullata*. This taxon may be a beneficiary of the human-mediated fish breeding, stocking and transportation during the last centuries (Balon 1995). Thousands of fish ponds were created in Europe in order to provide suitable habitats for fisheries (Van

Damme *et al.* 2007) being highly eutrophic with intense predation pressure and therefore ideal for *D. cucullata*.

Based on these assumptions we analyzed the population genetic structure of *D. cucullata*, *D. galeata*, and *D. longispina* to identify the influence of natural and human-mediated environmental changes on the effective gene flow among populations. Hence, we studied the clonal diversity and genetic differentiation within and among several populations belonging to one of the three syntopically occurring species across Europe using microsatellite and mitochondrial DNA. Both molecular markers detect different genetic patterns: Microsatellite DNA reveals fine-scale structures that enable the detection of population diversity and recent gene flow, while mitochondrial DNA has the ability to unravel historical processes such as the demographic development over time. In particular, we used these methods to detect if *D. galeata* and *D. cucullata* show a recent population expansion due to anthropogenic changes, like eutrophication of water bodies and increased handling and breeding of fish, and if *D. longispina* reveals amore ancient expansion, for example due to the formation of glacial lakes after the last ice age.

Material and Methods

Origin of studied populations and molecular procedures

We studied 44 natural populations including individuals that belong to *D. cucullata*, *D. galeata*, and *D. longispina*. The populations were sampled across a large geographic range covering Europe (see **Table 4-1**, **Figure 4-1**). Samples were collected using plankton nets with mesh sizes of 100-200 µm, in case of ponds and small lakes by oblique tows from the shore, in large lakes by vertical or oblique tows from a boat. After collection, the zooplankton was preserved in 70-96% ethanol. A subset of the populations was already published in a different study assessing the detailed population genetic structure of *D. longispina* (Thielsch *et al.* 2009). Further, the microsatellite markers used in this study were recently evaluated for their suitability for species identification and hybrid detection by using the same 44 populations (**Chapter 2**). Therefore, methods for DNA preparation, amplification and sequencing of the 12S ribosomal RNA gene as well as genotyping using twelve microsatellite loci (DaB10/14, DaB17/17, Dgm105, Dgm109, Dgm112, Dp196NB, Dp281NB, Dp519, SwiD6, SwiD12, SwiD14 and SwiD18; Brede *et al.* 2006) are described in detail in **Chapter 2** and **3**.

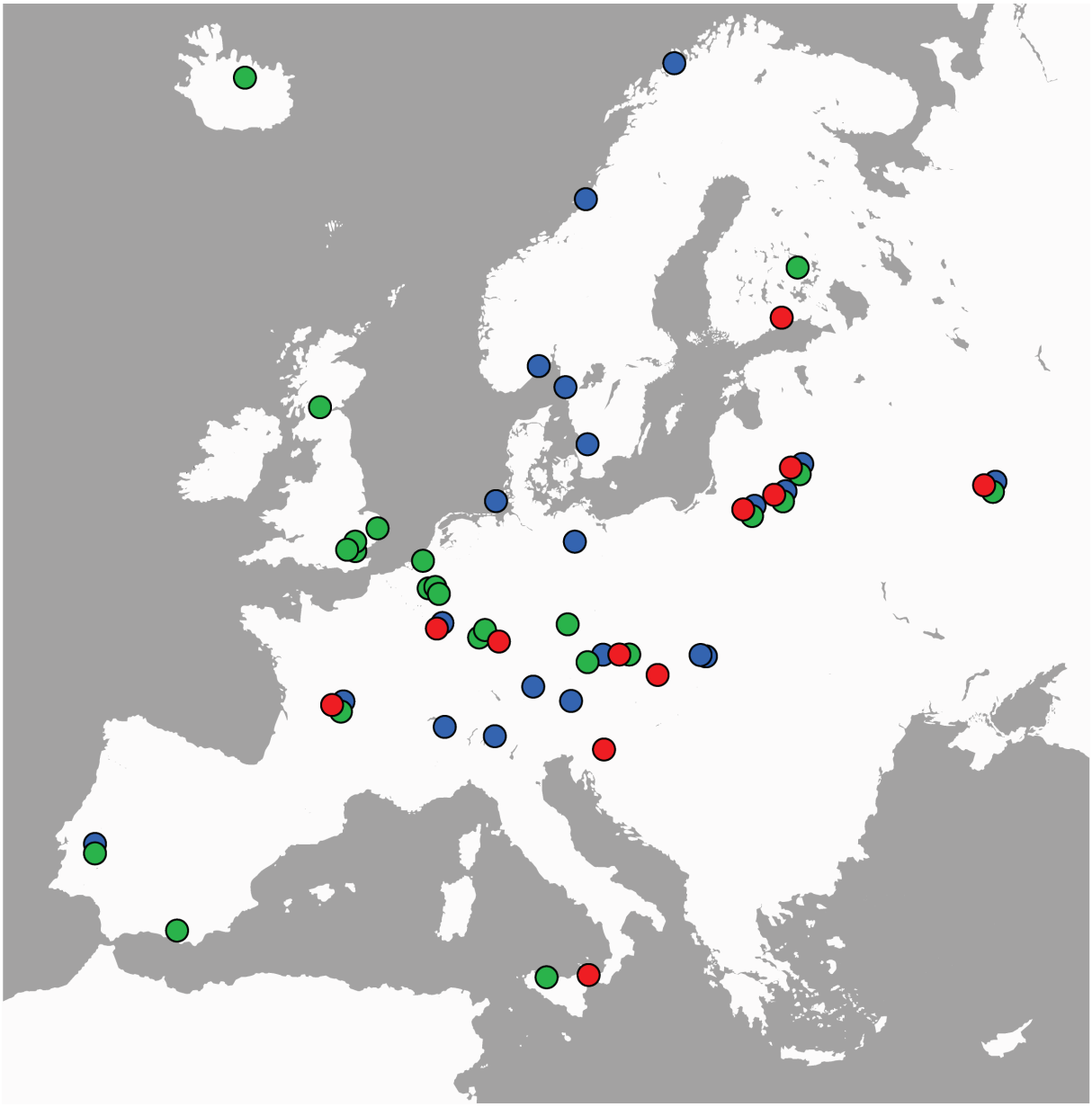

Figure 4-1 Approximate locations of the studied populations within Europe. Red circles represent *D. cucullata* populations, blue circles *D. longispina* populations, and green circles *D. galeata* populations.

Statistical analyses of mitochondrial DNA

Raw sequences were manually proofread using CHROMASPRO 1.5 (Technelysium Pty Ltd, 2003-2009) and aligned using ClustalW algorithm (Thompson *et al.* 1994) implemented in BIOEDIT (Hall 1999). We estimated the number of haplotypes and calculated summary statistics per species and population without considering alignment gaps: Haplotype diversity (*Hd*), the probability that two randomly chosen individuals have different haplotypes; nucleotide diversity (π), the average pairwise nucleotide difference between individuals within samples (Nei 1987); the extent of genetic differentiation between populations (N_{ST}; Lynch & Crease 1990); Fu and Li's D* (Fu & Li 1993) and Fu's F_S (Fu 1997), to test the hypothesis that all mutations are selectively neutral (Kimura 1983). We performed all tests in

DNASP 5.10.01 (Librado & Rozas 2009). Significance of F_S was determined by 1000 coalescent simulations of the neutral model, while that of D* was determined by the critical values presented in Fu & Li (1993), where *p* must be less than 0.02 to be significant due to the non-normal distribution of the statistic. D* and F_S are assumed to be zero when mating is random and populations have reached equilibrium. Negative values signify an excess of low frequency polymorphisms relative to expectation, indicating population size expansion, selective sweeps, or background selection; while positive values signify an excess of intermediate-frequency alleles, indicating a decrease in population size (bottleneck) or balancing selection. Fu (1997) found that F_S has more power to detect population growth and genetic hitchhiking than D*, while the opposite is true for background selection.

Isolation-by-distance (IBD) was tested using IBDWS (Jensen *et al.* 2005): Slatkin's (1993) similarity index M ($M = ((1/\Phi_{ST})-1)/4$; 10000 randomizations; gaps and missing data were ignored) was used as genetic distance measure and geographic estimates were measured in kilometers. Median joining networks were constructed for each species using NETWORK 4.6 (Bandelt *et al.* 1999).

The Bayesian skyline plot model (Drummond *et al.* 2005), as implemented in BEAST 1.6.2 (Drummond *et al.* 2002; Drummond & Rambaut 2007), uses standard Markov chain Monte Carlo (MCMC) sampling procedures to estimate a posterior distribution of effective population size through time directly from a sample of gene sequences. Unlike previous methods, the Bayesian skyline plot includes credibility intervals for the estimated effective population size at every point in time, back to the most recent common ancestor of the gene sequences. These credibility intervals represent both phylogenetic and coalescent uncertainty.

Two independent MCMC analyses with 2.5×10^8 steps for *D. cucullata* and 5×10^8 steps for *D. galeata* and *D. longispina* were performed sampling every 10000^{th} generation with a burn-in of 10%. Substitution models per species (*D. cucullata*: HKY, *D. galeata*: TN93, and *D. longispina*: TN93 + G) were determined using JMODELTEST and the Bayesian information criterion (Posada 2008). A strict molecular clock was used (Drummond *et al.* 2006) and we applied a strong prior (mean 2.3×10^{-8} substitutions per site and year, lower bound = 1×10^{-8}, upper bound = 4×10^{-8}) that corresponds to 2.3% sequence divergence per one million year (Brower 1994) within a reasonable range to all analyses. The hyperparameter m (number of grouped intervals) was set to 20 for each taxon.

Table 4-1 Data of natural populations studied using analysis of twelve microsatellite loci as well as sequencing of 12S rRNA gene. Names of populations are given together with country of origin, corresponding geographical coordinates, number of individuals used for this study using microsatellite markers ($N_{\mu sat}$) and 12S rDNA (N_{12S}) as well as amount of individuals belonging to either *D. galeata* (N_{gal}), *D. longispina* (N_{lon}), or *D. cucullata* (N_{cuc}); first number shows the assignment of individuals using microsatellite markers according to STRUCTURE 2.3.3 ($K = 3$; assignment probability > 0.95) and second number shows the number of individuals with sequences belonging to that particular taxon. The last columns indicate how many individuals per population belong to which hybrid class according to NEWHYBRIDS (assignment probability > 0.80): F_1 = F_1 hybrid, BC_{lon} = backcross with taxon *D. longispina*, BC_{gal} = backcross with *D. galeata*.

Population	Origin	Abbreviation	Longitude	Latitude	Sampling date	$N_{\mu sat}$	N_{12S}	N_{gal}	N_{lon}	N_{cuc}	GxC	LxC	LxG
Mondsee	Austria	AT-MS	47°50'26" N	13°22'48" E	2004 Oct 28	22	10	-/-	22/10	-/-			
Beringen	Belgium	BE-BE	51°00'20" N	05°17'20" E	2007 May 09	35	10	35/10	-/-	-/-			
Diest	Belgium	BE-DI	50°59'16" N	05°03'52" E	2007 May 09	45	10	43/10	-/-	-/-	2x F_1		
Maaseik	Belgium	BE-MA	51°05'49" N	05°48'17" E	2007 May 09	20	12	6/12	-/-	-/-	14x F_1		
St. Bernard	Switzerland	CH-SB	45°52'19" N	07°10'13" E	2005 Sep 06	32	13	-/-	32/13	-/-			
Brno	Czech Republic	CZ-BR	49°15'36" N	16°27'18" E	2004 Jul 20	39	12	5/1	-/-	34/11			
Římov	Czech Republic	CZ-RM	48°48'25" N	14°29'30" E		38	11	37/11	-/-	-/-			
Stanovice	Czech Republic	CZ-ST	50°10'30" N	12°53'00" E	2004 Jul 09	37	10	37/10	-/-	-/-			
Vranov	Czech Republic	CZ-VR	48°54'28" N	15°49'01" E	2004 Oct 11	32	10	-/-	23/10	-/-			4x F_1, 2x BC_{lon}
Helgoland	Germany	DE-HL	54°11'04" N	07°54'46" E		39	18	-/-	39/18	-/-			
Ismaning	Germany	DE-IS	48°13'15" N	11°46'19" E	2004 Sep 22	37	18	-/-	36/18	-/-			
Palmengarten	Germany	DE-PG	50°07'26" N	08°39'13" E	2008 Oct 10	62	10	62/10	-/-	-/-			
Stechlinsee	Germany	DE-SS	53°09'00" N	13°01'58" E	2004 May 10	31	10	-/-	31/10	-/-			
Trais-Horloff	Germany	DE-TH	50°27'00" N	08°54'10" E	2006 Jul 30	39	10	6/10	-/-	-/-			2x F_1, 31x BC_{gal}
Usingen	Germany	DE-US	50°20'37" N	08°30'26" E	2006 Aug 15	32	11	-/-	-/-	32/11			
Cogollos	Spain	ES-CO	37°12'36" N	02°50'06" W	2001 Sep 05	37	1	37/1	-/-	-/-			
Lake Pyhäjärvi	Finland	FI-PY	62°17'31" N	26°46'08" E		15	6	15/5	-/-	-/-			
Lake Vesijärvi	Finland	FI-VJ	60°59'38" N	25°37'45" E		33	10	-/-	-/-	33/10			
Etang de Bellebouche	France	FR-BB	46°47'07" N	01°18'31" E		60	0	42/-	1/-	6/-	11x F_1		
Loch Leven	Great Britain	GB-LO	56°12'00" N	03°22'48" W		37	11	37/11	-/-	-/-			
Hyde Park	Great Britain	GB-HP	51°30'23" N	00°10'20" W	2008 Sep 27	37	13	37/13	-/-	-/-			
Queens Garden	Great Britain	GB-QG	51°31'37" N	00°09'07" W	2008 Sep 28	36	10	36/10	-/-	-/-			
Rollesby	Great Britain	GB-RO	52°41'20" N	01°39'24" E	2006 Nov 30	38	11	23/11	-/-	-/-			13x F_1
Regents Park	Great Britain	GB-RP	51°31'41" N	00°09'32" W	2008 Sep 28	40	10	40/10	-/-	-/-			
Mývatn	Iceland	IS-MY	65°34'56" N	16°59'22" W	2006 Oct 06	38	11	38/11	-/-	-/-			
Lago Trearie	Italy	IT-LT	37°57'07" N	14°50'20" E	2006 Oct 08	38	11	-/-	-/-	-/11		38x F_1	
Lago di Piana degli Albanesi	Italy	IT-PD	37°58'37" N	13°18'03" E	2006 Nov 07	36	12	3/12	-/-	-/-	33x F_1		
Lago di Piano	Italy	IT-PI	46°02'20" N	09°09'48" E	1999 Jul 03	32	10	-/-	7/10	-/-			18x F_1, 1x BC_{gal}, 6x BC_{lon}
Lake Asveja	Lithuania	LT-AS	55°02'42" N	25°30'06" E	2006 Sep 21	111	0	33/-	57/-	14/-	6x F1		
Drabužis	Lithuania	LT-DR	54°34'08" N	24°39'03" E	1995 Sep 24	33	0	-/-	4/-	29/-			
Luodis	Lithuania	LT-LU	55°34'54" N	26°12'44" E	2006 Sep 21	40	10	-/1	1/-	38/9	1x F_1		
Esch-sur-Sûre	Luxembourg	LU-ES	49°54'10" N	05°52'30" E	2006 Sep 19	61	11	-/-	24/1	21/10			14x F_1
Delftse Houd	Netherlands	NL-DH	51°58'41" N	04°20'55" E	2001 Jun 16	29	9	29/9	-/-	-/-			
Goksjø	Norway	NO-GO	59°10'22" N	10°09'54" E	1999 Sep 01	41	10	-/-	38/10	-/-		1x F_1	1x BC_{longi}
Nordfjordvatn	Norway	NO-NV	69°16'33" N	19°01'15" E	2001 Sep 25	32	10	-/-	32/10	-/-			
Storveavatn	Norway	NO-SV	64°50'38" N	11°22'33" E	1998 Sep 08	24	10	-/-	24/10	-/-			
Maranhão	Portugal	PT-MA	39°02'55" N	07°55'47" W	NA	37	11	32/11	-/1	-/-			
Lake Glubokoe	Russia	RU-GL	55°45'13" N	36°30'15" E		88	12	33/8	22/-	-/4	25x F_1		1x F_1, 1x BC_{lon}
Göteburg	Sweden	SE-GB	57°42'00" N	12°00'00" E	2004 May 02	20	10	-/-	20/10	-/-			
Koarp	Sweden	SE-KO	56°22'48" N	13°07'30" E	2007 Aug 03	40	10	-/-	38/10	-/-			
Lake Smartinsko	Slovenia	SI-SJ	46°16'58" N	15°15'57" E		33	13	-/-	-/-	33/13			
Reservoir Dubník II	Slovakia	SK-DU	48°46'01" N	17°40'58" E	2006 Sep 24	39	11	-/-	-/-	39/11			
Nižné Jamnícke	Slovakia	SK-NJ	49°12'10" N	19°46'13" E	2000 Sep 24	40	13	-/-	40/13	-/-			
Satanie	Slovakia	SK-SA	49°10'15" N	20°03'50" E	2003 Sep 27	30	11	-/-	30/11	-/-			
Total						1715	442	666/187	521/165	279/90	92	39	94

Statistical analyses of microsatellite DNA

The affiliation of each studied individual to one of the three species was estimated using Bayesian assignment testing implemented in STRUCTURE 2.3.3 (Falush *et al.* 2003; Pritchard *et al.* 2000), and is explained and described for the studied dataset in **Chapter 2** (see also **Table 4-1**). The hybrid status of intermediate genotypes (according to STRUCTURE) was detected using NEWHYBRIDS 1.1 beta (Anderson & Thompson 2002), which identifies the posterior probabilities of each genotype if belonging to one of six classes (parental taxon 1, parental taxon 2, F_1 hybrid, F_2 hybrid, backcross with taxon 1, backcross with taxon 2). We tested three combinations: (1) using *D. galeata* and *D. cucullata* as well as their intermediates; (2) using *D. galeata* and *D. longispina* and intermediates, and (3) using *D. cucullata* and *D. longispina* and intermediates. For each combination a total of five independent runs with at least 500,000 iterations and default settings were conducted. For combination one and three we used only a subset of nine microsatellite loci (the loci Dgm112, DaB10/14, and SwiD12 were excluded; see **Chapter 2**). If an individual had a posterior probability of belonging to a specific genotype frequency class of 0.95 or higher, then it was scored as belonging to that particular class. We also tested to what extent the assignment increased if a decreased posterior probability threshold of 0.80 was applied on the data. Intermediate genotypes (probably representing hybrid genotypes) were excluded from further population genetic analyses to eliminate the effect of multispecies comparison on the intraspecific variability.

For taxon *D. cucullata* we only used a subset of nine loci. One locus was excluded due to non-amplification in most samples (Dgm112) and the other two loci were excluded because they were fixed for the same allele in all populations (Dp196NB, Dp519). Two more loci were monomorphic in many populations but showed little variation in others, therefore we kept them. In order to determine if a sufficient number of microsatellite loci has been scored to distinguish between individuals with different genotypes, we used the program MULTILOCUS (Agapow & Burt 2001) to randomly sample from 1 to 12 loci for *D. galeata* and *D. longispina* and from 1 to 9 loci for *D. cucullata* from the dataset (1000 replicates) and to calculate the number of different genotypes detectable. This allowed us to see whether scoring more loci is likely to increase the detection of multi-locus genotypes (MLGs), or whether we have reached a plateau.

Summary statistics of the twelve studied microsatellite loci (for *D. cucullata* a subset of nine loci) was estimated using GENALEX 6.41 (Peakall & Smouse 2006): number of MLGs; number of polymorphic loci (P); allelic diversity (A), the number of different alleles averaged

over all loci; private alleles (PA) per population within a species; observed and expected heterozygosity (H_o and H_e, respectively). Isolation-by-distance (IBD) for microsatellite data was tested using IBDWS (Jensen *et al.* 2005) as explained for mitochondrial DNA. Genetic differentiation among populations, based on the microsatellite markers, was assessed by means of D_{est} (Jost 2008), using the SMOGD software (Crawford 2010). Significant deviations from gametic (GE) and Hardy-Weinberg equilibria (HWE) were identified using GENEPOP 4.0 (Rousset 2008) which was further used to detect excess (HE) or deficit of heterozygotes (HD). These parameters were estimated with and without repeated identical multi-locus genotypes (except GE, which was only calculated without repeated MLG).

To identify the population genetic structure in each of the three species a model-based assignment test implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000) was applied. For *D. cucullata* the number of assumed populations (*K*) was set to *K* = 1-20 and for the other two species, *D. galeata* and *D. longispina*, at *K* = 1-30. A total of ten independent runs with 50,000 burn-in iterations and 450,000 MCMC steps were conducted at each value of *K* using the admixture model and correlated allele frequencies among populations. The method described by Evanno *et al.* (2005) implemented in STRUCTURE HARVESTER (Earl & von Holdt 2011) which employs an ad hoc statistic Δ*K* based on the rate of change in Ln P(D) between successive *K* values was used to detect the uppermost hierarchical level of population structure.

Results

Species and hybrid class assignment

In total we analyzed 44 populations including 1715 individuals using twelve microsatellite loci, and sequences of 12S rDNA were obtained from a subset of 442 individuals (**Table 4-1**). Assignment of individuals to the three species *D. cucullata*, *D. galeata*, and *D. longispina* was conducted using the microsatellite data and a Bayesian approach implemented in STRUCTURE. This resulted in 42 "pure species" populations. Several of the originally sampled populations constituted a mix of pure and hybrid genotypes which coexisted within the locality, but hybrid and backcross genotypes were not and only populations with more than 14 pure genotypes were considered for the assessment of population genetic structure: 8 *D. cucullata* populations (259 individuals), 18 *D. galeata* populations (646 individuals), and 16 *D. longispina* populations (508 individuals).

In total, 249 admixed genotypes were identified during species assignment tests (**Chapter 4-2**). Within the species pair *D. galeata-longispina* 115 admixed individuals were identified. Out of these, 1 was assigned to *D. galeata* in the NEWHYBRIDS analysis, 7 (9 if the 0.8 posterior probability threshold was considered) were identified as *D. longispina*, 49 (52) as F_1 hybrids, 32 as backcrosses with *D. galeata*, 8 (10) as backcrosses with *D. longispina*, and 18 (11) were not assigned to any of these classes. Within the species pair *D. cucullata-galeata* (93 individuals), 1 was identified as *D. galeata* and the remaining 92 as F_1 hybrids. Within the species pair *D. cucullata-longispina* (41 individuals), 1 was identified as *D. cucullata*, 38 (39) as F_1 hybrids, and the last individual was assigned to *D. longispina* only under the less strict posterior probability threshold.

Population genetic structure of Daphnia cucullata

We sequenced a total of 531 bp of mitochondrial 12S rDNA from 90 individual *D. cucullata* belonging to nine populations, obtaining 15 unique haplotypes due to 20 variable sites (3 singletons, 17 parsimony informative sites; see **Table 4-2**). The haplotype network calculated for this 12S mtDNA dataset is shown in **Figure 4-2**; two haplotypes were shared among populations: (1) among populations IT-LT and SK-DU; and (2) among LT-LU, LU-ES, and RU-GL; all other haplotypes found were distinct for one locality. Haplotype diversity (*Hd*) ranged from 0-0.75 (overall populations *Hd* of 0.90) and nucleotide diversity (π) ranged from 0-0.0066 within populations (overall populations = 0.0091; **Table 4-2**). Both F_S and D* values were not significant, neither within single nor overall populations and the values overall studied sequences were close to zero indicating no population growth, background selection or genetic hitchhiking. Also, the Bayesian skyline plot did not detect an increase in effective population size (**Figure 4-3**).

The number of microsatellite loci studied in *D. cucullata* was not sufficient to identify all multi-locus genotypes within this taxon (**Figure 4-4**) which is supported by the finding of five shared MLGs among populations. Nonetheless, we detected a high overall clonal diversity of 0.87 (MLG/number of studied individuals) after analyzing eight populations (259 individuals) with these nine microsatellite loci (see **Table 4-2**). We detected an average of 2.6 alleles per locus across all populations, with 1-10 private alleles in five populations. Expected and observed values for heterozygosity did not differ greatly, although half of the populations deviated significantly from HWE, mostly due to a deficit of heterozygotes. The other populations were in agreement with HWE and we observed little gametic disequilibrium among locus pairs in *D. cucullata*. Bayesian cluster analysis evaluated using STRUCTURE

HARVESTER suggests that $K = 2$ is adequate to describe the structure within the dataset. One cluster consists more or less of populations CZ-BR, DE-US, LU-ES, SI-SJ, and SK-DU, and the other cluster of the populations FI-VJ, LT-DR, LT-LU (**Figure 4-5**).

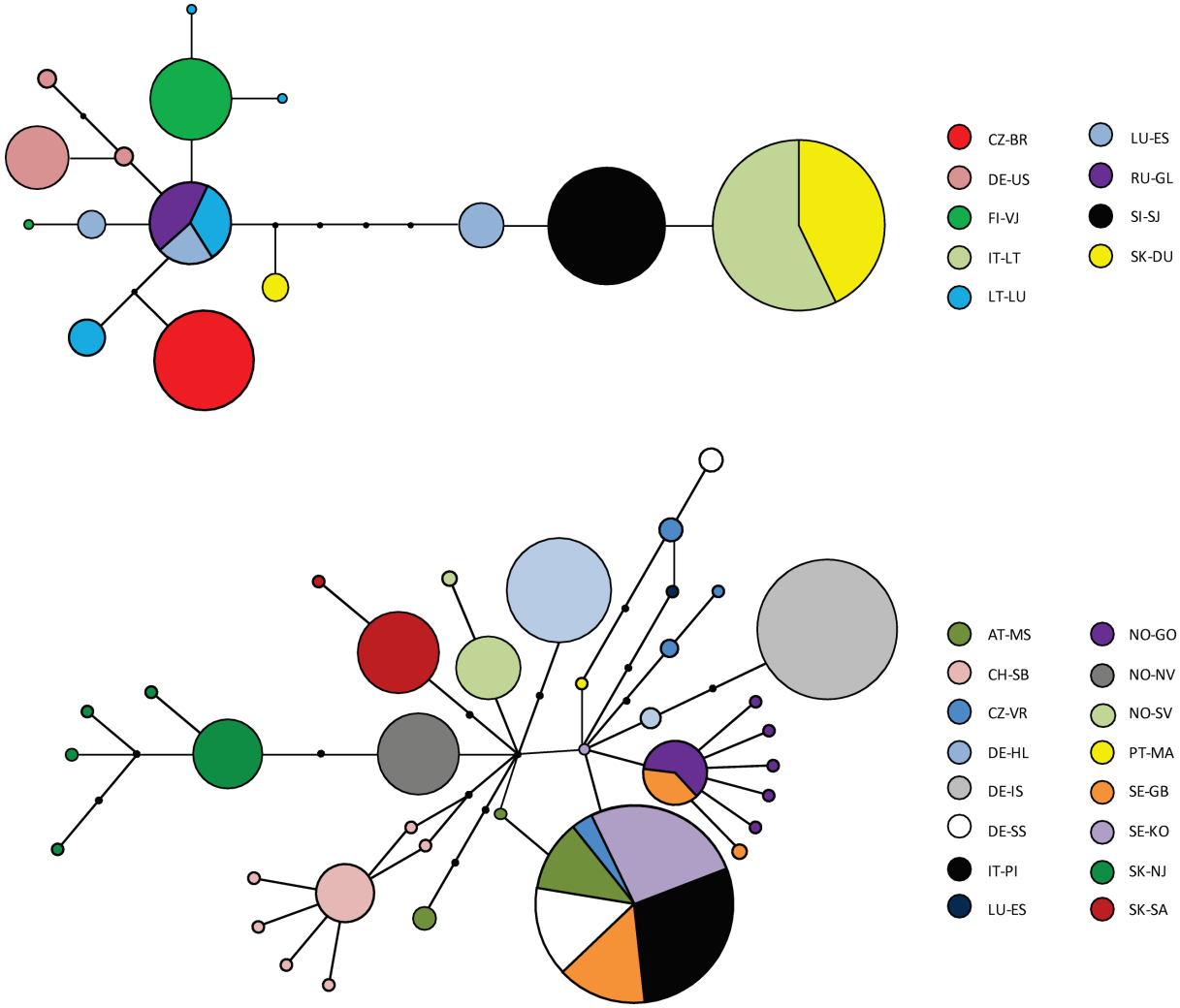

Figure 4-2 Median joining haplotype networks of 12S rDNA sequences of *D. cucullata* (upper network) and *D. longispina* (lower network). Sizes of the circles correspond to the number of sequences found for each haplotype and the colors correspond to the respective populations. Black dots indicate one mutation step, but no sequence that was recovered for this haplotype.

A test for genetic differentiation revealed contrasting patterns in both molecular markers. Calculated N_{ST} values for mitochondrial DNA ranged from 0.2 to 1.0 (overall value 0.768), while D_{est} values calculated for microsatellite data ranged from 0.006 to 0.159 with an overall value of 0.064. Also differences in isolation-by-distance tests were detected: no correlation between genetic and geographic distances ($r^2 = 0.031$, $p = 0.17$) was found for mitochondrial DNA, whereas the same test was significant for the microsatellite loci studied ($r^2 = 0.232$, $p = 0.01$).

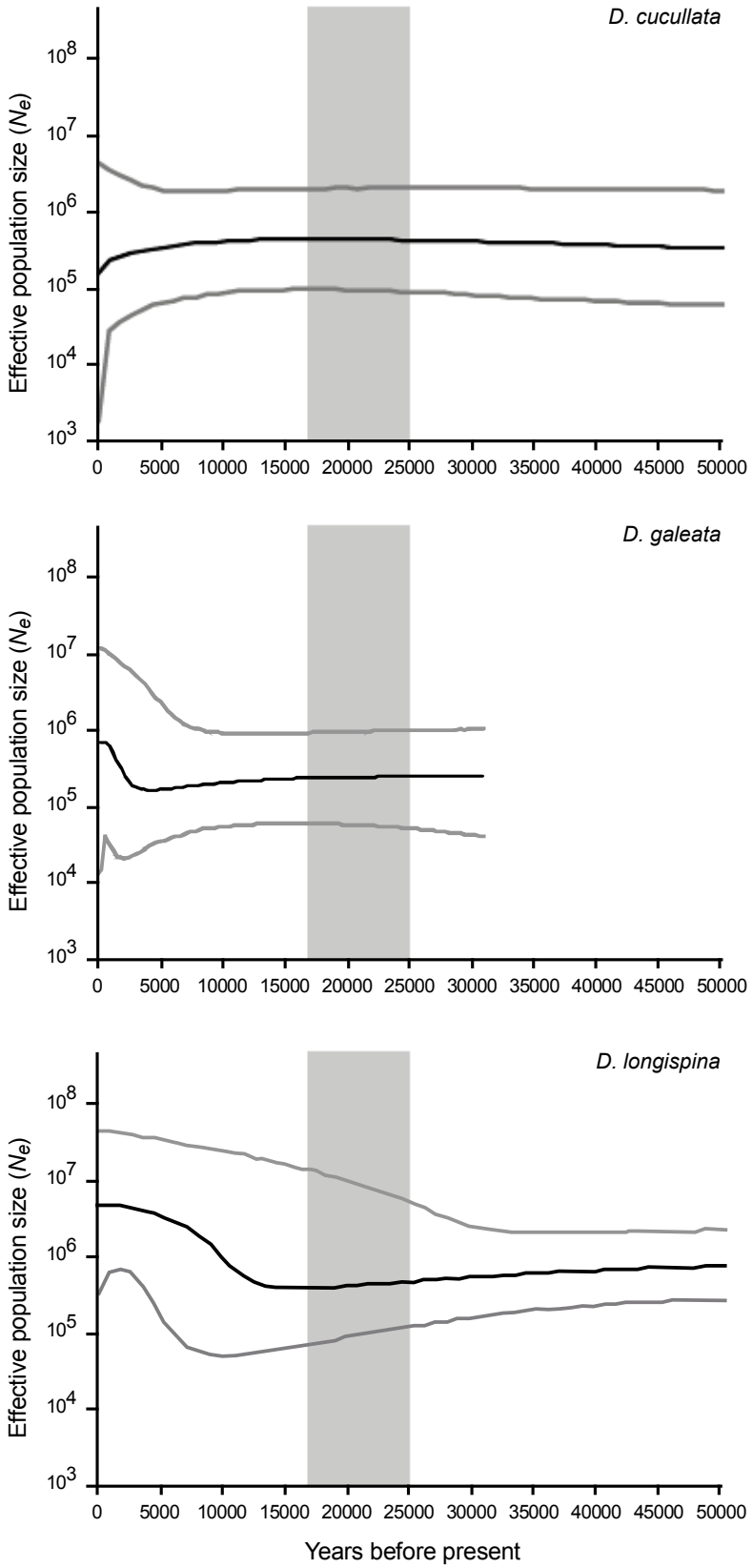

Figure 4-3 Reconstruction of the demographic past of all three species, *D. cucullata* (upper graph), *D. galeata* (middle graph), and *D. longispina* (lower graph) using Bayesian skyline plots. The analyses suggest a relative stable population size for *D. cucullata*, but an increase for *D. galeata* (2000-7000 years BP) and *D. longispina* (9000-30000 years BP). The black line represents the median and the grey lines the 95% confidences intervals. Marked in grey is the last glacial maximum.

Population genetic structure of Daphnia galeata

For *D. galeata* we sequenced a total of 491 bp of mitochondrial 12S mtDNA from 187 individuals belonging to 21 populations yielding 15 unique haplotypes due to 16 variable sites (5 singletons, 11 parsimony informative sites; see **Table 4-2**). In three populations (CZ-BR, ES-CO, and LT-LU) we recovered only one individual sequence, therefore, we excluded them from population comparisons, e.g. for detection of genetic differentiation. The haplotype network and the distribution of haplotypes over Europe is shown in **Figure 4-6**; six of the 15 detected haplotypes were shared by two or more populations: (1) among BE-BE, BE-MA, DE-PG, DE-TH, ES-CO, NL-DH, and PT-MA; (2) among BE-BE, CZ-RM, GB-HP, GB-QG, GB-RP, IT-PD, NL-DH, and RU-GL; (3) among BE-DI, BE-MA, CZ-RM, CZ-ST, DE-TH, GB-HP, GB-LO, GB-RO, and NL-DH; (4) among BE-MA, GB-QG, and GB-RP; (5) among CZ-RM and PT-MA; and (6) among CZ-RM, CZ-ST, and PT-MA. Haplotype diversity (*Hd*) ranged from 0-0.78 (overall populations *Hd* of 0.79) and nucleotide diversity (π) ranged from 0-0.0044 among populations (overall populations = 0.0042; **Table 4-2**). Both F_S and D* were not significant neither overall sequences nor within populations, but negative overall values were detected indicating population growth. Also, demographic past reconstruction using Bayesian skyline analysis suggests population expansion starting 2000-7000 years BP (**Figure 4-3**).

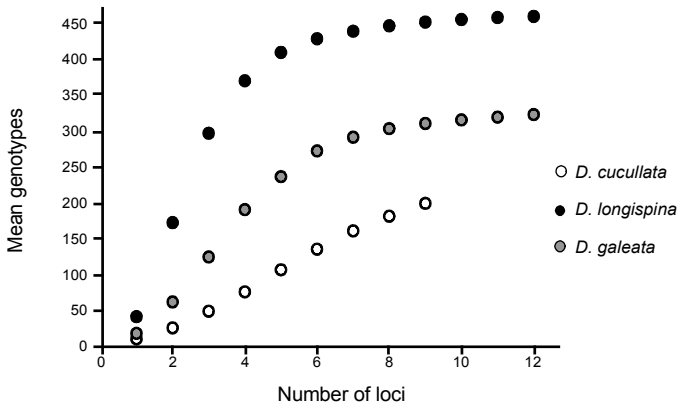

Figure 4-4 Evaluation of the amount of microsatellite loci scored for the detection of multi-locus genotypes. The y-axis represents the number of different MLGs that may be detected with a certain number of loci (given on x-axis).

The assessment of clonal diversity within 18 *D. galeata* populations (646 individuals) using twelve microsatellite loci revealed a comparatively low value of 0.55 (MLG/*N*), although six to eight loci seem sufficient to characterize all individuals (**Figure 4-4**). There was an average of 2.7 alleles across all populations with 1-3 private alleles found in ten populations. Expected

and observed values for heterozygosity did often differ significantly within populations; we observed an increased heterozygosity, which resulted in the significant deviation of HWE in many populations. After correcting for multiple MLG most populations were consistent with HWE; four populations deviated and two could not be tested due to the insufficient number of MLGs. We observed little gametic disequilibrium among locus pairs, but the number of linked loci was, compared to the other two species, higher in *D. galeata* populations (11% of locus pairs were linked). The Bayesian cluster analysis suggests that $K = 22$ is adequate to describe the structure within the dataset (**Figure 4-5**) if evaluating Ln P(D) and with STRUCTURE HARVESTER. Nine of the studied populations consisted to 80% or more of individuals forming one cluster. Individuals from the other nine populations were split into several clusters.

Stronger genetic differentiation patterns were detected using mitochondrial DNA, N_{ST} values ranged from 0 to 1 with an overall value of 0.677, compared to microsatellite DNA, D_{est} values ranges from 0.041 to 0.398 with an overall of 0.15. A Mantel test revealed no correlation ($r^2 = 0.003$, $p = 0.24$) of genetic divergence and geographic distance in mitochondrial DNA and only a marginally significant correlation in microsatellite DNA ($r^2 = 0.086$, $p = 0.04$).

Population genetic structure of Daphnia longispina

For *D. longispina* we sequenced a total of 505 bp of mitochondrial 12S mtDNA from 165 individuals belonging to 16 populations yielding 37 unique haplotypes due to 38 variable sites (12 singletons, 26 parsimony informative sites; see **Table 4-2**). In two populations (LU-ES and PT-MA) we recovered only one individual sequence; therefore, we excluded them from population comparisons. The haplotype network calculated for this 12S mtDNA dataset is shown in **Figure 4-2**; only two of the 37 detected haplotypes were shared by two or more populations: (1) among SE-KO, AT-MS, CZ-VR, DE-SS, IT-PI, and SE-GB; (2) among NO-GO and SE-GB. Haplotype diversity (*Hd*) ranged from 0-0.78 (overall *Hd* of 0.92) and nucleotide diversity (π) ranged from 0-0.0066 among populations (overall populations = 0.0089; **Table 4-2**). Both F_S and D* were neither significant overall sequences nor within most populations. We detected significant negative F_S values for CH-SB and NO-GO. Overall studied sequences high negative values were detected (more negative values were detected for F_S than for D*), which is indicative of population expansion or genetic hitchhiking. This is strongly supported by the Bayesian skyline analysis which suggests a strong population expansion starting 9000-30000 years BP (**Figure 4-3**).

Table 4-2 Summary statistics using twelve microsatellite loci or sequences of 12S rRNA gene of all *Daphnia cucullata* (only nine microsatellite loci), *D. galeata*, and *D. longispina* populations. Population abbreviation; number of individuals genotyped with microsatellites ($N_{\mu sat}$); number of multi-locus genotypes (MLG); clonal diversity (D = MLG/$N_{\mu sat}$); allelic diversity (A); number of polymorphic loci (P); number of private alleles (PA); expected (H_e) and observed (H_o) heterozygosity; deviations of Hardy-Weinberg equilibrium (HWE_{NC}); heterozygote deficit (HD_{NC}) or excess (HE_{NC}); deviations of gametic equilibrium (GE_{NC} = number of locus pair deviating from gametic equilibrium/number of locus pairs tested); number of individuals sequenced for 12S rDNA region (N_{12S}); number of haplotypes (N_{hap}); nucleotide diversity (π); haplotypic diversity (*Hd*); number of polymorphic sites (PS); Fu and Li's D* (overall: *D. cucullata* = 0.429, *D. galeata* = -2.096, *D. longispina* = -2.112); Fu's F_S (overall: *D. cucullata* = 0.039, *D. galeata* = -3.112, *D. longispina* = -15.988).

Population	$N_{\mu sat}$	MLG	D	A	P	PA	H_e	H_o	HWE_{NC}	HD_{NC}	HE_{NC}	GE_{NC}	N_{12S}	N_{hap}	π	*Hd*	PS	D*	F_S
D. cucullata																			
CZ-BR	34	33	0.97	2.56	0.78	3	0.24	0.19	**	***	n. s.	0.10	11	1	0.0000	0.00	0	NA	NA
DE-US	32	32	1.00	2.67	0.89	3	0.28	0.25	**	**	n. s.	0.00	11	3	0.0022	0.58	3	1.13	0.90
FI-VJ	33	31	0.94	2.67	0.56	1	0.22	0.21	**	n. s.	n. s.	0.10	10	2	0.0020	0.60	4	-1.13	0.60
FR-BB	6	6											0						
IT-LT	0	0											11	1	0.0000	0.00	0	NA	NA
LA-AS	14	14											0						
LT-DR	29	26	0.90	2.33	0.78	0	0.18	0.18	n. s.	n. s.	n. s.	0.14	0						
LT-LU	38	37	0.97	4.11	0.78	10	0.23	0.20	n. s.	***	n. s.	0.00	9	4	0.0041	0.75	6	-0.29	0.56
LU-ES	21	19	0.90	2.44	0.78	1	0.18	0.19	n. s.$^+$	n. s.	n. s.	0.00	10	2	0.0061	0.69	6	1.35	3.34
RU-GL	0	0											4	1	0.0000	0.00	0	NA	NA
SI-SJ	33	11	0.33	1.56	0.44	0	0.14	0.18	n. s.	n. s.	n. s.$^+$	0.00	13	1	0.0000	0.00	0	NA	NA
SK-DU	39	38	0.97	2.33	0.89	0	0.24	0.24	***	**	n. s.	0.00	11	2	0.0066	0.44	8	1.40	6.29
Total	279	247				18							90						
Average			0.87	2.58	0.74		0.22	0.21				0.04							
D. galeata																			
BE-BE	35	35	1.00	3.75	0.83	0	0.43	0.44	n. s.	n. s.	n. s.	0.07	10	2	0.0044	0.53	4	1.24	4.19
BE-DI	43	10	0.23	3.08	0.83	0	0.28	0.43	n. s.$^+$	n. s.	n. s.$^+$	0.13	10	1	0.0000	0.00	0	NA	NA
BE-MA	6	5											12	3	0.0016	0.32	3	0.09	0.18
CZ-BR	5	5											1	1	0.0000	0.00	0	NA	NA
CZ-RM	37	28	0.76	3.92	0.92	3	0.41	0.41	***	*	n. s.	0.24	11	5	0.0025	0.78	5	-1.45	-1.54
CZ-ST	37	17	0.46	3.00	0.83	0	0.36	0.41	n. s.$^+$	n. s.	n. s.$^+$	0.04	10	2	0.0010	0.47	1	0.80	0.82
DE-PG	62	2	0.03	2.25	0.83	0	0.30	0.58	NA$^+$	NA	NA$^+$	NA	10	1	0.0000	0.00	0	NA	NA
DE-TH	6	3											10	2	0.0008	0.20	2	-1.59	0.59
ES-CO	37	26	0.70	2.42	0.75	1	0.36	0.40	***	n. s.	n. s.	0.17	1	1	0.0000	0.00	0	NA	NA
FI-PY	15	15	1.00	2.33	0.75	1	0.33	0.38	n. s.	n. s.	n. s.	0.00	5	2	0.0016	0.40	2	-0.97	1.04
FR-BB	42	42	1.00	3.42	0.92	0	0.42	0.43	n. s.	n. s.	n. s.	0.02	0						
GB-LO	37	18	0.49	2.42	0.75	1	0.31	0.39	n. s.$^+$	n. s.	n. s.$^+$	0.17	11	1	0.0000	0.00	0	0.95	1.32
GB-HP	37	18	0.49	3.08	1.00	1	0.40	0.52	n. s.$^+$	n. s.	**	0.11	13	2	0.0012	0.28	2	NA	NA
GB-QG	36	11	0.31	1.83	0.67	0	0.27	0.41	n. s.$^+$	n. s.	***	0.07	10	3	0.0016	0.38	4	-1.92	0.06

GB-RO	23	11	0.48	2.58	0.75	2	0.26	0.35	n. s.$^{+}$	n. s.	n. s.$^{+}$	0.22	11	1	0.0000	0.00	0	NA	NA
GB-RP	40	24	0.60	2.50	0.83	0	0.35	0.42	n. s.$^{+}$	n. s.	*	0.11	10	2	0.0033	0.53	3	1.15	3.34
IS-MY	38	24	0.63	1.67	0.50	1	0.12	0.13	n. s.	n. s.	n. s.	0.00	11	3	0.0007	0.35	2	-1.66	-1.25
IT-PD	3	1											12	1	0.0000	0.00	0	NA	NA
LT-AS	33	2	0.06	1.50	0.42	0	0.21	0.31	NA^{+}	NA	NA^{+}	NA	0						
LT-LU	0												1	1	0.0000	0.00	0	NA	NA
NL-DH	29	11	0.38	3.33	0.83	1	0.39	0.46	*	n. s.	n. s.	0.38	9	3	0.0039	0.67	4	1.27	1.66
PT-MA	32	31	0.97	3.50	0.92	1	0.39	0.40	n. s.	n. s.	n. s.	0.11	11	3	0.0021	0.35	4	-0.40	0.62
RU-GL	33	12	0.36	2.75	0.83	1	0.42	0.58	***	n. s.	***	0.18	8	1	0.0000	0.00	0	NA	NA
Total	666	351				13							187						
Average			0.55	2.74	0.79		0.33	0.41				0.11							
D. longispina																			
AT-MS	22	21	0.95	3.17	0.92	1	0.38	0.39	n. s.	n. s.	n. s.	0.04	10	3	0.0055	0.64	5	1.17**	2.85
CH-SB	32	31	0.97	2.58	0.75	1	0.26	0.24	n. s.	*	n. s.	0.03	13	7	0.0019	0.73	6	-2.61	-5.09
CZ-VR	23	23	1.00	3.75	1.00	1	0.52	0.57	n. s.	n. s.	*	0.00	10	4	0.0074	0.71	8	0.80	2.09
DE-HL	39	15	0.38	2.75	1.00	0	0.46	0.63	n. s.	n. s.	***	0.08	18	2	0.0029	0.37	4	1.18	3.97
DE-IS	36	36	1.00	6.33	1.00	5	0.64	0.64	n. s.	n. s.	n. s.	0.03	18	1	0.0000	0.00	0	NA	NA
DE-SS	31	31	1.00	4.33	1.00	0	0.53	0.52	n. s.	n. s.	n. s.	0.05	10	2	0.0066	0.56	6	1.79**	5.80
FR-BB	1	1											0						
IT-PI	7	4											10	1	0.0000	0.00	0	NA	NA
LT-AS	57	57	1.00	6.67	1.00	7	0.55	0.54	n. s.	**	n. s.	0.03	0						
LT-DR	4	4											0						
LT-LU	1	1											0						
LU-ES	24	24	1.00	5.17	1.00	4	0.56	0.58	n. s.	n. s.	n. s.	0.02	1	1	0.0000	0.00	0	NA	NA
NO-GO	38	38	1.00	4.58	1.00	3	0.58	0.55	n. s.	*	n. s.	0.05	10	6	0.0020	0.78	5	-2.18	-3.88
NO-NV	24	24	1.00	3.33	1.00	2	0.49	0.50	n. s.	n. s.	n. s.	0.03	10	1	0.0000	0.00	0	NA	NA
NO-SV	32	32	1.00	2.75	0.75	0	0.24	0.25	n. s.	n. s.	n. s.	0.06	10	2	0.0007	0.36	1	0.68	0.42
PT-MA	0												1	1	0.0000	0.00	0	NA	NA
RU-GL	22	22	1.00	4.50	0.92	1	0.52	0.49	n. s.	n. s.	n. s.	0.02	0						
SE-GB	20	20	1.00	3.92	0.92	1	0.52	0.48	n. s.	n. s.	n. s.	0.04	10	3	0.0029	0.69	3	1.35	1.26
SE-KO	38	38	1.00	5.00	1.00	3	0.66	0.76	***	n. s.	***	0.09	10	2	0.0004	0.20	1	-1.35	-0.34
SK-NJ	40	40	1.00	3.83	1.00	1	0.40	0.40	n. s.	n. s.	n. s.	0.06	13	5	0.0023	0.54	6	-1.96	-1.40
SK-SA	30	9	0.30	1.33	0.25	0	0.12	0.16	n. s.	n. s.	n. s.	0.00	11	2	0.004	0.18	1	-1.40	-0.41
Total	521	471				30							165						
Average			0.91	4.00	0.91		0.46	0.48				0.04							

$_{NC}$ = given results are for the tests using populations without repeated MLG

$^{+}$ indicates that this population was either significantly deviating from HWE or obtained a significant heterozygote deficit or excess if using all individuals of each population for the tests

NA = not available; Significance level: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

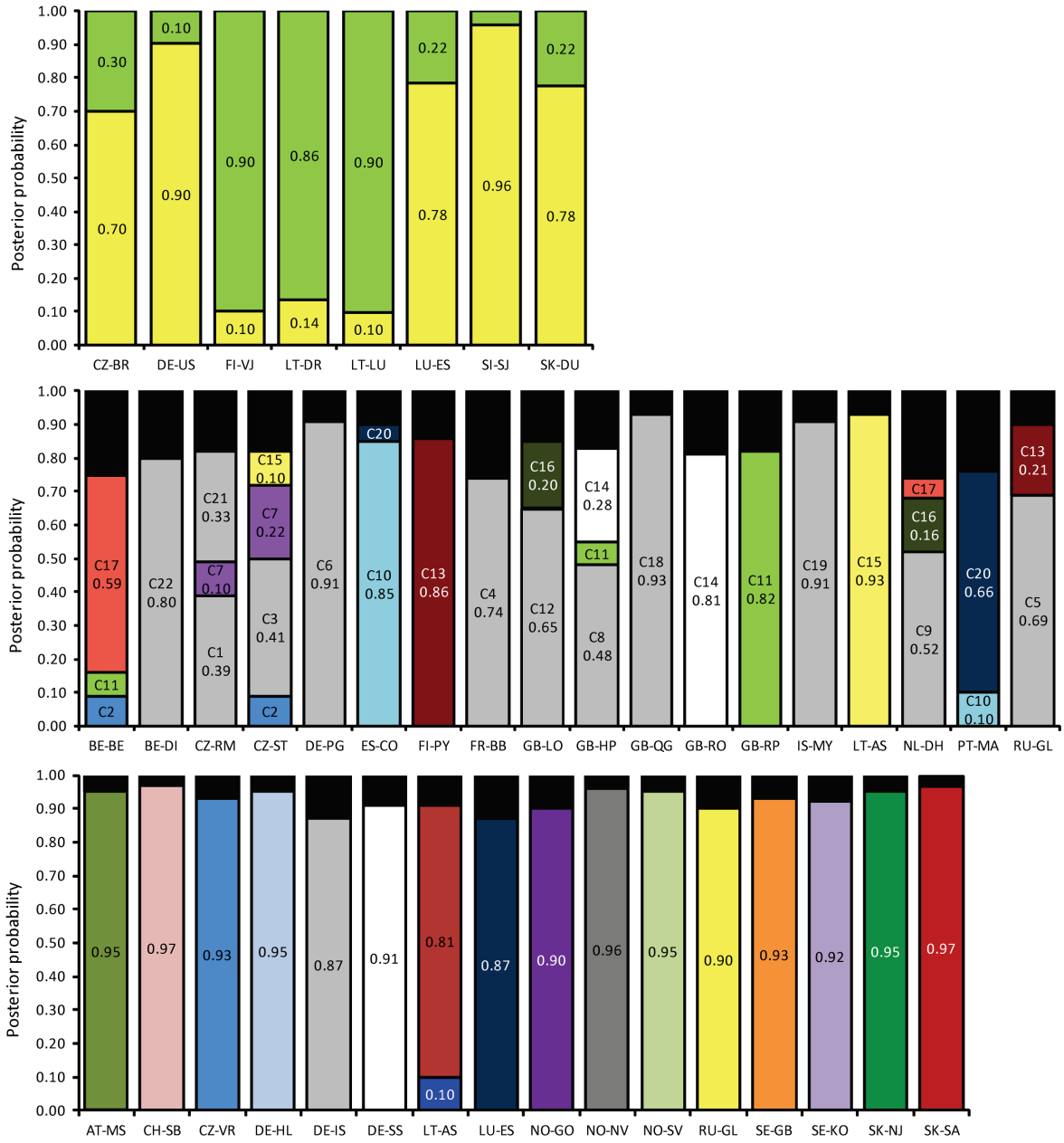

Figure 4-5 Population genetic structures of the three species using nuclear microsatellite DNA and a Bayesian assignment method implemented in the program STRUCTURE. First graph shows the populations of *D. cucullata* with $K = 2$. The population abbreviations are given on the x-axis and the posterior probability on the y-axis. The middle graph represents the 18 *D. galeata* populations with $K = 22$ clusters: individuals belonging to clusters that are colored are found in more than one populations while individuals belonging to clusters that are grey, are found mainly in one population; the clusters are labeled from C1-C22 often with the posterior probability given below. The bottom graph represents the *D. longispina* populations with $K = 17$: in each population we found one main cluster and only population LT-AS shows a substructure in two clusters. The black part in the *D. galeata* and *D. longispina* populations indicate the amount of genotypic information that belonged to different clusters, but the posterior probabilities were less than 0.05.

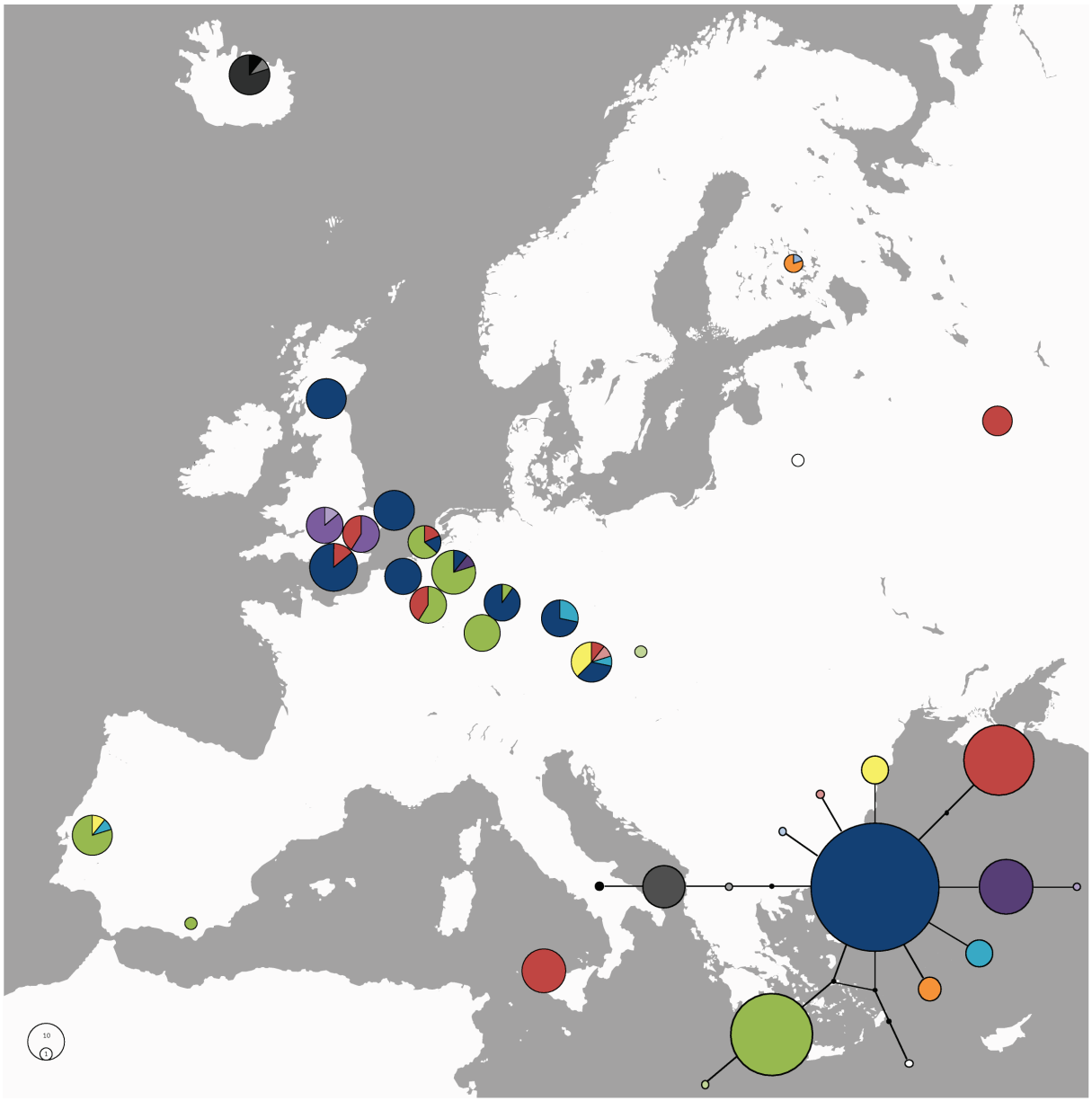

Figure 4-6 Median joining haplotype network of 12S rDNA sequences of *D. galeata* (lower right corner) and the distribution of haplotypes over Europe. Sizes of the circles correspond to the number of sequences found for each haplotype and the colors correspond to a certain haplotype. Black dots within the network indicate one mutation step, but no sequence that was recovered for this haplotype.

For assessing population genetic structure all twelve microsatellite loci were used for the 16 studied populations (**Table 4-2**). Clonal diversity (MLG/*N*) was high with an average of 0.91. There was an average of 4 alleles across all populations with 1-7 private alleles found in twelve populations and the study of six to eight loci was sufficient to characterize all individuals (**Figure 4-4**). Average expected and observed values for heterozygosity rarely differed, although we detected both significant excess and deficit of heterozygotes in single populations. All, but one (SE-KO), populations were consistent with HWE and we observed very little gametic disequilibrium among locus pairs. Bayesian cluster analysis suggests that $K = 17$ is adequate to describe the structure within the dataset using STRUCTURE HARVESTER

and the evaluation of Ln P(D). All of the studied populations belonged with 80% of their individuals, often more than 90%, to one cluster (see **Figure 4-5**). Only population LT-AS was divided into two clusters (one cluster with 81% and the other with 10%).

Calculated genetic differentiation was high using both molecular markers. In mitochondrial DNA the N_{ST} values ranged from 0 to 1 (overall value 0.73), while in the studied microsatellite data the D_{est} values ranged from 0.19 to 0.77 (overall value of 0.44). No isolation-by-distance was revealed using a Mantel test, neither in mitochondrial ($r^2 = 0.001$, $p = 0.64$) nor in microsatellite DNA ($r^2 = 0.062$, $p = 0.09$).

Discussion

Impact of historical events on population genetic structure

Daphnia longispina and *D. cucullata* inhabit quite contrasting environmental niches; while *D. longispina* often occurs in oligotrophic deep lakes or fishless water bodies is *D. cucullata* regularly found in eutrophic shallow habitats with high fish predation (Benzie 2005; Flößner 2000). Therefore, *Daphnia longispina* may have benefited from the retreat of the ice shield after the last ice age in Europe, as numerous glacial lakes with oligotrophic status formed. According to Hewitt (1996) the colonizers of these new populations, often founded by a few individuals and long-distance dispersal, would dominate the populations gene pool and without effective gene flow among these populations they would differentiate unconstrained. An early population expansion, probably starting soon after the last glacial maximum, was indeed derived from our sequence data (**Figure 4-3**, **Table 4-2**: F_S values) which would support the mentioned assumptions. Other mitochondrial DNA studies (Hamrová 2011; Hamrová *et al.* 2011; Petrusek *et al.* 2007; Petrusek *et al.* 2008; Thielsch *et al.* 2009) reveal large genetic divergence within this taxon which are in line with our results. Although 165 individual sequences out of 16 populations were analyzed, only two of the 37 detected haplotypes were shared among populations. This high genetic differentiation suggests monopolization (De Meester *et al.* 2002) after the founding of the respective populations. As older populations possess as much or more genetic structure than presumed younger populations the observed patterns (nuclear and mitochondrial) found in *D. longispina* hint that the ancient founding is probably still detectable, especially if considering studies concerning a narrower geographic range like the Tatra Mountains (Petrusek *et al.* 2007) or the Pyrenees (Hamrová 2011). The high occurrences of private alleles at the nuclear and mitochondrial DNA level also support this in general.

Impact and consequences of contemporary environmental changes on population genetic structure

Human-mediated environmental changes are assumed to have the highest impact during the last centuries and freshwater habitats have severely changed in particular since industrialization. Nutrient inflow increased in many water bodies and resulted in a shift from oligotrophic to eutrophic and even hypereutrophic status (Correll 1998). Further, fish stocking intensely amplified over the last decades (e.g. Hesthagen & Sandlund 2004; Knapp *et al.* 2001), although the breeding and transport of fish started already centuries ago (Balon 1995).
The consequences for the analyzed species may have been diverse. Studies revealed that *D. galeata* is a successful invader into recently eutrophicated (Brede *et al.* 2009; Jankowski & Straile 2003; Rellstab *et al.* 2011) and fish-stocked habitats (Cammarano & Manca 1997; Hamrová *et al.* 2011; Ishida *et al.* 2011; Wolinska *et al.* 2007). But also higher water temperature seems to result in an advantage of *D. galeata* over *D. longispina* (Keller *et al.* 2008). According to our mitochondrial DNA results a recent expansion is suggested (overall D* and F_S both negative although not significant); the Bayesian skyline analysis indicates a very recent increase in effective population size starting 2000-7000 years BP. Our data are in line with the results of further studies using also mitochondrial DNA markers (Dove 2005; Ishida & Taylor 2007b; Seidendorf 2002). However, another scenario might explain the obtained mitochondrial pattern. According to Avise *et al.* (1987) this kind of pattern is present if populations have had comparatively extensive and recent historical interconnections through gene flow. Nonetheless, the observed population differentiation in *D. galeata* (Ishida & Taylor 2007b: $F_{ST} = 0.71$, this study: $N_{ST} = 0.68$; Seidendorf 2002: $F_{ST} = 0.56$) would favor the population expansion over the gene flow scenario. The nuclear microsatellite data support the results from the mitochondrial DNA as populations are found to be highly heterogeneous (Dove 2005; this study). Still, as the results are accompanied by a relatively low level of genetic differentiation among populations (low D_{est} values, shared genetic clusters among populations) no discrimination can be made between effective gene flow among populations and recent expansion.
Less information is available for *D. cucullata* as it is less often sampled and analyzed than the other two species (e.g. Keller *et al.* 2008; Schwenk 1997). But *D. cucullata* is known to be well adapted to fish predation, especially due to its small body size. Consequently, localities with high fish densities, especially eutrophic ones, are often inhabited by *D. cucullata* which may have made this taxon to a beneficiary of the human-mediated fish breeding, stocking and

transportation during the last centuries (Balon 1995; Van Damme *et al.* 2007). However our results indicate that this taxon did not show signs for recent (or ancient) population expansion (**Table 4-2**; **Figure 4-3**; overall Fu and Li's D* as well as Fu's F_S). Further, the mitochondrial DNA obtained from nine populations revealed a highly structured pattern (overall N_{ST} = 0.77; **Figure 4-2**), with several distinct haplotypes within populations and very few haplotypes shared between populations. Although *D. cucullata* shows a very homogeneous within-population structure like *D. longispina*, only two nuclear genetic clusters were recorded over all populations which would suggest very high levels of ongoing gene flow especially if considering the low levels of genetic differentiation detected for microsatellite data. This would be in line with the above mentioned assumption, however, the microsatellite marker resolution does not seem to be sufficient to unravel the fine-scale genetic structure of *D. cucullata* as markers are less variable or even fixed for one allele (**Chapter 2**). If considering the high clonal diversity within populations together with the similarities in mitochondrial DNA patterns of *D. cucullata* and *D. longispina* we assume that *D. cucullata* population structure may also be shaped by a combination of persistent founding events and low effective gene flow among populations and that the addition of more variable microsatellite markers would probably reveal highly differentiated populations.

The recent anthropogenic impact did also affect *D. longispina*, although not detectable in our data, which resulted for example in a decrease of *D. longispina* populations in the Alps (Nevalainen *et al.* 2011). This shift is probably an indirect response to a cascade of limnological alterations, perhaps initiated by large forcing factors such as atmospheric pollution or fish introductions. Above that, recent studies demonstrate that human-made ecological changes like eutrophication support the invasion of *D. galeata* leading to increased hybridization events and eventually to introgression and change of population genetic structure in *D. longispina* (Brede *et al.* 2009; Jankowski & Straile 2003; Rellstab *et al.* 2011). Therefore, the long-term consequences of human impact on these taxa are uncertain and needs further attention.

Population clonal diversity in species of the D. longispina *complex*

By comparing several studies analyzing the population genetic structure of *D. galeata* and *D. longispina* a different overall pattern for both species emerges that does not seem to be highly dependent on biotic and abiotic characteristics of the habitat (e.g. habitat size, trophic status, predation pressure, parasitism, co-occurrence with other species) or timing and location of sample collection (Dove 2005; Hamrová 2011; Hamrová *et al.* 2011; Ishida &

Taylor 2007b; Petrusek *et al.* 2007; Petrusek *et al.* 2008; Ruthová 2008; Seidendorf 2002; Thielsch *et al.* 2009; Yin *et al.* 2012a; Yin *et al.* 2010).

D. galeata exhibits a lower clonal diversity than *D. longispina*. If summarizing all published information on populations studied with microsatellites (53 *D. galeata* populations and 49 *D. longispina*) an overall clonal diversity (MLG/*N*) of 0.61 was recovered for *D. galeata* (using 6-15 loci) and for *D. longispina* this value is close to 1 (0.92; using 9-15 loci) meaning that most analyzed individuals in this taxon have a unique multi-locus genotype (Dove 2005; Hamrová 2011; Hamrová *et al.* 2011; Ruthová 2008; Thielsch *et al.* 2009; Yin *et al.* 2012a; Yin *et al.* 2010). The low numbers of detected MLGs in *D. galeata* very likely represent low numbers of clonal lineages and are not observed because of insufficient marker resolution (see **Figure 4-4**). This suggests that clonal erosion has a larger impact on clonal diversity in *D. galeata* compared to *D. longispina*. One reason could be increased clonal selection, for example due to genotype x environment interactions (e.g. because of decreased fitness due to parasites in some genotypes; Yin *et al.* 2012a) or due to intrinsically superior genotypes (Reznick *et al.* 2000). Another reason for enhanced erosion is the length of the growing season (De Meester *et al.* 2006) and the according influence from genetic drift (Vanoverbeke & De Meester 2010) which is observed for example in overwintering populations (Hamrová *et al.* 2011). Overwintering populations that invest less in sexual reproduction result in a less established dormant egg bank compared to populations going regularly through a sexual cycle. In *D. longispina* the investment in and consequences of sexual reproduction are probably high and therefore clonal erosion was rarely detected (Hamrová 2011; Thielsch *et al.* 2009). However, clonal selection may also shape *D. longispina* populations as was for example shown by King *et al.* (1995) who detected genotype succession due to seasonal changes. As counteracting mechanism to clonal erosion, resurrection of genotypes from the egg bank throughout the growing season is discussed (Thielsch *et al.* 2009). Nonetheless, a recent study by Rother *et al.* (2010) detected only a short time window at which individuals hatched from ephippia and contributed to the population; but in their study they did not distinguish among taxa and the population consisted of *D. galeata* and hybrids with *D. longispina*. Furthermore, the studied population overwintered within the reservoir which might strongly limit the effects from the egg bank (Zeis *et al.* 2010). The assumptions made for *D. longispina* may, to a lesser extent, also apply for *D. cucullata*. As this is the first study using a larger set of *D. cucullata* population as well as a combination of high resolution nuclear markers and maternally inherited mitochondrial DNA only little comparisons can be made. The few earlier studies suggest a high number of clonal diversity within populations

(Ruthová 2008; Spaak 1996) which is in line with our results and let us assume also a high investment in sexual reproduction in *D. cucullata*.

Conclusions

Historical and contemporary environmental changes do seem to differently influence the distribution and abundance of the studied taxa. While *D. longispina* underwent a population expansion very likely fueled by the formation of glacial lakes after the last ice age, contemporary anthropogenic changes of freshwater habitats do rather enlarge *D. galeata* which is indicated by the observed results and recent literature. This current expansion of *D. galeata* may also be the reason for the frequently observed hybridization of this taxon with several other species of the *D. longispina* complex (Hobæk *et al.* 2004; Ishida *et al.* 2011; Schwenk 1993; Schwenk & Spaak 1995; Taylor *et al.* 2005). The often produced F_1 hybrids of *D. galeata* and *D. cucullata* (Schwenk 1997) that are competitively superior (Spaak 1996) may be a reason why there is no evidence for population growth or expansion in *D. cucullata* although the human-impacted freshwater habitats should have supported them.

The study of three widely distributed and often syntopically occurring species of the *Daphnia longispina* complex revealed different patterns in population genetic structure. Especially for the taxa *D. galeata* and *D. longispina*, for which further support was given by other studies, we may generalize that the population genetic structure in *D. galeata* indicates a lower investment in sexual reproduction, a higher impact of clonal erosion as well as recent gene flow or expansion, while *D. longispina* revealed highly diverse and differentiated populations. The first results of *D. cucullata* populations revealed less variable microsatellite marker that may hinder the detection of the real population structure, but high clonal diversity as well as high genetic differentiation of mitochondrial DNA was observed.

Acknowledgements

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Chapter 5

Priority effects and fitness differences determine the genetic structure during population build-up in *Daphnia*

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Abstract

During the initial stages of population build-up in new habitats, the time at which immigrants arrive may influence their contribution to the population and thus determine population genetic structure. While the numerical advantage associated with priority effects may promote dominance of the offspring of early colonizers, fitness differences associated with ecological differences among genotypes may potentially overwhelm these priority effects. We conducted an outdoor mesocosm experiment to test whether the sequence of arrival determines the relative contribution of genetic lineages (clones) to populations. A set of *D. galeata* clones was inoculated pairwise in different time sequences (two treatments: time advantage of approximately 1/3 or one generation; reciprocal treatments), and clonal abundances were monitored over time. In general we found that, after six weeks (four generations), clones with an initial time advantage had higher relative abundances than in the treatments with simultaneous inoculations. However, in some cases abundances of genetic lineages varied strongly when inoculated simultaneously, reflecting strong differences in fitness among clones in our experimental containers. In these cases, initial numerical advantages could be overwhelmed and the competitively strongest clone became dominant after nine months independently of inoculation sequence. Nonetheless, in other treatments we could still detect priority effects after nine months. Our results highlight the importance of two different processes that determine the fate of clonal lineages and ultimately determine population genetic structuring. First, the sequence of arrival is crucial, with an advantage of five days being already sufficient to dominate the population in specific cases. Second, fitness differences among clones, reflecting the degree to which they are pre-adapted to the environment, may overrule priority effects.

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Introduction

Since genetic markers have come into wide usage for population research, many studies in population biology and molecular ecology have assessed the genetic structure of natural (meta)populations and have studied the processes that alter gene flow and local adaptation (e.g., Hartl & Clark 2007). Founder events, where a population is founded by a few individuals resulting in a low level of genetic diversity and altered genotype frequencies in young populations, are discussed as one factor determining population genetic structure, especially during population build-up (Hartl & Clark 2007).

Where successive invasion events occur, the question arises, how different waves of succession impact local diversity and population structure. The null model would assume that all invading individuals contribute equally to the gene pool. There are, however, two processes that may lead to deviations from this null model. First, individuals are likely to vary either intrinsically (Khazaeli & Curtsinger 2010; Reznick *et al.* 2000) or in their response to local environmental conditions (studied for example in different animal organisms; insect *Rhopalosiphum*: Bieri *et al.* 2009; and rotifer *Brachionus*: Campillo *et al.* 2009; crustacean *Daphnia*: De Meester 1996) and thus have a different fitness. Subsequently, natural selection may result in a differential contribution of invading lineages to the population. Second, the sequence in which individuals arrive at a habitat may influence population genetic structure, because individuals that arrive early may profit from priority effects. These early colonizers have a numerical advantage and may, as they consume resources, change the habitat such that establishment success and subsequent growth of individuals arriving later is reduced (Chase 2003; Morin 1999). If the early colonists are given sufficient time, priority effects may be enhanced as the resident population genetically adapts to local environmental conditions (De Meester *et al.* 2002; Urban & De Meester 2009).

Interspecific priority effects (Chase 2003; Morin 1999) have been intensely studied in community ecology and have been reported in plants (Harper 1961; Körner *et al.* 2008), fungi (Kennedy & Bruns 2005; Kennedy *et al.* 2009) and animals (Dayton & Fitzgerald 2005; Louette & De Meester 2007; Rohlfs 2005). There are just a few controlled experimental studies on priority effects among lineages within a species (Eitam *et al.* 2005; Fukami *et al.* 2007; Geange & Stier 2010; van Gremberghe *et al.* 2009).

As it is difficult to observe the foundation of new populations in nature, several investigators studied the genetic structure of established populations and tried to infer their origin. Standing water bodies and island habitats have been particularly popular for this kind of study because they are limited in size and exhibit a specific history of succession. Zooplankton populations

have been studied intensely, especially since the genetic structure of different taxa revealed that the previously believed cosmopolitan distribution did not hold up (Frey 1982b; Hebert & Wilson 1994; Xu *et al.* 2009). Many studies revealed evidence for high local genetic differentiation and endemism, as found for *Daphnia* (Innes 1991; Thielsch *et al.* 2009), *Polyphemus* (Xu *et al.* 2009), *Brachionus* (Gómez & Carvalho 2000), *Artemia* (Muñoz *et al.* 2008) and *Paramphisopus* (Gouws & Stewart 2007). At the same time, many zooplankton species possess a high potential for passive dispersal as they produce so-called dormant eggs that withstand cold, heat and desiccation (Brendonck & De Meester 2003). Those dormant eggs may be recruited from the sediment to the water column of the same habitat, or may be dispersed by wind and water or carried along by animal vectors like waterfowl to another water body (Havel & Shurin 2004). Also short-distance dispersal of adults is possible and mostly accomplished through animal vectors (Allen 2007). This results in an often rapid colonization of new habitats (Louette & De Meester 2005).

Daphnia is a common genus in freshwater zooplankton communities with an almost world-wide distribution (Benzie 2005). Individuals of this genus reproduce via cyclic parthenogenesis (Zaffagnini 1987) enabling them to reproduce clonally in a fast and efficient way if environmental conditions are favourable. Additionally, *Daphnia* individuals are able to reproduce sexually, resulting in the production of dormant eggs. These ephippia favour dispersal in space and time (dormancy) as they can survive unfavourable environmental conditions (Brendonck & De Meester 2003).

Despite the high potential for dispersal, population genetic studies, using a variety of molecular techniques, often revealed high genetic differentiation among populations suggesting limited gene flow among populations or genetic drift (e.g. Hebert *et al.* 1993; Thielsch *et al.* 2009; Vanoverbeke *et al.* 2007). De Meester *et al.* (2002) proposed a concept, called *monopolization hypothesis,* to explain this paradox of low gene flow and high dispersal rates. The hypothesis states that rapid population growth (resulting in a numerical advantage) in combination with rapid local genetic adaptation (resulting in a fitness advantage) may result in strong and persistent priority effects during population build-up. The authors further argued that in organisms that produce dormant stages, the numerical effect is stabilized by the build-up of a large dormant propagules bank.

The aim of our study was to determine whether the relative abundances of different genotypes within a population are determined by sequence of colonization or by interclonal differences in performance in the habitat. To test the latter, we established a treatment where genotypes were inoculated in the experimental habitats at the same time. Further, we wanted to assess

the effect of the duration of the time lag on priority effects, and to what extent priority effects remain stable over a longer time period.

We present here the results of a colonization experiment, carried out in outdoor mesocosms using *Daphnia galeata* clones, in which the time of invasion is manipulated for several pairs of genotypes in order to investigate the occurrence of intraspecific priority effects and its impact on population genetic structure in newly founded populations. We chose three different times between the inoculation of the first and the second clone. In the first treatment, we inoculated both clones at the same time to quantify any differences in fitness among clones under the experimental conditions. Secondly and thirdly, we inoculated one clone five and fifteen days, respectively, after the other one. As the latter two treatments were done reciprocally for each pair of genotypes, we have five inoculation treatments per clone pair.

Material and Methods

Collection of clones

Daphnia galeata clones were selected from three different sampling sites in Belgium. Six of the eight clones (BE03, 04, 05, 10, 11 and 15) were sampled near Beringen (51°00'18''N, 5°18'17''E) and one was sampled from a lake near Maaseik (51°05'49'' N, 5°48'16''E; genotype MA12). Samples were collected on May 9th 2007 using a plankton net with a mesh size of 200 µm. Afterwards individuals were selected to establish clonal cultures in the laboratory. One additional clone (HE01), was hatched from sediments from a pond near Oud Heverlee (51°21'01.97''N, 3°19'49.58''E). The sediments of this pond were sampled in October 2006 and stored at 4 °C and in the dark until March 2007. The sample was then soaked in tap water and sieved through a 224 µm mesh. *Daphnia* ephippia were picked out, placed in demineralized tap water and hatched neonates were isolated. All experimental clones were raised in the laboratory under standardized conditions for at least two months prior to the start of the experiment to reduce maternal effects. They were cultured in ADaM (Klüttgen *et al.* 1994) with a light : dark cycle of 16 : 8 h, at around 18-20 °C and under optimal food conditions (1 mg C/L *Scenedesmus obliquus*).

Molecular identification of clones

First, we established whether we could differentiate the eight clones using molecular markers so that we could use them in the experiment in which they are combined and in which we needed to be able to assign individuals to genotypes. DNA preparation was conducted using

proteinase K digestion according to Schwenk *et al.* (1998). Further, we amplified twelve microsatellite loci (Dp281NB, SwiD14, DaB10/14, DaB17/17, Dp196NB, SwiD6, SwiD12, SwiD18, Dgm105, Dgm109, Dgm112 and Dp519; Brede *et al.* 2006) for several individuals of each clone according to the protocols published by Thielsch *et al.* (2009). Based on the result of this initial screening, we picked three loci out of the twelve analysed that were sufficiently variable to differentiate the multi-locus genotypes of the pairwise clonal combinations that we intended to use: HE01/BE03 (SwiD14), BE04/BE10 (SwiD12), BE05/BE11 (SwiD18) and BE15/MA12 (SwiD14).

Mesocosm experiment

The experiment was performed at the Aquatic Research ExperimeNtal Area (ARENA) at KULeuven (Heverlee, Belgium), using white conical plastic buckets comprising a volume of 100 L (Type: Spa code 030, $Ø_{Bottom}$ 410 mm, $Ø_{Top}$ 480 mm, height 645 mm). To fill the buckets we used 80 L of tap water. Populations were established using four clonal combinations with two clones per combination. Clones were either inoculated at the same time, or with a 5 or 15 days time lag. The latter treatments were carried out two ways, giving one or the other clone a time advantage. Each treatment was replicated three times. The design resulted in four clone combinations x 5 inoculation treatments x 3 replicates = 60 experimental buckets. The usage of eight different clones in four combinations allowed us to contrast differences in fitness and priority effects. The treatment in which clones were inoculated at the same time directly assessed fitness differences among clones. Treatments were randomly assigned to buckets. Buckets were covered with mosquito net to avoid contamination, e.g. by aquatic insects. Twenty-four hours after filling the barrels with water, we added 4 L of a 64 µm filtered suspension of a diverse phytoplankton community to each bucket. The algae stock was grown by sampling a species diverse phytoplankton suspension from two ponds nearby the experimental site in May 2007 and filtering it through a 30 µm net to remove zooplankton. Fourty litres of this algae mix were added to a large container with 560 L tap water which contained nutrients (3.48 g KH_2PO_4 and 34.8 g $NaNO_3$). In order to monitor algae concentration within this algae stock, the amount of chlorophyll *a* was determined in the laboratory every three to four days by measuring absorbance at 665 nm with a fluorimeter. This community was allowed to grow during approximately 50 days, after which 4 L of it were added to each experimental bucket.

The first inoculation of animals was carried out on July 9th 2007. We used 50 individuals per clone per inoculation (see **Table 5-S1**). During the experiment, various environmental

parameters were monitored (Chl_a [µg/L], temperature [°C], oxygen [mg/L], conductivity [µS/cm] and pH) every two weeks in the first phase of the experiment (until the first sampling) and then two more times (November 2007 and May 2008).

The first sampling campaign took place approximately 6 weeks after the second inoculation (**Table 5-S1**). We used a tube-sampler, which allowed us to sample the whole water column. We sampled 20% of the volume of the experimental bucket. The zooplankton was filtered over 64 µm plankton gauze. If there were less than 30 individuals in the initial sample, we sampled twice (10 out of 60 populations).

After the first sampling round, we left the buckets in the experimental field for a period of approximately seven more months, including a winter period, until May 2008. This resulted in a total experimental period of nine months. We then sampled all populations by emptying the buckets and taking all available plankton for morphological and genetic identification. From each container we collected on each sampling date 30 *D. galeata* individuals (if available) and determined their genotype.

Estimation of genotype abundances using microsatellite loci

Experimental individuals were preserved in ethanol. Prior to DNA preparation (Schwenk *et al.* 1998) individuals were incubated for 4-16 h at 4 °C in 1 mL TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) to remove the ethanol.

To assign individuals in our mesocosm experiments to a genotype, we amplified the one discriminatory locus depending on the clonal pair. The forward primers were labelled with the fluorescent dyes Alexa 647 (Invitrogen; SwiD14) and IRD700 (MWG; SwiD12 and SwiD18). PCR was performed in 0.2 mL tubes with 10 µL reaction volume containing 2.4 mM $MgCl_2$, 1x PCR buffer, 0.25 mM of each dNTP, 0.2 µM (SwiD14 and SwiD18) or 0.1 µM (SwiD12) of forward and reverse primer, 0.5 U (SwiD12 and SwiD14) or 1 U (SwiD18) *Taq* polymerase (Invitrogen), 0.2 mg/mL (for loci SwiD14 and SwiD18) or 0.1 mg/mL (for locus SwiD12) BSA (New England Biolabs), 8% DMSO (Roth) for locus SwiD18 and 2-4 µL prepared DNA. Cycling conditions for PCR started with a 3 min denaturing step at 95 °C followed by 35 cycles (26 cycles for locus SwiD14) of 1 min steps at 95 °C, at 55 °C (SwiD12, SwiD18) or 60 °C (SwiD14) and 72 °C. A final 7 min synthesis step at 72 °C completed all programs. Amplicons were diluted and electrophoresed on a CEQ 2000 (Beckman Coulter; denaturation at 90 °C for 2 min; injection at 2.0 kV for 30 sec; separation at 6.0 kV for 45 min) with a self-designed size standard based on Lambda phage DNA (Symonds & Lloyd 2004).

Statistical tests for priority effects

To visualize the frequency of the founder clones (time advantage of either 0, 5 or 15 days) we calculated a bean plot (Kampstra 2008) in R (R Development Core Team 2011) for the results of the first sampling after six weeks, if at least 30 individuals were collected and genotyped. Whereas all the data in the 5 and 15 day treatments represent only the abundance of "initial" clones, the 0 day treatment represents abundances of both clones as inoculation was simultaneously. Thus, the 0 day treatment results in perfectly symmetrical beans and serves as a neutral expectation for the priority treatments (5 and 15 days delay). A fair assessment of the strength of the priority effects can be made by comparing the shape of the beans across all treatments.

To test for significant changes in frequencies of genotypes among treatments we applied G statistics (Sokal & Rohlf 1995). We assumed two different scenarios. First, we tested the hypothesis (named *hypothesis 1:1*) that the relative abundance of the genotypes in each container remained the same (50% clone A and 50% clone B) irrespective of the treatment. Second, we took into account that genotypes might have different performances, thus we tested against the hypothesis that the relative abundance of the genotypes in the priority treatments remained the same with those of the treatment (0 days) where both clones were inoculated at the same time (*hypothesis day 0*). We calculated G-tests of goodness-of-fit for each individual experimental unit where the resulting G-values were the "individual G-values". For the results of the first sampling we further calculated total G, pooled G, and heterogeneity G. Total G was calculated for each treatment by adding up the three individual G-values of the three replicates of the treatment. Pooled G-values were calculated for each treatment by adding up the frequencies of the three replicates per treatments. To test whether individual G-values obtained from replicates of a given treatment were different we calculated heterogeneity G using pooled and total G-values.

To assess whether a time advantage of the founder clone resulted in general in an overall frequency increase was determined by pooling all observations for all genotypes in three different combinations and applying G-statistics: (i) for the results of the treatment 5 days, (ii) for the results of the treatment 15 days, and (iii) for the combined results of these treatments 5 and 15 days.

All G-values were calculated using an excel spreadsheet provided by J. H. McDonald (2009, *http://udel.edu/~mcdonald/statrepgtestgof.html*) and p-values for total and heterogeneity G were calculated using the webpage *http://www.stat.tamu.edu/~west/applets/chisqdemo.html*.

Table 5-1 Genotype abundances of 30 individuals selected from each container during the first and the second sampling. Founder = clone that was inoculated first; invader = clone that was inoculated second; *Δt* = time difference; six weeks = frequency of genotypes after six weeks of experimental time; Ind G = individual G value (p value in brackets; significant values marked in bold); Total G = total G values calculated from Ind G; Pool G = pooled G value obtained when datat of all replicates are pooled; Het G = heterogeneity G; Nine months = abundance of genotypes after nine months experimental time. $^{1:1}$ = *hypothesis 1:1* (comparison to expectation of equal fitness); ^{0d} = *hypothesis day 0* (comparison with frequencies observed when the two clones are inoculated simultaneously).

Founder	Invader	*Δt*	Six weeks								Nine months			
			BE05	BE11	Ind G $^{1:1}$	Total G $^{1:1}$	Pool G $^{1:1}$	Het G	Ind G ^{0d}	Total G ^{0d}	Pool G ^{0d}	BE05	BE11	Ind G $^{1:1}$	Ind G ^{0d}
BE05		0	14	16	0.133 (0.715)										
&		0	16	14	0.133 (0.715)	1.113 (0.291)	3.66 (0.301)	2.55 (0.279)							
BE11		0	10	20	3.398 (0.065)							15	15	0 (1.000)	
BE05	BE11	5	17	13	0.54 (0.465)				1.84 (0.175)						
		5	16	14	0.13 (0.715)	0.04 (0.833)	2.83 (0.419)	2.78 (0.249)	0.98 (0.321)	0.76 (0.382)	3.55 (0.314)				
		5	11	19	2.16 (0.142)				0.72 (0.396)						
		15	26	4	18.03 **(< 0.001)**				23.46 **(< 0.001)**						
		15	29	1	32.82 **(< 0.001)**	80.68 **(< 0.001)**	83.67 **(< 0.001)**	2.99 (0.224)	39.62 **(< 0.001)**	99.72 **(< 0.001)**	102.71 **(< 0.001)**				
		15	29	1	32.82 **(< 0.001)**				39.62 **(< 0.001)**			28	2	26.89 **(< 0.001)**	26.89 **(< 0.001)**
BE11	BE05	5	0	30	41.59 **(< 0.001)**				35.11 **(< 0.001)**			3	27	22.08 **(< 0.001)**	22.08 **(< 0.001)**
		5	4	26	18.03 **(< 0.001)**	86.15 **(< 0.001)**	92.44 **(< 0.001)**	6.29 **(0.043)**	13.38 **(< 0.001)**	69.00 **(< 0.001)**	75.30 **(< 0.001)**	1	29	32.82 **(< 0.001)**	32.82 **(< 0.001)**
		5	1	29	32.82 **(< 0.001)**				26.80 **(< 0.001)**						
		15	3	27	22.08 **(< 0.001)**				16.98 **(< 0.001)**						
		15	6	24	11.57 **(0.001)**	61.98 **(< 0.001)**	66.47 **(< 0.001)**	4.49 (0.105)	7.84 **(0.005)**	47.12 **(< 0.001)**	51.62 **(< 0.001)**				
		15	1	29	32.82 **(< 0.001)**				26.80 **(< 0.001)**			0	30	41.59 **(< 0.001)**	41.59 **(< 0.001)**
			BE03	**HE01**								**BE03**	**HE01**		
BE03		0	13	17	0.54 (0.465)										
&		0	16	14	0.13 (0.715)	0.40 (0.527)	1.20 (0.753)	0.80 (0.670)				0	30	41.59 **(< 0.001)**	
HE01		0	13	17	0.54 (0.465)							10	20	3.40 (0.070)	
BE03	HE01	5	21	9	4.94 **(0.026)**				6.65 **(0.010)**			24	6	11.57 **(< 0.001)**	58.08 **(< 0.001)**
		5	10	20	3.40 (0.065)	0.18 (0.673)	9.54 **(0.023)**	9.37 **(0.009)**	2.21 (0.137)	0.04 (0.838)	9.41 **(0.024)**	28	2	26.89 **(< 0.001)**	86.26 **(< 0.001)**
		5	12	18	1.21 (0.272)				0.55 (0.460)			3	27	22.08 **(< 0.001)**	1.10 (0.294)
		15	23	7	8.99 **(0.003)**				11.24 **(0.001)**			17	13	0.53 (0.465)	24.55 **(< 0.001)**
		15	22	8	6.79 **(0.009)**	13.17 **(< 0.001)**	16.32 **(0.001)**	3.15 (0.207)	8.78 **(0.003)**	18.06 **(< 0.001)**	21.21 **(< 0.001)**	4	26	11.57 **(< 0.001)**	0.26 (0.611)
		15	17	13	0.54 (0.465)				1.20 (0.274)						
HE01	BE03	5										2	28	26.89 **(< 0.001)**	2.70 (0.101)
		5	22	8	6.79 **(0.009)**	NA	NA	NA	8.78 **(0.003)**	NA	NA	20	10	3.40 (0.070)	37.05 **(< 0.001)**
		5	25	5	14.56 **(< 0.001)**				17.33 **(< 0.001)**			0	30	41.59 **(< 0.001)**	10.96 **(< 0.001)**
		15	4	26	18.03 **(< 0.001)**				15.25 **(< 0.001)**			4	26	18.03 **(< 0.001)**	0.26 (0.611)
		15	3	27	22.08 **(< 0.001)**	66.25 **(< 0.001)**	67.01 **(< 0.001)**	0.75 (0.687)	19.04 **(< 0.001)**	57.13 **(< 0.001)**	57.88 **(< 0.001)**				
		15	2	28	26.89 **(< 0.001)**				23.59 **(< 0.001)**						

			BE04	**BE10**								**BE04**	**BE10**					
BE10		0	1	29	32.82 (**< 0.001**)	34.69	46.54	11.85				0	30	41.59 (**< 0.001**)				
&		0	6	24	11.57 (**0.001**)	(**< 0.001**)	(**< 0.001**)	(**0.003**)				0	30	41.59 (**< 0.001**)				
BE04		0	11	19	2.16 (0.142)							0	30	41.59 (**< 0.001**)				
BE10	BE04	5	0	30	41.59 (**< 0.001**)			13.39 (**< 0.001**)				0	30	41.59 (**< 0.001**)	0 (1.000)			
		5	8	22	6.79 (**0.009**)	46.97	59.95	12.98	0.77 (0.379)	1.18	14.16	0	30	41.59 (**< 0.001**)	0 (1.000)			
		5	6	24	11.57 (**0.001**)	(**< 0.001**)	(**< 0.001**)	(**0.002**)	0.00 (1.000)	(0.277)	(**0.003**)	0	30	41.59 (**< 0.001**)	0 (1.000)			
		15	0	30	41.59 (**< 0.001**)	NA	NA	NA	13.39 (**< 0.001**)	NA	NA	0	30	41.59 (**< 0.001**)	0 (1.000)			
BE04	BE10	5	4	26	18.03 (**< 0.001**)	92.04	101.21	9.17	0.92 (0.338)	18.53	27.70	0	30	41.59 (**< 0.001**)	0 (1.000)			
		5	0	30	41.59 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	(**0.010**)	13.39 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	0	30	41.59 (**< 0.001**)	0 (1.000)			
		5	0	30	41.59 (**< 0.001**)				13.39 (**< 0.001**)			0	30	41.59 (**< 0.001**)	0 (1.000)			
		15	15	15	0.00 (1.000)				13.39 (**< 0.001**)									
		15	8	22	6.79 (**0.009**)	18.42	33.68	15.27	0.77 (0.379)	3.13	18.40	0	30	41.59 (**< 0.001**)	0 (1.000)			
						(**< 0.001**)	(**< 0.001**)	(**0.001**)		(0.077)	(**< 0.001**)							
		15	2	28	26.89 (**< 0.001**)				4.24 (**0.040**)									
			BE15	**MA12**								**BE15**	**MA12**					
BE15		0	30	0	41.59 (**< 0.001**)	66.25	74.17	7.92				30	0	41.59 (**< 0.001**)	0 (1.000)			
&		0	26	4	18.03 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	(**0.019**)				30	0	41.59 (**< 0.001**)	0 (1.000)			
MA12		0	25	5	14.56 (**< 0.001**)							30	0	41.59 (**< 0.001**)	0 (1.000)			
BE15	MA12	5	30	0	41.59 (**< 0.001**)	92.04	96.49	4.45	6.32 (**0.012**)	3.82	8.27	30	0	41.59 (**< 0.001**)	0 (1.000)			
		5	27	3	22.08 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	(0.108)	0.00 (1.000)	(0.051)	(**0.041**)	30	0	41.59 (**< 0.001**)	0 (1.000)			
		5	29	1	32.82 (**< 0.001**)				1.95 (0.163)			30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	30	0	41.59 (**< 0.001**)	124.77	124.77	0.00	6.32 (**0.012**)	18.97	18.97	30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	30	0	41.59 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	(1.000)	6.32 (**0.012**)	(**< 0.001**)	(**< 0.001**)	30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	30	0	41.59 (**< 0.001**)				6.32 (**0.012**)									
MA12	BE15	5	12	18	1.21 (0.272)	0.00	17.71	17.71	45.04 (**< 0.001**)	91.95	109.66							
		5	24	6	11.57 (**0.001**)	(1.000)	(**0.001**)	(**< 0.001**)	2.66 (0.103)	(**< 0.001**)	(**< 0.001**)	30	0	41.59 (**< 0.001**)	0 (1.000)			
		5	9	21	4.94 (**0.026**)				61.95 (**< 0.001**)			30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	17	13	0.54 (0.465)				22.40 (**< 0.001**)			30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	8	22	6.79 (**0.009**)	11.63	25.36	13.73	68.21 (**< 0.001**)	173.89	187.62	30	0	41.59 (**< 0.001**)	0 (1.000)			
		15	4	26	18.03 (**< 0.001**)	(**0.001**)	(**< 0.001**)	(**0.001**)	97.02 (**< 0.001**)	(**< 0.001**)	(**< 0.001**)	30	0	41.59 (**< 0.001**)	0 (1.000)			

Results

The first samples were taken after six weeks (September 2007), while the second set of samples was taken after nine months (May 2008; **Table 5-1** & **5-S1**). Bucket 4 was not included in the analysis of the six-week samples as we found less than 30 individuals even after sampling twice. In addition, buckets 29 and 30 were excluded completely as not enough juveniles were available for a second inoculation. Besides buckets 29 and 30, we had to exclude 19 more buckets of the nine-month samples as we did not retrieve 30 individuals from these experimental units. All statistical analyses were conducted without these buckets (6 weeks: 57 experimental units; 9 months: 39 experimental units).

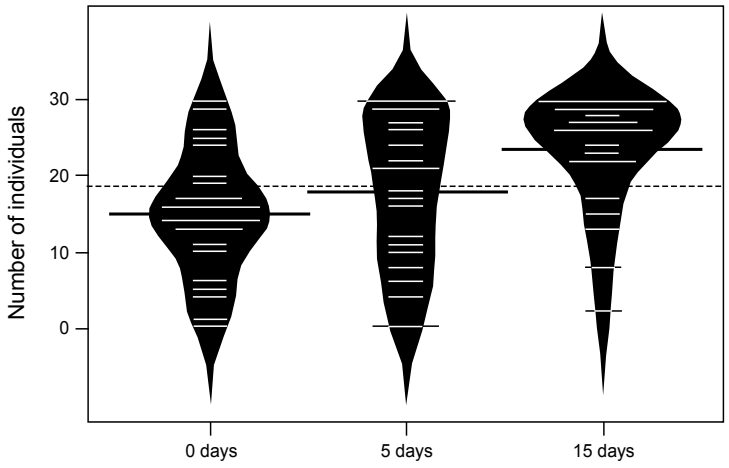

Figure 5-1 Bean plot visualizing frequency distribution of genotypes in the experiment. Data are grouped according to treatment (0 days: inoculated simultaneously with the partner of the clonal trial; 5 days: inoculated 5 days prior to the invader clone; 15 days: inoculated 15 days prior to the invader clone). The bean plot visualizes all data irrespective of clonal pair, and plots the number of individuals of a given clone (BE05, BE11, BE03, HE01, BE10, BE04, BE15 and MA12) retrieved in one experimental unit. For the 5 and 15 days treatments, the beans only visualize the abundances of the clone that is inoculated first (irrespective of clonal pair). For the 0 days beans, all data are plotted, resulting in symmetrical shape. Every individual observation is shown as a white single line of standardized length, with the total length of the line indicating how often the same observation was made in all trials. The bold black line indicated the average of the three treatments over all observations (all clonal pairs) and the dotted line gives the overall average. Each bean shows the distribution of the observations as a density shape.

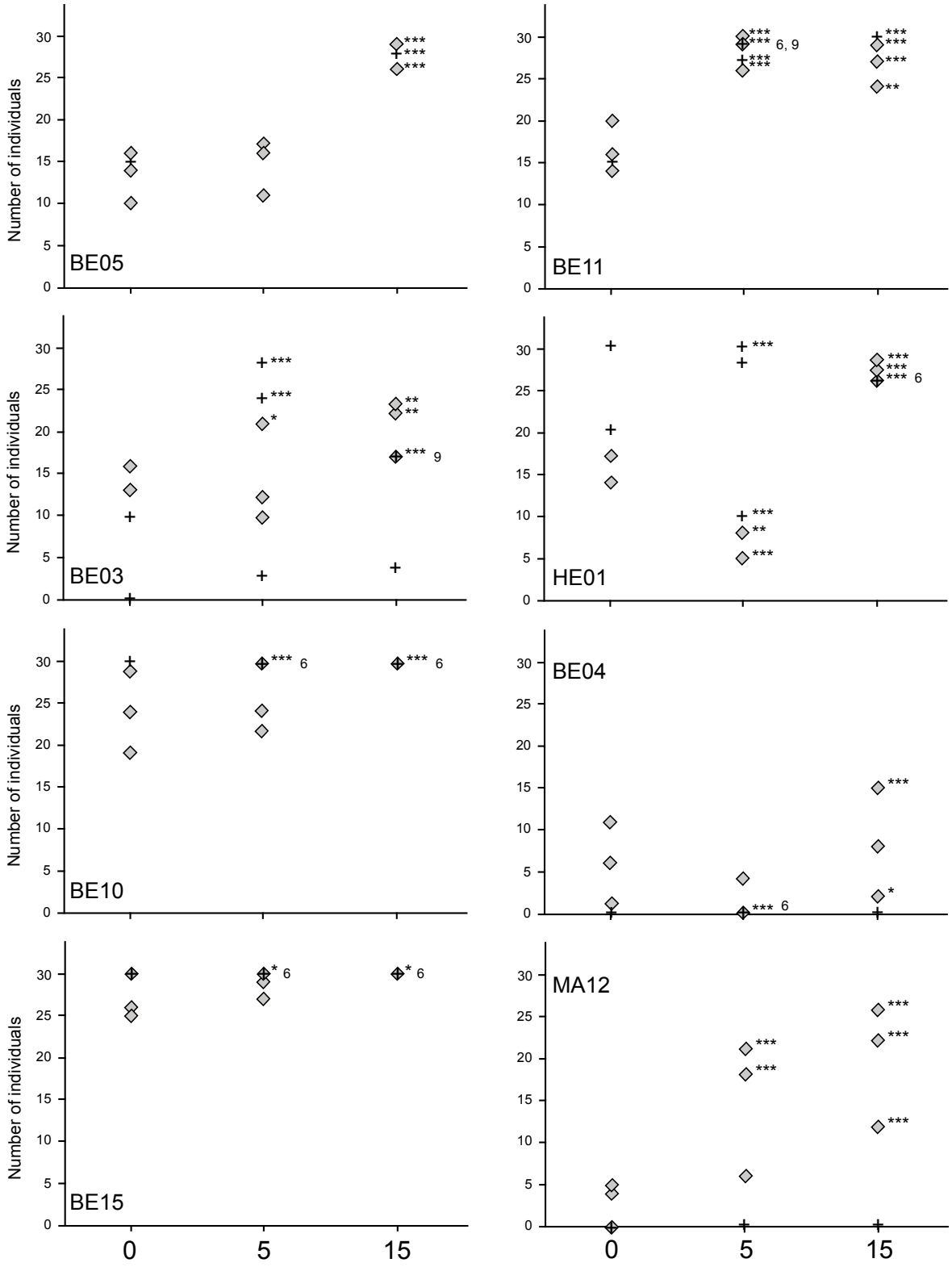

Figure 5-2 Frequency of *Daphnia* clones BE05, BE11, BE03, HE01, BE10, BE04, BE15 and MA12 in the three treatments (see **Table 5-1**): inoculated simultaneously with the other clone of the trial (0), 5 days prior to the invader clone (5), and 15 days prior to the invader clone (15). Grey rhombuses show data points from the first sampling (after 6 weeks) and black crosses represent data points from the second sampling (after 9 months). A significant increase in genotype abundances compared to the mean abundance of treatment 0 days is indicated for both sampling times: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. In ambiguous cases, with two overlaying data points, it is indicated by a number for which sampling moment the significant increase or decrease in abundance is observed (6 = six weeks, 9 = nine months).

Differences in competitive strength among genotypes

When clones were inoculated simultaneously, they showed variable differences in competitive strength (**Table 5-1**; significant deviations from *hypothesis 1:1* indicate strong fitness differences among genotypes). Clones BE05 and BE11 did not differ significantly in competitive strength when inoculated simultaneously, both after six weeks and after nine month (no significant G-values; **Table 5-1**). In clone pair BE03 and HE01, genotype abundances were not significantly different after six weeks of population build-up. However, competitive strength was shifted in favour of HE01 after nine months. In the trials with clones BE04 and BE10, clone BE10 showed to be competitively superior (all but one individual G-values significant, **Table 5-1**). This clone dominated (approximately 80%) after six weeks and had driven clone BE04 to extinction after nine months. A similar difference in competitive strength was observed in the trials between MA12 and BE15, with clone BE15 as superior competitor (all G-values significant, **Table 5-1**), representing approximately 90% of the individuals after six weeks and having driven clone MA12 to extinction after nine months.

Priority effects

Overall, we found that clones reached higher abundances if they were inoculated first (5 days: a total of 396 individuals of the founder genotype and 294 individuals of the invader genotype, summed across all clones, $G = 15.31$, $p < 0.001$; 15 days: 513 individuals of the founder genotype and 147 individuals of the invader genotype, $G = 214.91$, $p < 0.001$; 5+15 days combined: 909 individuals of the founder genotype and 441 individuals of the invader genotype, $G = 165.66$, $p < 0.001$; see also **Figure 5-1**). Priority effects for all clone combinations separately were evaluated by estimating goodness-of-fit G-tests for genotype frequency changes between treatment 0 days and treatment 5 or 15 days (*hypothesis 0 days*, see **Table 5-1** and **Figure 5-2**). In the trial with clone pair BE05 and BE11, which showed similar competitive strength when inoculated simultaneously, we observed a clear-cut priority effect when the first clone had a time advantage of 15 days. When inoculated first, both clones clearly dominated the population after six weeks (~ 90% abundance). After nine months, this pattern was maintained. When clone BE11 was inoculated five days prior to clone BE05, it also benefited from a clear priority effect, which was equally strong as when it was given 15 days of advantage. This did not hold when clone BE05 was inoculated 5 days prior to clone BE11, however. For clones BE03 and HE01, the pattern was not as consistent. When clone BE03 was inoculated first, a five days advantage did not lead to a priority effect,

but an advantage of 15 days resulted in a clear-cut priority effect after six weeks. After nine months, the pattern was different, with clone BE03 as the inferior partner in this clone pair except when it had an advantage of 5 days. When clone HE01 was inoculated first, a clear-cut priority effect was observed in the case of a 15 days delay before the invading genotype was inoculated, which was maintained after nine months. In the case of a 5 days advantage, the pattern was reversed after six weeks, but after nine months a priority effect was observed. In the competition between clones BE04 and BE10, a 15 days advantage of the superior clone resulted in extinction of the inferior clone within 6 weeks. A 5 days advantage did not result in a significant priority effect after six weeks. Inoculating the weaker clone (BE04) first (time advantage of 15 days) resulted in a compensation of the difference in competitive strength after six weeks. A 5 days advantage of the weaker clone did not lead to compensation and even reduced the relative abundance of this genotype after six weeks. After nine months, the inferior clone went extinct irrespective of inoculation order. Finally, in the competition trials with clones MA12 and BE15, giving a time advantage to the weaker competitor (clone MA12) resulted in a compensation of the difference in competitive strength after six weeks in the case of a 5 days advantage and in a slight dominance of this genotype in the case of a 15 days advantage. After nine months, the competitively inferior clone was driven to extinction irrespective of inoculation order.

Summarizing, after six weeks, we observed 11 cases of positive priority effects, three cases of absence of priority effects, and two cases of opposite responses in which the founder showed reduced abundance compared to the treatments with simultaneous inoculation. The data after nine months are less trustworthy because of lower numbers of replicates, but show four cases of priority effects and eight cases of no effect of inoculation order. These eight cases all refer to treatments in which the competitively superior clone drove the inferior clone to extinction independent of inoculation order. The overall emerging pattern is that priority effects are strong and long-lasting (at least over a period of nine months) when fitness differences are not large, while they are temporary at most when fitness differences among genotypes are large.

Discussion

Parameters impacting the abundances of genotypes during population build-up

Our aim was to determine to which extent the frequencies of different genotypes in a founder population are impacted by the succession sequence and by the differential fitness of those genotypes, respectively. In all clonal combinations we found changes in genotype abundances

in relation to the sequence of inoculation (with a time lag of 5 or 15 days) after approximately six weeks (four generations) of population build-up, suggesting that priority effects are indeed widespread. While numerically few, the results of other experimental studies dealing with priority effects on an intraspecific level (Eitam *et al.* 2005; Fukami *et al.* 2007; Geange & Stier 2010; van Gremberghe *et al.* 2009) are in line with our observations. For example, Geange & Stier (2010) recorded a higher mortality rate in late arriving reef fish of the species *Thalassoma quinquevittatum* that had a time disadvantage of 5 to 12 days, while Eitam *et al.* (2005) observed high rates of cannibalism and competition in *Salamandra salamandra*, where late-cohort larvae suffered from increased mortality relative to early-cohort larvae. As the two previously mentioned studies presented priority effects within a single generation, the study of van Gremberghe *et al.* (2009) recorded priority effects among different strains of the cyanobacterium *Microcystis* using an experimental approach over a time period encompassing several generations. In general, we found that priority effects are much more pronounced if the first colonizers had a 15 compared to a 5 days' time advantage. After six weeks, we observed a significant priority effect in 7 out of 8 comparisons when the founding genotype was given an advantage of 15 days, while this was the case in 2 out of 8 comparisons when a time advantage of only 5 days was given. Our data after nine months are less balanced as we lost some replicates, but overall, we also observed priority effects after this longer period and a less striking difference between the 5 days and the 15 days treatment (3 out of 8 cases in the 15 days treatment and 2 out of 7 for which we had data in the 5 days treatment; see **Figure 5-1**). In some cases (combination of BE05 and BE11 both ways) the patterns after nine months were reinforced compared to that after six weeks, suggesting that the priority effect was not a temporary advantage but rather a long-lasting effect. In other clonal trials (e.g. MA12 and BE15), the initial priority effect was overruled by the competitively superior genotype.

Overall, we observed a strong interdependence of differences in competitive ability and the strength of priority effects. We observed clear-cut and reciprocal priority effects in the trials with two clones that are competitively similar to each other (e.g. clones BE05 and BE11 and to a lesser extent clones BE03 and HE01), while priority effects were unclear or transient in the trials with combinations of clones that differed strongly in competitive strength. A numerical advantage through inoculation order as applied in our experiment was not sufficient to overrule strong differences in fitness among genotypes. In other words: the stronger the fitness differences among clones, the more deterministic their relative abundances are, and the less they are dependent on inoculation order. Importantly, our results indicate that if fitness differences are not strong, the priority effects are likely to become a higher impact and also

result in a longer-lasting effect. The low impact of priority effects on competitively superior clones are, however, likely an artefact of the fact that these clones (BE10 and BE15) even strongly dominate the population in the absence of a numerical advantage, so that the scope for increased dominance is small. This scope becomes larger as clones are competitively similar or slightly inferior to their competitor, and under these conditions priority effects are most pronounced. For clones that are strongly inferior competitors, nevertheless, an initial numerical advantage may very rapidly be annihilated and any priority effect may be lost. Clones BE04 in our trials may be a case at hand.

Priority effects and population genetic structure

Population genetic analyses of cyclically parthenogenetic zooplankton, like *Daphnia galeata*, often report high levels of among-population genetic differentiation and endemism (Gómez & Carvalho 2000; Gouws & Stewart 2007; Innes 1991; Muñoz *et al.* 2008; Thielsch *et al.* 2009; Xu *et al.* 2009), suggesting low levels of ongoing gene flow. Still, freshwater organisms have been shown to exhibit a relatively high dispersal capacity thanks to the production of dormant stages that can be passively transported via wind and waterfowl (Havel & Shurin 2004). De Meester *et al.* (2002) have proposed the *monopolization hypothesis* as an explanation for this paradox between low gene flow and high dispersal capacity. This hypothesis states that the observed patterns of high among-population genetic differentiation may be explained by a combination of stochastic and selection-driven processes. After historical colonisation from one or a few propagules, the rapid population growth rate combined with local genetic adaptation reduces effective gene flow by lowering the establishment success of late arriving genotypes (De Meester *et al.* 2002), resulting in an enhanced priority effect. Our study provides support for this hypothesis by showing that, if fitness differences among clones are not too strong, priority effects being caused by the order in which genotypes arrive in a target habitat can be important and result in long-lasting changes in genetic composition among populations. As local genetic adaptation increases fitness of residents compared to the average invading genotype (which was not studied here) one would expect that this observed priority effect in mesocosms would be even stronger in natural communities. Importantly, the time span over which we observed priority effects is very short: in some cases a long-lasting (nine months) effect is present already when a time advantage of 5 days is given, whereas a time advantage of 15 days resulted in strong priority effects in most of our experimental populations. This is in line with earlier results in which similarly strong priority effects were

observed at the interspecific level, in a study with different species of large-bodied cladocerans (Louette *et al.* 2007).

This large impact of even a short time advantage leads to the expectation that in nature a time difference of a few months or years is likely to have an important effect, especially given that local populations produce a large dormant egg bank. Juveniles hatch from egg banks when conditions become favourable, thus invasion is only possible during a very small time window each year during which population densities are relatively low. Ortells *et al.* (2011) studied the population genetic structure of *D. magna* over five growing seasons and detected a population with high numbers of invading clones for the first growing season but little evidence for further successful invasions during the following growing seasons. New alleles where detected at two studied microsatellite loci, but the frequencies were marginal. This illustrates that priority effects as observed in our experimental study seem to be an important factor in shaping genetic structure also in natural populations.

Importantly, though, our results also show the limitations of priority effects, which are dictated by the differences in fitness among clones. Our results indeed indicate that a superior genotype can overrule priority effects, consistent with observations by Ebert *et al.* (2002). This, however, highlights the importance of local genetic adaptation in explaining long-standing founder effects in nature (cf. *monopolization hypothesis*), as local genetic adaptation results in a decline in the probability that a genotype of higher fitness than the residents will arrive in a habitat.

Summary

Our results identified that two different processes interact strongly in determining population genetic structure during population build-up in the water flea *Daphnia*. First, priority effects play an important role, with an advantage of starting a few days earlier being sufficient for a genotype to dominate a population. Although we only compared two time lags, our results indicate that the more time a first colonist is given to grow before an invading genotype reaches the population, the stronger the impact of this advantage and the more likely that it becomes permanent. Even if we ignore differences in arrival time of genotypes within a given growing season, our results suggest that genotypes which colonize a new habitat in the first year may have a strong and potentially long-lasting advantage over later arriving genotypes, which may result in strong and persistent founder effects if the number of colonists each year is relatively low (e.g. Louette *et al.* 2007; Ortells *et al.* 2011). Second, it is well established that *Daphnia* genotypes can strongly differ in fitness in a given habitat, and clonal selection

may be very effective. Our results confirm this, and indicate that priority effects can be overwhelmed by intrinsic fitness differences among clones. During the initial stages of habitat colonization, these fitness differences may reduce the impact of founder events and may change their impact on population genetic structure by changing the identity of the clones that dominate in a habitat. As a population gets established and genetically adapts to local environmental conditions, however, fitness differences will tend to be biased in favour of residents and may as such stabilize the patterns of genetic differentiation resulting from founder events (De Meester *et al.* 2002). This at least is expected when environmental conditions remain stable. It is conceivable that invading genotypes may have higher fitness in the case when environmental conditions change. The degree to which this occurs needs further study, as it depends on the fitness profiles of genotypes in the regional genotype pool as well as on evolutionary potential of local populations, for instance, their capacity to rapidly track environmental change. Most studies in *Daphnia* suggest that rapid genetic tracking of environmental change is possible (e.g. Jansen *et al.* 2011; Van Doorslaer *et al.* 2009). For example, Van Doorslaer *et al.* (2009) did also show that local adaptation may reduce establishment success of pre-adapted genotypes from the region.

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General discussion

The main objectives of my thesis focused on the processes which alter the population genetic structure in cyclic parthenogenetic species of the subgenus *Hyalodaphnia*. After the successful development and evaluation of variable nuclear microsatellite markers (**Chapter 1**), which were suitable for species identification and hybrid class assignment (**Chapter 2**), I analyzed the population genetic structure of three *Daphnia* species (**Chapter 3** and **4**). The drivers impacting clonal diversity in and the genetic differentiation among cyclic parthenogenetic zooplankton populations have been debated frequently (Boileau *et al.* 1992; Boileau & Taylor 1994; De Meester 1996; De Meester *et al.* 2002; De Meester *et al.* 2006; Gómez *et al.* 2002) and are discussed within the scope of my thesis for the species *D. cucullata*, *D. galeata*, and *D. longispina*. My results revealed that a reappraisal might be appropriate, as they show that the population genetic structure of a species is not only explained by monopolization according to De Meester *et al.* (2002). An experimental study was conducted to gain deeper insights into the early stages of a population and uncovered that monopolization may be impaired by effective gene flow (**Chapter 5**). Therefore, I conclude that the age of a population as well as severe environmental changes must be considered when studying population genetic structure as they efficiently alter dispersal regimes.

Dynamic processes impact population genetic structures in *Daphnia* species

So far, the within population genetic structure of cyclic parthenogenetic zooplankton organisms was explained by three main factors: (I) the size of the dormant egg bank, (II) the length of the growing season, and (III) the strength of clonal selection (De Meester *et al.* 2006). These factors impact population clonal diversity, as a large dormant egg bank increases clonal diversity, while over time due to chance extinctions and clonal selection this diversity is eroded. The influence from gene flow is assumed to be negligible in well established populations, as reestablishment from the dormant egg bank together with parthenogenetic reproduction mode assure the fast achievement of carrying capacity with locally adapted genotypes (De Meester *et al.* 2002). These processes are shortly summarized in **Figure D-1**B, showing that established populations exhibit locally adapted genotypes

derived from a large dormant egg bank, contributing to clonal diversity which is eroded through time and selective pressures encountered in the habitat. Within a rather stable environment the population will reach an equilibrium resulting in the efficient impediment of effective gene flow and its consequences on population genetic structure. However, my study indicated that this concept should be extended as a number of biological processes may promote effective gene flow, which was further corroborated by recent empirical and experimental published evidence (Ebert *et al.* 2002; Louette & De Meester 2005; Ortells *et al.* 2011). Gene flow may be effective, because firstly, during the early phases of population build-up it will contribute to genetic variation necessary to facilitate local adaptation, and secondly, severe environmental changes annihilate the advantage of locally adapted genotypes within the pelagic population and in the dormant egg bank.

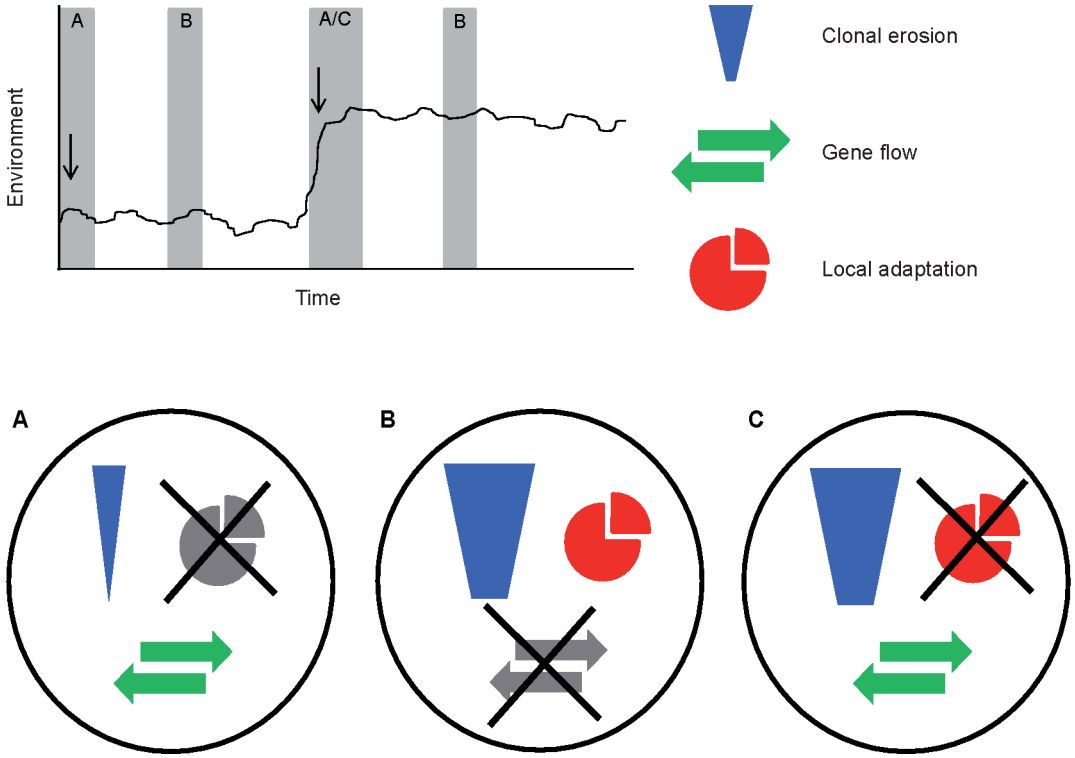

Figure D-1 Processes explaining population genetic structure in cyclic parthenogenetic zooplankton: (A) In newly founded populations clonal erosion acts strongly as only few genotypes are available, local adaptation has not been developed yet and gene flow may be common depending on the ecological niches, the competitive strength of genotypes, and the differential advantages from priority effects depending on the temporal succession of invasions; (B) Summary of the hypotheses of De Meester *et al.* (2002; 2006): within an established population under a relatively stable environment clonal diversity is impacted by clonal erosion (determined through the size of the dormant egg bank contributing genetic diversity and length of growing season as well as clonal selection eroding this diversity) and local adaptation thus successfully hindering gene flow; (C) In established populations confronted with severe environmental change local adaptation is strongly impaired and better pre-adapted genotypes may invade increasing effective gene flow; as the dormant egg bank is well established a high clonal diversity is expected in the beginning of the growing season, but clonal erosion will effectively reduce variation due to strong clonal selection. Black arrows indicate successful invasion events.

Gene Flow during population build-up

Under the assumptions of monopolization only few colonists found a population and successfully monopolize the available resources. This would result in a distinct population genetic structure, as the genetic diversity would be low due to the few colonizing genotypes. Although genetic variation may increase over time due to recombination and mutation events (Hartl & Clark 2007), the process of genetic drift will counteract, thus keeping allelic diversity low. This expected pattern was found, for example, by Wolf and Hobæk (1986) who detected low levels of genetic and clonal diversity as well as high fixation rates due to inbreeding in *Hyalodaphnia* populations. Nonetheless, I obtained empirical evidence, in particular for the taxon *D. longispina* (**Chapter 3**), for genetically diverse populations that exhibit high clonal diversity. In most populations the effects of clonal erosion remained undetected as populations started the growing season probably with thousands of unique genotypes, which was also observed in other studies focusing on cyclic parthenogens (Gómez *et al.* 2002; Hamrová 2011).

For a better understanding of the influences during the early stages of population build-up I conducted an outdoor mesocosm experiment (**Chapter 5**). I assessed the time period necessary between the first colonizer and the second invader to result in a priority effect and therefore in a clear advantage of the colonizer. Although I could show that already five days time advantage may be sufficient to successfully dominate a population, which is in line with monopolization, I also detected that the competitive strength of genotypes may overrule this effect. Although not observed in my experiment, early genotypes may alter the environmental conditions in a way that later arriving genotypes enjoy an advantage. A study by van Gremberghe *et al.* (2009) showed that first inoculated toxic *Microcystis* strains could facilitate the growth of later inoculated nontoxic strains under predation pressure.

A further aspect resulting in a disadvantage of the members in an early population is the severe erosion of genotypes until sexual reproduction is induced. Therefore, inbreeding is very likely in young populations and might result in an advantage for invading clones, as inbreeding depression was observed (Ebert *et al.* 2002). Moreover, not all genotypes invest in sexual reproduction; some may continue reproducing asexually even though cues for induction of sex are available (Keller & Spaak 2004). Also different investment in sexual reproduction among populations was observed (Allen & Lynch 2011; Hamrová *et al.* 2011), especially in permanent habitats, which would decrease the potential for local adaptation. As

sexual reproduction is in general observed annually, during the first years local adaptation establishment may be influenced by the invasion of better pre-adapted genotypes (Allen *et al.* 2010). The invasion success will further increase through temporal changes in environmental conditions as well as the occurrence of several ecological niches in one habitat. Changes in genotype abundances during the year (De Meester 1996; Jacobs 1982; King *et al.* 1995) and coexistence of generalist and specialist genotypes are often observed (De Meester 1996), indicating that the invasion of several pre-adapted genotypes may have occurred.

In summary (**Figure D-1**A), the high clonal diversity often observed may be explained if we relax the *monopolization hypothesis* until the populations are indeed locally adapted and sufficiently backed up by a dormant egg bank. As newly founded populations do not exhibit a dormant egg bank, the establishment of local adaptation is hindered. Clonal erosion is pronounced because the population consists only of few genotypes and induces inbreeding. Consequently, for the build-up of a new population a succession of invasions is necessary. Empirical evidence showed that early populations often represented an assemblage of several invaded genotypes and that the new genotypes invaded more successfully in consecutive growing seasons if the clonal diversity was low (Louette & De Meester 2005; Ortells *et al.* 2011).

Environmental change facilitating expansion and gene flow

My studies not only indicated that young populations may favor newly invading clones as they are lacking locally adapted genotypes, but further displayed that in already established populations the successful invasion of individuals is probable (**Chapter 4**). Under severe environmental changes the adaptive advantages of the residential genotypes get lost and they may become inferior to invading genotypes (**Figure D-1**C). Such events are probably rare as the common population consists of generalist and specialist genotypes (De Meester 1996), enabling the population to respond to certain changes over the seasons or years. However, long-lasting processes, like the advance and retreat of glaciers during the ice ages, were supposedly responsible for species range shifts and therefore changed their population genetic structure (Hewitt 1996). In addition, the impact of recent human-mediated changes in water bodies resulted also in severe shifts, e.g. from oligotrophic to eutrophic, or from low to high predation pressure. In **Chapter 4** I did show, that environmental changes influenced the expansion and gene flow patterns in the species *D. longispina* and *D. galeata*, which probably occurred due to the formation of glacial lakes after the retreat of the ice sheet (*D. longispina*)

and due to the human-mediated change of freshwater habitats (*D. galeata*). Further evidence provided from interspecific studies showed successful invasions of *D. galeata* after the eutrophication of lakes, which resulted in an negative abundance shift of the residing taxon *D. longispina* and in the successful interspecific hybridization and introgression (Brede *et al.* 2009; Rellstab *et al.* 2011), thus severely impacting the population genetic structure.

Monopolization versus gene flow: Synopsis

Overall, I conclude that the population genetic structure in cyclic parthenogenetic zooplankton species is impacted by various processes, partly described by De Meester *et al.* (2006), explaining clonal diversity within populations (clonal selection, growing season length, and dormant egg bank size) and by De Meester *et al.* (2002) explaining high genetic differentiation among populations (local adaptation, priority effects, dormant egg bank). First, I propose that the age of the population should be considered, as I could show that during population build-up the time of arrival is not sufficient to describe population assembly, but that the competitive strength of clones must be regarded as well (**Chapter 5**). This indicates that pre-adapted clones may substantially change the structure of a population if this population is not locally adapted at that moment in time. In addition, priority effects may be overruled and local adaption may be insufficient without sexual reproduction. Secondly, the genetic structure of established populations may be severely impacted by effective gene flow, if severe environmental changes alter the habitat of the locally adapted population (**Chapter 4**). This would catapult the residing population in a fitness valley enabling better pre-adapted genotypes to successfully invade the population. These may either replace the current population or contribute to the gene pool to generate new locally adapted genotypes.

Further implications deduced from the detected population genetic structure

Recent range expansion resulting in interspecific hybridization

The detected effective gene flow, including expansion, in the taxon *D. galeata* (**Chapter 4**) had seemingly serious consequences on interspecific interactions, as this may explain the fierce hybridization observed. Several hybridization partners of *D. galeata* are known, although *D. longispina* and *D. cucullata* are the most prominent (Schwenk 1993; Schwenk *et al.* 2000; Schwenk & Spaak 1995) and hybrids are recorded frequently. Further genetic evidence was also detected for interspecific hybridization of *D. galeata* with *D. lacustris* (Hobæk *et al.* 2004), *D. dentifera* (Ishida *et al.* 2011), and *D. mendotae* (Taylor & Hebert

1993a; Taylor *et al.* 2005). However, hybrids are less often documented. Also, during my studies I detected F_1 hybrids of *D. galeata-cucullata* and for the species pair *D. galeata-longispina* F_1 hybrids and backcrossed individuals with both parental species in many European locations (**Chapter 4**), which is consistent with the recent literature (Brede *et al.* 2009; Keller & Spaak 2004; Keller *et al.* 2008; Ruthová 2008; Schwenk *et al.* 2000). Even if the extent of hybridization seemed severe, the species pools are still distinct (**Chapter 2**, **3** and **4**; Keller *et al.* 2007), which might be explained by reduced fitness of hybrid genotypes in terms of sexual reproduction as they often produce empty ephippia (Carvalho & Wolf 1989). In addition, in the species pair *D. galeata-cucullata* little evidence for introgression was found (Schwenk *et al.* 1998), which might indicate efficient postzygotic reproductive barriers. However, as interspecific hybridization events among the species *D. galeata*, *D. cucullata*, and *D. longispina* are probably recent, the outcome is not conceivable yet. Increased evidence for introgression (Gießler & Englbrecht 2009; Taylor *et al.* 2005) already indicated severe changes in the genetic structure of several species, which may be even enhanced over time. Possible consequences are in general the extinction of parental species (Rhymer & Simberloff 1996) or the origin of new evolutionary genetic lineages through hybrid speciation (Mallet 2007); an example is very likely the taxon *D. mendotae* (Taylor & Hebert 1993b). Often interspecific hybridization enables the fast adaptive evolution to changing conditions (Brede 2008), which may observe in *D. galeata* through the accumulation of positive DNA polymorphism, as this taxon is the most ecologically plastic and widest distributed of the *D. longispina* species complex.

Monopolization favoring the origin of new evolutionary lineages

Another process may be deduced from my data. The observed genetic differentiation among *D. longispina* populations was very high, suggesting low levels of ongoing gene flow (**Chapter 3**). This pattern, which is in concordance with monopolization, may consequently lead to the development of several independent evolutionary lineages over time if invasion is indeed successfully hindered. In a recent survey of mitochondrial DNA variation I detected together with Adam Petrusek and Klaus Schwenk undescribed mitochondrial DNA variation that exhibited high levels of nucleotide divergence compared to any known species of the *D. longispina* species complex (~ 10%). Together with two more lineages recently published (Ishida *et al.* 2011; Petrusek *et al.* 2008) the number of lineages belonging to this species complex increased to eight. These new lineages, which do not seem to be abundantly distributed, were therefore just recently detected. Although other explanations are

conceivable, e.g. the occurrence of ancient polymorphisms (Clark 1997) or the sequencing of pseudogenes incorporated in the nuclear genome (Hazkani-Covo *et al.* 2010), the evolution of populations that effectively monopolized a water body is likely. This suggests that more divergent lineages await their discovery, probably in remote regions with little human impact.

Outlook

The results of my thesis show that human-mediated impact on freshwater systems may strongly influence the abundance and distribution of *Daphnia* species, thus influencing the genetic architecture due to intra- and interspecific gene flow. As these animals occupy a key position in the food web of a wide range of pond and lake ecosystems, which humans are dependent on, their future should matter. Consequently, information on their past, as presented in this thesis, is indispensable to thoroughly assess the changes observed today. In addition, data about the distributional range and in particular about the abundance of species are mandatory for the evaluation of recent and potential future changes. Although species of the highly studied genus *Daphnia* are, amongst others, ecologically well described (Benzie 2005; Flößner 2000; Lampert 2011; Peters & de Bernardi 1987), the actual distribution and abundance of taxa is only incompletely known. Especially understudied areas are the Eastern Palaearctic as well as the Afrotropic, though they are recently coming into focus of research (Ishida *et al.* 2011; Ishida & Taylor 2007a, b; Kotov *et al.* 2006; Mergeay *et al.* 2007). The recent detection of eight new mitochondrial DNA lineages emphasizes this lack of knowledge (Ishida *et al.* 2011; Petrusek *et al.* 2008; Petrusek *et al.* 2012). If these divergent lineages are indeed the result of independent evolutionary formation, the expected diversity is even higher. However, other explanations, like ancient polymorphism (Clark 1997) or the occurrence of pseudogenes (Hazkani-Covo *et al.* 2010), must be excluded first. Besides the mitochondrial DNA divergence, also high nuclear divergences were detected in *D. longispina* populations from southern Spain (**Chapter 3**). Mitochondrial DNA sequenced in these populations grouped with *D. longispina* mitochondrial sequences from other European locations (**Figure 3-2**). The different divergences detected in mitochondrial and nuclear DNA patterns suggest that the Spanish populations either represent an evolutionary entity at the dawn of speciation, or that these populations represent extreme geographical variants due to the highly unstable habitats they occupy (sand dune ponds with high UV radiation).

Although genetically divergent from *D. longispina*, I detected genetic evidence of hybridization of the Spanish populations with *D. galeata*. This again emphasizes the high interspecific hybridization potential of *D. galeata* probably induced through the recent expansion of this taxon. The consequences of the hybridization events are not conceivable yet and definitely need further attention. Backcrossed and introgressed individuals are detected increasingly in particular in the species pair *D. galeata-longispina* (Brede *et al.* 2009; Gießler & Englbrecht 2009), indicating the lack of sufficient reproductive barriers. Although the species gene pools are still distinct, this may change if this extensive interspecific gene flow is maintained. As fine-scale genetic markers are available, like the microsatellite DNA loci developed within this thesis (**Chapter 1** and **2**), the extent of hybridization and the distribution and abundance of hybrids should be monitored. Further, the past of hybridization events needs to be evaluated, as was recently achieved for a couple of lakes by the study of the dormant egg banks (Brede 2008; Brede *et al.* 2009; Rellstab *et al.* 2011). The extraction of sediment cores enables the analysis of dormant eggs stored for decades or even centuries (Brede 2008; Mergeay *et al.* 2007). As hybrids are sexually produced the history may be reconstructed by genetically studying the species status of individual eggs. This approach may reveal the consequences of human-made changes on the species composition, as was shown for example by Brede *et al.* (2009) in two European lakes. Above that, the study of dormant eggs from sediment cores will also give more information on monopolization of water bodies by cyclic parthenogenetic zooplankton species. In particular, the sensitivity to environmental change, as proposed in my thesis, may be analyzed as the successful inflow of new alleles will be detectable within the dormant egg bank.

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Supplement

Table 2-S1 Data of natural populations studied using twelve microsatellite loci. Names of populations are given together with country of origin, corresponding geographical coordinates, number of individuals used for this study (*N*) as well as amount of individuals belonging to either *D. galeata* (N_{gal}), *D. longispina* (N_{lon}), or *D. cucullata* (N_{cuc}) according to STRUCTURE 2.3.3 ($K = 3$; assignment probability > 0.95). Each pure species population (see N_{gal}, N_{lon}, N_{cuc}) with 15 or more individuals was analyzed using MICRO-CHECKER 2.3.3 to test for genotyping errors (GE_{gal}, GE_{lon}, and GE_{cuc}). Results (no = no indications for genotyping errors, # Indications for null alleles, § Indications for errors due to stuttering, NT = not tested) are given for each population and each locus (1 = DaB10/14; 2 = DaB17/17, 3 = Dgm105, 4 = Dgm109, 5 = Dgm112, 6 = Dp196NB, 7 = Dp281NB, 8 = Dp519, 9 = SwiD6, 10 = SwiD12, 11 = SwiD14, 12 = SwiD18).

Population	Origin	Latitude	Longitude	*N*	N_{gal}	GE_{gal}	N_{lon}	GE_{lon}	N_{cuc}	GE_{cuc}
Mondsee	Austria	47°50'26" N	13°22'48" E	22			22	no		
Beringen	Belgium	51°00'20" N	05°17'20" E	35	35	no				
Diest	Belgium	50°59'16" N	05°03'52" E	45	43	no				
Maaseik	Belgium	51°05'49" N	05°48'17" E	20	6	NT				
St. Bernard	Switzerland	45°52'19" N	07°10'13" E	32			32	6#, §		
Brno	Czech Republic	49°15'36" N	16°27'18" E	39	5	NT			34	1#, §, 4#, 12#
Římov	Czech Republic	48°48'25" N	14°29'30" E	38	37	7#, §, 9#				
Stanovice	Czech Republic	50°10'30" N	12°53'00" E	37	37	no				
Vranov	Czech Republic	48°54'28" N	15°49'01" E	32			23	no		
Helgoland	Germany	54°11'04" N	07°54'46" E	39			39	8#, §		
Ismaning	Germany	48°13'15" N	11°46'19" E	37			36	4#, 12#, §		
Palmengarten	Germany	50°07'26" N	08°39'13" E	62	62	5#, §				
Stechlinsee	Germany	53°09'00" N	13°01'58" E	31			31	no		
Trais-Horloff	Germany	50°27'00" N	08°54'10" E	39	6	NT				
Usingen	Germany	50°20'37" N	08°30'26" E	32					32	1#, §
Cogollos	Spain	37°12'36" N	02°50'06" W	37	37	5#, §				
Lake Pyhäjärvi	Finland	62°17'31" N	26°46'08" E	15	15	no				
Lake Vesijärvi	Finland	60°59'38" N	25°37'45" E	33					33	10#
Etang de Bellebouche	France	46°47'07" N	01°18'31" E	60	42	no	1	NT	6	NT
Loch Leven	Great Britain	56°12'00" N	03°22'48" W	37	37	no				
Hyde Park	Great Britain	51°30'23" N	00°10'20" W	37	37	no				
Queens Garden	Great Britain	51°31'37" N	00°09'07" W	36	36	no				
Rollesby	Great Britain	52°41'20" N	01°39'24" E	38	23	no				
Regents Park	Great Britain	51°31'41" N	00°09'32" W	40	40	no				
Mývatn	Iceland	65°34'56" N	16°59'22" W	38	38	no				

Lago Trearie	Italy	37°57'07" N	14°50'20" E	38						
Lago di Piana degli Albanesi	Italy	37°58'37" N	13°18'03" E	36	3	NT				
Lago di Piano	Italy	46°02'20" N	09°09'48" E	32			7	NT		
Lake Asveja	Lithuania	55°02'42" N	25°30'06" E	111	33	12[#, §]	57	8[#]	14	NT
Drabužis	Lithuania	54°34'08" N	24°39'03" E	33			4	NT	29	11[#]
Luodis	Lithuania	55°34'54" N	26°12'44" E	40			1	NT	38	10[#]
Esch-sur-Sûre	Luxembourg	49°54'10" N	05°52'30" E	61			24	no	21	no
Delftse Houd	Netherlands	51°58'41" N	04°20'55" E	29	29	4[#], 10[#]				
Goksjø	Norway	59°10'22" N	10°09'54" E	41			38	8[#]		
Nordfjordvatn	Norway	69°16'33" N	19°01'15" E	32			32	no		
Storveavatn	Norway	64°50'38" N	11°22'33" E	24			24	no		
Maranhão	Portugal	39°02'55" N	07°55'47" W	37	32	no				
Lake Glubokoe	Russia	55°45'13" N	36°30'15" E	88	33	no	22	8[#, §], 10[#, §]		
Göteburg	Sweden	57°42'00" N	12°00'00" E	20			20	4[#]		
Koarp	Sweden	56°22'48" N	13°07'30" E	40			38	no		
Lake Smartinsko	Slovenia	46°16'58" N	15°15'57" E	33					33	no
Reservoir Dubník II	Slovakia	48°46'01" N	17°40'58" E	39					39	1[#, §]
Nižné Jamnícke	Slovakia	49°12'10" N	19°46'13" E	40			40	no		
Satanie	Slovakia	49°10'15" N	20°03'50" E	30			30	no		
Total				1715	666		521		279	

Table 2-S2 Information about newly designed primers for amplification and sequencing of primer binding sites of seven microsatellite loci within clone C2. Locus names and studied primer binding sites are given together with sequences of newly designed primers (labeled as N01-N47; for = forward and rev = reverse). Successful combinations which resulted in an amplicon and further successful sequencing data are given. Each primer was tagged to either M13 (5'-GTAAAACGACGGCCAG-3') or T7 (5'-ATTATGCTGAGTGATATCCC-3') which were used for sequencing reaction.

Locus	Primer binding site	Primer sequences	Successful combination
DaB10/14	forward	N01for 5'-GTAAAACGACGGCCAG**TTGTGTATGTTTGATGCG**-3'	N01for + N03rev
		N02for 5'-GTAAAACGACGGCCAG**TGATGCGTGTTTAATGCC**-3'	N02for + N03rev
		N03rev 5'-ATTATGCTGAGTGATATCCC**GGAGAAGAGAACACCCAC**-3'	N02for + N04rev
		N04rev 5'-ATTATGCTGAGTGATATCCC**GAAATGCCGCCGTTGGGTCCG**-3'	
	reverse	N05for 5'-GTAAAACGACGGCCAG**ACAAGACGATGGGTATAGG**-3'	N05for + N08rev
		N06for 5'-GTAAAACGACGGCCAG**GCATCGGGAATAGACTAAAGG**-3'	N06for + N08rev
		N07rev 5'-ATTATGCTGAGTGATATCCC**TCAACTAACGTCCTCGAGG**-3'	
		N08rev 5'-ATTATGCTGAGTGATATCCC**CTCTCTAAGATTCTCCTCCG**-3'	
Dgm105	reverse	N09for 5'-GTAAAACGACGGCCAG**ATTAAAGGATAAGCC**-3'	0
		N10for 5'-GTAAAACGACGGCCAG**AGGATAAGCCTGACATGG**-3'	
		N11rev 5'-ATTATGCTGAGTGATATCCC**CCAACGGCAAACCGAATC**-3'	
		N12rev 5'-ATTATGCTGAGTGATATCCC**TTCCAAGCCAACGGCAAACC**-3'	
Dgm109	reverse	N13for 5'-GTAAAACGACGGCCAG**TTTCACATCTACTACACC**-3'	N14for + N16rev
		N14for 5'-GTAAAACGACGGCCAG**TATCTATACTGTTGTTGG**-3'	N14for + N15rev
		N15rev 5'-ATTATGCTGAGTGATATCCC**CTACGTCCCTGAAGAAGC**-3'	
		N16rev 5'-ATTATGCTGAGTGATATCCC**TAATCTTCTACGTCCCTG**-3'	
Dgm112	forward	N17for 5'-GTAAAACGACGGCCAG**ACTAATAGCTACATGGACATAG**-3'	N18for + N19rev
		N18for 5'-GTAAAACGACGGCCAG**GCGCCAAGAGTGTTGATAAG**-3'	N18for + N20rev
		N19rev 5'-ATTATGCTGAGTGATATCCC**CTCTCTGCTGTTTGTGTC**-3'	
		N20rev 5'-ATTATGCTGAGTGATATCCC**CTCACTCACTCTCTCTGC**-3'	
	reverse	N21for 5'-GTAAAACGACGGCCAG**GCTGCTGGTTGGATTGAAG**-3'	N21for + N23rev
		N22for 5'-GTAAAACGACGGCCAG**GGTTGGATTGAAGATTCAC**-3'	N21for + N24rev
		N23rev 5'-ATTATGCTGAGTGATATCCC**GGTGTATATGCACACATG**-3'	N22for + N23rev
		N24rev 5'-ATTATGCTGAGTGATATCCC**CGTTAGACGGTGTATATGC**-3'	N22for + N24rev

Dp196NB	forward	N25for 5'-GTAAAACGACGGCCAG**CAGCAGACGAGCCAAATT**-3' N26for 5'-GTAAAACGACGGCCAG**GAGCCAAATTCTCTTGGAG**-3' N27rev 5'-ATTATGCTGAGTGATATCCC**TTGTCAGCATTCTTTTCC**-3' N28rev 5'-ATTATGCTGAGTGATATCCC**GTTGTTGTCCTTTTGTCAGC**-3'	N25for + N28rev
	reverse	N29for 5'-GTAAAACGACGGCCAG**ACAAGCCAATGGCGAGTC**-3' N30for 5'-GTAAAACGACGGCCAG**CAAGCCAATGGCGAGTCTG**-3' N31rev 5'-ATTATGCTGAGTGATATCCC**CGGAAGCTTGGGATTCC**-3'	0
Dp519	forward	N32for 5'-GTAAAACGACGGCCAG**TGTGCCACTTATTTGTTGC**-3' N33for 5'-GTAAAACGACGGCCAG**CGGGGTGTTAAACACACACC**-3' N34rev 5'-ATTATGCTGAGTGATATCCC**ATACTGTTTGTCTATGTG**-3' N35rev 5'-ATTATGCTGAGTGATATCCC**TCTCTTGCACATATACTG**-3'	0
	reverse	N26for 5'-GTAAAACGACGGCCAG**ACTTTTAATTGCTGCAGG**-3' N37for 5'-GTAAAACGACGGCCAG**TTTAATTGCTGCAGGGAC**-3' N38rev 5'-ATTATGCTGAGTGATATCCC**CCAGACATCCAGTCGTCC**-3' N39rev 5'-ATTATGCTGAGTGATATCCC**CACACAAGGTGGTGGAGC**-3'	N37for + N39rev
SwiD14	forward	N40for 5'-GTAAAACGACGGCCAG**TTCACCCCACCACAGTCG**-3' N41for 5'-GTAAAACGACGGCCAG**ACCACAGTCGAGAATAGG**-3' N42rev 5'-ATTATGCTGAGTGATATCCC**GAGAGAAGGTTATTTTCG**-3' N43rev 5'-ATTATGCTGAGTGATATCCC**GAAGAGAGATGAGAGAAGG**-3'	0
	reverse	N44for 5'-GTAAAACGACGGCCAG**GTGTTTGCTGATTCGTTCG**-3' N45for 5'-GTAAAACGACGGCCAG**TGCTGATTCGTTCGTGCG**-3' N46rev 5'-ATTATGCTGAGTGATATCCC**GTGATGGGGATGGTGAGG**-3' N47rev 5'-ATTATGCTGAGTGATATCCC**ACTTTCAATTCCATGGCCG**-3'	N45for + N46rev N44for + N46rev

Table 5-S1 Inoculation scheme including the names of the founder and invader clones, the dates of inoculation and the time difference between the founder and invader in days (Time). Also shown are the dates of the first and final sampling. If a container had to be sampled twice due to few individual, the date is given in brackets.

Bucket number	Founder clone	Inoculation date of the founder (2007)	Invader clone	Inoculation date of the invader (2007)	Time	First sampling (2007)	Final sampling (2008)
01	HE01	09.07.	BE03	09.07.	0	31.08. (01.09.)	22.05.
02	HE01	09.07.	BE03	09.07.	0	31.08.	22.05.
03	HE01	09.07.	BE03	09.07.	0	30.08.	22.05.
04	HE01	18.07.	BE03	23.07.	5	29.08.	22.05.
05	HE01	09.07.	BE03	14.07.	5	30.08.	22.05.
06	HE01	09.07.	BE03	14.07.	5	30.08. (01.09.)	22.05.
07	HE01	09.07.	BE03	24.07.	15	31.08. (01.09.)	22.05.
08	HE01	09.07.	BE03	24.07.	15	31.08.	22.05.
09	HE01	09.07.	BE03	24.07.	15	29.08.	22.05.
10	BE03	09.07.	HE01	14.07.	5	31.08.	22.05.
11	BE03	09.07.	HE01	14.07.	5	31.08.	22.05.
12	BE03	09.07.	HE01	14.07.	5	31.08.	22.05.
13	BE03	09.07.	HE01	24.07.	15	31.08.	22.05.
14	BE03	09.07.	HE01	24.07.	15	30.08.	22.05.
15	BE03	09.07.	HE01	24.07.	15	01.09.	22.05.
16	BE04	09.07.	BE10	09.07.	0	31.08.	23.05.
17	BE04	09.07.	BE10	09.07.	0	30.08.	23.05.
18	BE04	09.07.	BE10	09.07.	0	01.09.	23.05.
19	BE04	18.07.	BE10	23.07.	5	29.08.	23.05.
20	BE04	09.07.	BE10	14.07.	5	30.08.	23.05.
21	BE04	09.07.	BE10	14.07.	5	30.08.	23.05.
22	BE04	09.07.	BE10	24.07.	15	30.08.	23.05.
23	BE04	09.07.	BE10	24.07.	15	30.08.	23.05.
24	BE04	09.07.	BE10	24.07.	15	31.08.	23.05.
25	BE10	09.07.	BE04	14.07.	5	30.08.	23.05.
26	BE10	18.07.	BE04	23.07.	5	29.08. (01.09.)	23.05.
27	BE10	09.07.	BE04	14.07.	5	31.08.	23.05.
28	BE10	09.07.	BE04	24.07.	15	31.08.	23.05.
29	BE10	09.07.	BE04	No inoculation	15	31.08.	23.05.
30	BE10	09.07.	BE04	No inoculation	15	31.08.	23.05.

31	BE11	09.07.	BE05	09.07.	0	31.08.	24.05.
32	BE11	09.07.	BE05	09.07.	0	31.08.	24.05.
33	BE11	21.07.	BE05	21.07.	0	31.08.	24.05.
34	BE11	17.07.	BE05	22.07.	5	30.08. (01.09.)	24.05.
35	BE11	17.07.	BE05	22.07.	5	30.08. (01.09.)	24.05.
36	BE11	17.07.	BE05	22.07.	5	29.08.	24.05.
37	BE11	09.07.	BE05	24.07.	15	31.08.	24.05.
38	BE11	09.07.	BE05	24.07.	15	31.08.	24.05.
39	BE11	09.07.	BE05	24.07.	15	30.08.	24.05.
40	BE05	18.07.	BE11	23.07.	5	29.08.	24.05.
41	BE05	21.07.	BE11	26.07.	5	29.08.	24.05.
42	BE05	21.07.	BE11	26.07.	5	30.08. (01.09.)	24.05.
43	BE05	09.07.	BE11	24.07.	15	31.08. (01.09.)	24.05.
44	BE05	09.07.	BE11	24.07.	15	30.08.	24.05.
45	BE05	09.07.	BE11	24.07.	15	30.08.	24.05.
46	BE15	12.07.	MA12	12.07.	0	30.08.	25.05.
47	BE15	12.07.	MA12	12.07.	0	01.09.	24.05.
48	BE15	12.07.	MA12	12.07.	0	30.08.	25.05.
49	BE15	12.07.	MA12	17.07.	5	29.08.	25.05.
50	BE15	12.07.	MA12	17.07.	5	30.08.	25.05.
51	BE15	12.07.	MA12	17.07.	5	31.08.	24.05.
52	BE15	09.07.	MA12	24.07.	15	30.08.	25.05.
53	BE15	09.07.	MA12	24.07.	15	29.08.	25.05.
54	BE15	09.07.	MA12	24.07.	15	01.09.	24.05.
55	MA12	09.07.	BE15	14.07.	5	01.09.	24.05.
56	MA12	09.07.	BE15	14.07.	5	29.08.	25.05.
57	MA12	12.07.	BE15	17.07.	5	31.08.	25.05.
58	MA12	09.07.	BE15	24.07.	15	31.08.	25.05.
59	MA12	09.07.	BE15	24.07.	15	31.08. (01.09.)	25.05.
60	MA12	09.07.	BE15	24.07.	15	31.08. (01.09.)	25.05.

List of abbreviations

°C	degree Celsius
%	percent
12S	12S rDNA
A	adenine
A	allelic diversity
ADaM	Aachener *Daphnia* medium
ALF	Automated Laser Fluorescence
AMOVA	Analysis of Molecular Variance
ARENA	Aquatic Research ExperimeNtal Area
AT-MS or MS	Mondsee, Austria
BA	Badajoz, Spain
BC	before Christ
BC_{gal}	backcross with *D. galeata*
BC_{lon}	backcross with *D. longispina*
BE-BE or BE	Beringen, Belgium
BE-DI	Diest, Belgium
BE-MA or MA	Maaseik, Belgium
BiK^F	Biodiversity and Climate Research Centre, Frankfurt am Main
BIOPOOL	abbreviation for the project "Connectivity, dispersal and priority effects as drivers of biodiversity and ecosystem function in pond and pool communities"
BP	before present
bp	base pair
BSA	Bovine Serum Albumin
C	cytosine
C	Carbon
C2	*D. cucullata* clone isolated from Tjeukemeer
CG1, 4, 5, 6, 8	interspecific hybrids between C2 and G1
CGL µsat kit	*D. cucullata*, *D. galeata*, and *D. longispina* microsatellite marker set
Chl_a	Chlorophyll a
CH-SB or SB	St. Bernard, Switzerland
cm	centimeter
COXI	Cytochrome c oxidase subunit I
cuc	*D. cucullata*
CytB	Cytochrome B
CZ-BR or BR	Brno, Czech Republic
CZ-RM	Řimov, Czech Republic

CZ-ST or ST	Stanovice, Czech Republic
CZ-VR or VR	Vranov, Czech Republic
D	MLG/*N*
D.	*Daphnia*
D*	D* according to Fu and Li (1993)
Δt	time difference
DAAD	German Academic Exchange Service
DaB	microsatellite abbreviation; Ender *et al.* (1996)
DE-HL	Helgoland, Germany
DE-IS or IS	Ismaning, Germany
DE-PG	Palmengarten, Germany
DE-SS or SS	Stechlinsee, Germany
DE-TH	Trais-Horloff, Germany
DE-US or US	Usingen, Germany
D_{est}	genetic differentiation measurement according to Jost (2008)
DFG	German Science Foundation
Div	genetic diversity
Dgm	*Daphnia galeata mendotae*, microsatellite abbreviation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Dp	*Daphnia pulicaria*, microsatellite abbreviation
E	East
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
ES-CO or CO	Cogollos, Spain
et al.	and others
F_1	first filial generation of an interspecific cross
F_{IS}	inbreeding coefficient
FI-VJ	Vesijärvi, Finland
F_S	F_S according to Fu (1997)
F_{ST}	genetic differentiation measurement
FCA	Factorial correspondence analysis
G	guanine
G1	*D. galeata* clone isolated from Tjeukemeer
gal	*D. galeata*
GB-HP	Hyde Park, Great Britain
GB-LO	Loch Leven, Great Britain
GB-QG	Queens Garden, Great Britain
GB-RO	Rollesby, Great Britain
GB-RP	Regents Park, Great Britain
GE	gametic equilibrium

G_{ST}	genetic differentiation measurement
GxC	hybrid between *D. galeata* and *D. cucullata*
h	*D. hyalina*
h	hours
hap	haplotype
Hd	haplotype diversity
HD	deficit of heterozygotes
H_e or H_{exp}	expected heterozygosity
HE	excess of heterozygotes
HE	Heverlee, Belgium
Het G	heterogeneity G value
HKY	Hasegawa, Kishino and Yano; substitution model
H_o or H_{obs}	observed heterozygosity
HWE	Hardy-Weinberg equilibrium
IBD	Isolation-by-distance
IBDWS	Isolation-by-distance web service
i.e.	id est
Ind G	individual G value
IT-LT	Lago Trearie, Italy
IT-PD	Lago di Piana degli Albanesi, Italy
IT-PI or PI	Lago di Piano, Italy
ITS	Internal transcribed spacer
IUPAC	International Union of Pure and Applied Chemistry
RFLP	Restriction fragment length polymorphism
K	number of assumed populations (Software STRUCTURE)
K	wobble base for guanine or thymine
KH_2PO_4	monopotassium phosphate
km^2	square kilometer
kV	kilovolt
L	Liter
LAKES	abbreviation for the project “Phylogeography and dispersal in aquatic organisms”
LD_{prop}	proportion of loci significantly deviating from GE
Ln P(D)	log probability of data
LOEWE	Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz
lon or ls	*D. longispina*
LT-DR or DR	Drabužis, Lithuania
LT-LU	Luodis, Lithuania
LU-ES	Esch-sur-Sûre, Luxembourg
LxC	hybrid between *D. longispina* and *D. cucullata*
LxG	hybrid between *D. longispina* and *D. galeata*
λ	Lambda

M	wobble base for adenine or cytosine
M	mole
M	similarity index
MA	Maaseik, Belgium
MCMC	Markov chain Monte Carlo
mg	milligram
$MgCl_2$	magnesium cloride
min	minute
mL	milliliter
MLG	multi-locus genotypes
MLG/*N*	clonal diversity
mM	milli mole
mtDNA	mitochondrial DNA
µg	microgram
µL	micro liter
µm	micro meter
µM	micro mole
µS	micro Siemens
µsat	microsatellite
N	North
N	number of individuals
n.c.	not calculated
NA	not available
$NaNO_3$	sodium nitrate
NC	no clones
ncDNA	nuclear DNA
N_e	effective population size
NL-DH or DH	Delftse Houd, The Netherlands
no.	number
NO	no amplicon obtained
NO-GO or GO	Goksjø, Norway
NO-NV or NO	Nordfjordvatn, Norway
NO-SV or SV	Storveavatn, Norway
nos.	numbers
NP-40	detergent
N_{ST}	genetic differentiation measure according to Lynch and Crease (1990)
NT	not tested
π	nucleotide diversity
P	number of polymorphic loci
p	probability
PA	private alleles
PCR	Polymerase chain reaction

PS	number of polymorphic sites
PT-MA	Maranhão, Portugal
Φ_{ST}	genetic differentiation measurement
r	*D. rosea*
®	registered trademark
RAPD	Random amplified polymorphism
rDNA	ribosomal DNA
Rep.	Republic
rRNA	ribosomal ribonucleic acid
RU-GL	Glubokoe, Russia
r^2	coefficient of determination
SD	standard deviation
sec	second
SE-GB or GB	Göteborg, Sweden
SE-KO	Koarp, Sweden
SI-SJ	Smartinsko, Slovenia
SK-DU	Dubník II, Slovakia
SK-NJ or NJ	Nižné Jamnícke, Slovakia
SK-SA or SA	Satanie, Slovakia
STRs	short tandem repeats
SwiD	microsatellite abbreviation; Brede *et al.* (2006)
T	thymine
t0	no scoring errors, PCR artifacts or mutations
t3	threshold at the third distance class
*T*a	annealing temperature
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TM	trademark
TN93	Tamura and Nei 1993; substitution model
TN93 + G	TN93 gamma distributed
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
U	Unit
W	West
WOC	without clones
z	*D. zschokkei*
ZH	Zahillo, Spain

Curriculum Vitae

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Education

07/2009 - 01/2012	Scientific researcher in the department of Molecular Ecology at the University of Koblenz-Landau, Landau, Germany
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02/2006 - 06/2006	Scientific assistant in the project: "Mutation rates of microsatellites and flanking regions" in the department of Ecology and Evolution at the Goethe University, Frankfurt am Main, Germany
12/2005	Diploma in Biological Sciences, Goethe University, Frankfurt am Main, Germany
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Declaration

I herewith declare that this thesis reports my independent work on "Population genetic structure in European *Hyalodaphnia* species: Monopolization versus gene flow". All used resources are specified and contributions from other authors and third parties are fully acknowledged.

This or another thesis have never been previously submitted in part or in whole to another academic or non-academic institution.

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Place, date Signature

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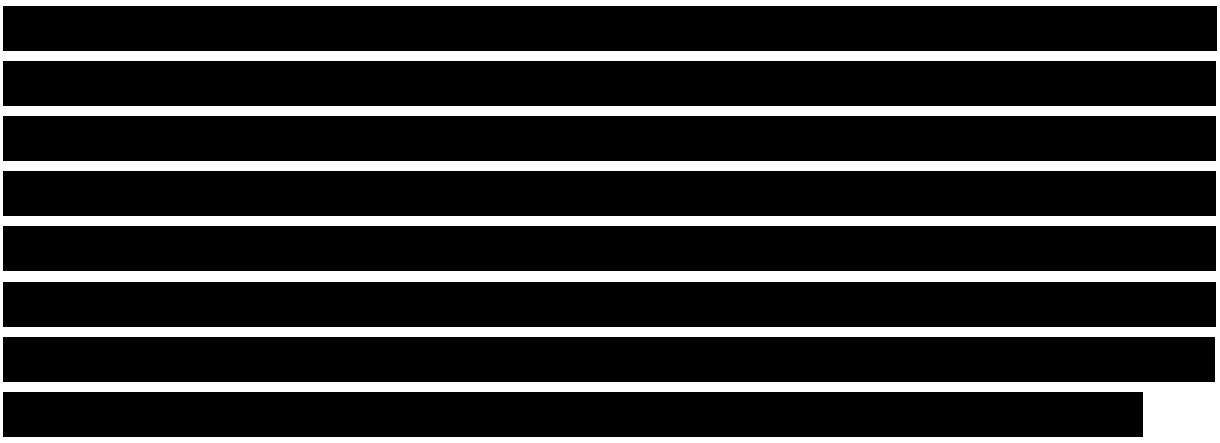

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