Morphological variation and genetic diversity of *Triops cancriformis* (Crustacea: Notostraca) and their potential for understanding the influence of postglacial distribution and habitat fragmentation

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PD Dr. H. Heilmeier (TU Bergakademie Freiberg)
Dr. Africa Gómez (University of Hull)
Dr. Bernd Hänfling (University of Hull)

Weitere Personen waren an der Abfassung der vorliegenden Arbeit nicht beteiligt. Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen. Weitere Personen haben von mir keine geldwerten Leistungen für Arbeiten erhalten, die nicht als solche kenntlich gemacht worden sind.
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Thorid Zierold

Summary

Triops cancriformis (Crustacea: Notostraca) occurs in ephemeral habitats like rain pools or floodplain pools distributed over a large geographical range. The named habitats are disturbed by human impacts and, consequently, *T. cancriformis* is endangered throughout its distribution range. In the present thesis the populated habitats and threats are characterised and further morphological and genetic variations detected among and within European populations are reported. On the basis of recent investigations it is shown that *T. cancriformis* subspecies separation is hampered by an individual variability which points to the necessity of species revision. The analysis of mitochondrial gene sequence data suggests that the species has colonised most of Europe very recently. The advantage of a complex reproductive strategy in *T. cancriformis* in this process is discussed. The population structure resolved with nuclear DNA markers highlights that there is low allelic diversity among and within populations compared to other Branchiopoda (Daphnia). By means of the present study it can be shown that habitat conservation is most important to protect *T. cancriformis.*

Zusammenfassung

Triops cancriformis (Crustacea: Notostraca) ist in ephemeren Gewässern wie Regenpfützen oder Überschwemmungstümpel in Flussauen verschiedener geographischer Bereiche zu finden. Anthropogene Eingriffe zerstören die genannten primären Lebensräume und gefährden somit auch die Existenz von *T. cancriformis*. In der vorliegenden Arbeit werden die besiedelten Habitate sowie deren Bedrohungen charakterisiert. Weiterhin werden morphologische und genetische Variationen zwischen und innerhalb der Populationen untersucht. Die vorliegende Arbeit zeigt, dass die europäischen *T. cancriformis* Vorkommen aus einer nacheiszeitlichen Besiedlung stammen, die Art einen komplexen Reproduktions-modus aufweist und eine Revision der Unterarten erforderlich ist. Anhand der hier zusammengestellten Daten wird ferner dargestellt, dass der Habitatschutz entscheidend für den Erhalt von *T. cancriformis* ist.

Preface

Again and again inundations of water and severe flooding events distress our civilisation. On the one hand, this is a catastrophe for humans, but on the other hand, it creates ideal conditions for species adapted to ephemeral waters like inundated pools. One animal group that is superbly adapted to the change from terrestrial to aquatic conditions is large branchiopods. Large branchiopods like *Triops cancriformis* are endangered by increasing urbanization and by agricultural use of floodplains. Since *Triops* is an unselective feeder that ingests dead organic material, the group increases the rate of decomposition of the detritus. The significant position of large branchiopods at the beginning of the food chain guarantees the existence of other species in the food chain. The protection of large branchiopods will automatically support the existence of intact floodplains and thus reduce the threats of flooding and inundation for human civilisations.

The present dissertation will report about ecological, morphological and genetic research topics. The results will lead into action modules for habitat and species conservation. The research was kindly supported by the German Environmental Foundation (DBU, project-no.: 20002/243). The thesis at hand is an important part of the research topic of the Biology / Ecology group of the Department of Biological Science in the Technischen Universität Bergakademie Freiberg *‚population ecology in fragmented landscapes'*.

Vorwort

Immer wieder suchen Überschwemmungen und Hochwasserereignisse die Zivilisation heim. Was einerseits für den Menschen eine Katastrophe ist, liefert andererseits ideale Bedingungen für Lebewesen, die in temporären Gewässern wie Überschwemmungstümpel oder Pfützen leben. Eine Tiergruppe, die ausgezeichnet an den Wechsel von terrestrischen zu aquatischen Bedingungen angepasst ist, sind die Großen Branchiopoden. Durch zunehmenden Auenverbau und intensive Landwirtschaft in Flussauen sind die Großen Branchiopoden wie *Triops cancriformis* stark gefährdet. Ihre Eigenschaft sich als nicht-selektive Fresser auch von abgestorbenem organischem Material zu ernähren, beschleunigt die Humuszersetzung. Durch exponierte Stellung am Beginn der Nahrungskette bilden die Großen Branchiopoden außerdem die Existenzgrundlage für viele weitere Tierarten. Darüber hinaus trägt der Schutz dieser Tiergruppe automatisch zur Erhaltung intakter Flussauen bei und reduziert damit ebenso Hochwassergefahren für die Zivilisation.

In der vorliegenden Dissertation wurden ökologische, morphologische und genetische Fragestellungen untersucht, die in Handlungsempfehlungen für den Habitat- und Artenschutz münden. Für die Finanzierung dieses Forschungsvorhabens konnte die Autorin die Deutsche Bundesstiftung (DBU, Projekt-Nr. 20002/243) gewinnen. Die vorliegende Arbeit ist wichtiger Bestandteil des in der Arbeitsgruppe Biologie / Ökologie am Institut für Biowissenschaften der Technischen Universität Bergakademie Freiberg aufgestellten Forschungsschwerpunktes *‚Populations-ökologie in fragmentierten Landschaften’*.

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My thesis was greatly improved by the efforts of Dr. Africa Gómez and Dr. Bernd Hänfling (University of Hull, Biological Department, Molecular Ecology and Fisheries Genetics group) that splendidly supervised me during my research in the University of Hull and critically checked the chapter on phylogeography in the present work.

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Bei meinem Lehrer, Herrn PD Hermann Heilmeier (TU Bergakademie Freiberg, Institut für Biowissenschaften, Arbeitsgruppe Biologie/Ökologie) möchte ich mich für das stete Interesse am Fortgang meiner Arbeit, die positive Einflussnahme auf meine wissenschaftliche Entwicklung sowie für die bereichernde Diskussion des Manuskriptes der vorliegenden Arbeit bedanken.

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Thesis aims and outline

"Life has no rehearsals, only performances"

Darwin (1809 – 1882)

The present thesis focuses on the tadpole shrimp *Triops cancriformis* which is an endangered Crustacean species inhabiting ephemeral freshwater bodies. To frame the central question of this thesis '*are there phenotypic and genotypic variation in T. cancriformis populations and what are they telling us'*, we can consider what might be the relevant factors for variation. The simple question demands a broad investigation on morphological characters and genetic structure on different scales (mitochondrial and nuclear). Morphological studies are intended to deduce characteristics appropriate for population subdivision or at least for morphotype identification. The genetic approach is separated into a phylogeography framework based on mitochondrial markers and a detailed population genetic study using nuclear markers. The question requires also ecological studies on habitat characteristics, species distribution and reproductive strategies.

As with humans, no two individuals of *T. cancriformis* are exactly identical. In the most basic terms this variation can be attributed to three sources: genetics, environment and the interaction between genetics and environment. The genetic component of an individual consists of the DNA inherited from two parents or transferred from one parent in the case of hermaphrodites or clones. DNA acts as a blueprint for the construction of proteins which control the development and function

of the individual. The environmental influences on a tadpole shrimp are extremely variable in form and function. Understanding the nature of variation between individuals and groups of individuals (populations) and how this occurs is valuable for a number of reasons. For the present work the investigations of variation are first of scientific interest and second of necessity for the conservation of the species or populations.

This thesis consists of seven chapters presenting the data collected during my PhD research programme.

CHAPTER 1 starts by reviewing the present knowledge on *T. cancriformis*. In particular it introduces the phylogeny, anatomy, ontogeny, reproductive strategy, habitat characteristics and species-distribution patterns which are germane to an understanding of the remarkable ability of *Triops* to survive over millions of years in ephemeral habitats. Second, basic information is provided about the study of phenotypic variation within *T. cancriformis.* This part will also visualize the morphological differences between the currently recognized subspecies of *T. cancriformis.* Third and fourth, the principles of phylogeographic and population-genetics studies are explained corresponding to their relevance for *T. cancriformis*. This is followed by an explanation of molecular methods and methodologies necessary for genetic analysis of populations both on phylogeographic and on population genetic levels. The general introduction will be closed with a brief review of the various approaches of conservation management.

In CHAPTER 2, the characteristic ecological features of large branchiopod habitats obtained from field, laboratory and literature studies are summarized. This chapter will give an overview of inhabited ecological niches considering both primary and secondary habitats. Additionally major threats are reviewed to understand the need for official protection regulations.

CHAPTER 3 highlights the results of morphological variation in *T. cancriformis.* Here morphological differences among and within regional populations are investigated which results in suggestion for revision of the currently recognised subspecies of *T. cancriformis*. Furthermore a review is given about the observed reproductive strategies within *T. cancriformis*.

In CHAPTER 4 use was made of the fact that mitochondrial sequence data contain information about demographic history of species. The screening of nucleotide sequence variation in cytochrome c oxidase I (COI) and 16S rDNA in European *T. cancriformis* populations including the currently recognised subspecies will be depicted in detail. Additionally the importance of various reproductive strategies is discussed in relation to range expansion after glacial ages.

CHAPTER 5 describes the process of microsatellite identification, sequencing and design of microsatellite markers for *T. cancriformis*. The results illustrate the problematic issues of this procedure.

In CHAPTER 6, the question of what (finer) spatial scale reveals about structure among *T. cancriformis* populations is addressed. To be able to detect subtle levels of differentiation, microsatellites were identified and applied for the first time in *Triops cancriformis* population genetic approaches. In this chapter the genetic structure of *T. cancriformis* populations throughout the European samples will be characterized to deduce how habitat isolation and geographic variation of reproductive strategy influence the population structuring.

In CHAPTER 7, a broad review of the various approaches of conservation ecology will be given based on the results of the present thesis. Detailed theoretical and practical information in the field of conservation management are illustrated in several boxes. Furthermore, the different threats on ephemeral habitats influencing the management action will be reviewed.

Chapter 1

General Introduction

Survival on earth is a surprisingly tricky business.

(B. Bryson, 1951[*])

1.1 *Triops cancriformis* (Crustacea: Notostraca)

Notostracan records date back to the Carboniferous and possibly up to the Devonian period (Guthörl, 1934; Wallossek, 1993, 1995; Kelber, 1998). In fact, there are Upper Triassic *Triops* fossils from Germany which are almost indistinguishable from the extant *Triops cancriformis* (Tröger et al., 1984; Kelber, 1999) and thus *Triops* is considered to be one of the best examples of evolutionary stasis or 'living fossils' (Fisher, 1990; Futuyma, 1990; Suno-Uchi et al., 1997; King & Hanner, 1998; Kleesattle, 2001).

The next paragraphs will highlight the present knowledge on the phylogeny, anatomy, reproductive strategy, habitat characteristics and distribution pattern which are germane to understand the remarkable ability of *Triops* to survive over millions of years in ephemeral habitats.

[*] Bryson, B. (2004) A Short History of Nearly Everything. London: Black Swan.

1.1.1 Phylogeny

Based on different morphological features and on genetic data, the Notostraca collapse with Laevicaudata and Spinicaudata in the group of Phyllopoda. Phyllopoda and Anostraca are belonging to the group of Branchiopoda. Anostraca, Notostraca, Spinicaudata and Laevicaudata are known as "large branchiopods" (Löffler, 1993).
The monophyletic origin of the major phyllopod taxa on Notostraca has been genetically proved by Spears and Abele (2000) and Braband et al. (2002). The position of Laevicaudata within Phyllopoda is under debate (Olesen, 1995, 1998; Spears & Abele, 2000; Martin & Davis, 2001). A hypothesis which favours a sister group relationship between Laevicaudata and all remaining Phyllopoda is shown in Figure 1-1.

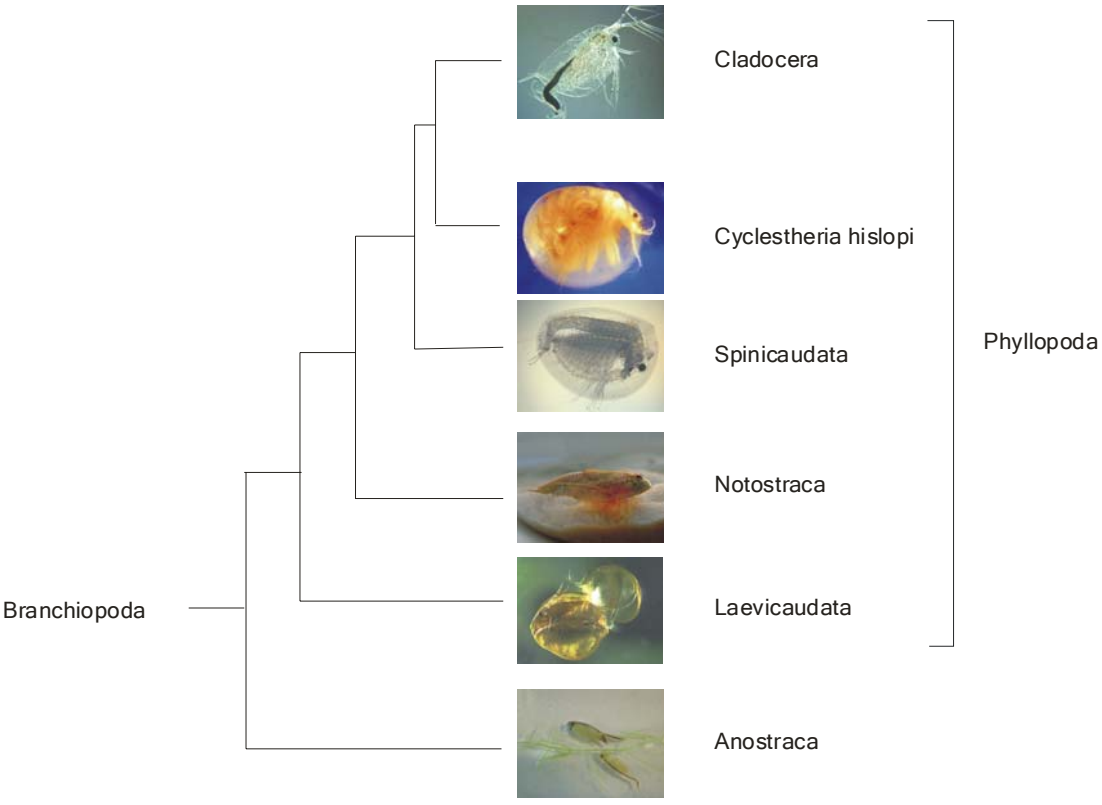

Figure 1-1: Phylogenetic relationships of the Branchiopoda based on Braband et al. (2002). Anostraca and Phyllopoda are well supported groups of the monophyletic Branchiopoda. The tree shows the sister group relationship between Laevicaudata and the remaining Phyllopoda. The close relationship between *Cyclestheria hislopi* and the Cladocera support for monophyletic origin of the Cladoceromorpha.

1.1.2 Anatomy

The large branchiopods bauplan is characterized by varying number of segments and appendages on thorax and abdomen, the latter usually lacking appendages, the

presence or absence of carapace or cephalic shield, the phyllopodous appendages, reduced or absent maxillules and maxillae and by paired compound eyes and single simple eyes (Brusca & Brusca, 1990).

The body of Notostraca (tadpole shrimps) consists of a large number of segments (Figure 1-2). The first eleven segments each bear one pair of appendages ventrally, and together comprise the thorax. In females the 11th limbs protopodit and endopodit are transformed to an egg pouch (the egg bearing leg). The abdomen consists of 'rings', each of them being formed by fusion of several segments. The number of post-thoracic, or abdominal, segments ('rings') is variable. A single anterior ring is characterized by several pairs of appendages, whereas the posterior rings lack appendages (Schäffer, 1756; Longhurst, 1955). The telson is equipped with long caudal rami (furca) which can become a very long filament. The gonopores are situated on the last thoracomere. A broad oval chitin shield fused only with the head covers thorax and part of the abdomen. Paired sessile complex eyes and a single simple eye lie close together near the anterior midline (Brusca & Brusca, 1990). The antennules are very small and hide under the shield. The antennae are almost completely stunted. The whole trunk consists of up to 42 segments (Figure 1-2). The notostracan species *T. cancriformis* can reach a body size of 10 cm (Kaestner, 1967).

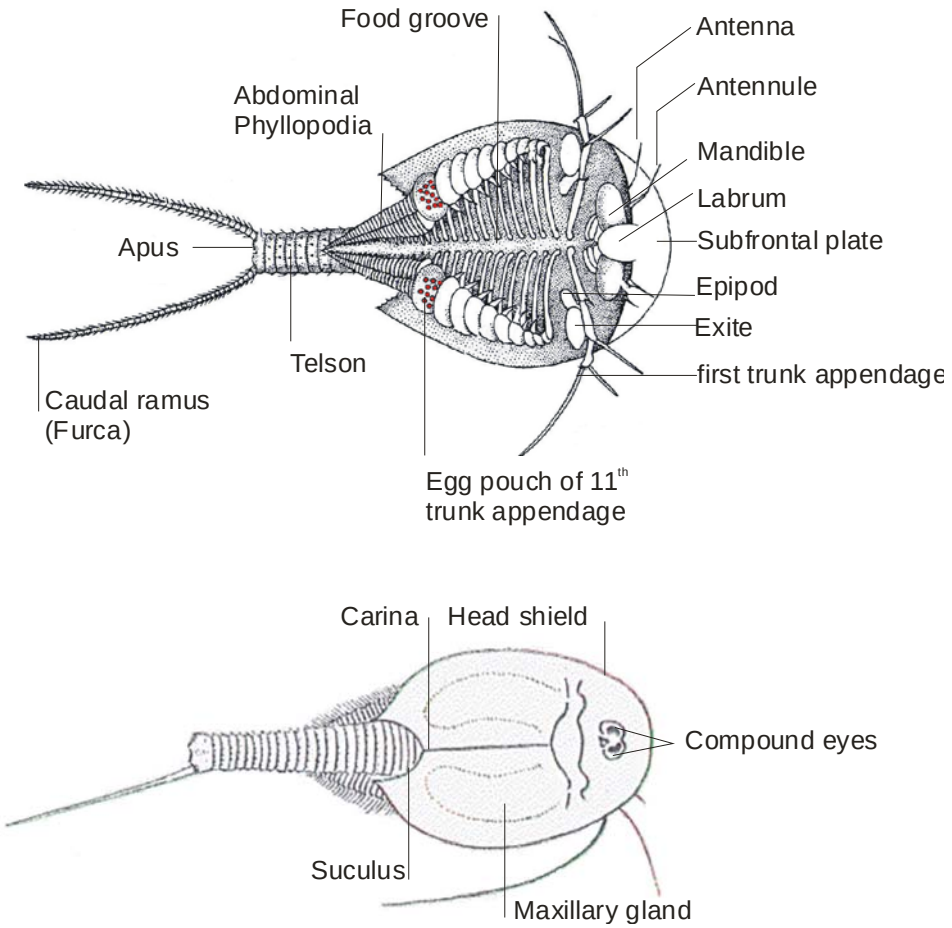

Figure 1-2: External anatomy of *Triops cancriformis*; ventral view of *T. cancriformis* female (upper) and dorsal view (lower) (modified after Brusca & Brusca 1990).

1.1.3 Ontogeny

The large branchiopod *T. cancriformis* presents adaptations to desiccation, the main one being the production of thick-walled resting cysts (Figure 1-3). A high number of resting cysts is laid into the pond sediment or is fixed on plants during the adulthood (Thiéry et al., 1995). As illustrated by Thiery & Gasc (1991) the clutch size depends on the diameter of the cyst. According to this the number of cysts in one egg pouch of *T. cancriformis* (cysts about 360 – 400 µm) averages 60. From the enormous amount of cysts released some are able to hatch without drought period caused by reduced osmotic pressure in the medium (Longhurst, 1955; Thiel, 1963; Brendonck, 1996). The drought resistant portion of cysts undergoes an extreme form of diapause. Diapause is the halting of embryonic development and thus the interruption of the life functions (Brendonck, 1996; Fryer, 1996; Belmonte, 1998). During this resting time the embryo is protected by different (cement) layers against

drying out, UV radiation and pressure (Scanabissi Sabelli & Tommasini, 1992; Thiéry et al., 1995).

Figure 1-3: Three resting cysts of *Triops cancriformis* on millimetre paper; photo Zierold.

When the habitat changes again from terrestrial to aquatic phase and the environmental conditions are suitable (pH, temperature, osmotic pressure, light), a proportion of the cysts will hatch (bed hedging). The remaining unhatched cysts in the soil are an effective insurance for the tadpole shrimps to maintain their populations under unpredictable fluctuations of environmental conditions, that is a resting cyst bank is formed and the large branchiopod population can survive the terrestrial period of the habitat (Thiery, 1997). It is not yet completely understood which of the eggs hatch after one period of drying and which after several periods. The cysts from *T. cancriformis* will hatch within a temperature range of 15°C to 30°C after two to three days of flooding. Depending on temperature and light the development of the swimming larvae (nauplius) to the juvenile stage (Figure 1-4) takes place within several hours to days (Hempel-Zawitkowska & Klekowski, 1968; Eder, 2002; Zierold, 2002; Moeller, 2003; Zierold, 2003).

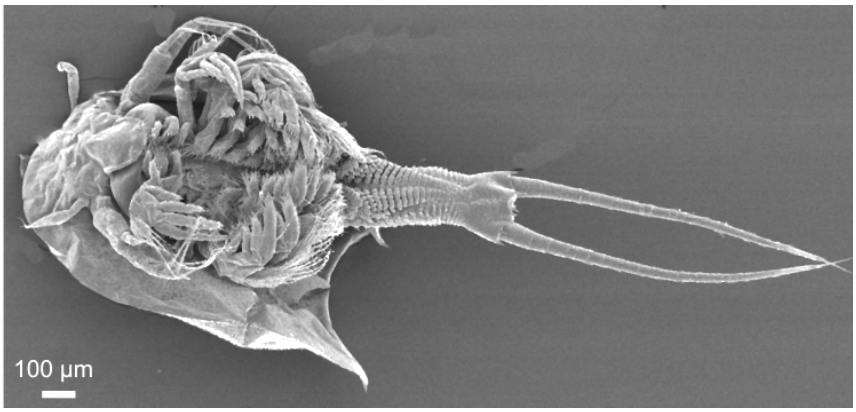

Figure 1-4: Scanning electron microscope image of juvenile *Triops cancriformis* just after metamorphosis; photo Zierold, supported by COBICE.

After repeated shedding of the skin the juvenile individuals attain the adult stage and reproduce. The shell of the fresh cysts has an alveolar layer - a spongy matrix of hundreds of tiny, interconnected chambers - which is filled with fluid

(Thiéry et al., 1995). Therefore the cysts remain on the bottom of the pond. During the terrestrial period the cyst shell fluid will be replaced by air (Hempel-Zawitkowska, 1967). With the next hydration the cysts will float to the surface (if they are not buried too deep in the sediment) and be exposed to the sunlight, which is one important trigger for nauplius hatching (Flößner, 1972; Wiggins et al., 1980; Frank, 1986; Zierold, 2002)

1.1.4 Reproductive strategy

Female and male of *T. cancriformis* can be differentiated by sexual dimorphism. Females are identified by the egg pouch on the 11th thoracic appendage, males by their absence. Confirmation of the sex of males might be possible with two additional criteria: the greater number of apodous terminal body rings and the relative length of endites 5 and 6 of the second thoracic leg (Sassaman, 1991; Engelmann et al., 1997). The copulation process of *T. cancriformis* is reported by Hotovy (1937). The author reported that the male swims with the ventral side up under the female and adheres with the abdomen to the female's shield (Figure 1-5).

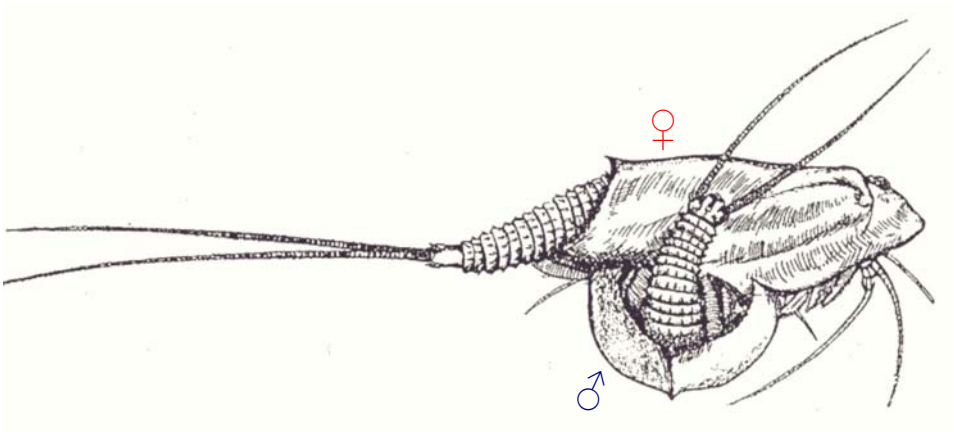

Figure 1-5: Lateral view of copulation of *Triops cancriformis* (after Hotovy (1937)

T. cancriformis has evolved a mixed reproductive system which includes unisexual (parthenogenesis or selfing hermaphrodites), female biased and bisexual populations (Brauer, 1872; von Siebold, 1873; Hotovy, 1937; Longhurst, 1954; Zaffagnini & Trentini, 1980; Sassaman, 1991; Browne, 1992; Engelmann et al., 1997). Furthermore a wide range of male:female ratio variation has been observed (Figure 1-6). Within *T. c. cancriformis* unisexual populations and female biased populations with male:female ratio between 0.8 to 28.1% occur in central and

northern Europe (Kozubowsky, 1857; von Siebold, 1871; Braem, 1916; Abonyi, 1926; Gaschott, 1928; Hotovy, 1937; Peres, 1939; Hempel-Zawitkowska, 1968; Zaffagnini & Trentini, 1980; Heidecke & Neumann, 1987; Engelmann et al., 1996; Engelmann et al., 1997; Engelmann & Hahn, 2005; Scanabissi Sabelli et al., 2005). Equal or male biased sex ratios have been described in *T. c. simplex* and *T. c. mauritanicus* (Alonso, 1996; Machado et al., 1999a; Boix et al., 2002). In the Italian Peninsula only unisexual populations have been found (Zaffagnini & Trentini, 1980; Scanabissi Sabelli et al., 2005). The histological examinations of females from several unisexual populations have revealed the presence of ovotestis (Longhurst 1955, Zaffagnini & Trentini 1980) although the examination of females from a female-biased population in Germany revealed no traces of testicular tissue (Engelmann et al., 1997). In conclusion, the reproductive mode of unisexual and female biased populations remains unclear. It may be possible that the female biased populations show a androdioecy mating system as it has been reported for the Spinicaudata *Eulimnadia texana* (Weeks & Bernhardt, 2004). Androdioecy is a rare form of reproduction, only found in a few plant and animal species, wherein males co-exist with hermaphrodites (Weeks et al., 2006).

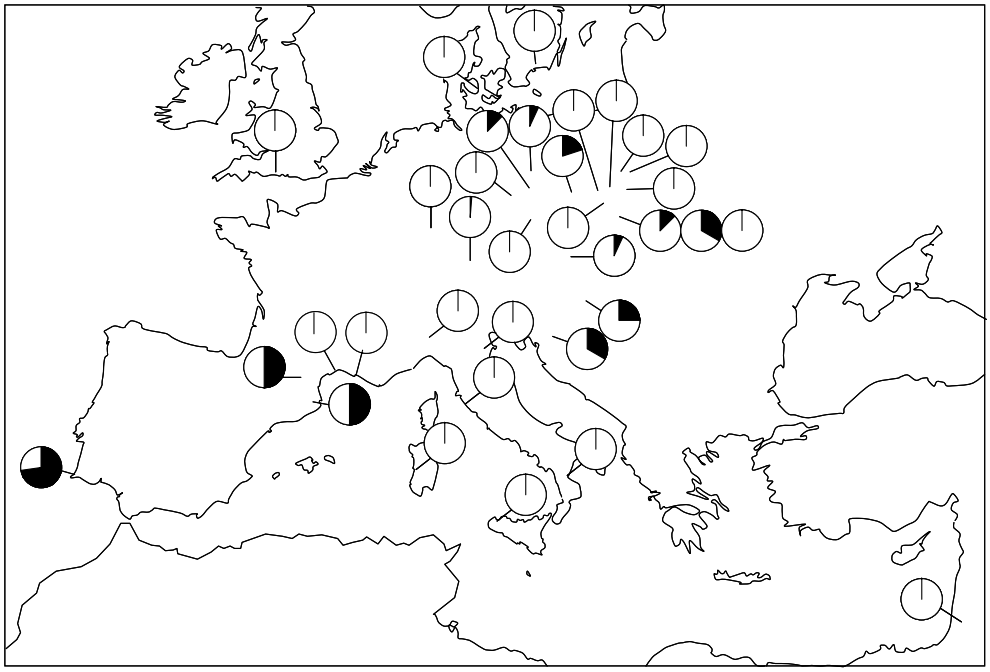

Figure 1-6: Overview of male:female ratios (male percentages are the filled sections in pie charts) of *Triops cancriformis* populations in Europe (figure based on data summarized in Table 1-1).

Table 1-1: Number of *Triops cancriformis* males and females and the male proportion given per pond

location information	n_{male}	n_{female}	prop. of males	references
Austria, Neusiedler Lake, Kaiserlacke	0	11	0.0000	p.d.
Croatia	9	34	0.2093	1
France, Camargue	0	9	0.0000	2
France, Baillargues (Montpellier)	0	200	0.000	
Germany, Bavaria	8	1000	0.0079	3
Germany, Bavaria, Augsburg	7	568	0.0122	4
Germany, Königswartha, fish pond area	3	119	0.0246	p.d.
Germany, Lacoma, fish pond area	2	28	0.0667	p.d.
Great Britain, New Forest, Godshill pond	0	19	0.0000	p.d.
Hungary, Lake Balaton	7	19	0.2692	5
Hungary, Lake Balaton	15	45	0.2500	5
Japan	0	20	0.0000	2
Poland, Breslau (April 1867)	29	88	0.2479	6
Poland, Breslau (April/June 1865)	114	912	0.1111	6
Poland, Breslau (July 1864)	2	9	0.1818	1
Poland, Breslau (July 1879)	5	19	0.2083	1
Poland, Cracow (1857)	16	144	0.1000	7
Poland, Cracow (1858)	154	395	0.2805	7
Poland, Debina	0	10	0.0000	8
Poland, Jaktorów	0	40	0.0000	8
Poland, Jarosy	0	10	0.0000	8
Poland, Klomnice	0	15	0.0000	8
Poland, Kludno	0	20	0.0000	8
Poland, Zabieniec	0	40	0.0000	8
Portugal, Algarve	54	22	0.7105	9
Portugal, Algarve	19	23	0.4524	9
Spain, Plat d'Espolla	1723	1775	0.4927	10

[1] (Braem, 1893), [2] (Akita, 1976), [3] (Heidecke & Neumann, 1987), [4] (Gaschott, 1928), [5] (Abonyi, 1926), [6] (von Siebold, 1871), [7] (Kozubowsky, 1857), [8] (Hempel-Zawitkowska, 1968), [9] (Machado et al., 1999b), [10] (Boix, 2002), [p.d.] personal data

1.1.5 Habitat

Triops cancriformis occurs in ephemeral (seasonal, temporary or vernal) freshwater bodies (Longhurst, 1955; Brtek & Thiery, 1995). The ephemeral character depends on geographical location, but may result from the ebb and flow of flooding rivers and streams, heavy precipitation events and groundwater elevations. The defining characteristics of such pools and puddles (natural origin) and ponds (human origin) are that they periodically dry up and do not (in most cases) contain fish. Drying may occur annually or only in drought years. In general ponds dry most often in late summer or early fall. The wet-dry cycle prevents fish from becoming established, allowing favourable breeding and rearing habitat for amphibians, crustaceans, and insects. Ephemeral water bodies provide a window of necessity for these species to survive. Another characteristic of ephemeral waters is that the soil on the bottom is often quite compact. Periodic drying allows leaves and dead plants that have

accumulated in the wetland to decompose. Ephemeral ponds in natural environment often indicated by plants like bulrushes (*Juncus*) and sedges (*Carex*).

1.1.6 Distribution pattern

The distribution of large branchiopods is affected by their drought-resistance cysts, which become efficient agents of passive dispersal, so that populations occur on remote islands, and are apparently found wherever there are suitable habitats (Longhurst, 1955). The diapausing cysts may be dispersed by wind, water or birds, which regularly visit seasonal water bodies (Càceres & Soluk, 2002; Figuerola & Green, 2002; Figuerola et al., 2003; Green & Figuerola, 2005; Green et al., 2005). Furthermore the extremely sticky eggs could presumably adhere to land animals (Longhurst, 1955; Frank, 1986; Gottwald & Eder, 1999; Bohonak & Roderick, 2001; Coulson et al., 2002). Thus, the high dispersal abilities afforded by their diapausing eggs and the possibility of unisexual reproduction seemingly account for the wide distribution of *T. cancriformis* including occurrences in Japan, North and South Africa, Europe and South Asia. The species has a thermophilic character (Gaschott, 1928; Engelmann et al., 1988; Eder et al., 1997) and seems to be absent from cold regions without a drought period. The summarizing study on geographic distribution of *T. cancriformis* (Brtek & Thiery, 1995) indicates that the species is currently not found north of 65˚ latitude. The three *T. cancriformis* subspecies which are currently recognized show a distinct geographic distribution pattern (Longhurst, 1955; Brtek & Thiery, 1995; Alonso, 1996). The nominal subspecies *T. c. cancriformis* has a wide distribution range, from Europe, western Russia, and the Middle East to northern India and Japan (Longhurst, 1955; Akita, 1976; Zaffagnini & Trentini, 1980; Suno-Uchi et al., 1997), but has not been reported from China and Korea (Umetsu et al., 2002). Information according to the geographic distribution of *T. c. simplex* differs between authors. Longhurst (1955) reported this subspecies only from northern Africa, from Ceuta to Egypt. Brtek & Thiery (1995) indicated the occurrences of *T. c. simplex* for Spain and northern Africa (Algeria). Thiery (1996) reported also occurrences from Arabian Peninsula, where it was found at a single locality in Yemen. Finally *T. c. mauritanicus* (Ghigi, 1921; Margalef) occurs in southwestern Spain, South Portugal, Morocco and Tangier (Alonso & Alcaraz, 1984; Machado et al., 1999a). In Figure 1-7 the occurrence of *T. cancriformis* subspecies has been

summarized based on literature studies (Burmeister, 1988; Brtek & Thiery, 1995; Eder & Hödl, 1996; Eder et al., 1996; Maeda-Martínez et al., 1997; Petrov & Petrov, 1997; Lanfranco, 2001; Pérez-Bote, 2004; Engelmann & Hahn, 2005).

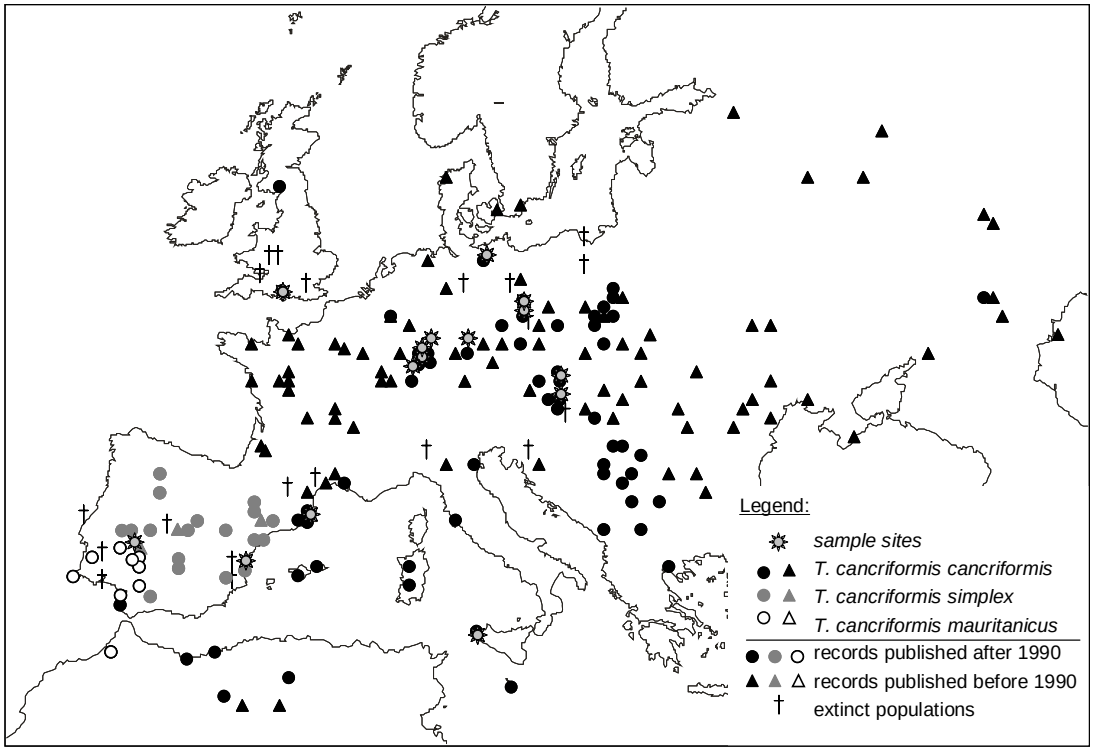

Figure 1-7: Distribution pattern of *Triops cancriformis* subspecies based on literature studies (for references see text); records before and after 1990 as well as known species extinction illustrated.

Currently *T. cancriformis* populations occur along major rivers like Danube, Elbe, March and Rhine as well as in rice field and isolated rain pools and puddles. Further information published before 1990 refer to occurrences from rivers Dnepr, Dnestr, Oka, Volga and also from the Romania plain along the river Danube and from several locations in Poland (indicated as a triangle, Figure 1-7).

Despite its wide distribution, *T. cancriformis* is an endangered species, mainly caused by loss of ephemeral habitats. Those natural areas have been partly destroyed or degraded through the direct or indirect action of mankind.

1.2 Subspecies differentiation and morphological variation

Morphological separation of notostracan species is hampered by high inter-individual variability which resulted in subspecies-introduction. This chapter will highlight the morphological characters used to differentiate *Triops cancriformis* subspecies and will further present the knowledge of morphological variation among and within *T. cancriformis* populations.

1.2.1 Subspecies classification

Within *T. cancriformis* three geographically restricted subspecies occur: *T. c. mauritanicus* (Ghigi 1921), *T. c. simplex* (Ghigi 1921) and *T. c. cancriformis* (Bosc, 1801). The subspecies of *T. cancriformis* differ by the presence or absence of spines along the dorsal carina, as well as the size and number of small posterior spines (Longhurst, 1955; Petrov & Cvetkovic, 1999) (Figure 1-8). In *T. c. mauritanicus* the carina bears a number of teeth posteriorly, the largest often sub-equal to the terminal spine and the furcal spines are very large. The subspecies *T. c. simplex* is equipped with a smooth carina in the front of the terminal spine and with small furcal spines. Longhurst (1955) recognized that these features are not as invariant as in subspecies *T. c. mauritanicus*. The nominal subspecies *T. c. cancriformis* shows in front of terminal spine along the dorsal carina a few small teeth (generally 2-3, but also up to 10 observed).

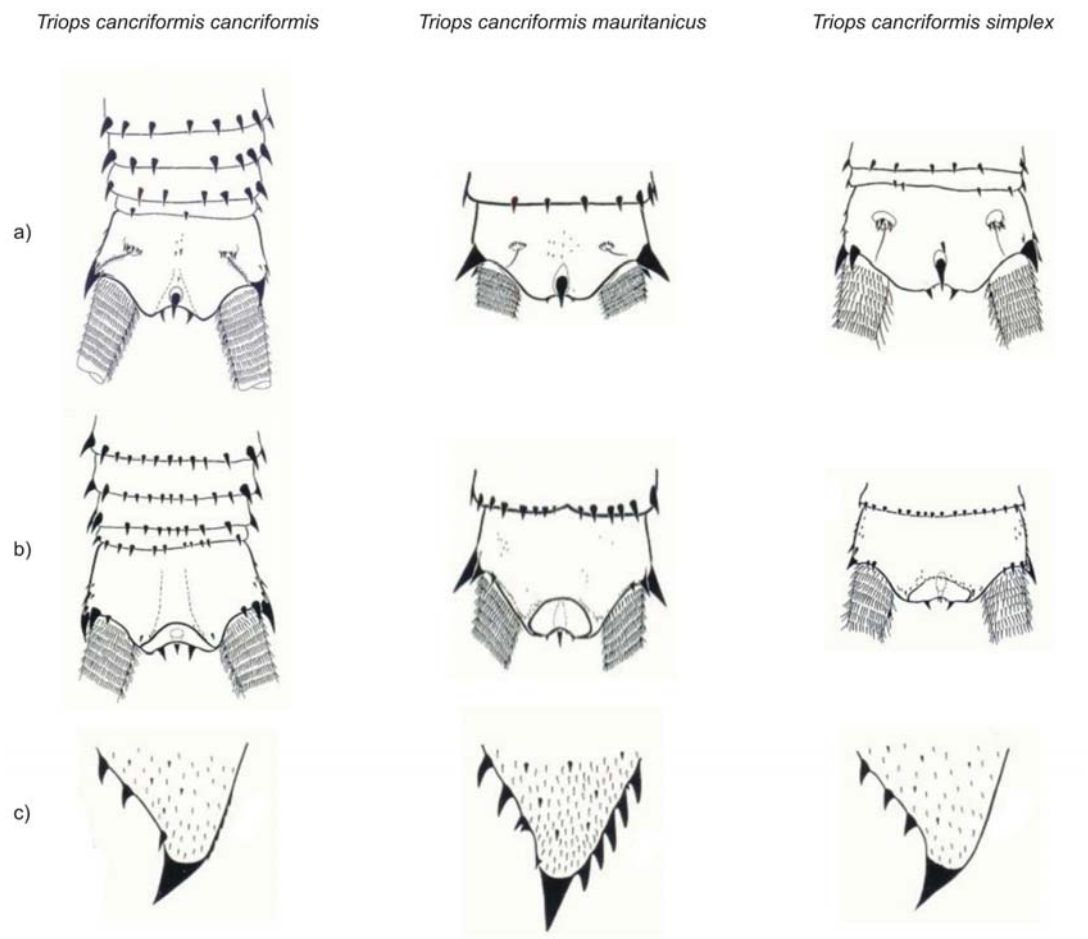

Figure 1-8: Morphological characteristic of *Triops cancriformi*s subspecies; dorsal (a) and ventral (b) view of the telson, and the (c) part of the carina (Alonso, 1996)

1.2.2 Morphological variation

As shown by Hempel-Zawitkowska (1968) morphological characters of *T. cancriformis* have a remarkable individual variability. Scientists have been debating whether the morphological differences between *T. c. simplex* and *T. c. cancriformis* represent species variety or not (Linder, 1952a; Longhurst, 1955). Characters applied in morphological studies on *T. cancriformis* are summarized in (Table 1-2).

Table 1-2: Morphological characters used to distinguish between *Triops cancriformis* populations

characters	**literature**
Head shield length [cm]	(Longhurst, 1955; Hempel-Zawitkowska, 1968; Suno-Uchi et al., 1997; Petrov & Cvetkovic, 1999)
Head shield width [cm]	(Hempel-Zawitkowska, 1968; Suno-Uchi et al., 1997; Petrov & Cvetkovic, 1999)
Head shield length-width ratio	(Longhurst, 1955; Hempel-Zawitkowska, 1968; Petrov & Cvetkovic, 1999)
No. of body segments	(Linder, 1952a; Longhurst, 1955; Petrov & Cvetkovic, 1999)
No. of uncovered segments	(Longhurst, 1955; Petrov & Cvetkovic, 1999)
No. of appendages	(Linder, 1952a; Longhurst, 1955; Petrov & Cvetkovic, 1999)
No. of legless segments	(Linder, 1952b; Longhurst, 1955; Hempel-Zawitkowska, 1968)
No. of sulcal spines	(Petrov & Cvetkovic, 1999)
No. of carina spines	(Petrov & Cvetkovic, 1999)
Shape of the dorsal organ	(Petrov & Cvetkovic, 1999)
Abnormal body rings	(Linder, 1947)

Shape and size of the **head shield** (often named as carapace; and rarely also tergit) varies considerably in *T. cancriformis*. Longhurst (1955) reviewed that the growth of the carapace from the earliest metanauplius stage is probably isometric, so the ratio of carapace length to total length remains constant throughout growth. The carapace is little affected by the preservation fluid and thus can give an almost real image of the animal size. The carapace length is generally measured along the mid-dorsal line (carina). The greatest width of the carapace could be measured in a straight line. But, because of the height of the carapace this method is not reasonably accurate. Linder (1952b) suggested to measure the greatest width from the carina to both sides of the head shield margin. The head shield in general seems to be more flattened in males than in females (Longhurst, 1955; Meintjes et al., 1994).

The dorsal **carina** frequently ends in a spine and may bear smaller spines along its length (Figure 1-9). Longhurst (1955) reviewed that this carapace feature varies even within a population.

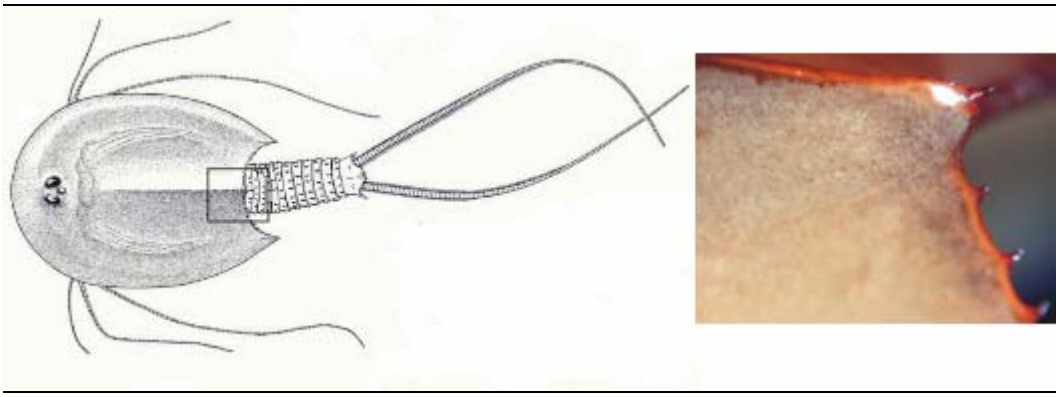

Figure 1-9: The mid-line of the notostracans shield (carina) ends in a large spin (terminal spine). The carina itself may bear several smaller spines (drawing Alonso 1996, photo Zierold)

In adult notostracan species the **dorsal surface** of the head bears a pair of compound eyes, an ocellus, and the dorsal organ (Longhurst, 1955). After Longhurst (1955) the dorsal organ is a character which is correlated with the number of segments and therefore a character which may vary between or within populations. The author mentioned that in *T. cancriformis* the usual shape of the dorsal organ is round, but in some of the longer bodied individuals it approaches the triangular form which is known from most specimens of *Triops granarius* (Figure 1-10).

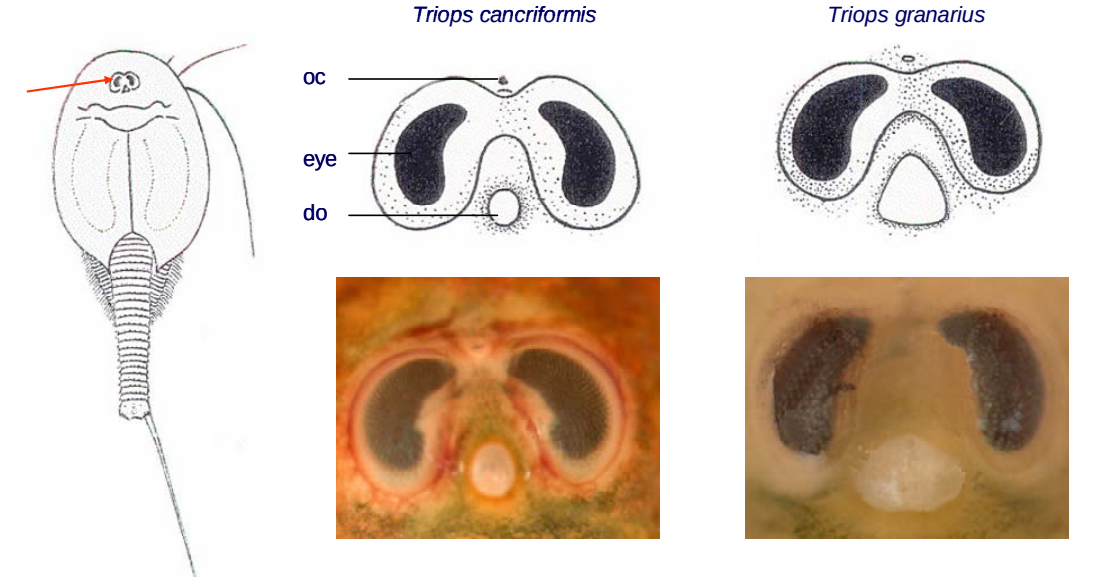

Figure 1-10: Comparison of the dorsal surface of *Triops cancriformis* and *Triops granarius*. The dorsal surface of Notostraca is composed of a pair of compound eyes (eye), an ocellus (oc), and the dorsal organ (do) (photo Zierold, drawing modified after Longhurst 1955).

For the postmaxillary part of *Triops* the terms ‘segment’ and ‘body ring’ are used. The varying number of **legless segments** has been described by several authors (Linder, 1952b; Longhurst, 1955; Sassaman et al., 1997) as a varying character within and between populations. In *Triops longicaudatus* the number of total body rings and the legless rings are used to discriminate between sexual and asexual reproductive populations. The hypothesis is that gonochoric and androdioecious specimens are longer than unisexual specimens (Murugan et al., 2002). Furthermore Linder (1952b) studied the phenomenon of abnormal body rings at legless segments of *T. cancriformis*. The author suggests that there is a connection between incomplete body-rings (Figure 1-11). However, the occurrence of abnormal rings is not yet understood. But they have to be counted if legless segments are studied. Otherwise a wrong impression of variation will be obtained.

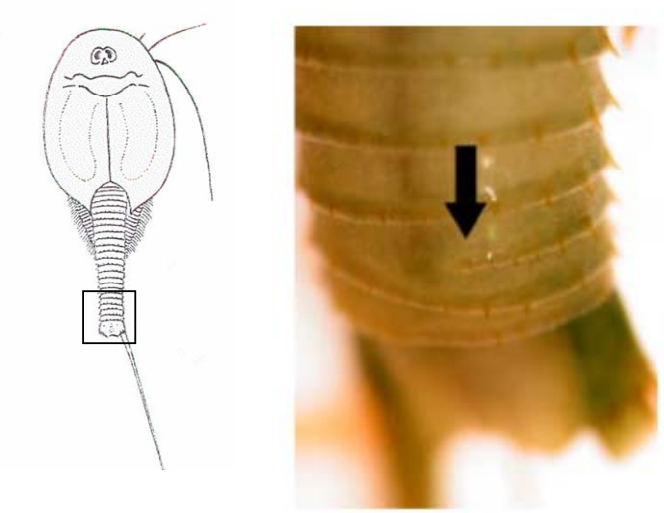

Figure 1-11: Arrow indicating the location of abnormal segments along the legless segments of *Triops cancriformis* (drawing Longhurst 1955, photo Palmer)

The telson is heavily chitinized and thus very little subjected to the influence of preservation fluid. The **telson** bears an armature of spines on both dorsal and ventral surfaces (Figure 1-12). The spines on the dorsal part of the telson are separated into marginal spines and central spine both very obvious, posterior marginal spines, median spines and finally around the dorsal sensory setae are *setal spines* (Figure 1-12).

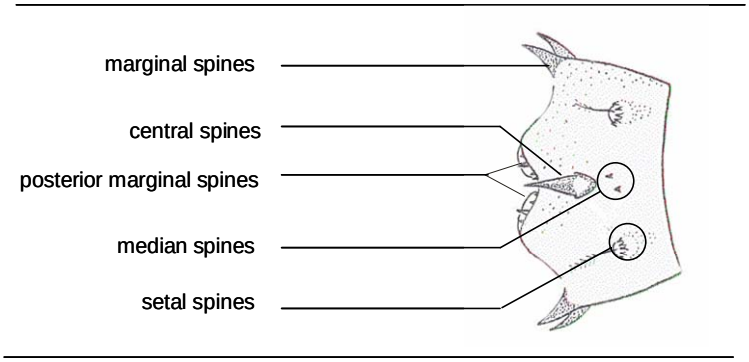

Figure 1-12: Classification of dorsal telson spines of *T. cancriformis* (drawing modified after Longhurst (1955))

The illustrated morphological variation of *T. cancriformis* populations was used to investigate the phenotype variation of European populations (see chapter 3). The correlation between phenotype and genotype variation will be discussed in chapter 4.

1.3 Phylogeography

Historical biogeography aims to reconstruct origins, dispersal, patterns of endemism, vicariance and eventual extinction of populations and communities (Van Veller et al., 1999; Randi, 2000; Arbogast & Kenagy, 2001). Avise et al. (1987) defined phylogeography as the study concerned with the principles and processes governing the geographical distribution of genealogical lineages, especially those at intraspecific level. According to Lowe et al. (2004) phylogeography interprets the historical processes that may have left their evolutionary signature on the present geographic distributions of genetic traits. For phylogeographic studies individuals are sampled from throughout the geographical range of a species, and the mtDNA genome is characterized for each individual, either through restriction fragment analysis or direct sequencing. The resulting haplotypes are then used to infer a phylogeny, or gene tree, which reflects the evolutionary relationships of the individuals and populations sampled. By combining the resulting gene trees with the geographical location from which each individual was sampled, one can elucidate the geographical distributions of major gene lineages (monophyletic clades) that comprise the gene tree (Arbogast & Kenagy, 2001).

An ideal marker for the phylogeographic reconstruction is the mitochondrial DNA (mtDNA). As illustrated in Figure 1-13 branchiopods mtDNA is a molecule of less than 16 kilobases (kb) (Umetsu et al., 2002).

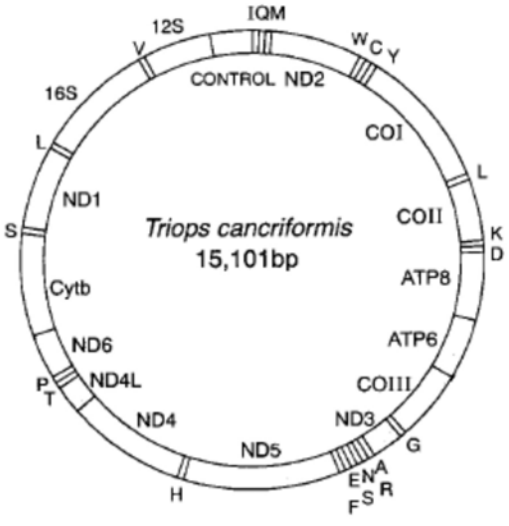

Figure 1-13: Genetic map of. T. cancriformis mtDNA. The tRNAs are identified by the 1-letter aa code (Umetsu et al., 2002).

Due to the effective maternal inheritance even within breeding populations mtDNA lineages are genetically isolated from one another, such that any observed homologies in genetic structure presumably result from historical connection in a matriarchal genealogy (Avise, 1992). The high popularity of mtDNA for studies of animal populations is also due to its relatively high rate of base substitutions. The mtDNA sequences evolve, on average, 5-10 times faster than nuclear genes (Brown et al., 1982; Hillis et al., 1996). Closely related mtDNA sequences differ mainly in transitions (Ti) rather than in transversions (Tv), and values of Ti:Tv ratios can be 10-20 or higher in intraspecific comparisons (Brown et al., 1982; Randi, 2000). Third positions of the codons, synonymous sites and the hypervariable parts of the control regions can evolve 10-20 times faster than average rates of mtDNA coding sequences. The rates of mtDNA evolution vary considerably among taxa (Martin & Palumbi, 1993) as well as among genes within taxa (Zhu et al., 1994; Schubart et al., 1998). The average overall mtDNA divergence rate has been estimated about 0.41-2% per million years (Myrs) in invertebrates (Cunningham et al., 1992; Klicka & Zink, 1998; Jarman & Elliot, 2000; Hewitt, 2004). Schubart et al. (1998) established among the 16S rDNA and Cytochrom oxidase *I* genes of Jamiacan crabs (*Sesarmoides reticulatum*, Grapsidae) a evolution rate of 0.88% per Myrs for 16S rDNA and 2.33% for COI. For Anaspididae (Crustacea: Malacostraca) 16S rDNA genes Jarman & Elliott (2000) reported a substitution rate of 0.78% per Myrs. However Cunningham et al. (1992) established for king crabs a smaller rate (0.41% per Myrs).

Assuming the molecular clock with the estimated evolutionary rates one can estimate time of population/species differentiation. This process of divergence was forced, for example, by Quaternary ice ages, when species ranges became fragmented because at the glacial-maxima populations would persist only in isolated refugia. These populations remained relatively small for thousands or tens of thousands of years, and differentiated genetically because of lineage sorting and occasional new mutations. In Europe these refugia particularly have been Iberia, Italy, the Balkans and Caucasus where the climate was relatively buffered against the glacial cycles (Hewitt, 2000; Beebee & Rowe, 2004). After the ice sheets retreated, populations at the northern limits of the refugial range expanded into often large areas of suitable territory (leading edge expansion) (Hewitt, 2000). Hewitt (2000)

subdivided the postglacial colonisation into three broad colonisation patterns, where the grasshopper, the hedgehog and the bear may serve as paradigms (compare Figure 1-14). Modelling and simulation of range expansion possibilities are inferred from phylogeographic analysis, genetic diversity estimates, and biogeographical data (Beebee & Rowe, 2004).

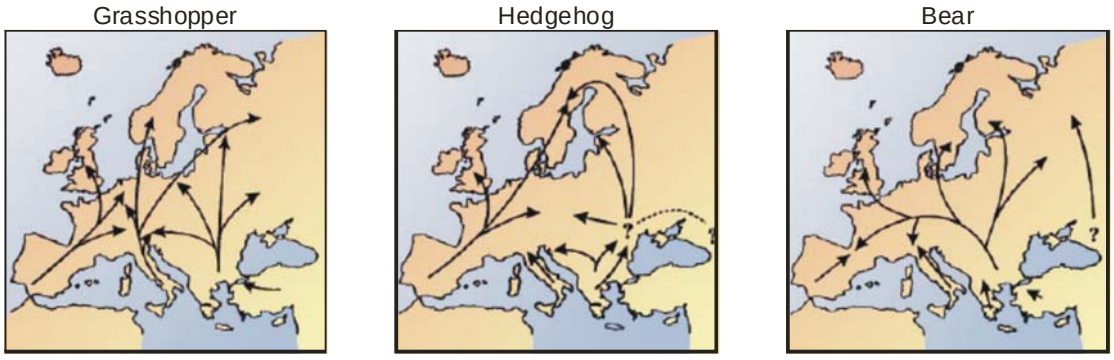

Figure 1-14: Paradigmatic postglacial colonisations from Southern Europe deduced from DNA differences for the grasshopper, *Chorthippus parallelus*, the hedgehog, *Erinaceus europeus/concolor* and the bear, *Ursos arctos*. Main refugia are located in Iberia, Italy, the Balkans and Caucasus which contributed differently to the repopulation of northern parts (Hewitt, 2000).

In front of this background mitochondrial DNA (mtDNA) phylogeography of European *T. cancriformis* populations was investigated to deduce genotype variation and the process of range expansion after glaciers have retreated (see chapter 4).

1.4 Population genetics

One of the universal attributes of natural populations is that organisms differ in phenotype with respect to many traits. Population genetics must deal with this phenotypic diversity, and especially with that portion of the diversity that is caused by differences in genotype. In particular, the field of population genetics has set for itself the tasks of determining how much genetic variation exists in natural populations and of explaining its origin, maintenance, and evolutionary importance (Hartl & Clark, 1997).

The ability to estimate genetic relatedness within and between populations has been applied to different areas of research, including (i) population conservation (FitzSimmons et al., 1995; Haig, 1998; Ciofi & Bruford, 1999; Moritz, 2002), (ii) estimation of dispersal (Schlötterer & Pemberton, 1998; Beaumont, 1999; Hutchinston & Templeton, 1999) and (iii) evolution of reproductive mode (Graham, 1982; Havel & Hebert, 1993; Hughes, 1998; Tregenza et al., 2000; Scanabissi Sabelli & Mondini, 2002; Wolf, 2005).

For nearly 50 years, the workhorse method revealing genetic variation has been electrophoresis because small differences in rate of migration in an electrophoretic field can be used to distinguish between nearly identical macromolecules (Richardson et al., 1986; Hartl & Clark, 1997). Primarily protein electrophoresis is applied to study allozymes variation in populations. Like polypeptides, DNA fragments can be separated by electrophoresis. Different methods based on comparison of fragmented DNA patterns have been established in the field of population genetics (Hartl & Clark, 1997; Hoelzel, 2002). The present work of population genetics (see chapter 5 and 6) focuses on microsatellite analysis. Microsatellites are highly polymorphic DNA markers with discrete loci and codominant alleles (Schlötterer, 2002) and thus able to detect genetic diversity within and among populations. Genetic diversity is the variety of alleles and genotypes present in a population.

Genetic diversity at a single locus is characterized by allelic diversity, expected and observed heterozygosity (Nei, 1972; 1973; 1978; Frankham et al., 2002; Schlötterer, 2002). Allelic diversity is defined as the average number of alleles per locus (Hartl & Clark, 1997; Frankham et al., 2002). Allele and genotype frequencies at an

autosomal locus attain equilibrium after one generation in large, random mating populations when there are no perturbing forces (no mutation, migration or selection). This equilibrium is referred to as the Hardy-Weinberg equilibrium (HWE). Deviations from HWE genotype frequencies provide information to detect inbreeding, population fragmentation, migration and selection. Therefore, fragmented populations with restricted gene flow show deficiencies of heterozygotes compared to Hardy-Weinberg expectations. Heterozygosity is a parameter indicating the structure and even history of a population. Large populations of naturally outbreeding species usually have extensive heterozygosity, but it is mostly reduced in small populations and species of conservation concern (Frankham et al., 2002). Loss of allelic variation and heterozygosity is caused by inbreeding. But also chance effects like passive dispersal, colonization, and genetic drift play an important role of genetics e.g. in freshwater pond populations. Thus, the larger the subpopulation, and the more recently it has been isolated, the smaller is the inbreeding effect. Inbreeding, defined as the mating of relatives, reduces the frequency of heterozygotes compared to random mating (Figure 1-15).

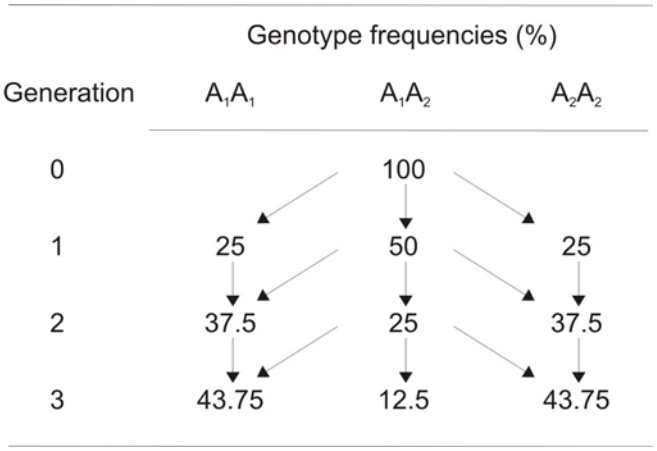

Figure 1-15: Effect of self-fertilization on genotype frequencies. The frequencies of heterozygotes halves with each generation of selfing, modified after Frankham et al. (2002).

Differences between observed and expected numbers of heterozygosities will occur by chance. To determine if the differences are of statistical significance, the deviation between observed and expected numbers (heterozygosity) is tested using a Chi-square test (Frankham et al., 2002). The larger the differences between observed and expected numbers, the larger the Chi-square value. To study hierarchical structure of random mating populations (showing significant genetic divergences) statistical methods like F-statistics are available (Wright, 1951; Nei, 1972). Recently advanced models have been developed to estimate population differentiation and

structure accurately also in the case of clonal reproduction (de Meeûs & Balloux, 2005).

In the thesis at hand, microsatellite analysis was applied to study the genetic structure of European *T. cancriformis* populations and further to investigate the rate of inbreeding and to deduce the reproductive strategy present in unisexual *T. cancriformis* populations.

1.5 Methodology - Molecular genetic analysis of populations

Population geneticists are relying increasingly on the study of DNA variation between and within populations. Thus (i) high quality DNA must be isolated from the specimens, (ii) the relevant region of the template DNA must be amplified and (iii) finally the PCR fragments have to be separated properly.

1.5.1 DNA extraction

DNA can be extracted from tissue that are fresh, frozen, dried, or stored in ethanol or buffers (Sambrook & Russell, 2002). The DNA extraction involves a sequence of several steps (Hoelzel, 2002). The procedure often begins with grinding or mechanical pulverization to separate cells and destroy cell membranes and/or cell walls, while leaving the nucleus intact. The tissue is then immersed in a solution containing a detergent that lyses the nuclear membrane, and a proteinase that denaturates proteins e.g. RNAses. In a next step proteins are separated from nucleic acids by extraction with organic compounds. After that DNA is purified from the reagents in the extraction buffer by ethanol precipitation and finally concentrated for use in subsequent analysis. In this study methods for total cellular DNA isolation were applied, followed by subsequent selection of subsets of the genome by PCR.

1.5.2 Polymerase Chain Reaction

Since its invention by Kary Mullis in 1983 the Polymerase Chain Reaction (PCR) has become a mainstay of molecular ecology and population genetic research (Newton & Graham, 1994). Defined segments of small amounts of template DNA can be amplified to high quantity in a very short time. The principle behind PCR is illustrated in Figure 1-16. Short ‘oligonucleotide’ sequences (primers) are designed, complementary to regions flanking either side of the sequences of interest. The PCR reaction mixture includes the primers in great excess to the template DNA, buffer, DNA polymerase, and free nucleotides. The template DNA is denatured at 93°C-95°C and then cooled to allow the primers to anneal (depending on primer sequence, but usually between 40-65°C); then temperature is adjusted to the optimal

temperature for the DNA polymerase for an extension phase (usually 72°C), this cycle is repeated (25-40 times). Thus, the expansion of amplified DNA is roughly exponential. The PCR technique is now so pervasive in molecular biology that it is difficult to think of life without it. Here, PCR techniques were applied to amplify microsatellites as well as cytochrome *c* oxidase subunit I (COI) and 16S rDNA genes for further sequencing reactions.

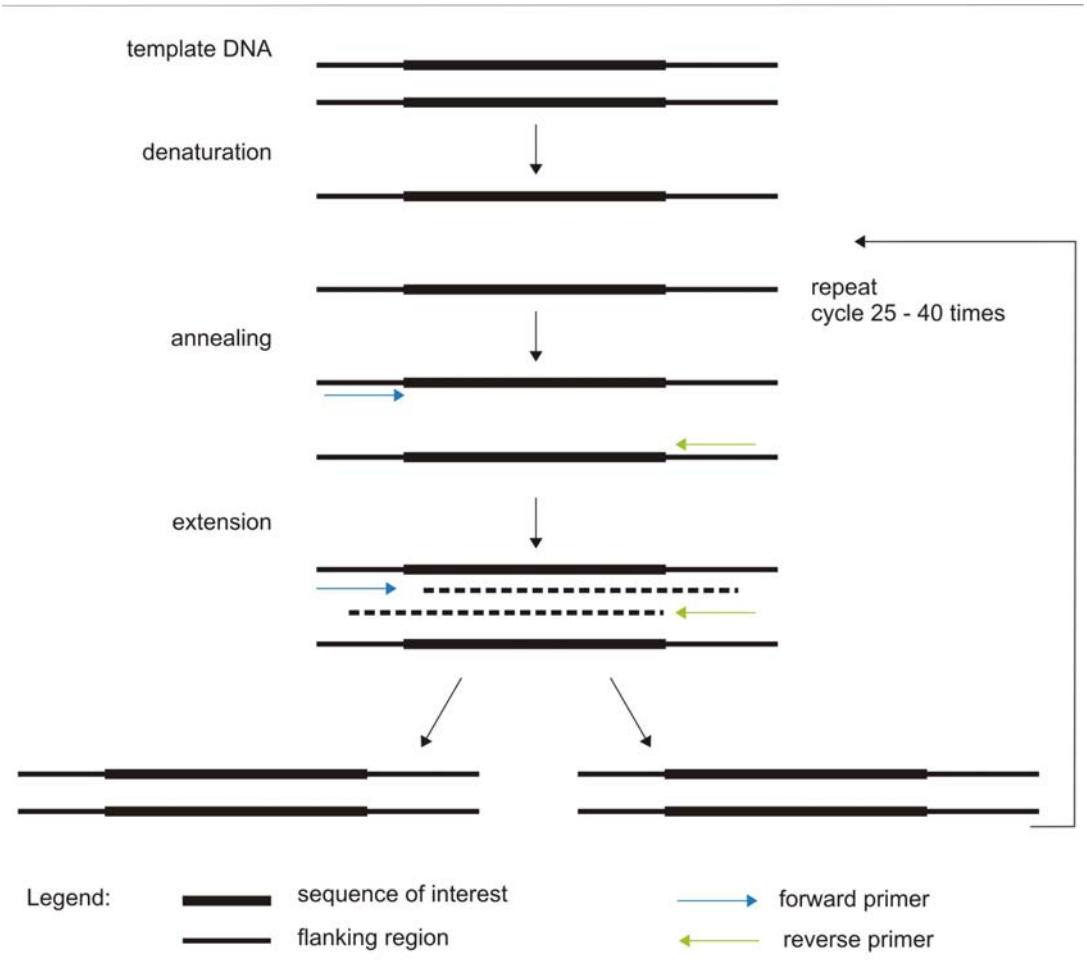

Figure 1-16: Principle of polymerase chain reaction (Hoelzel, 2002).

1.5.3 Electrophoresis

Electrophoresis is defined as separation of macromolecules in a gel matrix in an electrophoretic field. The migration of the DNA fragments depends on their length, charge and secondary structure. DNA fragments migrate to the positively charged anode due to the negative charge of the phosphates along the DNA-backbone. The

resulting bands are visualized by different staining procedures (e.g. ethidiumbromide, silver) and/or illumination by UV light (Richardson et al., 1986).

1.5.4 Cloning and sequencing

Cloning is essentially the introduction of a foreign piece of DNA (insert) into an autonomously replicating DNA molecule, called a vector (Sambrook & Russell, 2002). The vectors containing the foreign DNA are introduced into a suitable host so that it may be propagated (e.g. *Escherichia coli* bacteria). As referred by Hillis et al. (1996) the method to obtain sequence data (sequencing) can be subdivided into four basic steps: First, a particular target sequence must be identified that contains an appropriate amount of variation across species or individuals for the problem that is to be addressed. In the present thesis cytochrome *c* oxidase subunit I and 16S rDNA gene were sequenced to distinguish and identify *T. cancriformis* genotypes. Second, many copies of the target sequence must be isolated and purified from each individual to be examined. Third, the purified DNA (or, rarely, RNA) must be sequenced. Finally, homologous sequences must be aligned and analysed.

1.5.5 Microsatellite analysis

Microsatellites are a special class of tandem repeat loci that involve a base motif of 1-6 bp, such as 'AC', repeated up to about 100 times (Tautz, 1989). This class of nuclear marker is typically found in rapidly evolving, noncoding DNA (intron) (Figure 1-17).

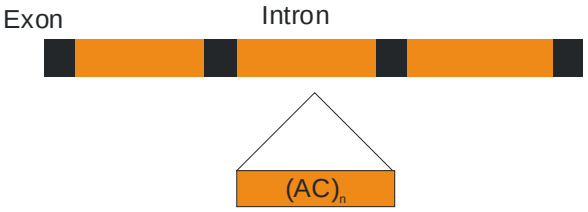

Figure 1-17: Genomic DNA is a complex molecule of coding (exons) and non-coding (introns) regions. Microsatellite sequences, like 'AC' repeats of n-times, predominate within introns (outline Zierold).

Several studies on tandem repeat loci indicate exceptionally high mutation rates, with minimal rates as high as 10^{-3} to 10^{-4} mutations per site (Levinson & Gutman, 1987; Goldstein et al., 1995; Ellegren et al., 1997; Goldstein & Schlötterer, 1999; Schlötterer, 2000; Calabrese et al., 2001; Schlötterer, 2002). The eukaryote genome is highly interspersed with microsatellites. Poly(G) and poly(A) are the simplest of the microsatellites, while poly(AC) is by far the most frequent, appearing

as 5-10x10^4 individual islets in the mammalian genome (Hamada & Kakunaga, 1982). Many other microsatellites have been reported, among them: poly(TC), poly(CAC), and poly (GATA) (Tautz & Renz, 1984; Tòth et al., 2000; Zane et al., 2002). Because of this and the features that they are highly polymorphic, codominant genetic markers, abundant and ubiquitous in eukaryote genomes, these loci have become the marker of choice for many types of population genetic studies (Goldstein & Schlötterer, 1999; Schlötterer, 2002). The Polymerase Chain Reaction (PCR) can be used to amplify microsatellite rich DNA regions. The PCR products are electrophoresed and visualized, allowing precise determination of length polymorphisms. The detected length polymorphism is defined by the variable number of repeated motifs. These markers can be compared reliably across gels, which allows for comparison among large numbers of individuals.

To use microsatellite analysis one has to identify the flanking sequence, which is necessary for the specific microsatellite primer design. These primers work only in fairly close related species or even subspecies. Thus, for every species which has not been studied yet by microsatellite analysis (i) microsatellite regions have to be found and (ii) microsatellite primers have to be developed and optimized.

In chapter 5 the process of microsatellite isolation on DNA of *T. cancriformis* is described and the application of microsatellite primers to analyse the population genetic structure of the study species is given in chapter 6.

1.6 Conservation biology and management

The science of conservation biology seeks to provide the information about our natural world that will enable the sustainable management of genes, species and communities and to maintain the biodiversity that characterises the richness of our planet (Pullin, 2002).

Conservation Biology hypotheses imply that anthropogenic habitat fragmentation reduces local population size and increases the isolation of population (Saunders et al., 1991; Young et al., 1996; Young & Clarke, 2000). Additionally small compared to large populations have increased extinction risks because of environmental, demographic or genetic stochasticity. Moreover, in small populations chances of mating close relatives increase (Voigt & Klaus, 2003). This often results in inbreeding depression, reduced individual fitness and also reduced heterozygosity (Storfer, 1999).

For Conservation Biology we have to answer the question what kind of landscape and population structure should be achieved to minimize the extinction of species and the loss of genetic variation within populations. Several interdisciplinary analyses are necessary to answer this question: (i) analysis of species interactions on broad geographical scale, (ii) characterization of population structure, (iii) definition of components of fitness, (iv) reproductive modes present and finally (v) management-unit definition for the species under study (Thompson, 1996; Lienert et al., 2002; Moritz, 2002; Pullin, 2002; Schmitt & Seitz, 2002).

The establishment of conservation-management action plans depends upon the statute of law. The protection and sustainable use of 'biological diversity' was manifested in the United Nations Conference on Environment and Development held in Rio de Janeiro, Brazil, in 1992. Besides this broad convention, many countries produced their own action plans for both habitats and species. Additionally, nature conservation laws and guidelines manage the protection of habitats and species for each country. The international and national agendas advanced the development of methods for describing and prioritizing areas for protection or rehabilitation. The methods include analyses of species and genetic diversity, reproductive mode as well as the study of connectivity across a mosaic of habitats (Moritz, 2002). Furthermore experiments for conservation breeding and reintroduction of species evolved.

Based on the broad investigation of *T. cancriformis,* which will be outlined in the thesis at hand, management action tools and priorities for the conservation of ephemeral habitats and thus for the protection of the species under study will be proposed.

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Chapter 2

Characterization of large branchiopod habitats*

We have short time to stay, as you,
We have as short a Spring,
As quick a growth to meet decay,
As you or any thing.
(Robert Herrick, 1591 – 1674)

Abstract

Temporary pools are unusual habitats, neither truly aquatic nor truly terrestrial, where alternating phases of flooding and drying out, as well as their isolation, favour the establishment of unique and diverse plant and animal communities. Crustaceans are important components of the invertebrate fauna of temporary waters, among them large branchiopods are the most prominent group, since their occurrence is confined to these habitats. The present report highlights the plasticity of *Triops cancriformis* indicated by its broad range of settled ecological niches. Despite ecological adaptations it is shown that tadpole shrimps are endangered mainly by human impacts on primary habitats. As a basis for conservation decisions threats to ephemeral habitat are summarized.

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2.1 Introduction

The notostracan genus *Triops* inhabits ephemeral fresh water bodies exclusively. Such habitats are also known as temporary or astatic pools and puddles (natural origin) or even ponds (human origin). Based on the Ramsar definition, temporary water bodies are small (generally <10 ha), shallow wetlands characterised by alternating phases of drought and flood and by a very self-contained hydrology. They dry out for long enough to exclude the more commonplace plant and animal communities which are characteristic of more permanent wetlands (Anonymus, 2002). However, temporary waters vary considerably depending on the biogeographical and climatic region (Keeley & Zedler, 1998; Blaustein & Schwartz, 2001; Friedman & Lee, 2002; Grillas et al., 2004b). The ecological characteristics of ephemeral habitats depend on the hydrological regime, soil type, nature of the underlying rocks and physicochemical characteristics of the water. The flora has developed remarkable adaptations for survival: a wide range of different sizes, growth forms, modes of reproduction and life-history strategies (Scholnick, 1994; Heilmeier & Hartung, 2001; Grillas et al., 2004a; Heilmeier et al., 2005). The fauna has also had to adapt to the same constraints, with the result that these habitats support a diverse genetic heritage of great value: there are many rare species here and many have unique ways of life (Boileau & Taylor, 1994; Grillas et al., 2004a; Biebighauser, 2005). Branchiopods constitute a very important group in ephemeral waters, with rare or localised subpecies (Herbst, 1982; Simon, 1987; Lanfranco, 1995; Alonso, 1996; Blum, 1998; Simovich, 1998; Hödl & Eder, 2001; Grillas et al., 2004b).

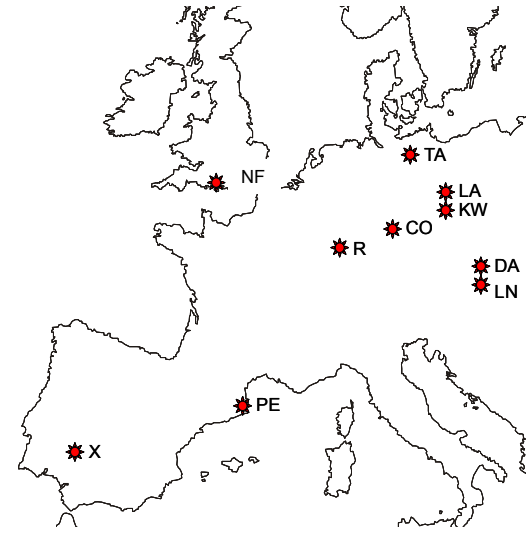
Nowadays ephemeral habitats and their inhabitants are extremely vulnerable, being subject to innumerable threats. These include drainage, reclamation for agricultural or urban development, filling up with garbage or waste, pollution by fertilizers or pesticides, unsustainable agriculture or cattle raising, and even bad management decisions for conservation purposes (Alonso & Alcaraz, 1984; Simon et al., 1994; Hughes, 1997b; Engelmann & Hahn, 2005; Günther et al., 2005). Thus, habitat fragmentation and destruction result in reduction of population sizes which may lead to an increased risk of extirpation (Lande & Barrowclough, 1987; Harrison

& Hastings, 1996). In fact, the notostracan species *Triops cancriformis* is considered as critically endangered according to the 1999 IUCN draft criteria (IUCN, 1999) though the species is not listed in the Flora-Fauna-Habitat guidelines (Der Rat der Europäischen Gemeinschaften, 1992). For *T. cancriformis* a statistically significant decrease of occurrences have been reported for several countries (Schembri, 1989; Simon et al., 1994; Hödl & Eder, 2001; Trust, 2006; UNEP, 2006). Furthermore the tadpole shrimp *T. cancriformis* is targeted as a priority species for conservation action under the UK Biodiversity Action Plan (UK BAP) (Maitland, 1995). To protect the endangered habitats as well as the species occurring in it, detailed ecological information on habitats is necessary.

The present paper provides an overview of habitat characteristics which enable the occurrence *T. cancriformis*. It further highlights major threats for ephemeral habitats and thus for the studied species itself.

2.2 Database

Ten ephemeral water bodies in inundated floodplains, isolated ponds and puddles and fish nursery pools in Europe were characterized using own and literature data (Figure 2-1). These habitats were chosen in order to study morphology and genetics of *Triops cancriformis* as presented in this work later.



Legend of studied ephemeral waters:

Region ID	Region	Reference
CO	Coburg	Engelmann & Hahn, 2005
DA	Danube	Eder et al., 1997
KW	Königswartha	Langner, 1985; Zierold, 2003
LA	Lacoma	personal data
LN	Neusiedler Lake	Eder et al., 1996
NF	New Forest	Hughes, 1997a
PE	Plat d'Espolla	Boix et al., 2002
R	Rhine	Dannapfel & Schätzle, 1988
TA	Tannen	personal data
X	Extremadura	Pérez-Bote, 2004

Figure 2-1: Location of characterized ephemeral habitats of *Triops cancriformis* and data source.

Based on the anthropogenic influence on the hydrological regime, the ephemeral water bodies can be classified into natural and those managed by humans. The latter type includes fish ponds. The natural pools have further been classified into 'Mediterranean pools' and 'Central European pools'. The Mediterranean pools include transitional coastal wetland and groundwater elevation waters. The Central European pools are separated into groundwater elevations, riparian pools and rain pools and puddles.

Based on literature data and field studies habitats were characterized by their geographical position, hydrology, surrounding area, pool/pond size and depth, chemical and biological features. Furthermore possible habitat threats were summarized as a basis for conservation decisions.

2.3 Review

2.3.1 Description of reviewed *Triops cancriformis* habitats

2.3.1.1 Ponds managed by humans

Königswartha (51°19' N, 14° 21 E). The Fishery area is located in the "Oberlausitzer Heide- und Teichlandschaft" a UNESCO biosphere reserve of 30.000 ha. The territory of Königswartha (Saxony, Germany) counts over 80 fish ponds including nursery ponds. Most of the fish ponds are artificial (Biosphärenreservat Oberlausitzer Heide- und Teichlandschaft, 2001; Zweckverband Naturschutzregion Neiße & Projektgruppe "Naturschutzprojekt Niederspree-Hammerstadt", 2003). The ponds are regularly drained of in October (Figure 2-2) and filled up in May of the following year to rear young carps (Langner, 1985). The origin of *T. cancriformis* in these ponds has not been clarified yet. It is most likely that the large branchiopod species have been introduced with fish transports from Hungary and Austria. However natural distribution from former occurrences along the Oder River or the Elbe River or their tributaries has to be taken into consideration. As observed during the examinations between 2000 and 2004, *T. cancriformis* often co-occurs with *Limnadia lenticularis* (Branchiopoda: Spinicaudata) and individual densities range from 266 to 2500 per square meter (Engelmann et al., 1988; Zierold, 2003). The 30 m by 20 m ponds have a depth of about 100 cm to 150 cm. The 2001 report of the fishery department (Saxony Institution of Agriculture) shows that water temperature in the nursery ponds fluctuated between 17.4°C in May after flooding, 24.2°C in August and 12.4°C in September before drain off. The pH value varied between 10.6 and 6.9 and conductivity ranged from 480 to 620 µS cm^{-1}.

Figure 2-2: Dried out fishery pond in Königswartha; photo Zierold.

Lacoma (51°47'N, 14°23'E)**.** The fishery area lies in the near neighbourhood of Cottbus (Brandenburg, Germany). Numerous endangered species occurred in the wide pond landscape until 2004 (Figure 2-3). Today the area is part of the opencast mining and thus none of the unique habitats exists any longer. *T. cancriformis* may have been introduced by fish transports either from Königswartha or from Hungary and Austria (Danube, Neusielder Lake) (Langner, 2003). The species reached a population sizes up to 1000 individuals per square meter (Langner, 2003). No co-occurrence with *Limnadia lenticularis* was observed. The ponds were of irregular size (up to 8000 m^2) and depth varied between 100 cm and 150 cm.

Figure 2-3: Fishery pond in the area Lacoma just before drain off due to opencast mining photo Zierold.

2.3.1.2 Natural pools

Central European pools

Ephemeral habitats in the area of **Coburg** (50°15' N, 10°58' E) and **Tannen** (53°34' N, 11°26') are located in former military training areas in Germany. Both territories contain large open areas with depressions created by tanks and other vehicles. Within those depressions water infiltration is inhibited by soil compaction. Thus waterlogged depressions establish through heavy rain in summer and early autumn. Pools holding water for at least three to four weeks are inhabited by *T. cancriformis*.

Rhine (49°00' N, 08°16 E)**.** The Rhine floodplain area between Mannheim and Rastatt can be characterized by regular floods and fluctuations of ground-water level which are correlated to the river-water level. Floods are caused by glacier thaw and snow melt in the Alps during June/July as well as by heavy rain falls in September/October (Dannapfel & Schätzle, 1988). The water level fluctuates within 5 m (Dannapfel & Schätzle, 1988). Ephemeral habitats along the river between Karlsruhe and Mainz establish either in floodplain depressions or behind dikes. The latter ones are fed by ground-water elevation. The ponds vary in size (ranging from 10 m^2 up to 1500 m^2) and depth (ranging from 30 to 80 cm). *T. cancriformis* appears during May and September (Simon, 1987; Dannapfel & Schätzle, 1988) mainly south of Mannheim (Daxlanden, Hagenbach, Ibersheim, Neuburg) (Figure 2-4).

Figure 2-4: Dried out *Triops cancriformis* habitat in the Rhine riparian area near Neuburg. The open character is achieved by yearly ploughing of deep furrows; photo Zierold.

Danube (48°10' N, 16°58' E)**.** With a length of almost 2900 km and a catchment area of approximately 800.000 km^2, the Danube is the second largest river of Europe. Highwaters of Danube river are significantly affected by the Alpine Inn river. Snowmelts in the Alps and heavy rains in summer usually cause highwaters in July and August (Schiemer, 1987), favouring the presence of eurythermal and thermophilic branchiopod species in astatic pools including *Branchipus schaefferi* (Anostraca), *T. cancriformis* (Notostraca), *Lynceus brachyurus*, *Limnadia lenticularis*, *Imnadia yeyetta* und *Leptestheria dahalacensis* (Spinicaudata) (Eder & Hödl, 1996; Eder et al., 1997; Gottwald & Eder, 1999). Due to their high large branchiopod diversity the "Blumengang-depression" (Figure 2-5) with a size of about 5 ha between the tributaries of Morava and Danube is one of the most important habitats (Eder & Hödl, 1996; Hödl & Eder, 1996).

Figure 2-5: The "Blumengang-depression" during midsummer; photo Zierold.

Neusiedler Lake (47°48' N, 16°51 E). Neusiedler Lake, a shallow alkaline water lake, straddles the border between Austria and Hungary. The lake's area (340 km^2) and depth (average 1.5 m) vary considerably with the seasonal rain falls. This part of eastern Austria wide lowlands biogeographically belongs to the Pannonian region, where a continental, semi-arid climate with high precipitation in spring and autumn favours the existence of astatic water bodies (Figure 2-6). The high sodium carbonate content most probably originated from salt-rich tertiary and quaternary

sediments (Löffler, 1982). Those lonely and desolate alkaline pans are not only a site of a sanctuary for rare and migratory birds which have been protected since 1935, but also for a number of large branchiopod species (Eder et al., 1996). *T. cancriformis* occurs in the so-called alkaline white pools but also in the black pools with high humus content (Eder et al., 1996). Additionally, the species is found in rain pools established in cart tracks. The habitats lie within extensive agricultural meadows or cornfields.

Figure 2-6: A wide pond in the neighbourhood of Neusiedler Lake (Kaiserlacke; photo Zierold).

New Forest (50°55' N, 01°45' W)**.** The Godshill pond is about 30 m long and very shallow (less than 60 cm) (Fox, 1948) and localized on plateau gravels which are common throughout the New Forest. The gravel presumably has a clay admixture to hold the water (Tadpole shrimp conservation group, 1995). Conditions at the pond were studied in detail by Khalaf & MacDonald (1975) and Hughes (1997a). The pond dries out (depending on the rainfall) between April and August/September, when it is covered with grass. The water level fluctuates during the winter. It does develop ice on the margins, but never completely freezes as the livestock continues to use it as a watering hole. The next nearest similar pond is about a quarter of a mile away from the pond. The pond is visited by livestock and their dung is deposited in the water which most strongly affects the water chemistry (Table 2-1). The land use in the neighbouring area has not changed for many years – which is grazing for ponies, sheep and cattle.

Table 2-1: Physochemical details of Godshill pond, as recorded by Khalaf & MacDonald (1975) and picture of the pond near Fordingbridge (Zierold, 2002).

Parameter	Value
Dry periods (weeks)	3
Water temperature	0.5 - 28.0°C
Mud temperature	0.5 - 26.0°C
Dissolved oxygen	4 -14 mg l^{-1}
Conductivity	930 µS cm^{-1}
Sodium	120 mg l^{-1}
Potassium	25 mg l^{-1}
Calcium	27.0 mg l^{-1}
Magnesium	3.5 mg l^{-1}
Chloride	8.0 mg l^{-1}

Mediterranean pools

Plat d'Espolla (Northeast Iberian Peninsula, 42°09'06" N, 02°46'01' W)**.** Espolla temporary pond with a maximum surface of 3.13 ha and a maximum depth of 4 m is located in the Banyoles karstic area (NE Iberian Peninsula) and fed by groundwater elevations. It has the same groundwater supply as Lake Banyoles and the other pools in the area. As reported by Boix et al. (2002) groundwater reaches the pond at high temperature (19°C) and low oxygen concentration (3 mg l^{-1} approx.), but mean values of pond water are 17.3°C and 7.4 mg l^{-1} respectively (Figure 2-7, Table 2-2).

Figure 2-7: Ground water elevation pond Plat d'Espolla in the Banyoles karstic area of the NE Iberian peninsula (photos Centre de Recursos Pedagògics del Pla de l'Estany, 2002).

Table 2-2: Water characteristics of the Espolla temporary pond during 1996–1997. The median is included due to the high values of chlorophyll a and dissolved oxygen reached in the last days of the hydroperiod, when the pond was drying (Boix et al., 2002).

Parameter	Range	Mean	Median
Temperature (°C)	5.60 33.00	17.30	16.20
Conductivity (µS cm^{-1})	212.00 1046.00	895.00	892.0
pH	6.50 8.70	7.70	7.70
Chlorophyll a (µg l^{-1})	0.14 350.42	13.09	2.93
Dissolved oxygen	4.10 14.60	7.60	6.80

The flooding dynamics of this pool is irregular as reported by Boix et al. (2002). There are two complete hydroperiods (autumn and spring) separated by a period of total desiccation during some years. It is not, however, uncommon to have years with only one complete hydroperiod, in late autumn or winter, and occasionally no flooding at all. The animal community richness is dominated by insects, with 82 taxa, followed by crustaceans (14 taxa) and amphibians (11 taxa).

Extremadura (39°27'N, 06°15'W)**.** Extremadura is located in the southwest of the Iberian Peninsula and is dominated by a Mediterranean-type climate with high values of precipitation in spring and autumn. About 90 % of the annual rain (450 – 1000 mm a^{-1}) falls between November and April. This rainfall regime favours the presence of temporary waters (Pérez-Bote, 2004). Land use is based mainly on a dry agriculture scheme (cereals, sunflower, olives, and vineyards predominate), together with holm-oak ("dehesas", 43% of the area of Extremadura), a semi-natural, open-forest habitat typical of the southwestern Iberian Peninsula (Pérez-Bote, 2004). The subspecies *T. c. mauritanicus* (Ghigi 1921) is located mainly fairly deep in turbid waters, with abundant aquatic vegetation (*Ranunculus* sp.) (Pérez-Bote, 2004).

2.3.2 Review of ephemeral ponds inhabited by *Triops cancriformis*

Defenceless notostracan inhabit species-poor, unrivalled ecological niches which are, in fact, ephemeral water bodies. Three different natural types of ephemeral water bodies and one astatic habitat managed by humans were classified in the present review (Table 2-3). Natural ponds have been identified as groundwater elevation pools, pools in inundated floodplains and rain pools. The different ephemeral water bodies occurred either in open areas or grassy lands including fallow ground, but also

extensively used cornfields. Fish nursery ponds are artificial habitats where aquatic and terrestrial phase are controlled by human.

Table 2-3: Overview and important characteristics of ephemeral water bodies inhabited by *Triops cancriformis* (data based on own studies between 2002 and 2005)

	Groundwater elevation pools	**Floodplain pools**	**Rain pools**	**Fish nursery ponds**
Definition	Temporary water bodies that are not connected with a permanent river but filled by groundwater due to increased ground water level.	Pools that are temporarily connected with a permanent stream or river as a result of high waters in spring or late summer run-offs.	A temporary pool that accumulates surface water in an isolated basin that at no time during the year has either an inlet or an outlet; water is entirely absent from the surface part of the year.	Carp nursery ponds that are drained of between October and May.
Peculiarity	Pools occur often on the landside of dikes.	The fauna resembles that of temporary pools, but may also include species introduced by surface connection from permanent waters.	The pool could not receive any animal inhabitants from adjacent stream by way of surface water connection. Solidified soil underground prevents infiltration of surface water.	Seed of winter barleys spread over the dried pond and is allowed to grow to a size of 10 cm (in the next spring). Thus ponds have high nutrient content right after filling with water.
Pond extension	Shallow waters of less than 60 cm water depth	Broad water bodies of various depths in riparian depressions and dips.	Often small puddles in former military areas, but also broad pans in the Seewinkel.	Ponds are less than 150 cm in depth but of various sizes.
Flooded areas	fallow ground, grassland, cornfields	Pasture, grassland, fallow ground and corn fields	Field track, depressions in former military areas with underground of less permeable soils	Artificial ponds
Hydrological regime	Depends on highwater events (snowmelt, heavy rain)	Depends on highwater events (snowmelt, heavy rain)	Depends on rain and surface run-off	Ponds have an annual cycle of 6 months wet phase and 6 months dry phase
Examples	Neuburg (Rhine riparian); Kaiserlacke (Neusiedler Lake), Plat d'Espolla (Banyoles karstic area)	"Blumengang"-depression (Danube)	Former military areas in neighbourhood to Tannen and Coburg; Godshill pond (New Forest) Extremadura	Königswartha (Bautzen) Lacoma (Cottbus)

2.3.3 Threats of ephemeral habitats

Reasons for the rare and declining occurrences of *T. cancriformis* have been discussed by various authors. One important review focusing on causes of endangerment was recently published by Günther et al. (2005). The work classified the endangerments for *T. cancriformis* into different complexes including three high impact complexes farming, construction and river engineering/shipping; further complexes of less impact including forestry, nature conservation, fishing, disturbance in military areas, direct removal, species-specific or habitat-specific biological risks and natural processes. The study (Günther et al., 2005) further identified 41 threats for large branchiopod species in Germany. The evaluation of the reports given by Boix et al. (2002), Eder & Hödl (1996), Engelmann & Hahn (2005), Günther et al. (2005), Herbst (1982), Königstedt (1993; 1994), Machado *et al.* (1999), Petrov & Petrov (1997), Ružičkova et al. (2004), Simon (1987), Simon et al. (1993) as well as own studies resulted in a long list of endangerments for *T. cancriformis* (the classification of complexes of endangerments were adopted from Günther et al. (2005) (see Box 2-1 and 2-2)

Box 2-1	Threats of high impact of ephemeral water bodies inhabited by *Triops cancriformis* (references see text)

Farming

- drainage of wet meadows and small water bodies
- lowering of groundwater level
- conversion of floodplain meadows into arable lands
- regulation and interruption of natural floodplain dynamic
- use as watering place for farming and artificial deepening for cattle raising
- nutrient inflow
- scattering of pesticides, herbicides and lime in floodplains

Construction

- drainage of wet meadows and small water bodies
- construction of housing estates, commercial and industrial centres
- establishment of new water bodies/streams/canals

Engineering/shipping

- river straightening
- interruption of natural floodplain dynamic
- sand and gravel extraction
- dam up of floods
- establishment of new water bodies/streams/canals
- opencast mining

Box 2-2	Threats of lower impact of ephemeral water bodies inhabited by *Triops cancriformis* (references see text)

Forestry
- lowering of groundwater level
- conversion of floodplain meadows for reforestation
- dike construction

Nature Conservation
- dike construction
- transformation into permanent water bodies
- close-down of farming without controlling the natural succession
- management decisions which focus on short-term conservation purposes
- shrub or perennial herbaceous vegetation encroachment

Environmental emissions
- destruction of small water bodies through sediment in-filling, waste deposit or other organic material input

Fishing
- eutrophication through intensive piscicultura

Disturbance in military areas
- succession in former military areas

Direct removal
- dike construction
- opencast mining

Species specific or habitat specific biological risks and natural processes
- natural infrequency
- default of floods and heavy rain
- hostile landscape matrix
- unawareness of branchiopods in public mind

2.4 Discussion

Recent *T. cancriformis* habitats cover a wide range including rainwater and ground water elevation ponds, brackish pans but also fishery ponds or rice fields. Those habitats share similarities, but are also various. Similar is the change in terrestrial and aquatic phase of the habitat, whereas the source of water, the duration of inundation or waterlogged phase and the timing of these phases is various (Keeley & Zedler, 1998; Williams, 2006). Additionally habitat substrate and thus water chemistry may vary considerably among those habitat types(Zierold, 2005). In consequence *T. cancriformis* shows a broad ecological plasticity which is known from other large branchiopods (Petrov & Cvetkovic, 1999; Zierold, 2002).

Compared to fossil Branchiopod records (Tasch, 1963; Kelber, 1998, 1999) recent habitats share correspondence including the astatic hydrological regime and that they are standing water bodies. In detail Lower Permian geological profiles showing Notostracan records indicate seasonal cycle of warm dry and warm wet climate. As notostracan were also reported from colder climates (Morell, 2005) one could conclude that water availability is the limiting factor for the establishment of large branchiopod habitats and presence or absence of vegetation as reported in Maitland (1995) are of less impact on the occurrence of large branchiopod. Hempel-Zawitkowska (1967) has defined three of the main habitat requirements of *T. cancriformis* as (i) optimum temperatures of 15-23 °C during the growth period, (ii) ponds must be filled with water for long enough to allow full development (i.e. 3-4 weeks) and (iii) high food availability. From first habitat modelling results it can be concluded that the occurrence of large branchiopods depends to a large extent on water chemistry (e.g. nitrate concentration, pH value) and water depths (Hochmuth, 2006).

The geological information on fossil Notostraca leads to the assumption that former niches for Triopsidiae are represented by Playas (depressions with alumina or marl) filled by monsoon rain. Similar rain pools and puddles are observed today in California (Keeley & Zedler, 1998), France (Mathias, 1937; Nourisson & Thiery, 1988), Spain (Alonso, 1996; Machado et al., 1999; Pérez-Bote, 2004), Maltese Islands (Lanfranco, 1995, 2001), Morocco (Thiery, 1986, 1991). It is most likely that floodplains became appropriate license for large branchiopod occurrences after

glacial ages when rivers and streams created wide lowlands appropriate for establishment of depressions inundated by snow and glacier melt and seasonal precipitation. In those primary niches algae plague dominates whereby no further vegetation establishes (Heidecke & Neumann, 1987). In fact recent ponds in inundated floodplains, which are considered to be of primary state, showed patches of uncovered soil or sparely vegetative structure. Beside the primary habitats secondary habitats including groundwater elevation ponds behind dikes, rice fields, rainwater ponds in military training areas and fish nursery ponds are important for species conservation considering the large population size and resting egg bank (Thiery, 1997).

Even though the large branchiopods have a remarkable ability to colonize new areas and have drought resistant cysts that lie dormant for decades (Carlisle, 1968), their primary habitats are seriously threatened and often destroyed by agricultural and urban development. In view of this background a broad investigation covering morphological, phylogeographic and population genetic studies on *T. cancriformis* was performed. Such research is very urgent as the knowledge of large branchiopods distribution, ecology and morphological and genetic variation is still incomplete, as there is little tradition of surveying the fauna of ephemeral water bodies on a broader scale. The analysis of spatial-temporal variability of population structure will present how consistent (in the term of male:female ratio, phenotype and genotype) are large branchiopods present in individual ephemeral waters. This information will further be applied to define management units for conservation biology (see chapter 7) to preserve large branchiopods in general and to look closer into phylogeography (see chapter 4) and population-genetics (see chapter 6).

Conclusion

The combined analysis of habitat characteristics and threats has great potential to reveal privileged ecological niches from which conservation management action plans may be inferred. The review delineated that large branchiopod occurrences cover a broad range of primary habitats (natural origin) but also secondary habitats (human origin). The long list of threats may indicate the necessity of official laws and long term conservation management.

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Chapter 3

Morphological variation in *Triops cancriformis* (Crustacea: Notostraca)

Taxonomy is described as a science and sometimes as an art,
but really it's a battleground.
(B. Bryson, 1951[*])

Abstract

Lineages which exhibit little morphological change over geologic time are evolutionarily and ecologically interesting, but often taxonomically difficult to handle. Such lineage is represented by the Notostraca, an ancient order of Crustacea. Morphological separation of notostracan species is hampered by high inter-individual variability. In this study morphological characters of the Notostraca *Triops cancriformis* have been investigated to clarify the amount of variation among and within populations and to compare the geographic distribution of phenotypes with respect to currently recognized subspecies. It is clearly shown that *T. c. mauritanicus* is distinct from the other two subspecies, whereas *T. c. simplex* populations from Spain do not warrant statistical significant separation as distinct subspecies with respect to the nominal *T. c. cancriformis*.

[*]Bryson, B. (2004) A Short History of Nearly Everything London: Black Swan.

3.1 Introduction

Fossil records indicate that the morphologies of many organisms remained unchanged over long periods of time, such as *Baiera taeniata*, *Selaginellites gudbierii*, *Mesolimulus walchi* or *Osmunda trimmelkam*. However, Levinton (1988) claims that fossil species are rarely biological species, and that morphological change is not always related to speciation. Thus, for the group of opponents morphological stasis is not a species-level property.

Maynard Smith (1983) explained stasis as a consequence of stabilizing selection which favours a special morpho- and genotype. The author suggested that it may be impossible that environments have not changed over long periods of time, but if population moves, physical conditions could remain constant and morphology might be maintained by selection. An alternative view of stasis given by Eldredge & Gould (1972) is focusing on punctuated equilibrium. The basic idea is that there are limits to the way morphology can change, which are determined by the way organisms develop. A further hypothesis claims that a widespread population that has achieved a complex adaptation to its environment will not be subjected to directional selection because gene flow from many different parts of the range would prevent evolution (Mayr, 1963).

Extant representatives of lineages having survived over long period of time with minimal morphological change are referred to as ‘living fossils’ (Fisher, 1990; Mantovani et al., 2004). One such taxon is the European tadpole shrimp *Triops cancriformis* (Crustacea: Branchiopoda: Notostraca), which inhabits temporary waterbodies such as freshwater pools in inundated floodplains, rainpools, and puddles in rice fields (Brtek & Thiery, 1995; Machado et al., 1999a). Evidence for a wide Notostracan distribution before the Pleistocene can be derived from an abundant fossil record dating back to the Carboniferous or possibly up to the Devonian period (Guthörl, 1934; Wallossek, 1993, 1995; Kelber, 1998). In fact, the striking morphological similarity of some Upper Triassic *Triops* sp. fossils from Germany and extant *T. cancriformis* (Tröger et al., 1984; Kelber, 1999) makes this notostracan Crustacea one of the best examples of evolutionary stasis (Fisher, 1990; Futuyma, 1990; Suno-Uchi et al., 1997; King & Hanner, 1998; Kleesattle, 2001).

The recent distribution of large branchiopods is effected by their drought-resistance cysts, which are efficient agents of passive dispersal. Thus, the high dispersal abilities afforded by their diapausing cysts and the possibility of unisexual reproduction seemingly account for the wide distribution of *T. cancriformis* including occurrences in Japan, North and South Africa, Europe and South Asia. The species has a thermophilic character (Gaschott, 1928; Engelmann et al., 1988; Eder et al., 1997) and seems to be absent from cold regions without a drought period. *T. cancriformis* is not found in latitudes north of 65˚N at present (Brtek & Thiery, 1995).

Three *T. cancriformis* subspecies are currently recognized (Longhurst, 1955). The nominal subspecies *T. c. cancriformis* has a wide distribution range, from Europe, western Russia, and the Middle East to northern India and Japan (Longhurst, 1955; Zaffagnini & Trentini, 1980; Suno-Uchi et al., 1997) but until now there are no records from China and Korea (Umetsu et al., 2002). Authors disagree on the geographic distribution of *T. c. simplex*. Longhurst (1955) reported this subspecies only from northern Africa, from Ceuta to Egypt. In contrast, Brtek & Thiery (1995) indicate the occurrences of *T. c. simplex* for Spain and northern Africa (Algeria), and Thiery (1996) reported it in Yemen (Arabian Peninsula). Finally, *T. c. mauritanicus* (Ghigi, 1921) occurs in southwestern Spain, South Portugal, and western Morocco (Alonso & Alcaraz, 1984) (Figure 3-1).

Morphological studies on *T. cancriformis* by Longhurst (1955) show that the number of segments and the number of legs may vary within taxonomic species. In contrast the basic architecture, such as the fundamental shapes of head shield, telson, legs, dorsal organ and the presence of furca are well preserved among the taxonomic species (fossil as well as recent).

The purpose of this study is to prove whether the subspecies differentiation is supported by morphological features. Additionally the morphological variation between and within populations (on regional and local scale) will be measured to elucidate the occurrence of geographical phenotypes and finally the relationship of the surveyed populations will be deduced.

3.2 Material and methods

3.2.1 Populations sampled

Ethanol-conserved specimens of *T. cancriformis* from 10 regions in Germany (Königswartha, Lacoma, Rhine, Tannen), Austria (Kaiserlacke next to Neusiedler Lake), Great Britain (Godshill pond within New Forest) and Spain (El Puig, Extremadura, Plat d'Espolla and Ullal de Baldovi) were used for morphological analysis (Table 3-1, Figure 3-1). The individuals from one region were treated as one population (regional population). For Königswartha, Lacoma and Rhine the populations were further divided into local populations (compare Table 3-1). Samples were either reared in the laboratory from sediment containing cysts of *T. cancriformis* and preserved in ethanol after reaching sexual maturity ('sediment' in Table 3-1), collected in the field and preserved immediately in the field or provided as conserved material from Natural History Museums or private persons (as it was the case for samples from Tannen, Plat d'Espolla, El Puig and Ullal de Baldovi) ('preserved life' Table 3-1). Live specimens could be collected only from the local populations Kw13, Kw25, Kw27 and Kw28 (Königswartha, Germany).

Table 3-1: Study site information including name and identification for regional and local population, sample size, n, geographic location, original material and species identification (T.c.c. = *Triops cancriformis cancriformis* (determined by the author), T.c.s. = *T. c. simplex* and T.c.m. = *T. c. mauritanicus*)

Country	Regional population		Local population		n	Geographic location	Material	subpecies
	ID	Region	ID	Location				
Austria	LN	Neusiedler Lake	-		11	47°48' N, 16°51' E	sediment	T.c.c.
Germany	KW	fish pond area Königswartha, Saxony	Kw11	fish pond 11	3	51°19' N, 14°21' E	sediment	T.c.c.
			Kw12	fish pond 12	19	51°19' N, 14°21' E	sediment	T.c.c.
			Kw13	fish pond 13	4	51°19' N, 14°21' E	preserved live	T.c.c.
			Kw21	fish pond 21	15	51°19' N, 14°21' E	sediment	T.c.c.
			Kw24	fish pond 24	2	51°19' N, 14°21' E	preserved live	T.c.c.
			Kw25	fish pond 25	6	51°19' N, 14°21' E	preserved live	T.c.c.
			Kw27	fish pond 27	15	51°19' N, 14°21' E	preserved live	T.c.c.
			Kw28	fish pond 28	50	51°19' N, 14°21' E	preserved live	T.c.c.
	LA	fish pond area Lacoma, Brandenburg	La1	fish pond 1	15	51°47' N, 14°23' E	sediment	T.c.c.
			La2	fish pond 2	15	51°47' N, 14°23' E	sediment	T.c.c.
			La3	fish pond 3	6	51°47' N, 14°23' E	sediment	T.c.c.
	R	Rhine, Rhineland-Palatinate	Da	Daxlander Au	3	48°59' N, 08°17' E	sediment	T.c.c.
			Ib	Ibersheim	2	49°43' N, 08°25' E	sediment	T.c.c.
			Ne	Neuburg	5	48°59' N, 08°16' E	sediment	T.c.c.
			Ha	Hagenbach	1	49°00' N, 08°16' E	sediment	T.c.c.
	TA	Tannen, Mecklenburg-Western Pomerania	-	-	6	53°34' N, 11°26' E	preserved live	T.c.c.
Great Britain	NF	Godshill pond, New Forest	-	-	18	50°55' N, 01°45' W	sediment	T.c.c.
Spain	PE	Plat d'Espolla, Banyoles karstic area	-	-	16	42°10' N, 02°46' E	preserved live	T.c.s.*
	EP	El Puig, Valencia	-	-	4	38°05' N, 12°42' E	preserved live	T.c.s.*
	UB	Ullal de Baldovi, Albufera de valencia Natural Park	-	-	5	no data	preserved live	T.c.s.*
	X	Extremadura, Laguna de la Gitanilla	-	-	2	39°27' N, 06°15' W	preserved live	T.c.m.*

* according to Alonso (1996) and Boix et al. (2002)

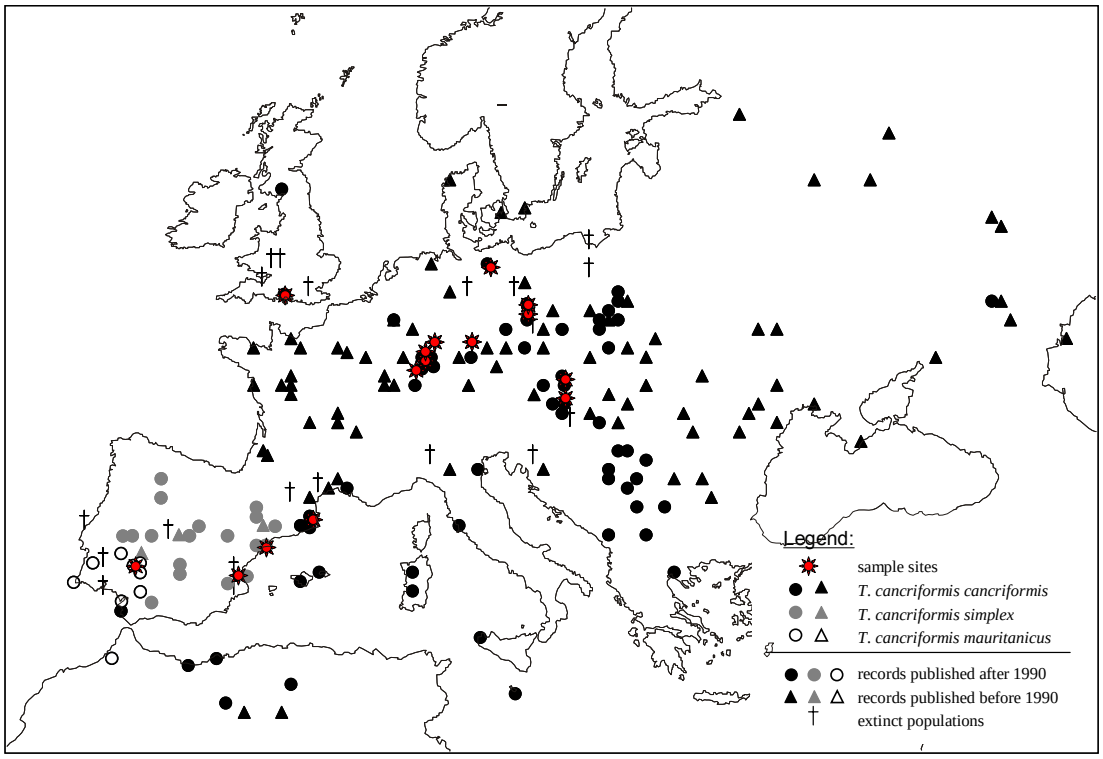

Figure 3-1: Map of European *Triops cancriformis* distribution including subspecies occurrence and the surveyed localities corresponding to Table 3-1; distribution data based on Brtek & Thiery (1995), Alonso (1996), Morell (2005) and own observations during 2000 and 2004.

3.2.2 Rearing conditions

Most of the samples were obtained from laboratory rearing experiments with sediment containing *T. cancriformis* cysts. Subsamples of sediment (50-100 mg) from sampled populations were hydrated in separate 5 l aquaria using 3 l distilled water. The aquaria were housed at room temperature (20°C) and ambient day-night rhythm. The hatched specimens were fed with dry-fish food (VITA®) and allowed to grow at least until sexual maturity. The specimens were preserved in ethanol for further investigations. According to Longhurst (1955) no effect on the morphology had to be expected under diverse conditions of temperature and food. Thus small variations in food supply and water temperature are negligible. To exclude influences on morphology due to vessel size, only 5 litre tanks were applied.

3.2.3 Morphological character

3.2.3.1 Quantitative characters

The preserved individuals were scored for the following characteristics: total number of cysts in the pouches (only for females), head shield length (measured from the anterior margin to the mid-dorsal terminus), eye extension, furca length (posterior margin of the telson to the tip of the furca-vertical), total body length (anterior margin to the tip of the furca-vertical), number of abdominal terminal body rings, number of terminal body rings lacking appendages (legless segments) and number of carina spines (in the central row of the head shield) (Figure 3-2). The quantitative character 'number of carina spine' was transferred into a qualitative classification (compare Figure 3-3). The ratio (rSE) between the two parameter head shield length and eye extension was calculated to achieve a standardized length parameter. Many of the named characters above have been investigated within some detail by Linder (1952a) and Longhurst (1955), and were therefore selected in this study. Due to the fact that after the 9th instar of *T. cancriformis* the number of segments and the number which are apodous may be taken as fixed in an individual, these could validly be used as characters in animals of more than 3 – 4 mm in median carapace length (Longhurst, 1955).

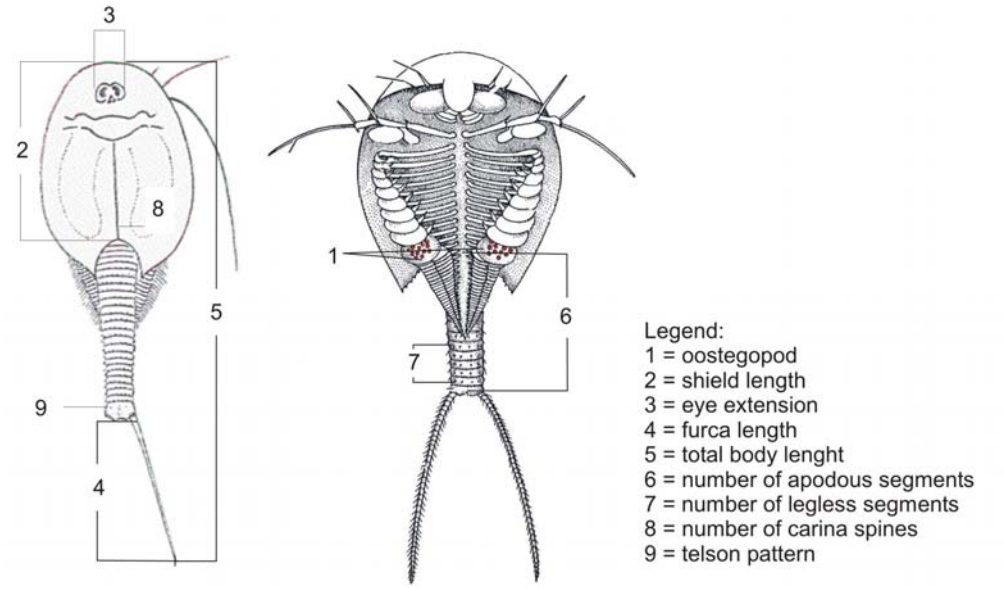

Figure 3-2: Morphological parameters included in the surveyed populations of *Triops cancriformis* (drawings modified after Longhurst 1955).

Length parameters were measured with millimetre paper. Counting was performed using a binocular stereomicroscope unit including digital documentation facilities (Plan Apo 1x WD 70, NIKON).

The morphological parameter furca length and total body length have not been included in comparative analysis because of preservation artefacts (contraction of soft body through ethanol preservation) and damaged or missing furca.

3.2.3.2 Qualitative characters

Since few data are available reporting on the presence of males of *T. cancriformis* populations all surveyed individuals were checked for their sex.

As described by Longhurst (1955) and Alonso (1996) the carina and telson spine pattern vary mainly between subspecies. Therefore qualitative characters were introduced to differentiate the observed pattern for both carina and telson. Based on the counted carina spines three carina patterns could be distinguished: smooth, rough and toothed (Figure 3-3).

carina type: smooth (no spines)	carina type: rough (between 1 and10 spines)	carina type: toothed (spines over the complete carina)

Figure 3-3: Definition of three carina types; pictures Zierold, 2004.

The observed telson patterns were distinguished into four categories: ‘one central spine and some small spines behind’, ‘one central spine’, ‘one central and a number of small spines behind’ and ‘one central spine with an obvious spine behind’ (Figure 3-4).

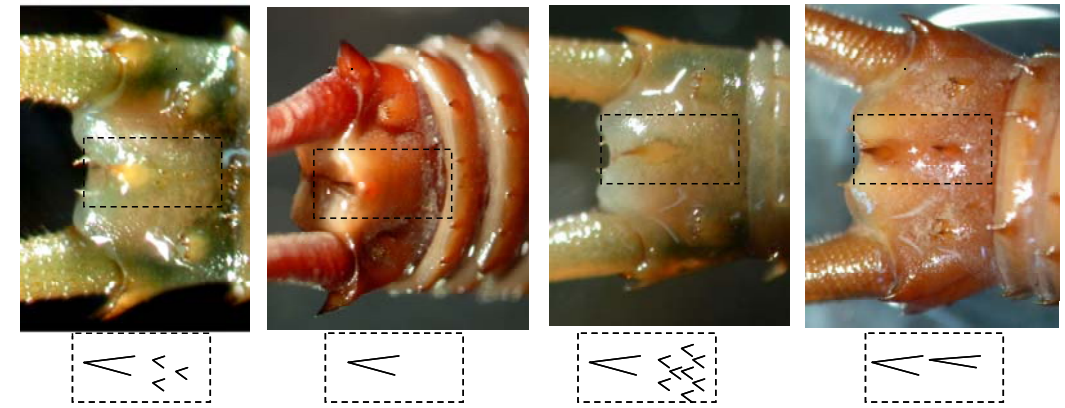

Figure 3-4: Definition of four telson types based on the sketch derived from the figures (Zierold, 2005)

3.2.4 Reproductive strategy

Sex ratio (male:female) was determined for all *T. cancriformis* populations sampled in this study. The geographic distribution of sex ratios was analysed using a combination of data from this study and published data for 22 European populations. In the analysis only literature data that provided either sample sizes and the sex ratio, or the raw numbers of males and females per sample were included. Populations were assigned to one of four qualitative patterns of sex ratio variation according to Sassaman (1991): type I (male biased), type II (equality), type III (female biased) and type IV (unisexual).

3.2.5 Statistical and distribution analysis

General characteristics of the dataset were described by summary statistics including mean, median, minimum, maximum, range, standard deviation and variance calculated in STATGRAPHICS plus 5.0 (Statistical Graphics Corp. 1994-2004). Homogeneity of variance in a set of samples (homoscedasticity) was tested by calculation of *p*-values for Cochran's C, Bartlett's test, Levene's test as well as the critical value for Hartley's test. In the case of heteroscedasticity (inequality of variances among samples) the Kruskal-Wallis test was used to calculate the

significance of sample differentiation. If *p*-values were less than 0.05, significant differences within the analysed dataset occur. Multiple comparisons were used to determine exactly which groups (on regional and local scale) are different. To determine the probability of the group differences Dunn's Test from SIGMAPLOT (Jandel Corporation, 1992-1995) was performed. Dunn's test lists the difference of rank means, computes the Q test statistics, and displays whether or not $P < 0.05$, for each group pair.

Significantly different characters among the surveyed populations (regions) were used to create a data matrix (indicating the presence or absence of the character) for Cladistic analysis performed with TNT version 1.0 (Goloboff et al., 2003). Default settings were applied for parsimony tree calculations (random seed 2756, bootstrapping = 1000), and strict consensus tree was performed using 50% cut-off criteria.

Binomial (chi-square) tests were used to test whether the sex ratio of each population was significantly different from an equal sex ratio. If no significant differences were found, the population was considered of type II. If it was significantly large, it was considered type I, and if significantly small, it was regarded as type III. Following Sassaman (1991) the type IV (unisexuality) was only assumed, if more than 50 individuals were sexed and all were found to be females.

3.3 Results

A total of 223 *T. cancriformis* individuals from one region in Austria, 4 different regions in Germany, one region in Great Britain and four different regions in Spain were investigated (Table 3-1). The dataset was composed of 215 females and 8 males.

3.3.1 Morphological variation within populations

For both paragraphs on quantitative and qualitative characters, the observed variation will first be described for those regional populations that were further divided into local populations. After that variation between the remaining regional populations will be considered.

3.3.1.1 Quantitative characters

The determined quantitative characters for local and regional populations are summarized in Table 3-2. The eight local populations from the region Königswartha resulted in 103 adult and 15 juvenile specimens in total. Among the adult specimens the shortest head shield was 10 mm and the longest 27 mm. The pairwise comparison of head shield length resulted in significant differences for four combinations of local populations (compare Table 3-3, signs below diagonal). Three significant differences were observed comparing the ratio between head shield length and eye extension (rSE) among local populations (compare Table 3-3, signs over diagonal).

Table 3-2: Variation of quantitative morphological features including head shield length, ratio of head shield length and eye extension, number of abdominal segments, number of legless segments and number of carina spines given for regional (KW = Königswartha, LA = Lacoma, R = Rhine, LN = Neusiedler Lake, TA = Tannen, NF = New Forest, EP = El Puig, PE = Plat d' Espolla, UB = Ullal de Baldovi, X = Extremadura) and local populations (different pond numbers for KW and LA, Da = Daxlander Au, Hg = Hagenbach, I = Ibersheim, Ne = Neuburg); n = number of surveyed individuals, mean = calculated average, S.D. = standard deviation, CV% = coefficient of variation given in percent and range = observed minimum and maximum.

Population		**Length of head shield (only adults)**					**head shield length-eye extension ratio**					**number of abdominal segments**					**number of legless segments**					**number of spines**				
regional	local	n	mean	S.D.	CV %	range	n	mean	S.D.	CV%	range	n	mean	S.D.	CV%	range	n	mean	S.D.	CV%	range	n	mean	S.D.	CV%	range
KW		103	17.37	3.39	19.52	10 to 27	77	6.87	1.29	18.78	4.66 to 12.22	77	22.39	0.88	3.93	20 to 24	78	4.97	0.39	7.85	4 to 6	77	0.51	0.87	170.59	0 to 3
	Kw11	3	18.00	1.70	9.44	16 to 19	3	7.31	0.87	11.90	6.33 to 8.00	3	22.33	1.53	6.85	21 to 24	3	5.33	0.58	10.88	5 to 6	3	1.00	1.00	100.00	0 to 2
	Kw12	19	17.63	4.06	23.03	11 to 23	18	6.72	0.92	13.69	4.80 to 8.64	18	21.78	0.81	3.72	20 to 23	19	4.89	0.57	11.66	4 to 6	18	0.11	0.47	427.27	0 to 2
	Kw13	4	20.50	2.08	83.20	18 to 23	4	7.32	1.17	15.98	6.00 to 8.40	4	22.25	0.50	2.25	22 to 23	4	5.00	0.00	0.00	5 only	4	0.00	0.00	-	0 only
	Kw21	19	14.31	1.57	10.97	11 to 17	19	6.43	0.61	9.49	5.36 to 7.50	19	22.37	1.01	4.51	21 to 24	19	4.84	0.37	7.64	4 to 5	19	0.37	0.83	224.32	0 to 3
	Kw24	2	15.50	4.95	31.94	12 to 19	2	6.17	0.23	3.73	6.00 to 6.33	2	23.00	0.00	0.00	23 to 23	2	5.00	0.00	0.00	5 only	2	0.00	0.00	-	1 only
	Kw25	6	12.33	2.50	20.28	10 to 15	6	5.92	1.92	32.43	4.66 to 7.50	4	22.75	0.50	2.20	22 to 23	4	5.00	0.00	0.00	5 only	4	0.00	0.00	-	0 only
	Kw27	15	18.86	2.64	14.00	13 to 22	14	7.33	0.90	12.28	6.00 to 9.50	14	22.64	0.63	2.78	22 to 24	14	5.00	0.00	0.00	5 only	14	0.71	0.91	128.17	0 to 2
	Kw28	50	18.24	3.00	16.45	13 to 27	13	7.81	1.67	21.38	6.00 to 12.22	13	22.85	0.69	3.02	21 to 24	13	5.15	0.38	7.38	5 to 6	13	1.15	1.14	99.13	0 to 3
LA		15	14.60	2.26	15.48	10 to 19	20	5.87	1.05	17.89	4.00 to 8.82	17	21.8	0.56	2.57	21 to 23	18	5.28	0.46	8.71	5 to 6	18	0.00	0.00	-	0 only
	La1	4	14.75	3.30	22.37	11 to 19	7	6.23	1.44	23.11	5.00 to 8.82	4	21.50	1.00	4.65	21 to 23	5	5.60	0.55	9.82	5 to 6	5	0.00	0.00	-	0 only
	La2	9	14.89	1.36	9.13	13 to 17	9	5.77	0.66	11.44	5.00 to 7.00	9	21.89	0.33	1.51	21 to 22	9	5.22	0.44	8.43	5 to 6	9	0.00	0.00	-	0 only
	La3	2	13.00	4.26	32.77	10 to 16	4	5.49	1.05	19.13	4.00 to 6.40	4	21.75	0.50	2.30	21 to 22	4	5.00	0.00	0.00	5 only	4	0.00	0.00	-	0 only
R		9	16.11	3.82	23.71	11 to 23	11	6.99	1.01	14.45	5.65 to 8.89	11	22.00	0.45	2.05	21 to 22	11	5.00	0.00	0.00	5 only	11	0.45	0.93	206.67	0 to 3
	Da	2	18.00	2.83	15.72	16 to 20	3	7.50	0.87	11.60	6.50 to 8.0	3	22.00	0.00	0.00	22 only	3	5.00	0.00	0.00	5 only	3	0.33	0.58	175.76	0 to 1
	Hg	1	17.00	-	-	17 only	1	6.80	-	-	6.80	1	23.00	0.00	0.00	23 only	1	5.00	0.00	0.00	5 only	1	1.00	0.00	0.00	1 only
	I	2	14.50	3.57	24.62	12 to 17	2	6.40	0.57	8.91	6.00 to 6.80	2	21.50	0.71	3.30	21 to 22	2	5.00	0.00	0.00	5 only	2	0.00	0.00	-	0 oly
	Ne	4	15.75	5.25	33.33	11 to 23	5	6.90	1.31	18.99	5.65 to 8.89	5	22.00	0.00	0.00	22 only	5	5.00	0.00	0.00	5 only	5	0.60	1.34	223.33	0 to 3
LN		5	15.33	3.11	20.29	13 to 20	11	6.23	0.67	10.75	5.56 to 8.00	11	21.91	0.30	1.37	21 to 22	11	5.09	0.30	5.89	5 to 6	11	0.00	0.00	-	0 only
TA		0	-	-	-	-	0	-	-	-	-	0	-	-	-	-	6	5.00	0.00	0.00	5 only	6	0.00	0.00	-	0 only
NF		4	17.00	2.45	14.41	14 to 20	18	6.01	0.54	8.99	5.00 to 7.14	18	21.83	0.38	1.74	21 to 22	18	5.06	0.24	4.75	5 to 6	18	1.28	1.41	110.16	0 to 5
EP		4	32.50	2.08	6.41	30 to 35	4	7.92	0.89	11.24	6.67 to 8.75	4	21.75	0.50	2.30	21 to 22	4	5.00	0.00	0.00	5 only	4	2.50	0.58	23.20	2 to 3
PE		4	12.25	1.26	10.29	11 to 14	16	6.23	0.65	10.43	5.00 to 7.33	16	21.94	0.77	3.51	21 to 23	15	7.07	0.26	3.68	7 to 8	16	2.33	1.81	77.68	0 to 7
UB		5	16.80	2.05	12.20	15 to 20	5	5.95	0.49	8.24	5.36 to 6.67	5	20.60	1.95	9.47	18 to 22	5	5.00	0.00	0.00	5 only	5	2.00	0.71	35.50	1 to 3
X		0	-	-		-	1	6.00	0.00	0.00	-	0	-	-	-	-	1	5.00	0.00	0.00	-	2	<70	-	-	<70

Table 3-3: Matrix with 5% significant differences in head shield length (star below diagonal) and ratio of head shield length to eye-extension (circle over diagonal) among eight local populations from Königswartha

	Kw11	Kw12	Kw13	Kw21	Kw24	Kw25	Kw27	Kw28
Kw11	-							
Kw12		-						
Kw13			-			°		
Kw21			*	-				
Kw24					-			
Kw25			*			-	°	°
Kw27				*		*	-	
Kw28								-

Within the local populations from the region Lacoma 15 adult and 5 juvenile specimens were investigated. However no significant differences were found among the local populations. Therefore the (regional) population Lacoma can be characterized as homogeneous. No specimens from Lacoma did bear any spines along the carina. The Rhine population included only 9 adult and 2 juvenile specimens. Due to the very small sample sizes no comparison at the local scale was performed. The average shield length was 16.11±3.82 mm (n adult = 9, ranging from 11 to 23 mm) long. The Rhine population was characterized by a constant number of legless segments (compare Table 3-2).

This paragraph highlights morphological characters for those regional populations which were not subdivided into local populations. Samples from Neusiedler Lake included 5 adult and 6 juvenile individuals. Within this population no carina spines could be observed. No carina spines were also observed for the six adult samples from Tannen. The New Forest population with 4 adult and 14 juvenile individuals was heterogeneous for all characters. The El Puig population, made up of only four adults, was characterized by remarkably large head shields in comparison to all other populations (ranging from 30 to 35 mm). Population Plat d' Espolla included four adult and 12 juvenile individuals. This population could be characterized by up to eight legless segments which were so far not observed in other populations. Other characters for this population were heterogeneous. Counting of legless segments for five adult specimens from Ullal de Baldovi resulted in a constant value. Characteristic for this population was also the small number of abdominal segments which were observed in two individuals. The two individuals from Extremadura were marked by high number of carina spines.

3.3.1.2 Qualitative characters

In Table 3-4 determined qualitative characters are summarized for regional and local populations. Heterogeneous carina patterns including smooth and rough type were observed within local Königswartha populations. The telson pattern was heterogeneous for one half of the local Königswartha populations. For the other half one central spine was observed exclusively. The statistical tests for both carina and telson type showed a significant *p*-value (Kruskal-Wallis test for carina type p = 0.004; Chi^2 test for telson type $Chi^2 = 34.32$, df = 14, p = 0.002), indicating that the observed patterns for a particular case are related to distinct local populations (Figure 3-5).

Table 3-4: Variation of qualitative morphological features including carina and telson pattern for regional (KW = Königswartha, LA = Lacoma, R = Rhine, LN = Neusiedler Lake, TA = Tannen, NF = New Forest, EP = El Puig, PE = Plat d' Espolla, UB = Ullal de Baldovi, X = Extremadura) and local populations (different pond numbers for KW and LA, Da = Daxlander Au, Hg = Hagenbach, I = Ibersheim, Ne = Neuburg)

Population		Carina spines				Telson type				
regional	local	n	toothed	rough	smooth	n	1	2	3	4
KW		77	0	23	54	77	11	54	0	12
	Kw11	3	0	2	1	3	0	2	0	1
	Kw12	18	0	1	17	18	3	11	0	4
	Kw13	4	0	0	4	4	0	4	0	0
	Kw21	19	0	4	15	19	7	5	0	7
	Kw24	2	0	2	0	2	0	2	0	0
	Kw25	4	0	0	4	4	1	3	0	0
	Kw27	14	0	6	8	14	0	14	0	0
	Kw28	13	0	8	5	13	0	13	0	0
LA		18	0	0	18	18	1	4	0	13
	La1	5	0	0	5	5	0	3	0	2
	La2	9	0	0	9	9	1	0	0	8
	La3	4	0	0	4	4	0	1	0	3
R		11	0	3	8	11	3	3	0	5
	Da	3	0	1	2	3	0	1	0	2
	Hg	1	0	1	0	1	0	0	0	1
	I	2	0	0	2	2	1	0	0	1
	Ne	5	0	1	4	5	2	2	0	1
LN		11	0	0	11	11	3	4	0	4
TA		6	0	0	6	n.d.	n.d.	n.d.	n.d.	n.d.
NF		18	0	11	7	9	2	0	7	0
EP		4	0	4	0	4	1	0	3	0
PE		16	0	4	12	16	0	9	0	7
UB		5	0	5	0	5	0	1	2	2
X		2	2	0	0	1	0	1	0	0

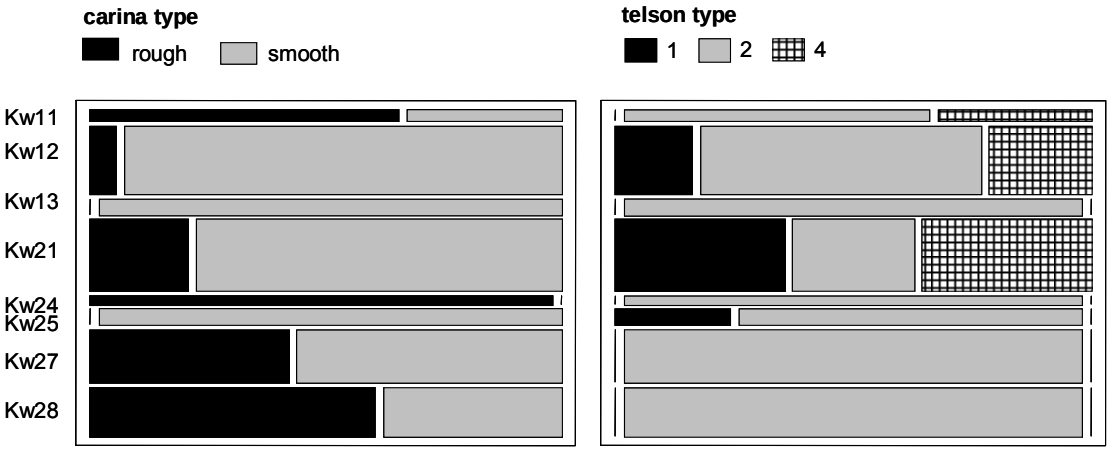

Figure 3-5: Morphological variation among eight local Königswartha populations considering number of carina spines (left, rough = 1-10 carina spines, smooth = no carina spines) and telson type (right, type 1 = one central spine and some small median spines, type 2 = one central spine, type 4= one central spine and an obvious median spine behind). The width of the bars represents the sample size included in the analysis.

All specimens of the local populations from Lacoma were homogeneous for carina type but heterogeneous for telson type (Table 3-4). However telson variation was not significant among local population (Chi^2 = 7.29, df = 4, p = 0.121). As like as for quantitative characters Lacoma can be characterized as a homogeneous group.

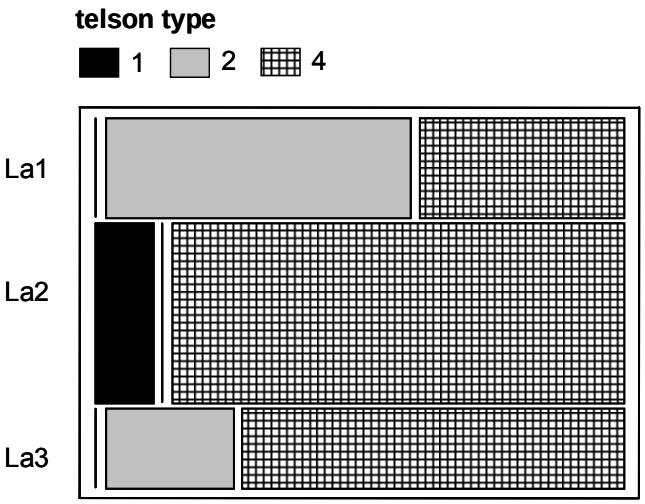

Figure 3-6: Morphological variation among three local Lacoma populations considering telson type (right, type 1 = one central spine and some small median spines, type 2 = one central spine, type 4= one central spine and an obvious median spine behind). The width of the bars represents the sample size included in the analysis.

The Rhine population showed a mixed carina and telson pattern (Table 3-4) (due to small sample size statistical test were not performed).

Only smooth carina type was observed within samples from Neusiedler Lake and Tannen. Rough and smooth carina could be detected in New Forest and Plat d'Espolla population. All of the El Puig and Ullal de Baldovi specimens showed a

rough carina. And the Extremadura sample was the single one with toothed carina type.

3.3.2 Morphological variation among regional populations

3.3.2.1 Quantitative characters

El Puig specimens showed highly significant (Kruskal-Wallis test p = 0.0005) differences by their enormous head shield length from all other populations. Considering the ratio between head shield length and eye-extension (rSE) El Puig differ significantly only from Lacoma (Kruskal-Wallis test p = 0.016). Regarding the number of abdominal segments none of the pairwise comparisons resulted in significant differences, however less than 20 segments were observed exclusively within the population Ullal de Baldovi. Among the surveyed populations the number of legless segments differed significantly (Kruskal-Wallis test *p*-value < 0.0001) (Figure 3-7). Considering the segments lacking appendages significant differences were found among regional populations (Kruskal-Wallis test p < 0.0001). Based on this character two clusters could be identified: one comprising populations with an average of 5.03±0.03 segments lacking appendages (n = 146) and a second collapsing the Spanish populations Plat d'Espolla and Extremadura with an average of 7.06±0.09 segments lacking appendages (n = 16).

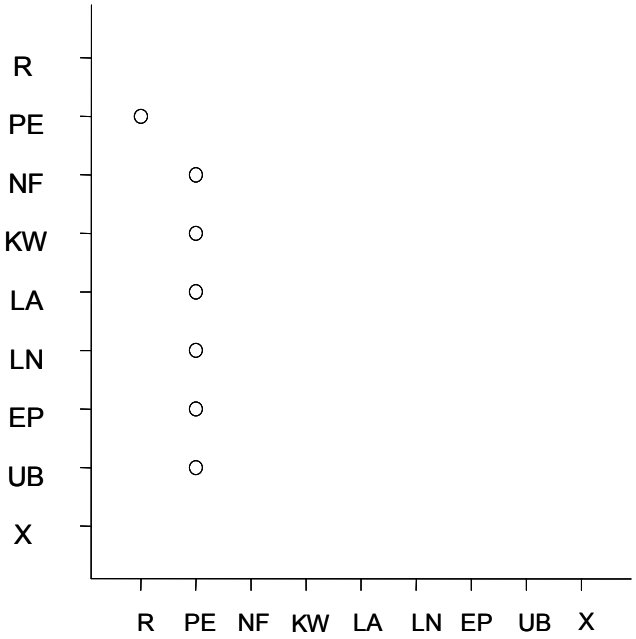

Figure 3-7: Multiple comparison graph indicating significant different populations (R = Rhine, PE = Plat d'Espolla, NF = New Forest, KW = Königswartha, LA = Lacoma, LN = Neusiedler Lake, EP = El Puig, UB = Ullal de Baldovi, X = Extremadura) considering the number of legless segments.

3.3.2.2 Qualitative characters

Considering the carina type significant differences where found among regional populations, indicated by *p*-value less than 0.0001 (Kruskal-Wallis test). In consequence four clusters emerged: (1) smooth carina exclusively including Lacoma and Neusiedler Lake, (2) heterogeneous carina pattern including smooth and rough type (Königswartha, New Forest, Plat d'Espolla and Rhine), (3) rough carina type exclusively represented by El Puig and Ullal de Baldovi and (4) finally the toothed carina type within the Extremadura individual (Table 3-4). Within the total sample 4 different telson types were observed. However, the surveyed populations (regional level) did not differ significantly from one another.

3.3.2.3 Morphotype distribution and relationships

The morphological differences among the regional populations are illustrated in Figure 3-8. Extremadura population, representing the *T. c. mauritanicus* subspecies, is a single geographic restricted morphotype which can be characterized by a toothed carina. The two southern populations at the East Iberian coast surprisingly show rough carina only. And Neusiedler Lake and Lacoma are characterized by smooth carina only. Between the two subspecies *T. c. cancriformis* and *T. c. simplex* no clear separation including morphological features as well as geographic distribution could be identified.

Based on the morphological features number of carina spines, number of legless segments, telson pattern and head shield length-eye distance ratio a parsimony dendrogram calculated (Figure 3-9). As shown in the consensus tree (Figure 3-9 B) specimens from Extremadura (X) differ significantly from all other populations. Bootstrap support was less than 50% for the remaining branches thus in the consensus tree *T. c. cancriformis* and *T. c. simplex* specimens collapse together in one single group. However the Ullal de Baldovi (UB) population differs from remaining populations by the morphological combination of five legless segments, a rough carina and the absence of telson type 1 in all of the investigated individuals (Figure 3-9 A). The other two Spanish populations collapse together with Central European populations (Figure 3-9 A). As illustrated in the parsimony tree the populations Lacoma and Neusiedler Lake are most different from all other populations within the *T. c. cancriformis/simplex* lineage (compare Figure 3-8).

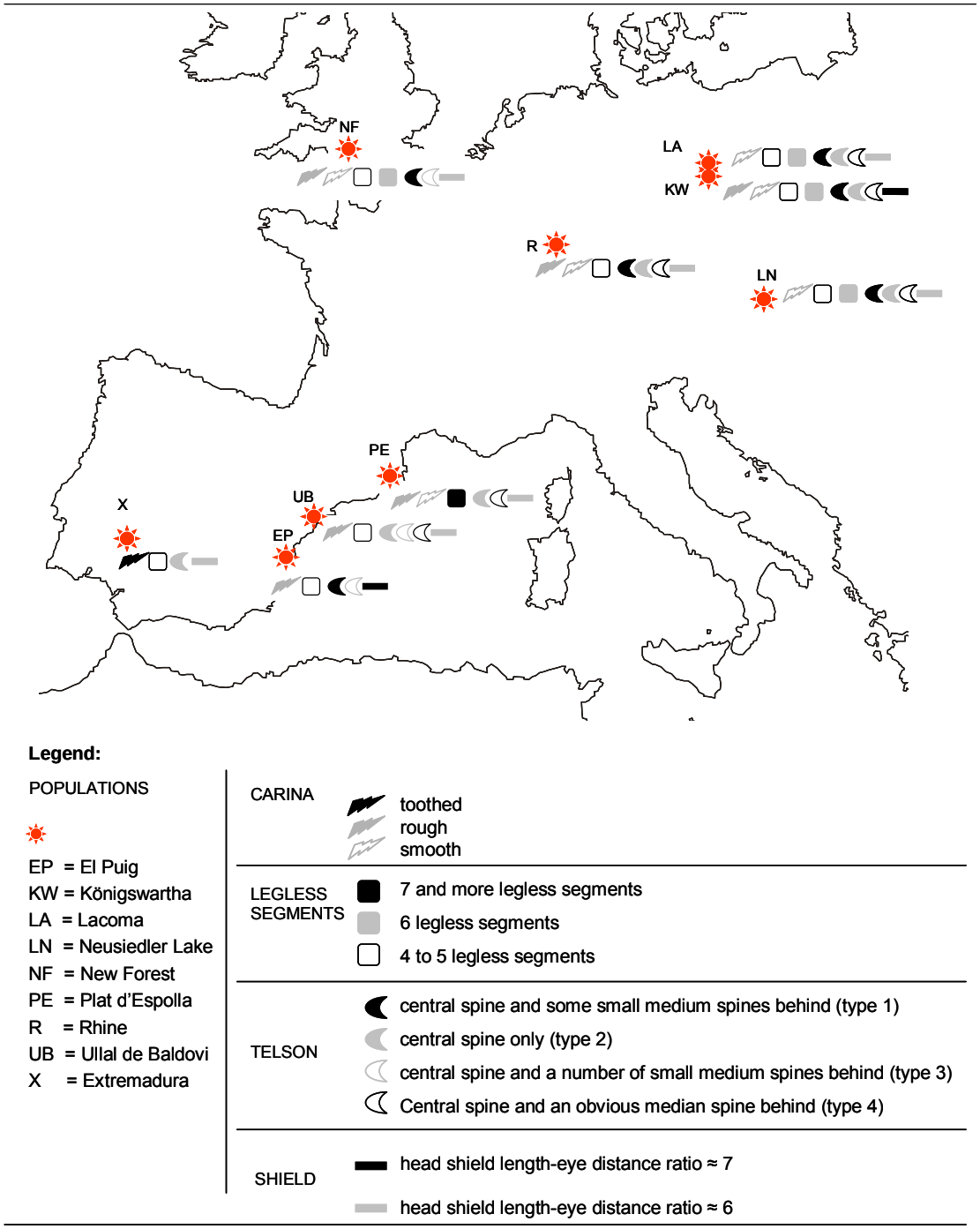

Figure 3-8: Distribution of *Triops cancriformis* morphotypes including the morphological variation of carina patterns, number of legless segments, telson pattern and head shield length-eye distance ratio for the studied populations.

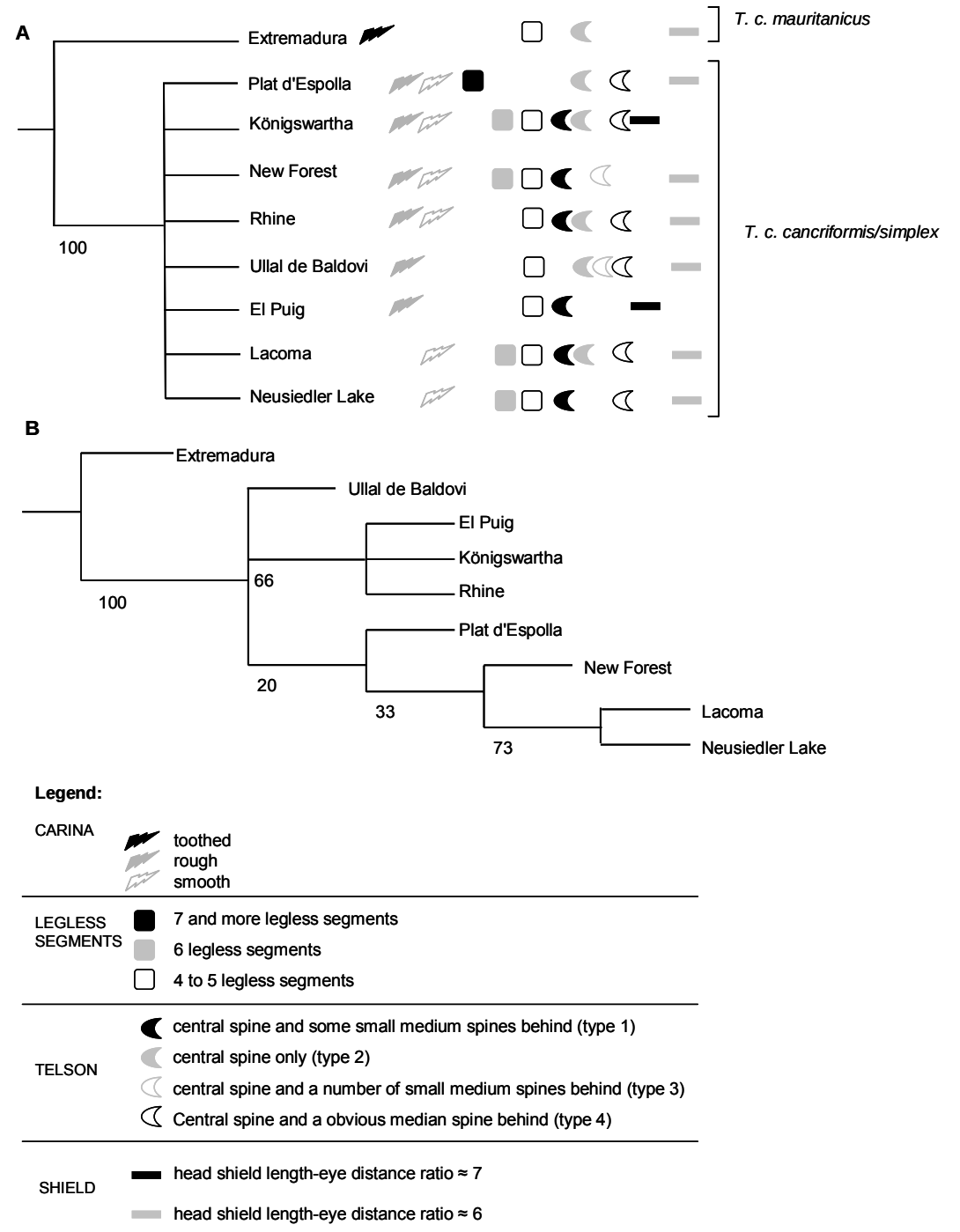

Figure 3-9: Morphological relationship of *Triops cancriformis* based on Parsimony analysis of 13 character codes performed with TNT version 1.0, (A) parsimony tree showing bootstrap support, (B) consensus tree based on 100 trees and using 50% cut-off criteria.

3.3.3 Reproductive strategy

The reanalysis of sex ratios in *T. cancriformis* based on own and literature data indicated that equal sex ratio indicating bisexual reproduction was only found in Iberian populations, described as *T. c. mauritanicus* and *T. c. simplex* (Table 3-5).

Female biased *T. cancriformis* populations occur throughout central and northern Europe (Germany, Austria, Croatia, France, Hungary, Great Britain and Poland). For several locations (Austria, Great Britain, France, Poland and Japan) males have never been reported. Additionally no males have been reported from Italian populations (Zaffagnini & Trentini, 1980; Cesari et al., 2004). However, no absolute numbers of investigated individuals are published and thus no test could be performed to infer the sex ratio type for the Italian populations. In all cases, small (<50) or unreported sample sizes did not provide unequivocal evidence that these populations are actually unisexual, as opposed to having strongly female biased sex ratios.

Table 3-5: Reproductive strategy in *Triops cancriformis* populations from different locations throughout Europe. Numbers (n) of individual males and females and the male proportion per pond is given. The inferred type of sex ratio according to Sassaman (1991) is provided: I = male biased, II = equality and III = female biased, IV = unisexuality

location information	n_{male}	n_{female}	prop. of males	p value (Chi-Square)	sex ratio	ref.
Austria, Neusiedler Lake, Kaiserlacke	0	11	0.0000	0.0009	III	p.d.
Croatia	9	34	0.2093	0.0001	III	1
France, Camargue	0	9	0.0000	0.0027	III	2
France, Baillargues (Montpellier)	0	200	0.000	<0.0001	IV	3
Germany, Bavaria	8	1000	0.0079	<0.0001	III	4
Germany, Bavaria, Augsburg	7	568	0.0122	<0.0001	III	5
Germany, Königswartha, fish pond area	3	119	0.0246	<0.0001	III	p.d.
Germany, Lacoma, fish pond area	2	28	0.0667	<0.0001	III	p.d.
Great Britain, New Forest, Godshill pond	0	19	0.0000	<0.0001	III	p.d.
Hungary, Lake Balaton	7	19	0.2692	0.0186	III	6
Hungary, Lake Balaton	15	45	0.2500	0.0001	III	6
Japan	0	20	0.0000	<0.0001	III	2
Poland, Wroclaw (April 1867)	29	88	0.2479	<0.0001	III	7
Poland, Wroclaw (April/June 1865)	114	912	0.1111	<0.0001	III	7
Poland, Wroclaw (July 1864)	2	9	0.1818	0.0348	III	1
Poland, Wroclaw (July 1879)	5	19	0.2083	0.0043	III	1
Poland, Cracow (1857)	16	144	0.1000	<0.0001	III	8
Poland, Cracow (1858)	154	395	0.2805	<0.0001	III	8
Poland, Debina	0	10	0.0000	0.0016	III	9
Poland, Jaktorów	0	40	0.0000	<0.0001	III	9
Poland, Jarosy	0	10	0.0000	0.0016	III	9
Poland, Klomnice	0	15	0.0000	0.0001	III	9
Poland, Kludno	0	20	0.0000	<0.0001	III	9
Poland, Zabieniec	0	40	0.0000	<0.0001	III	9
Portugal, Algarve	54	22	0.7105	0.0002	**I**	10
Portugal, Algarve	19	23	0.4524	0.5371	**II**	10
Spain, Plat d'Espolla	1723	1775	0.4927	0.3872	**II**	11

* [1] Braem (1893), [2] Akita (1976), [3] Mathias (1937) [4] Heidecke & Neumann (1987), [5] Gaschott (1928), [6] Abonyi (1926), [7] von Siebold (1871), [8] Kozubowsky (1857), [9] Hempel-Zawitkowska & Klekowski (1968), [10] Machado et al. (1999b), [11] Boix (2002), p.d. = personal data from this study

3.4 Discussion

3.4.1 Systematics of *Triops cancriformis*

The traditionally accepted subdivision of *Triops cancriformis* into three subspecies (geographical races) *sensu* Longhurst (1955) is not supported by the data of this study. *T. c. mauritanicus* forms a monophyletic group. More specimens of this subspecies, covering the whole distribution range (Brtek & Thiery, 1995), have to be investigated to decide whether the type '*mauritanicus*' is a species of its own as it has been described originally by Ghigi (1921). Despite a few differences the specimens from Eastern Iberian Peninsula (El Puig, Ullal de Baldovi and Plat d'Espolla) identified as *T. c. simplex* could not (significantly) be separated from the nominal subspecies *T. c. cancriformis*. The subspecies specific character including carina pattern and telson pattern were not unambiguous enough to differentiate between the subspecies as described by Gauthier (1934), Longhurst (1955) and Alonso (1996). In detail, individuals with typical characters for *T. c. simplex* (smooth carina and a telson bearing a central spine and a single obvious spine behind) were also identified in the Austrian, Britain and German populations. One explanation for this variation is that, assuming a smooth carina pattern is decisive for *T. c. simplex* identification all these Central European groups represent this subspecies. This leads to the assumption that the distribution range expanded from North and Middle Spain as described by Longhurst (1955) to Central Europe. Otherwise the subspecies classification is not longer valid. Accepting this hypothesis one could conclude that *T. cancriformis* is a heterogeneous species considering morphology pattern and reproductive strategies. To prove this, future research has to focus on cross breeding/fertility tests and genetic analyses based on high resolution markers.

3.4.2 Morphological diversity

Among the investigated qualitative characters the highest variance was observed in the number of carina spines. Similar results have been reported by Petrov & Cvetkovic (1999). The authors found a *T. c. mauritanicus* like specimen with up to 34 spines along the last third of the carina within their studied populations from Yugoslavia. The 'head shield length-eye distance ratio' was hardly variable

(coefficient of variance, CV, ranging from 0.00 to 23.15%) compared to other quantitative characters. The present study shows that the growth of the head shield in relation to the eye distance is isometric. Such a relationship has been observed between head shield length and total body length. However, the total body length was not included in the statistical analysis as this character is influenced by ethanol preservation. The head shield size of adult notostracan individuals varied between 10 mm and 35 mm, a fact that had already been noted by other authors (Longhurst, 1955; Machado et al., 1999b). Less variance was further observed of the number of abdominal segments (CV ranging from 0.00 to 9.47%) and the number of legless segments (CV ranging from 0.00 to 11.66%). These results are congruent with those reported by Linder (1952b), Longhurst (1955) and Hempel-Zawitkowska (1968) where the number of abdominal segments ranged from 20 to 24 and the segments lacking appendages ranged from 5 to 7. The authors mentioned that the smaller number is often related to unisexual populations. In fact Linder (1952b) found within a Swedish pure female population of *T. cancriformis* 32 specimens with 5 legless segments and only a single female with 6 legless segments. The qualitative character 'telson pattern' which has been reported as a perfect systematic feature (Longhurst, 1955) showed some variance, but no evidence for subspecies systematics was found. In general, there is less morphological variability within *T. cancriformis* which corresponds well to the results reported for species of nearly morphological stasis (Suno-Uchi et al., 1997).

Morphological data showed that the *T. (c.) mauritanicus* samples from south-western Spain represented a separate group compared to the other *T. cancriformis* populations. This corresponds to results of the genetic analysis of mitochondrial DNA which are summarized in chapter 4. It may be possible that those groups of comparable morphology also share common evolutionary history. In this sense one hypothesis explaining the close relationship between the fish pond population Lacoma and Neusiedler Lake is simply, that Lacoma was founded by specimens (cysts) from Austria. This may have happened during carp fish transports (personal communication Langner 2003). Genetic data of mitochondrial analysis (16S rDNA) also support the close relationship between Lacoma and Neusiedler Lake as both populations share the same genotype (see chapter 4).

The Königswartha population, as another large branchiopod occurrence in a pisci-culture area, shows in addition to morphological characters observed in Lacoma a rough carina pattern. This was observed also in natural occurrences from Rhine, New Forest or East Iberian populations. Therefore, it could be suggested that the Königswartha population was either influenced by different 'founder' events or geographic isolation and the occurrence of males was responsible for heterogeneous features. The different morphological features observed within Königswartha are congruent with the identification of three distinct cytochrome *c* oxidase subunit I haplotypes (see chapter 4). Overall each surveyed location is characterized by a special combination of morphological features. Before one could conclude distinct morphotypes further populations have to be investigated focusing mainly on the two features carina pattern and number of legless segments.

3.4.3 Reproductive strategy

The recorded sex ratios indicate bisexual reproduction for Iberian Peninsula whereas female biased population with male proportions up to 0.3 occur throughout Central Europe. No males have been reported from Italian populations. Furthermore histological examinations of females resulted in functional ovary without testes lobes (Scanabissi Sabelli *et al.*, 2005). However, more samples are necessary to prove the hypothesis that all Italian populations are parthenogenetic. This illustrates that *T. cancriformis* is a species with complex reproductive strategy showing variation in sex ratio with the latitude, that is ''bisexual in the south, irregular occurrence of males in central Europe, and hermaphroditism in the north'' (Longhurst, 1954) and possibly parthenogenesis in Italy (Scanabissi Sabelli & Mondini, 2002). The hypothesis of hermaphroditic vs. parthenogenetic reproduction in unisexual *T. cancriformis* populations has been controversially discussed by Engelmann et al. (1996) and Zaffagnini & Trentini (1980). To reveal this open dispute extensive research is necessary covering histological examinations, breeding tests and also genetic approaches (the method of choice seems to be microsatellite, see chapter 6).

Besides sex ratio related to latitude, Thiery (1978) noticed that there is a high variability in the sex ratio of Notostraca, which may be due to a differential micro-distribution of sexes in the habitat and different male and female life span. The latter fact has also been observed by other authors (Petrov & Cvetkovic, 1996; Machado et

al., 1999b; Boix et al., 2002). However, more studies on the distribution of sexes and on male, female, and hypothetical hermaphroditic gametogenesis have to be performed so that the gamete functionality and the true sexuality of this species can be resolved.

Conclusion

The purpose of the present paper was to show the variability in morphological features within different populations and to state whether the subdivision into three subspecies is valid as described by Longhurst (1955). The results sowed that *T. c. mauritanicus* was significantly separated from the other *T. cancriformis* populations. Additionally no evidence was found to distinguish between subspecies *T. c. simplex* and *T. c. cancriformis*. Within *T. cancriformis* complex reproductive strategy occurs which seems to be linked with geographical latitude.

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Chapter 4

Mitochondrial DNA phylogeography of the 'living fossil' *Triops cancriformis* (Crustacea: Branchiopoda: Notostraca)*

"Evolution is not a force but a process. Not a cause but a law."
(Darwin, 1809 – 1882)

Abstract

The Notostraca is a small but ancient crustacean order characterised by a range of reproductive systems. The present paper reports about the investigation of the mitochondrial DNA phylogeography of the tadpole shrimp *Triops cancriformis*, a 'living fossil' with bisexual, female-biased and unisexual populations. Nucleotide sequence variation in cytochrome *c* oxidase subunit I (COI) and 16S rDNA genes was screened in European populations including the three recognised subspecies. The large sequence divergence in both genes COI and 16S between *T. c. cancriformis* and *T. c. mauritanicus* strongly supports their status as separate species. However, no mtDNA differentiation was found between *T. c. cancriformis* and *T. c. simplex*. The low, but geographically structured mtDNA sequence variation in *T. c. cancriformis/simplex* suggests a recent origin for European populations. Some support for a range expansion involving unisexual or female biased populations was found, but further research is needed to resolve the evolution of *T. cancriformis* reproductive strategies.

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4.1 Introduction

The widespread occurrence of outcrossing sexual reproduction has puzzled evolutionary biologists since Darwin, as unisexual reproduction (either through self-fertilisation or parthenogenesis) poses an immediate advantage to organisms (Bell, 1982; Otto & Lenormand, 2002). Mixed reproductive strategies allow a unique direct comparison of the relative advantages of different reproductive modes (Bell, 1982; Peck et al., 1998). Mixed strategies often have a geographic component (e.g. 'geographical parthenogenesis') which reflects the interplay between historical and selective factors (Kearney, 2005). In spite of this, ecological correlates have been intensively sought to explain such geographic patterns adaptively (Peck et al., 1998; Kearney, 2005), and the phylogeographic context of mixed reproductive patterns has only recently begun to be investigated (Moritz, 1991; Schon et al., 2000; Law & Crespi, 2002).

The climatic oscillations of the Pleistocene (the 'Ice Ages') were associated with cycles of population expansion and decline for most temperate organisms (Bennet et al., 1991; Brown & Lomolino, 1998; Adams, 2004). Such geographical range dynamics left a recognisable signature in the genetic diversity of extant populations the detection and interpretation of which is the main focus of studies in the field of phylogeography (Avise, 2000; Hewitt, 2000). Indeed, genetic markers have been used to make inferences about population subdivision, location of glacial refugia, sources of recolonization of Northern Europe and contact zones for a wide array of plants and animals (Hewitt, 2000; Hewitt, 2004). The process of recolonization of the newly open habitats (through receded glacier) in northern areas during interglacial periods might have provided a crucial advantage for passively dispersing unisexual organisms. As a single unisexual individual can initiate a new population, they enjoy a colonisation advantage (Baker's law) (Baker, 1955; Baker, 1967). As a consequence, thermophile unisexual organisms (either parthenogenetic or selfing hermaphrodites) are often found in areas that were covered by glaciers or permafrost, and that, therefore, must have been recolonized after the last glacial maximum leading to an association between geographic distribution and reproductive mode (Kearney, 2005).

Androdioecy is a rare mixed reproductive strategy in which populations consist of hermaphrodites and males, with highly variable sex ratios (Pannel, 2002). Androdioecy has been described in just a few animals: some rhabditid nematodes (including *Caenorhabditis elegans*) (Stewart & Philips, 2002), the fish *Rivulus marmoratus* (Turner et al., 1992) and the two crustacean genera, *Eulimnadia* (Sassaman & Weeks, 1993) and *Triops* (Sassaman, 1991). Unlike plants, hermaphrodites of androdioecious animals cannot cross-fertilise each other. Therefore, outcrossing depends exclusively on the presence of males in the population. A large body of theoretical and empirical research has focused on the evolution and maintenance of this reproductive system (Otto et al., 1993; Pannel, 2002) suggesting that androdioecy has evolved from dioecy rather than through invasion of males into hermaphroditic population.

The Eurasian tadpole shrimp, *Triops cancriformis* (Bosc 1801) (Crustacea: Branchiopoda: Notostraca) inhabits temporary freshwater bodies like rain pools (Brtek & Thiery, 1995; Machado et al., 1999a). Based on morphological data, three subspecies are currently recognized (Longhurst, 1955a). *Triops c. cancriformis* is widely distributed, its area includes Europe, western Russia, and the Middle East to northern India and Japan (Longhurst, 1955b; Zaffagnini & Trentini, 1980; Suno-Uchi et al., 1997), but not China and Korea (Umetsu et al., 2002). *T. c. simplex* occurs in Spain and north Africa, from Ceuta to Egypt and Yemen (Arabian Peninsula) (Longhurst, 1955b; Brtek & Thiery, 1995; Thiery, 1996). Finally, *T. c. mauritanicus* (Ghigi 1921) occurs in the southwestern Iberian Peninsula and western Morocco (Alonso & Alcaraz, 1984). The species is thermophilic (Gaschott, 1928; Engelmann et al., 1988; Eder et al., 1997) and seems to be absent from cold regions without a drought period. At present, *T. cancriformis* is not found in latitudes north of 65°N (Brtek & Thiery, 1995).

As other notostracans, *T. cancriformis* has a mixed reproductive strategy which includes unisexual (selfing hermaphrodites or parthenogenetic individuals), female biased and bisexual populations (Zaffagnini & Trentini, 1980; Sassaman, 1991) (Figure 4-1). Both *T. c. simplex* and *T. c. mauritanicus* are considered to be bisexual, with equal or male biased sex ratios (Machado et al., 1999a; Boix et al., 2002). Male biased sex ratios are considered to be secondary, due to differential predation of the larger-sized females (Boix et al., 2002). In central and northern

Europe, *T. c. cancriformis* populations are unisexual or female biased with a wide range of sex ratio (male proportions) (von Siebold, 1871; Abonyi, 1926; Hempel-Zawitkowska, 1968; Zaffagnini & Trentini, 1980; Engelmann et al., 1996; Engelmann & Hahn, 2005; Scanabissi Sabelli et al., 2005). Interestingly, in the Italian Peninsula no males have been reported (Zaffagnini & Trentini, 1980; Scanabissi Sabelli et al., 2005).

The reproductive mode of such female-biased and unisexual populations is controversial although most evidence suggests that these populations are androdioecious, as in the American species *T. longicaudatus* and *T. newberryi* (Sassaman et al., 1997). The histological examination of females from several unisexual populations revealed the presence of ovotestes, and therefore confirmed the presence of hermaphroditism (Longhurst, 1954; Zaffagnini & Trentini, 1980). However, females from a female-biased population in Germany had no testicular tissue (Engelmann et al., 1997) suggesting that parthenogenetic reproduction in some populations cannot be completely ruled out. Preliminary microsatellite data (see chapter 6) indicates significant heterozygote deficiencies in many populations, lending support to androdioecy.

In the present paper the term ’female’ is used in a wide sense, as individuals described as females can actually be hermaphrodites.

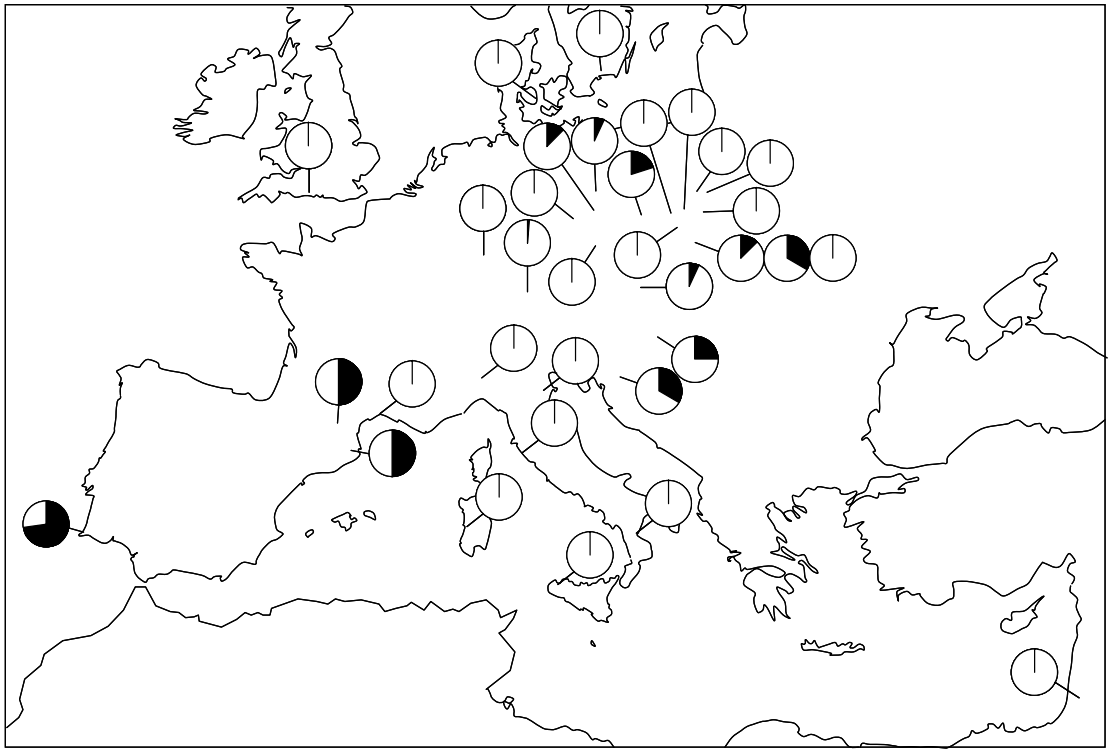

Figure 4-1: Overview of recent sex ratios (male percentages are the filled sections in pie charts) of *Triops cancriformis* populations in Europe.

Evidence for a wide notostracan distribution before the Pleistocene can be derived from an abundant fossil record dating back to the Carboniferous or possibly up to the Devonian period (Guthörl, 1934; Trusheim, 1937; Gall, 1971). In fact, the striking morphological similarity of some Upper Triassic *Triops* sp. fossils from Germany and extant *T. cancriformis* (Trusheim, 1931, 1937; Tröger et al., 1984; Kelber, 1999) makes this notostracan one of the best examples of evolutionary stasis or 'living fossils' (Fisher, 1990; Futuyma, 1990; Suno-Uchi et al., 1997; King & Hanner, 1998; Kleesattle, 2001).

The distribution of large branchiopods is effected by their drought-resistance cysts, which are efficient agents of passive dispersal. Populations may occur on remote islands, and are apparently found wherever suitable habitats are available (Longhurst, 1955a). The diapausing and – shortly after laying – extremely sticky eggs might be dispersed by wind, water or birds (Càceres & Soluk, 2002; Green & Figuerola, 2005) or by other land animals such as amphibians or hoofed animals (Frank, 1986; Thiery, 1991; Coulson et al., 2002). Thus, the high dispersal abilities afforded by their diapausing cysts and the possibility of unisexual reproduction seemingly account for the wide distribution of *T. cancriformis*.

Given the geographical variation in its mixed reproductive system and its thermophilic character, the phylogeography of *T. cancriformis* could potentially illustrate the differential impact of Pleistocene climatic oscillations on the distribution of the different reproductive strategies. In particular, and in agreement with Longhurst (1955b) it can be hypothesized that unisexual lineages or female-biased lineages should be more abundant in areas which were covered with ice sheets or unsuitable habitats (e.g. permafrost) during glacial maxima, and that therefore must have been recently colonised.

An ideal marker for the phylogeographic reconstruction is the mitochondrial DNA (mtDNA) which is effectively maternally inherited. Thus even within breeding populations, mtDNA lineages are genetically isolated from one another, such that any observed homologies in genetic structure presumably result from historical connection in a matriarchal genealogy (Avise, 1992).

The chapter at hand presents a phylogeographic survey of European *T. cancriformis* based on cytochrome *c* oxidase subunit I (COI) as well as small ribosomal RNA subunit (16S) sequence data and puts these data in the context of reproductive strategy variation. Data concerning the sex-ratio in this species will be reviewed and reanalysed. Finally, it will be addressed the phylogeny of the genus *Triops* using sequence information from the COI and 16S genes to obtain information on the evolution of its reproductive strategy variation. The results provide insights into population diversification and have implications for the taxonomy and the evolution of reproductive mode in *Triops*.

4.2 Material and methods

4.2.1 Sampled populations

Twenty-one water bodies in inundated floodplains, isolated ponds and puddles and fish nursery pools in Europe were sampled between 2000 and 2004 (Table 4-1, see Figure 4-1). Samples included specimens collected using a dip net (5 mm pore diameter), and sediment containing resting cysts from dry ponds where the species was known to occur.

Most samples belong to the nominal subspecies, but a small number of samples from the other two recognised subspecies *T. c. mauritanicus* and *T. c. simplex*, both from Spain (Figure 4-2).

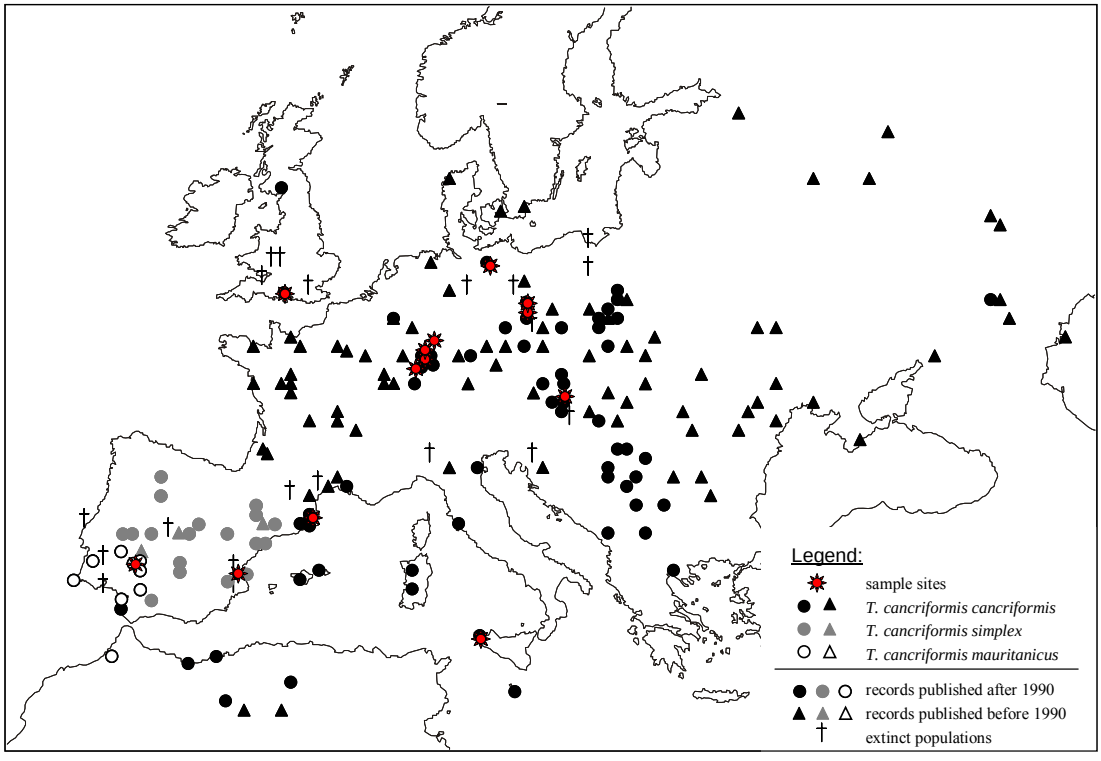

Figure 4-2: *Triops cancriformis* distribution and locations of sample sites for genetic analyses based on mitochondrial markers.

In total 60 *Triops cancriformis* specimens, representing 20 populations, were sampled for cytochrome *c* oxidase gene I (COI) analyses (Table 4-1). This included 8 localities in fishery areas (Königswartha and Lacoma), 4 localities along the river

Rhine (Daxlander Au, Ibersheim, Neuburg, Hagenbach), 2 localities in former military areas (Tannen, Coburg).

Table 4-1: Overview of *Triops cancriformis* samples investigated in the mitochondrial DNA sequence analysis. Country, population identification and geographic location are summarized. Number of analysed samples (n) is given for cytochrom c oxidase subunit I gene analyses and in 16S rDNA analysis (values in parentheses)

Country	**Regional population**		**Local population**		**n COI (16S)**	**Geographic location**	**Material**	**subpecies**
	ID	Region	ID	Location				
Austria	DN	Danube, Bumengang	-		1	48°10' N, 16°58' E		T.c.c.
	LN	Neusiedler Lake, Kaiserlacke	-		3 (1)	47°48' N, 16°51' E	sediment	T.c.c.
Germany	KW	fish pond area Königswartha, Saxony	Kw11	fish pond 11	3 (1)	51°19' N, 14°21' E	sediment	T.c.c.
			Kw12	fish pond 12	3 (1)	51°19' N, 14°21' E	sediment	T.c.c.
			Kw21	fish pond 21	3	51°19' N, 14°21' E	sediment	T.c.c.
			Kw27	fish pond 27	3	51°19' N, 14°21' E	preserved live	T.c.c.
			Kw28	fish pond 28	4	51°19' N, 14°21' E	preserved live	T.c.c.
	LA	fish pond area Lacoma, Brandenburg	La1	fish pond 1	2 (1)	51°47' N, 14°23' E	sediment	T.c.c.
			La2	fish pond 2	3	51°47' N, 14°23' E	sediment	T.c.c.
			La3	fish pond 3	2	51°47' N, 14°23' E	sediment	T.c.c.
	R	Rhine, Rhineland-Palatinate	Da	Daxlander Au	3	48°59' N, 08°17' E	sediment	T.c.c.
			Ib	Ibersheim	2	49°43' N, 08°25' E	sediment	T.c.c.
			Ne	Neuburg	3	48°59' N, 08°16' E	sediment	T.c.c.
			Ha	Hagenbach	3	49°00' N, 08°16' E	sediment	T.c.c.
	TA	Tannen, Mecklenburg-Western Pomerania	-	-	5	53°34' N, 11°26' E	preserved live	T.c.c.
Great Britain	NF	Godshill pond, New Forest	-	-	3 (1)	50°55' N, 01°45' W	sediment	T.c.c.
Italy	SC	Monte Cofano, Sicily		-	3 (1)	38°05' N, 12°42' E	preserved live	T.c.c.
Spain	PE	Plat d'Espolla, Banyoles karstic area	-	-	3 (1)	42°10' N, 02°46' E	preserved live	T.c.s.*
	EP	El Puig, Valencia	-	-	3 (1)	38°05' N, 12°42' E	preserved live	T.c.s.*
	X	Extremadura, Laguna de la Gitanilla	-	-	2 (2)	39°27' N, 06°15' W	preserved live	T.c.m.*

* according to Alonso (1996) and Boix et al. (2002)

To rank the genetic diversity resulting from the COI analysis, the 16S rDNA has been investigated afterwards. Therefore, from each resulting COI haplotype one representative specimens of each pond collapsed in that haplotype was used for the 16S analyses (compare Table 4-1). This led to a total of 10 *T. cancriformis* specimens sampled from Austria, Germany, Great Britain, Italy and Spain. Additionally, sequences available from GenBank for other Triops species and also for Lepidurus species were included; thus 40 sequences could be analysed for the 16S rDNA (Table 4-2).

Table 4-2: List of sequences applied in the 16S rDNA analysis in addition to own data

Species	**sequence source/reference**
Triops cancriformis	AB084514, AY115613, AY159571 to AY159579, TcanJapan (Suno-Uchi, 1997)
Triops granarius	AF200963 to AY200971, AY115612; TgraJapan (Suno-Uchi, 1997)
Triops longicaudatus	AY115605 to AY115611, AY159580 and AY159581; TlonTakamatsu , TlonTsuda, TlTriopsw (Mantovani et al. 2004)
Lepidurus apus lubbocki	AY159582 and AY159583
Lepidurus apus apus	AY159584
Lepidurus lemmoni	AY115614
Lepidurus arcticus	AY159585

4.2.2 Reproductive mode

Sex ratio (male proportion) was determined for all *T. cancriformis* populations sampled in this study. The geographic distribution of sex ratios was analysed using a combination of data from this study and published data for 16 European populations. Only literature data were included that provided either sample sizes and the sex ratio, or the raw numbers of males and females per sample. Populations were assigned to one of four qualitative patterns of sex ratio variation according to Sassaman (1991): Type I (male biased), Type II (equality), Type III (female biased) and type IV (unisexual). Binomial (chi-square) tests were used to test whether the sex ratio of each population was significantly different from an equal sex ratio. If it was not found to be different, the population was considered of type II, if significantly large (male:female ratio), it was considered type I, and if significantly small (male:female ratio), it was regarded as type III. Following Sassaman (1991) the type IV

(unisexuality) was only assumed when there were >50 individuals sexed and all were found to be females.

4.2.3 DNA amplification and sequencing

Total genomic DNA was isolated from ethanol preserved tissue using commercial DNA extraction kits (Invisorb Spin Forensic Kit, Invitek; PureGene Kit, Gentra Systems). For some populations, DNA was extracted from resting eggs which were isolated from the sediment following procedures described in Gómez & Carvalho (2000).

Two regions of mitochondrial DNA (mtDNA) were selected in order to provide appropriate resolution both on intra- and inter-specific levels and to facilitate inclusion of published sequences. Cytochrome *c* oxidase subunit I gene (COI) usually provides sufficient level of intra-specific variation to address phylogeographic questions. The gene for the small ribosomal RNA subunit (16S) often shows little variability within species but because of its wide application in phylogenetic studies a large number of published sequences are available. Amplifications of both genes were performed in 20 µl final volume containing 2 µl template DNA (10-40 µg/ml), 1.5 mM $MgCl_2$, 200 µM of each nucleotide, 100 µM of each primer, 0.01 U of *Taq* DNA polymerase (Bioline) and 1x NH_4-PCR Buffer (Bioline). The following cycling conditions were used for both COI and 16S: 3 min denaturating at 93°C, 35 cycles of 45 s at 94°C, 45 s at 50°C and 1 min at 72°C and finally 5 min extension at 72°C. To amplify 710 bp of the COI gene the primers LCO1490 and HCO2198 (Folmer et al., 1994) were used, and for 16S rDNA amplification Sar and Sbr primers were applied (Palumbi, 1996). PCR products were sequenced directly using a Beckman CEQ8000 capillary sequencer and the Beckman DTCS Quick Start Sequencing kit. The sequences were eye checked with the CEQ8000 data analyser and initially aligned with Clustal W (Thompson et al., 1994) and finally adjusted by hand using the sequence chromatogram. All 16S and COI sequences available on GenBank for *T. cancriformis* and representatives of Notostraca were downloaded and analysed together with our dataset. Thus, in total 61 sequences for 16S and 64 sequences for COI were analysed.

4.2.4 Phylogenetic reconstructions

Phylogenetic and molecular evolutionary analyses of COI and 16S rDNA datasets were performed with the Bayesian inference algorithm in MRBAYES 3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), and maximum likelihood (ML) algorithm provided by PHYML 2.4.4 (Guindon & Gascuel, 2003). The best fitting evolution models were selected using the stepwise hierarchical likelihood-ratio test in the program ModelGenerator version0.6 (Keane *et al.*, 2004). For COI ML analyses, the GTR+Γ model of sequence evolution and four gamma distributed rate categories, and for 16S rDNA TVM+Γ and four gamma-distributed rate categories were found to be the best fitting models and used in the analysis. Branch support of the ML tree was assessed by 1000 bootstrap pseudo-replications. The Bayesian search was partitioned by codon positions with a 4 by 4 nucleotide model for each codon position with 6 nucleotide substitution rates. The default priors in MrBayes were modified according to the best fitting model of sequence evolution. This included parameters for the substitution model (command: revmatpr) and the nucleotide frequencies rate (command: statefreqpr). For the COI data analyses revmatpr was set to variable=dirichlet (0.91, 7.79, 4.23, 0.56, 12.23, 1.0) and statefreqpr was set to variable=dirichlet (0.27, 0.19, 0.19, 0.34). To analyse the 16S data in MrBayes the priors were modified as follows: revmatpr which was set to variable=dirichlet (0.00, 4.25, 2.09, 0.19, 4.25, 1.0) and statefreqpr which was set to variable=dirichlet (0.32, 0.12, 0.21, 0.35). Two simultaneous Metropolis-coupled Markov chain Monte Carlo analyses were run each with four chains (3 ‘heated’) for 1,000,000 generations, sampling trees and parameters every 100 generations. We discarded the first 250,000 generations (2,500 trees) on each run as ‘burnin’ after visually confirming chain convergence from plots of likelihood against generation. The 50% consensus tree was obtained from the 35,000 trees from both runs sampled after the initial burn-in period.

Nodes in the ML-tree were considered to be well supported, if they showed at least 70% bootstrap support (Hillis & Bull, 1993). Similarly, strong branch support was inferred in the Bayesian tree with probabilities over 80% (Wittingham et al., 2002). Trees were displayed with NJPlot (Pierrière & Gouy, 1996). Unrooted parsimony networks were constructed using PAUP* 4.0b8 (Swofford, 1998).

4.2.5 Estimation of divergence times

In order to obtain a rough estimation of divergence times for selected pairs of taxa molecular clock calibrations inferred for crustaceans were used. Knowlton and Weigt (1998) calibrated the same COI fragment that were used here for sister species of mangrove snapping shrimp (*Alpheus*) that diverged at the time of closure of the Panama strait. This calibration, appropriate for less than 20% sequence divergence, is equivalent to 1.4% sequence divergence per million years. Faster evolutionary rates were obtained in Jamaican land crabs (1.66-2.33 percent sequence divergence per million years) (Schubart et al., 1998). For 16S, the calibration of 0.65-0.88% sequence divergence per million years on land crabs (*Sessarma*) in Jamaica was used (Schubart et al., 1998), which encompasses the calibration of 0.78 percent sequence divergence per million years obtained by Jarman & Elliot (2000) for Anaspid crustaceans.

To be able to use these molecular clock calibrations corrected average pairwise genetic divergence estimates between haplotypes by the Kimura's two parameter model using Mega version 3.1 were used (Kumar et al., 2004).

4.3 Results

4.3.1 Mitochondrial DNA diversity

4.3.1.1 Based on cytochrom *c* oxidase subunit I gene data

A total of 60 *T. cancriformis* specimens were sequenced for COI with two to four specimens per pond. The alignment included 600 bp, and after collapsing identical sequences we identified five haplotypes (designed A to E, Table 4-3, haplotype sequences will be provided by the author if requested). A sixth haplotype was represented by the only COI sequence available from GenBank for *T. cancriformis*, obtained from Japan.

Table 4-3: COI haplotypes of *Triops cancriformis* including the number of populations and specimens collapsed in each haplotype and the list of united (redundant) sequences from analysed specimens (haplotype sequences will be provided by the author if requested)

Haplotype	number of samples	combined samples per haplotype	subspecies
A	11	Kw11Tc1, Kw11Tc2, Kw11Tc3, Kw21Tc9, Kw27Tc1, Kw27Tc4, Kw27Tc13, Kw28Tc3, Kw28Tc4, Kw28Tc30, Kw28Tc32	T.c.c
B	12	Kw12Tc4, Kw 21Tc1, COTc8, COTc11, DaTc2, DaTc3, PETc2, PETc5, PETc9, EPTc1, EPTc2, EPTc3	T.c.c./T.c.s.
C	32	DNTc1, LNTc5, LNTc6, LNTc12, NFTc2, NFTc6, NFTc18, COTc1, DaTc1, HgTc1, HgTc2, HgTc3, IbTc1, IbTc2, Kw12Tc14, Kw12Tc17, Kw21Tc4, La1Tc2, La1Tc6, La2Tc1, La2Tc7, La2Tc13, La3Tc4, La3Tc5, NeTc1, NeTc4, NeTc5, TATc1, TATc2, TATc3, TATc10, TATc11	T.c.c.
D	3	SCTc1, SCTc2, SCTc3	T.c.c.
E	2	XTc1, XTc2	T.c.m.

Most haplotypes (A, B, C and D) were found among *T. c. cancriformis* individuals. The two Iberian specimens of *T. c. mauritanicus* shared haplotype E and *T. c. simplex* specimens shared haplotype B. The number of haplotypes per population ranged from 1 to 3 (average 1.2). The most diverse pond was the fishery pond number 21 in Königswartha (Germany) with 3 haplotypes, however, 17 ponds contained a single haplotype (Figure 4-3).

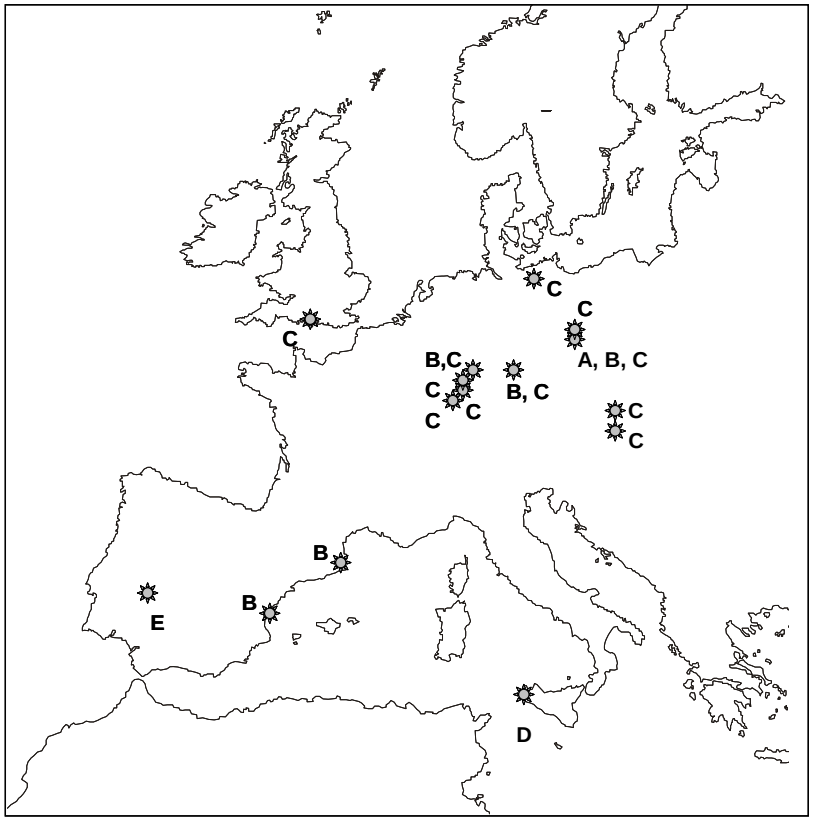

Figure 4-3: Geographic distribution of identified *Triops cancriformis* COI haplotypes (A to E).

Overall 65 variable sites were identified among all *T. cancriformis* haplotypes two of which were parsimony informative (Table 4-4). Excluding the *T. c. mauritanicus* haplotype, 14 sites remained variable and one site was parsimony informative. Overall, 42 transitions were located at third position, none at second and 11 at first position. There were 11 transversions at third codon position, one at second and none at first. There were no aminoacid replacements involving haplotypes A-D, but in haplotype E the transitions at position 98 G-A, 329 A-G, and 470 A-G at first codon positions and one transition at second codon position (position 336 T-G) resulted in amino acid replacements. Sequences were moderately A+T rich (mean AT content = 62.2%). The mean sequence diversity was 0.0135 (S.E. 0.0020) across all haplotypes (A to E) and 0.0065 (S.E 0.0018) within the *T. cancriformis/simplex* (A to D).

Table 4-4: Polymorphic positions (written vertically) of 5 *Triops cancriformis* COI haplotypes (including the subspecies *Triops cancriformis mauritanicus* collapsed in haplotype E)

Haplotype	Position																				
	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
	1	2	2	3	5	7	7	7	8	9	0	1	1	2	3	5	6	7	7	7	9
	0	5	6	4	0	0	6	9	8	8	0	5	8	4	0	1	3	2	5	8	9
A	A	G	C	T	T	G	A	T	C	G	T	G	C	A	C	G	C	G	G	A	T
B	.	A	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	A	A	.	.
C	.	A	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	A	A	.	.
D	.	A	.	.	.	A	.	.	.	.	C	A	.	.	.	.	.	A	A	.	C
E	G	T	T	C	C	A	T	C	T	A	.	A	T	G	T	A	T	A	A	T	.

Haplotype	Position (continued)																					
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3
	0	0	1	1	2	5	5	5	6	6	7	7	8	9	9	2	2	2	3	3	3	4
	5	8	4	7	3	0	4	9	3	6	1	4	0	2	8	5	8	9	1	4	6	0
A	A	C	C	C	T	C	T	C	T	T	A	A	T	G	A	G	A	A	A	C	T	C
B	.	.	.	.	.	.	.	.	.	.	.	.	C	A	G	.	.	.	.	.	.	.
C	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.
D	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.
E	G	T	T	A	C	T	C	T	C	C	G	G	.	A	.	A	G	G	G	G	G	A

Haplotype	Position (continued)																					
	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5
	4	4	5	9	0	0	0	1	6	7	7	8	9	9	0	0	1	2	2	4	7	8
	3	6	8	1	4	6	9	2	1	0	5	1	0	3	2	8	8	0	9	4	1	3
A	T	T	C	A	T	T	T	C	C	A	A	T	G	G	T	T	C	G	T	T	T	A
B	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.
C	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.
D	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	C	.	.	.
E	C	C	T	.	C	G	G	.	T	G	T	C	T	A	C	A	T	A	C	C	C	T

Pair-wise nucleotide differences between the five *T. cancriformis* haplotypes ranged from 1 to 61 mutations which translate into a maximum corrected distance of 19.39% (haplotype A from Germany, vs. haplotype E from Extremadura, Spain) (Table 4-5). The mean corrected sequence divergence between the haplotypes A to D was 1.32%. The mean corrected genetic distance between *T. c. mauritanicus* and *T. c. cancriformis/simplex* lineage is 17.79%. The corrected sequence divergences between the other *Triops* species ranged from 29 to 60%.

Table 4-5: Corrected Kimura 2-parameter distance matrix for COI gene of the 5 identified *Triops cancriformis* haplotypes, the Japanese sequence of *T. c. cancriformis* published by Umetsu et al. (2002), as well as *T. australiensis*, *T. longicaudatus*, *T. granarius* and *Lepidurus* sp.

COI haplotype/species		1	2	3	4	5	6	7	8	9
A	1	-								
B	2	0.02070	-							
C	3	0.01866	0.00171	-						
D	4	0.02253	0.00878	0.00696	-					
E	5	0.19385	0.17303	0.16902	0.17560	-				
T. c. c. Japan	6	0.01665	0.00346	0.00171	0.00518	0.16505	-			
Triops australiensis	7	0.42637	0.40697	0.40007	0.41442	0.47303	0.39667	-		
Triops longicaudatus	8	0.37036	0.35328	0.35962	0.35862	0.40864	0.35329	0.29113	-	
Triops granarius	9	0.60260	0.58997	0.58069	0.57171	0.60219	0.57611	0.39643	0.36498	-
Lepidurus sp.	10	0.70167	0.67020	0.66510	0.67014	0.76007	0.66002	0.72667	0.67703	0.56651

4.3.1.2 16S rDNA gene

Sequences were obtained from ten individuals, which had been previously selected in order to represent different populations and different COI haplotypes. These sequences were aligned with 41 sequences available to date from Genbank, including 12 sequences of *Triops cancriformis*, 11 sequences of *Triops granarius,* 12 sequences of *Triops longicaudatus*, one *Triops australiensis* sequence and 5 sequences of *Lepidurus* species. The overlap between all sequences permitted the analysis of a 437 bp fragment including for indels (gaps). Note that there were two indels from published sequences AY159571 (TCer1) and AY 159572 (TCer2) respectively, which were not included in our analysis as they were outside the analysed fragment. After examination of the initial alignment, the Japanese *T. cancriformis* and *T. granarius* (TgraJapan) sequences from Suno-Uchi et al. (1997) were excluded from the final dataset, as they show shared indels which were not present in any other sequence.

The final 16S dataset included seven *T. cancriformis* haplotypes with 18 variable nucleotide sites. A total of 105 variable sites and 78 parsimony informative sites were found in the alignment. The *T. cancriformis* sequence from Umetsu et al. (2002) (AB084514) differ from all other specimens of both genera by a single transversion at position 53 (G-A) (Table 4-6). The analysed 16S rDNA sequences are moderately A+T rich (mean AT content = 66.9%). The mean sequence diversity for the 20 *T. cancriformis* sequences collapsed in haplotypes 1 to 7 is 0.0056 (S.E. 0.014) (sequences will provided by the author if requested). Considering exclusively the European *T. cancriformis/simplex* haplotypes (1 to 5) the diversity is nearly 2.5 times smaller than the latter one (0.0023, S.E. 0.0012).

Table 4-6: 16S rDNA haplotypes of *Triops cancriformis* including the number of populations and specimens collapsed in each haplotype and the information about variable sites (positions written vertically) (sequences will be provided by the author if requested)

Haplotype*	Number of samples	Position																	
		0	0	0	1	1	2	2	2	2	2	3	3	3	3	3	3	3
		4	5	8	2	6	0	3	3	4	4	8	1	1	1	2	4	4	9
		3	3	3	6	9	8	7	9	0	6	1	2	7	9	9	6	7	5
1	8	C	G	G	T	A	G	A	C	T	C	G	T	A	T	C	G	C	A
2	3	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
3	1	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
4	3	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
5	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
6	1	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7	1	T	.	.	G	G	A	G	T	C	T	A	.	G	A	T	A	T	.

*
1 AY159572, AY159573, AY159576, TcanKw-A, TcanKw-B, TcanNF-C, TcanPE-B, TcanEP-B
2 AY159574, TcanSicily
3 AY159575,
4 AY159577, AY159578, AY115613),
5 AY159579, TcanLaC, TcanNskC),
6 AB084514),
7 TmauX-E

Pairwise nucleotide differences between the seven haplotypes ranged from 1 to 16. The maximum corrected sequence divergence of 4.54% was found between haplotype 6 (Japan) and 7 (Iberian *T. c. mauritanicus*). The mean of the corrected sequence divergence between the two detected lineages *T. c. cancriformis*/*simplex* (haplotype 1 to 6) and the *T. c. mauritanicus* haplotype is 3.94%. The mean corrected sequence divergence between the haplotypes belonging to the *T. c. cancriformis*/*simplex* lineage comes to 0.58% (haplotypes 1 to 6) and 0.42% (with only the European haplotypes: 1 to 5).

Table 4-7: Kimura 2-parameter distance matrix for 16S rDNA haplotypes of the analyses *Triops cancriformis* specimens.

16S haplotype/species		1	2	3	4	5	6
haplotype 1	1	-					
haplotype 2	2	0.00474	-				
haplotype 3	3	0.00232	0.00235	-			
haplotype 4	4	0.00232	0.00715	0.00468	-		
haplotype 5	5	0.00233	0.00717	0.00469	0.00469	-	
haplotype 6	6	0.00711	0.01218	0.00956	0.00955	0.00957	-
haplotype 7	7	0.03630	0.04260	0.03919	0.03348	0.03929	0.04539

Haplotype legend: **1** AY159572, AY159573, AY159576, TcanKw-A, TcanKw-B, TcanNF-C, TcanPE-B, TcanEP-B, **2** AY159574, TcanSicily, **3** AY159575, **4** AY159577, AY159578, AY115613), **5** AY159579, TcanLaC, TcanNskC), **6** AB084514), **7** TmauX-E

4.3.2 Estimation of divergence times

Divergence times were estimated for two events. First, the time of divergence of the *T. c. cancriformis/simplex* lineage from *T. c. mauritanicus*. Second, the time of the last common ancestor of all the *T. c. cancriformis/simplex* haplotypes were estimated in order to obtain an approximation of the age of the lineage in Europe.

Using the crustacean clock calibrations of Knowlton and Weigt (1998) and Schubart et al. (1998) for COI and the calibration of Schubart et al. (1998) and Jarman & Elliot (2000) for 16S estimates of the divergence time of the lineages *T. c. cancriformis* and *T. c. cancriformis/simplex* of approximately 10 to 16 mya for COI and of 4 to 10 mya for 16S were obtained. The divergence time among all *T. c. cancriformis/simplex* haplotypes was estimated as 0.46-0.76 mya for COI and as 0.66-1.41 mya for 16S.

4.3.3 Phylogenetic relationships

4.3.3.1 Based on cytochrome *c* oxidase subunit I gene data

Maximum Likelihood and Bayesian analyses produced nearly identical topologies (Figure 4-4). Within *T. cancriformis*, there are two divergent branches, one representing *T. c. mauritanicus* (represented by haplotype E) and the other comprising a well-supported lineage of closely related haplotypes grouping *T. c. cancriformis* and *T. c. simplex*. Within the latter lineage *T. c. simplex* and *T. c. cancriformis* are not represented by different genetic clades (Figure 4-4) as individuals of *T. c. simplex* from the East Iberian coast (El Puig, Plat d'Espolla) shared haplotype B with six German *T. c. cancriformis* individuals from four sites.

The T. c. cancriformis/simplex clade and *T. c. mauritanicus* together formed a well-supported monophyletic clade. In contrast, the relationships of *T. cancriformis*, *T. longicaudatus* and *T. australiensis* could not be resolved in the COI dataset. The three latter species however, form a well supported sister clade to the basal *T. granarius*.

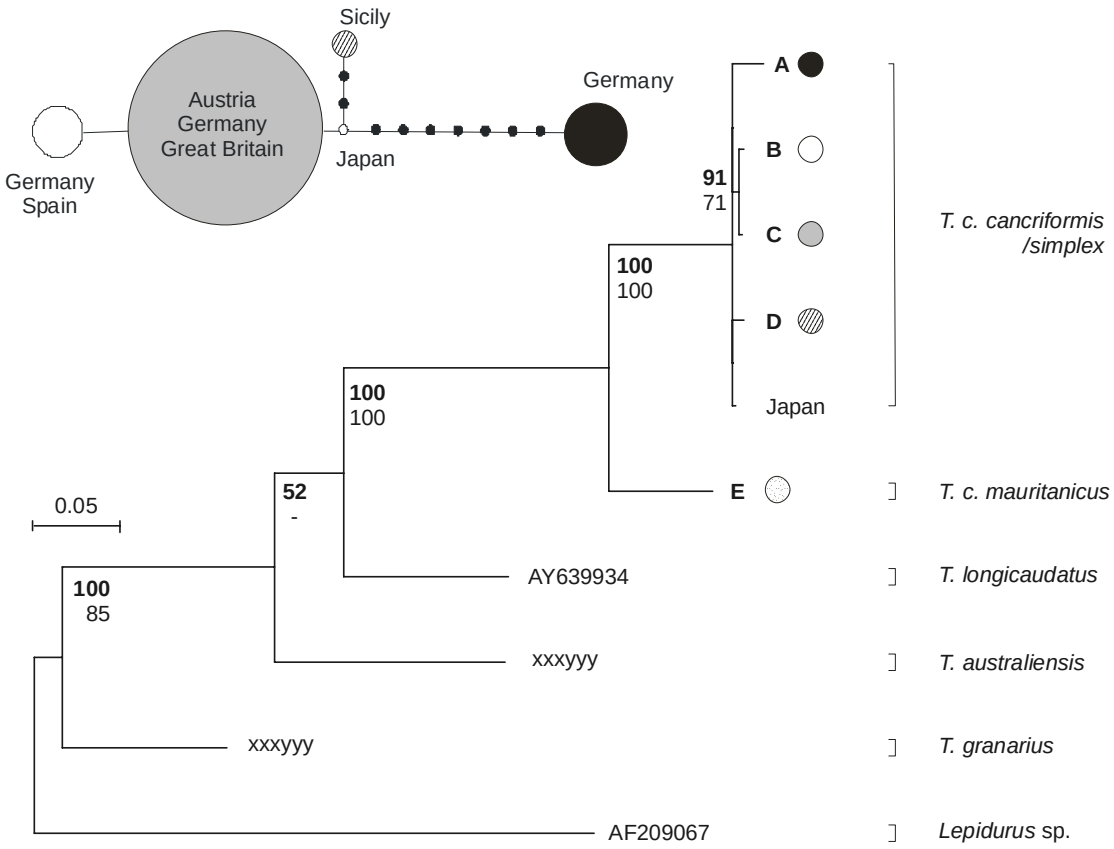

Figure 4-4: Maximum parsimony network and phylogenetic reconstruction of *Triops* based on COI data. The diameter of each circle which represents a single haplotype is proportional to the number of individuals belonging to that haplotype (A = 11, B = 9, C = 32, D = 3, Japan = 1). Each line between haplotypes represents a mutation. In the phylogram, values in bold at major branches are posterior probabilities in the Bayesian analysis; plain numbers indicate the bootstrap support in the Maximum-likelihood analysis (only values over 50% are shown). The tree is rooted with the *Lepidurus* sp. Sequence (xxxyyy indicate that the accession numbers for these sequences are not published yet).

4.3.3.2 Based on 16S data

Maximum Likelihood and Bayesian analyses produced identical topologies with nearly the same bootstrap support (Figure 4-5). *T. cancriformis* is subdivided into two lineages supported by high bootstrap values. One of them is represented by haplotype 7, representing *T. c. mauritanicus*. The other lineage combines the *T. c. cancriformis* and T. c. *simplex* specimens (with 6 haplotypes). As in the COI dataset, haplotypes are closely related and differ by a few substitutions only.

The two Notostracan genera *Triops* and *Lepidurus* formed highly supported monophyletic clades. Within the genus *Triops* all four species appear to be reciprocally monophyletic with high bootstrap support. The relationships between the species could be clearly resolved, and the analysis supported a close relationship

between *T. australiensis* and *T. longicaudatus*, and a well supported sister relationship of these with *T. granarius*.

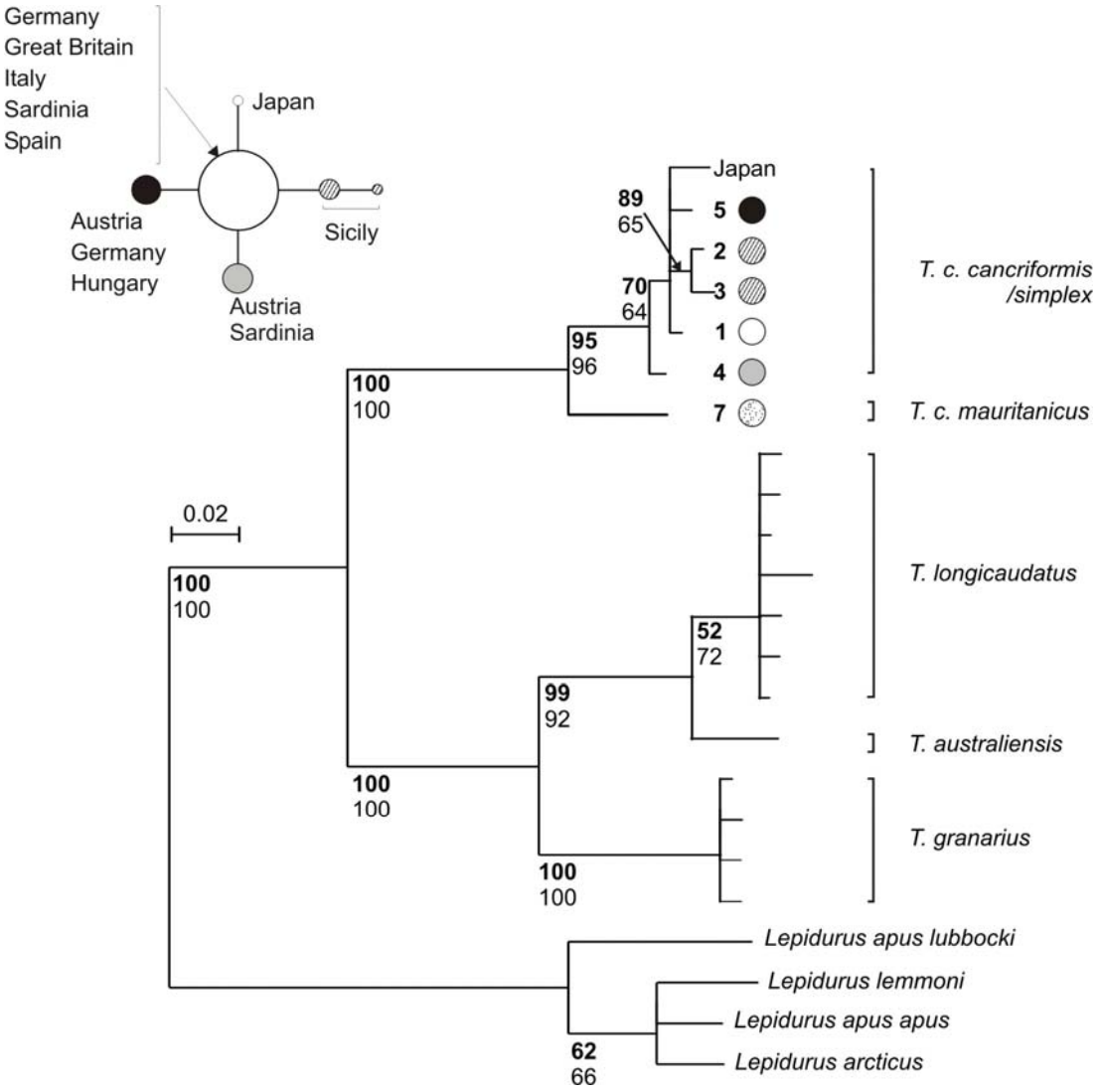

Figure 4-5: Maximum parsimony network and phylogenetic reconstruction of Triops based on 16S rDNA data. The diameter of each circle which represents a single haplotype is proportional to the number of individuals belonging to that haplotype (1 = 8, 2 = 2, 3 = 1, 4 = 3, 5 = 3, 6 = 1). Each line between haplotypes represents a mutation. In the phylogram, values in bold by major branches are posterior probabilities in the Bayesian analysis; plain numbers indicate the bootstrap support in the Maximum-likelihood analysis (only values over 50% are shown). The tree is midpoint rooted.

4.3.4 Phylogeography and reproductive mode

The reanalysis of sex ratios in *T. cancriformis* based on own and literature data suggested that equal sex ratio indicative of bisexual reproduction was only found in Iberian populations (Table 4-8). Female biased populations occur throughout central and northern Europe (Germany, Austria, Croatia, Hungary, Great Britain and Poland,

Table 2). For several locations in Austria, Great Britain, France, Italy, Poland and Japan males have never been reported. However in most cases small (<50) or unreported sample sizes did not allow us to conclude that these populations are actually unisexual, as opposed to having strongly female biased sex ratios. Only a French locality had enough published sample sizes to conclude unisexual reproduction (Table 4-8).

Table 4-8: Numbers (n) of *Triops cancriformis* males and females and the male proportion given per pond. The inferred type of sex ratio according to Sassaman 1991 is provided: I, male biased, II equality, III female biased, IV unisexuality

location information	n_{male}	n_{female}	prop. of males	p value (Chi-Square)	sex ratio	reference
Austria, Neusiedler Lake, Kaiserlacke	0	11	0.0000	0.0009	III	p.d.
Croatia	9	34	0.2093	0.0001	III	1
France, Camargue	0	9	0.0000	0.0027	III	2
France, Baillargues (Montpellier)	0	200	0.000	<0.0001	IV	3
Germany, Bavaria	8	1000	0.0079	<0.0001	III	4
Germany, Bavaria, Augsburg	7	568	0.0122	<0.0001	III	5
Germany, Königswartha, fish pond area	3	119	0.0246	<0.0001	III	p.d.
Germany, Lacoma, fish pond area	2	28	0.0667	<0.0001	III	p.d.
Great Britain, New Forest, Godshill pond	0	19	0.0000	<0.0001	III	p.d.
Hungary, Lake Balaton	7	19	0.2692	0.0186	III	6
Hungary, Lake Balaton	15	45	0.2500	0.0001	III	6
Japan	0	20	0.0000	<0.0001	III	2
Poland, Wroclaw (April 1867)	29	88	0.2479	<0.0001	III	7
Poland, Wroclaw (April/June 1865)	114	912	0.1111	<0.0001	III	7
Poland, Wroclaw (July 1864)	2	9	0.1818	0.0348	III	1
Poland, Wroclaw (July 1879)	5	19	0.2083	0.0043	III	1
Poland, Cracow (1857)	16	144	0.1000	<0.0001	III	8
Poland, Cracow (1858)	154	395	0.2805	<0.0001	III	8
Poland, Debina	0	10	0.0000	0.0016	III	9
Poland, Jaktorów	0	40	0.0000	<0.0001	III	9
Poland, Jarosy	0	10	0.0000	0.0016	III	9
Poland, Klomnice	0	15	0.0000	0.0001	III	9
Poland, Kludno	0	20	0.0000	<0.0001	III	9
Poland, Zabieniec	0	40	0.0000	<0.0001	III	9
Portugal, Algarve	54	22	0.7105	0.0002	**I**	10
Portugal, Algarve	19	23	0.4524	0.5371	**II**	10
Spain, Plat d'Espolla	1723	1775	0.4927	0.3872	**II**	11

* [1] Braem (1893), [2] Akita (1976), [3] (Mathias, 1937) [4] Heidecke & Neumann (1987), [5] Gaschott (1928), [6] Abonyi (1926), [7] von Siebold (1871), [8] Kozubowsky (1857), [9] Hempel-Zawitkowska & Klekowski (1968), [10] Machado et al. (1999b), [11] Boix (2002), p.d. = personal data

The haplotype networks (in Figure 4-4 and Figure 4-5) show the abundance and geographic distribution of the 16S rDNA and COI haplotypes. Despite the low level of variability, some geographical structure was found (Figure 4-6). Based on

COI data two geographically restricted haplotypes of *T. c. cancriformis* were identified. Haplotype A was only found in the fish nursery area of Königswartha (Germany) in populations with very low male proportions. Haplotype B was geographically widespread and was found in eastern Spain and Germany (Figure 4-6 A). One of the Spanish populations, Plat d'Espolla, has an equal sex ratio (Table 4-8) whereas at least one other population containing haplotype B (Königswartha) has a very low male proportion. The most common and widespread haplotype, C, was found in Austria, Great Britain and Germany exclusively in populations with low male proportions. The Japanese sequence differs from haplotype C in a single mutation and no males have been reported from this population (Akita, 1976). Haplotype D was only found in Sicily (Italy). No sufficient data on reproductive mode are available from Sicilian populations.

The 16S rDNA haplotype distribution shows a similar pattern. However, only the Sicilian haplotypes (2 and 3) were geographically restricted. The most common (haplotype 1) is shared by eight individuals originating from populations in Italy (including Tuscany, Ferrara and Sardinia), Germany (fishery ponds Königswartha), Great Britain (New Forest) and Spain (El Puig, Plat d'Espolla). Haplotype 4 was found in Austria (Morava, Danube) and Sardinia (Italy) and haplotype 5 observed in Austria (Neusiedler Lake), Germany (fishery pond Lacoma) and Hungary (Apaj).

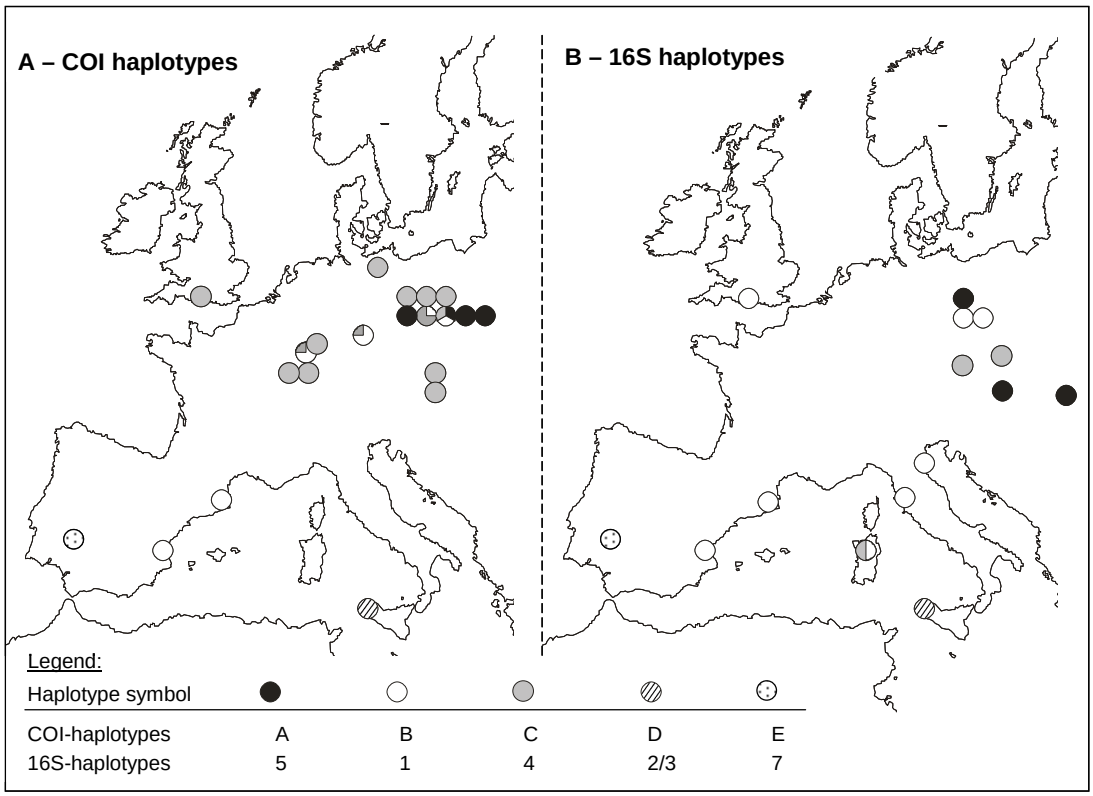

Figure 4-6: Comparison of COI and 16S *Triops cancriformis* haplotypes detected in the present study.

4.4 Discussion

4.4.1 Systematics of Notostraca and taxonomy of the genus Triops

The traditionally accepted subdivision of *T. cancriformis* into three subspecies *sensu* Longhurst (Longhurst, 1955a) is not supported by the presented data. First, the identification of genetic differences in the dataset that would justify a subspecies status for *T. c. cancriformis* and *T. c. simplex* failed. This is in line with the observation that there are no consistent morphological differences between populations from both subspecies (compare chapter 3). Therefore it can be proposed that the subspecies *T. c. simplex* and *T. c. cancriformis* have to be revised. Second, a large sequence divergence between *T. c. mauritanicus* and *T. cancriformis* for both COI (17.79%) and 16S (3.94%), indicating that both lineages have had long separate evolutionary histories was identified. In fact, the level of sequence divergence for COI is of a magnitude found in interspecific comparisons in Crustacea (Costa et al., 2005). This and the morphological divergence of *T. c. mauritanicus* (significant differences in the dorsal carina spines at 95% confidence interval, compare chapter 3) suggests that the original species status given by Ghigi (1921) should be reinstated. *T. cancriformis* and *T. mauritanicus* form a strongly supported monophyletic clade within the genus and therefore can be considered sister taxa.

The data further indicate that the genus *Triops* is monophyletic with respect to other Notostracan taxa. Therefore a split of *Triops* as suggested by Murugan et al. (2002) and Mantovani et al. (2004) does not appear justified on phylogenetic grounds. The analysis of COI data suggests that *T. granarius* is the sister taxon to the other *Triops* species (Figure 4-4). Furthermore, although *T. cancriformis, T. australiensis* and *T. longicaudatus* appear as separate clades, COI lacked resolution to clarify their relationships. In contrast, the 16S analysis resolves well the phylogenetic relationship within the genus. Indeed, there is a strongly supported sister relationship between *T. australiensis* and *T. longicaudatus*, with the clade formed by both taxa appearing as sisters to *T. granarius*. *T. cancriformis* forms a well supported sister clade to the other three *Triops* species (Figure 4-5). In addition, the 16S analysis strongly supports the monophyletic status of *Triops* and of

Lepidurus, respectively. The discrepant findings for *T. granarius* in COI *versus* 16S genes might be due to different origin of the specimen (COI from Namibia, 16S from Japan). However, more data on intraspecific differentiation within *T. granarius* are needed before final conclusions can be drawn.

Our phylogenetic reconstruction of the evolution of Notostraca allows us to make some inferences on the evolution of the reproductive strategy in this group. *T. (cancriformis) mauritanicus*, *T. australiensis* and *T. granarius* are considered to be bisexual, with separate males and females and generally balanced sex ratios (Sassaman, 1991; Machado et al., 1999b). In contrast, reproductive mode variation including bisexuality, unisexuality, androdioecy or a combination of them has been found in *T. cancriformis (cancriformis/simplex), T. newberryi* and *T. longicaudatus,* and in some *Lepidurus* species (*L. apus apus*, *L. arcticus*) (Sassaman, 1991). The phylogenetic analysis revealed that species showing reproductive strategy variation did not form a monophyletic lineage (Figure 4-7). If sexual reproduction with separate males and females was ancestral state in *Triops*, alternative reproductive strategies would need to have evolved separately at least twice and, in the case of *T. cancriformis*, relatively recently. Sexual reproduction with separate males and females has been shown to be the ancestral state in other organisms with androdioecy (*Limnadia*, *Caenorhabditis* and several plants) (Weeks & Sassaman, 1990; Pannel, 2002; Kiontke et al., 2004). However, the similarity in the mechanism of androdioecy in both species of *Triops* and *Lepidurus*, with the presence of testis lobes scattered in the ovary of otherwise female individuals (Longhurst, 1954) is remarkable.

A possible explanation for the independent evolution of alternative reproductive modes in the Notostraca may lay in the colonisation advantage afforded by unisexuality. In fact, the four species with androdioecious and unisexual populations occur in Northern hemisphere latitudes where populations are likely to have suffered population range contractions and expansions during the Pleistocene. Such repeated range expansions might have provided a strong selective advantage during colonisation for unisexual reproductive strategies. In conclusion notostracans have a remarkable plasticity regarding reproductive strategy. Further research is required to reveal whether this reproductive plasticity provides an explanation for the extraordinary persistence of this group of organisms.

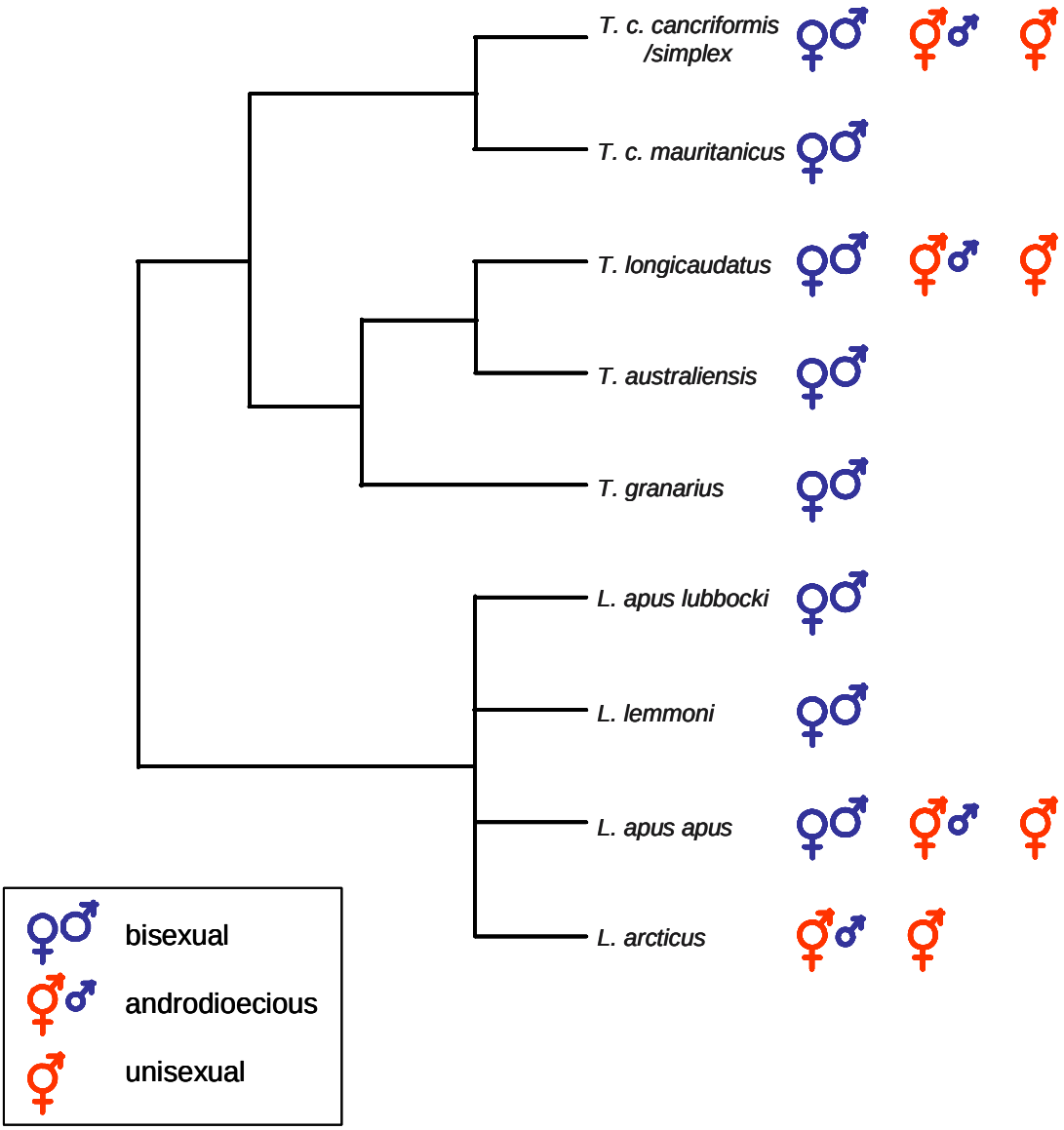

Figure 4-7: Phylogram showing the phylogenetic relationships of Notostraca species and their reproductive mode (see inset) (phylogenetic relationships are derived from 16S data)

4.4.2 Phylogeography of *Triops cancriformis* and mode of reproduction

Both 16S and CO1 data showed that the *T.* (*c.*) *mauritanicus* samples from south-west Spain represented a highly divergent lineage compared to the remaining *T. cancriformis* sequences. Although the exact timing of the split is tentative since the calibration assumed a general molecular clock within Crustaceans, it clearly predates the Pleistocene and possibly the Pliocene. The most salient geological event in the European continent during this time is the Messinian salinity crisis (ca. 6 mya) in the transition between both Miocene and Pliocene. At this time the Mediterranean

suffered complete or nearly complete desiccation due to a combination of low precipitation and isolation of the Mediterranean and Atlantic Ocean due to tectonic changes in the Gibraltar straits (Krijgsman et al., 1999). Range contraction and subsequent isolation of *Triops* lineages at this time might be most likely due to cold/dry conditions or hot/very humid conditions. Such a deep phylogenetic split within the Iberian Peninsula has also been observed in other taxa e.g. toad (*Bombina bombina*) and hedgehog (*Erinaceus europeus/concolor*) (Hewitt, 1996), grasshopper (*Chorthippus parallelus*) (Cooper et al., 1995) as well as in the bear (*Ursos arctos*) (Beebee & Rowe, 2004), but also for plants e.g. European oak (Bennet et al., 1991; Ferris et al., 1998; Hewitt, 1999). It indicates either the existence of several independent refugia across the Iberian Peninsula (Gómez & Lunt, 2006) or colonisation from North Africa (Martínez-Solana, 2004; Veith et al., 2004). For European recolonization several refugia are of particular, ie. interest Iberia, Italy, the Balkans and Caucasus where the climate was relatively buffered against the glacial cycles (Hewitt, 2000; Beebee & Rowe, 2004). After the ice sheets retreated, populations at the northern limits of the refugial range expanded into often large areas of suitable territory (leading edge expansion) (Hewitt, 2000).

Haplotype diversity and sequence divergence were generally low within *T. c. cancriformis/simplex*, but about 2.5 times higher within the COI data compared to the 16S rDNA data. Such low sequence and haplotype diversity across a wide geographic range suggests that the species has colonised most of Europe very recently. Indeed, according to our molecular clock estimations the age of the *T. c. cancriformis/simplex* clade is consistent with European colonisation in an interglacial of the first half of the Pleistocene. The question of the origin of the species in Europe cannot be resolved until a more comprehensive sampling across the range is obtained.

Since 16S sequence divergence within *T. c. cancriformis/simplex lineage* was low and sample size insufficient for phylogeographic interpretation the following discussion is mainly based on the COI data. Although only four COI haplotypes where found across the *T. c. cancriformis/simplex* lineage, their distribution showed some geographical associations. The most divergent haplotype (A) is restricted to the most Eastern sites and co-occurs with other haplotypes in the German fishery ponds. This could either represent ancient polymorphism – or more likely – secondary

(postglacial) contact between isolated lineages from different isolated refugia as seen in many other European taxa in this geographical area (Hewitt, 1996). Analyses of populations from the Balcans/Middle East are required to resolve this issue.

The two most widely distributed COI *T. cancriformis* haplotypes (B and C) are closely related, differing by only one mutation. Populations containing these haplotypes show either equal sex ratios (eastern Spain) or contain low male proportions with evidence for unisexual reproduction (Longhurst, 1954) (Central and northern Europe). In addition, haplotype C is the most widespread, suggesting that it was linked to a range expansion involving female-biased or unisexual populations. Based on the data it may be suggested that such female biased populations were established recently from a single refuge which could have been located in the western or Central Mediterranean. Genetic investigations on additional samples from the Iberian Peninsula, Italian Peninsula and from southern France will be needed to resolve this issue. Indeed, such a scenario would fit to the one described by Longhurst (1955a) who suggested that during the Pleistocene glaciations habitats suitable for *Triops*, such as ephemeral ponds, were present in southern parts of Europe only and northern and Central European populations should be descendants of unisexual lineages due to their colonisation advantage.

Conclusion

The present paper provides the first phylogeographic analysis of *T. cancriformis* in Europe. The data strongly support the specific status of the subspecies *T. mauritanicus*; a bisexual taxon restricted to south-western Spain and Morocco so far regarded as a subspecies of *T. cancriformis*. Molecular clock calibrations put the split between both lineages in the Miocene. Regarding the lineage *T. cancriformis*, so far separated into two subspecies *T. c. cancriformis* and T. c. s*implex*, which retains a mixed reproductive mode, a very low level of diversity is found consistent with a relatively recent age in Europe, around the first half of the Pleistocene. The outlined review of reproductive strategy indicates widespread distribution of female-biased populations. The female biased reproductive mode seems linked to a recent range expansion in Central and northern Europe. Our data clarifies the phylogenetic relationships of the genus *Triops* and suggests that reproductive mode variation has arisen several times in the evolution of this taxon. This makes *T. cancriformis* a good model to investigate the evolution of the mixed reproductive strategies.

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[in the paperback version you will find the appendix with COI- and 16S- haplotype sequences on the following pages]

Chapter 5

Microsatellite isolation in *Triops cancriformis* (Crustacea: Notostraca)*

"Microsatellite loci are numerous, highly variable,
well distributed through the genome,
give high quality data, and are easy to use.
Is there a catch? Unfortunately, yes.
Before one can use microsatellite loci, one has to find them."
(Queller et al., 1993)

Abstract

Simple and compound dinucleotide microsatellite motifs were isolated from *Triops cancriformis* template DNA with the method of selective hybridisation. However, application and redesign of microsatellite primers did not result in an applicable polymorphic microsatellite marker.

* The isolated microsatellite motifs have been published in Zierold, T. (2004) Winners of the flood - Ecology and genetics of Large Branchiopods. Ecdysiast **23**: 21-22.

5.1 Introduction

Evolutionary studies and also population genetics benefit greatly from the availability of genetic markers capable of revealing population structure, migration events, parentage and other genetic relationships among individuals (Weber, 1990; National Research Council, 1992; FitzSimmons et al., 1995; Blouin, 2003; Brumfield et al., 2003).

To date, a number of different techniques have been applied to the task of establishing genetic relatedness. One of the oldest techniques is the establishment of pedigrees based on inferring parentage from behavioural observations. This method may not detect surreptitious mating or even different reproductive modes including parthenogenesis and/or hermaphroditism as they are typical in large branchiopods. Later allozyme studies were applied to investigate genetic differentiation. Studies from arthropods (Anostraca, Daphnia) show that allozyme markers are appropriate for species differentiation (Richardson et al., 1986; Abatzopoulos et al., 2002; Adamowicz et al., 2004), but there is usually too little variation to estimate relatedness within-group or for parentage analysis (Queller et al., 1993; Strassmann et al., 1996; Limburg, 2000). DNA fingerprinting has proved to be a reliable technique for parentage exclusion within families and groups, but it is not appropriate for examining larger groups or across groups because of the high variability of minisatellite loci and the difficulties associated with comparisons between gels. However, only a small proportion of minisatellites are amenable to amplification by PCR, usually because the size of the tandem repeat exceeds PCR limitations. In contrast, essentially all microsatellite loci are amplifiable by PCR. Microsatellite markers come remarkably close to fitting in the model of an ideal marker for intraspecific relatedness estimations and parentage assignment, as they are codominant, selectively neutral and highly polymorph (Litt & Luty, 1989; Schlötterer & Pemberton, 1998; Goldstein & Schlötterer, 1999).

Prior to using microsatellite markers, they must be identified and characterised in the taxonomic group of interest. It is unlikely that known primers amplify the same locus (region within the genome) across related taxa unless the microsatellite region is flanked by highly conserved sequences where primer sites are

located (Goldstein & Schlötterer, 1999). In practice, a quite surprisingly useful number of primers work between species in different families as documented in songbirds (McDonald & Potts, 1994), marine turtles (FitzSimmons *et al.*, 1997), whales (Schlötterer *et al.*, 1991) and harbour seals (Coltman *et al.*, 1996) and also Daphnia (Colbourne et al., 2004). However, microsatellite primers established for several *Daphnia* species (Dpu7, Dpu16, Dma3/2, Dma11), which are closely related to Notostraca, did not work successfully in preliminary studies and no microsatellite primers for the species of interest were known at the time of starting this work. Thus, microsatellite primers had to be developed '*de novo*' for the species of interest to evaluate differentiation and substructure between geographically close and distant populations and to analyse the reproductive strategy present in unisexual *Triops cancriformis* populations (see chapter 6).

In this chapter the process of microsatellite isolation, primer development and optimization will be described using *T. cancriformis* (Branchiopoda) as study species.

5.2 Material and methods

The method applied for microsatellite isolation in *T. cancriformis* was based on protocols described by Glenn et al. (2000) and Zane et al. (2002).

The principle of microsatellite isolation illustrated in Figure 5-1 can be separated into (1) DNA fragmentation using a restriction enzyme, (2-3) adapter ligation and DNA amplification, (4) selective hybridisation with biotin-labelled microsatellite probes, (5-6) separation of microsatellite-rich DNA from non-microsatellite DNA and amplification of microsatellite rich DNA, (7) ligation of microsatellite fragments into a plasmid vector and transformation of bacterial cells with ligation product, (8) screening for positive clones and plasmid preparation from positive clones, (9) sequencing and finally (10) primer design and optimization. Detailed methodological information for these single steps of microsatellite isolation will be described as follows.

5.2.1 DNA fragmentation

About one or two phyllopodial limbs per individual were used as a source of DNA for extraction with a commercial kit (Machery & Nagel, Düren; NucleoSpin Tissue, protocol 4). The DNA concentration was measured applying TBS-380 Minifluorometer (Biocompare) using PicoGreen reagent. Extracted DNA from *T. cancriformis* specimens from Königswartha (Germany) was further applied for microsatellite isolation. The enzymatic DNA fragmentation was performed including 16 µl of the basis DNA from *T. cancriformis* (90 µg/ml), 20 U *Mbo*I (Fermentas) and 2 µl of 10x reaction buffer (R+; Fermentas). After vortexing the reaction mix was incubated on a thermoblock for 4 h at 37 °C. That was followed by enzyme inactivation (65°C for 20 minutes) and purification of the reaction mix with an Easy Pure kit (Biozym Diagnostik, Oldendorf).

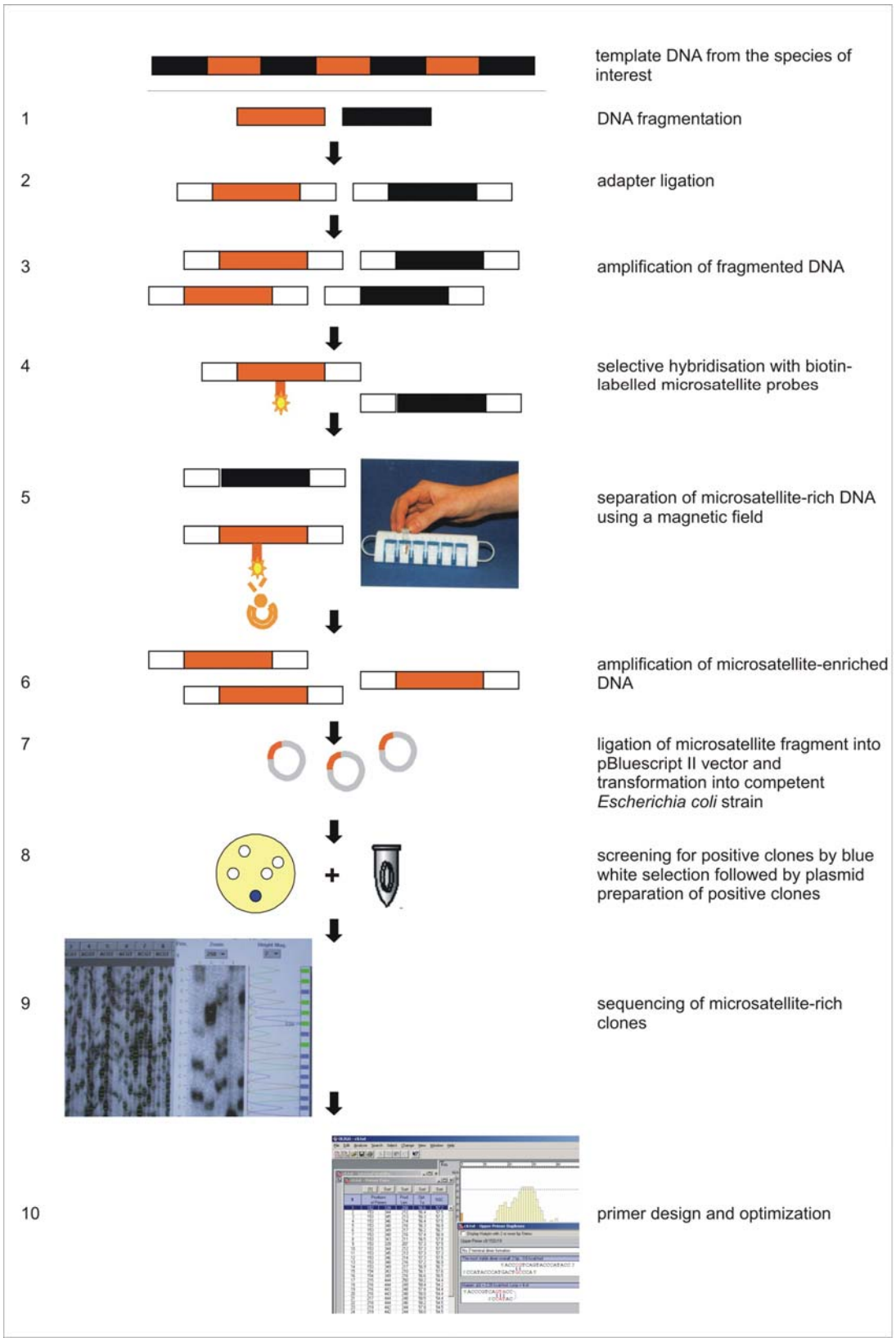

Figure 5-1: Process of microsatellite isolation using selective hybridisation (Zierold, modified after Glenn et al. (2000) and Zane et al. (2002))

5.2.2 Adapter ligation and DNA amplification

The fractionated genomic DNA was linked to an adapter using the GATC cohesive ends produced by *Mbo*I digestion. The adapter was produced by hybridisation of phosphorylated Sau3AantiT7 (5'-GAT CCT ATA GTG AGT CGT ATT A-3') and T7-oligonucleotides (5'-TAA TAC GAC TCA CTA TAG-3').

The phosphorylisation reaction, composed of 10 µM Sau3AantiT7, 1 U Kinase (Fermentas) and 1.2 µl of appropriate Kinase buffer (Fermenas), was incubated for 1 h at 37°C. The hybridisation of T7-oligonucleotides and Sau3AantiT7 was performed in a total volume of 25 µl containing 4 µM Sau3AantiT7 (phosphorylised), 4 µM T7 oligonucleotide, 1 M NaCl, 100 mM Tris pH 7.6 and 1 mM EDTA. The hybridisation reaction mix was placed on a thermoblock running a program from 20°C to 80°C and back within 1 hour. Thereafter the adapter could be used for ligation to the genomic DNA.

The adapter ligation was performed in a total volume of 25 µl containing 19 µl fragmented *T. cancriformis* DNA (about 70-90 µg/ml), 0.8 µM of the adapter hybrid and 1 U T4-Ligase. The vortexed reaction mix was incubated over night at 17°C. To increase the amount of potential microsatellite DNA the complex of adapter ligated with genomic DNA was amplified with T7 olignucleotide (T7-primer 5'-TAA TAC GAC TCA CTA TAG-3'). The amplification was performed in 50 µl final volume containing 2.5 µl template DNA (adapter-ligated-DNA), 0.5 µM T7-primer and 45 µl 1,1xPCR Master Mix (ABgene, Epsom, Surey). Reaction mixes were amplified using the following cycling conditions: 2.5 minutes denaturation at 95°C, 55°C for 30 seconds and 72°C for 5 min (one cycle), followed by 34 cycles of 30 seconds denaturation at 95°C, 55°C for 30 seconds, after the cycles the reaction was incubated for final step of 72°C extension for 5 minutes. The success of the PCR (product I) was controlled on a 1.5% agarose gel. If a broad smear was visible on the stained gel, the next step – selective enrichment of microsatellite DNA – followed.

5.2.3 Selective hybridisation with biotin labelled probes

For the selective hybridisation step, biotin-labelled synthetic dinucleotide microsatellite probe targets ($(AC)_{10}$ and $(GA)_{10}$) were used. These microsatellite motifs are referred to be common in Crustacea (Zane *et al.*, 2002). An aliquot of 25

µl of the PCR product I was denaturated at 95 °C for 5 minutes followed by the addition of 0.5 µM biotinylated microsatellite probe targets. Finally, 6x sodium chloride-sodium citrate hybridisation buffer (SSC) was added to a final concentration of 5.1x. The hybridisation reaction was performed first as referred to by Glenn et al. (2000) and Strassmann et al. (1996) for 30 minutes at 66°C in a hybridisation oven. However, the hybridisation conditions suggested by Lieckfeldt et al. (2001) turned out to be more efficient. Thus, the hybridisation later was performed as follows: The hybridisation reaction was first mixed including PCR product I, 6xSSC and microsatellite target probes. After a denaturation step of only 1 minute at 95°C a two minute incubation at 66°C followed and then the reaction was immediately placed on ice. After this step the biotinylated probe should be hybridized onto complementary DNA fragments containing microsatellites.

5.2.4 Separation of microsatellite-rich DNA and amplification

Biotinylted oligonucleotides and their hybrids were collected on Streptavidin coated magnetic beads (Dynabeads M-280, Dynal Biotech ASA, Oslo) following the manufacturer protocols. After that the complex of streptavidin coated magnetic beads, biotin-labelled microsatellite target probe and enriched DNA fragment containing the complementary microsatellite motive was collected from the reaction mixture using a batch magnetic separator. Thereafter the remaining solution was removed carefully without taking any of the beads. DNA bound unspecifically was washed off the magnetic beads by washing twice with 2xSSC at room temperature and four times at 65°C for about 30 seconds for each washing step. The separation of washing solution and magnetic beads was performed on the batch magnetic separator (Dynal Biotech, Norway). The bond between probe and DNA was solved by adding 100 µl of 200 mM sodium hydroxide solution and incubating at 37°C for a maximum of 15 minutes. After that the sample was neutralized by adding 36 µl of 800 mM hydrochloride acid and 10 µl of 1 M Tris solution (pH 8). Finally the microsatellite-enriched DNA was separated from the Dynabeads on the batch magnetic separator and pipetted into a new tube.

The extracted microsatellite DNA was amplified using the conditions mentioned above for unspecific DNA amplification. The resulting PCR product (II) was separated on a 1.5% agarose gel to check the success of the isolation procedure.

5.2.5 Ligation and transformation

The PCR product (II) was purified by precipitation with ammonium acetate and adapters were removed by *Mbo*I digestion. The resulting microsatellite DNA-fragments were ligated into a *Bam*HI digested and dephosphorylated pBluescript II (KS+) vector and finally transformed into competent *Escherichia coli* DH5α cells according to the protocol of Sambrook & Russell (2002).

5.2.6 Screening for positive clones

Blue-white detection (based on X-Gal and IPTG) was applied for isolation of resulting clones, where white clones represent cells with inserts. For that purpose two Petri dishes (LB-agar with 50 µg/ml Ampicillin) were prepared by taping a paper matrix defining a grid of 200 squares under each of them. After that each white colony was replated with sterile toothpicks onto the same grid cell of the two prepared Petri dishes. The colonies were allowed to grow over night at 37°C. One series of dishes was used to lift colonies onto hybridisation membranes (Nylon – plus, Roth); the other series was kept at 4°C and used later to inoculate new cultures for plasmid isolation (Minipreps). The Nylon membrane was later subjected to hybridisation with microsatellite probes used for enrichment before (Hoelzel, 2002).

5.2.7 Plasmid preparation and sequencing

Colonies that showed a strong hybridization signal when probing with AC-repeat arrays were used for minipreps (Flexiprep-Kit, Pharmacia-Biotech). The cycle sequencing with an IRDye 800 labelled T7 and T3 primers were performed separately for each of the four bases following the instruction provided by MBI Fermentas, (CycleReader Auto DNA Sequencing Kit #K1721; Quick Protocol QP01). The sequencing reaction was loaded with 1 µl of the sequencing amplican on a sequencing gel of a LI-COR Sequencer (Model 4200 – 11G). The obtained

sequences were checked by eye for correct identification using the provided sequencing software e-Seq (LI-COR, Version of 1999). The alignment of forward and reverse sequences was performed in SeqManII 4.03 (DNASTAR Inc. 1999).

5.2.8 Microsatellite primer design and -optimization

Pairs of primers were designed for sequences containing microsatellite sequences using Primer3 web based software (Rozen & Skaletsky, 1996) and Oligo6 Primer Analysis Software – Version 6.67 (Wojciech & Piotr Rychlik, 1989-2003 Wojciech Rychlik). For the primer design suggestions referred to by Coyne et al. (2001), Lunt et al. (1999) and Newton & Graham (1994) were taken into account. Designed primers were first ordered unlabeled from Sigma-Aldrich-Chemie GmbH and metabion Gesellschaft für angewandte Biotechnologie mbH. Fluorescently labelled primers for analyses of polymorphism with LI-COR (TU Bergakademie Freibrg) or ALF Express™ (University of Hull) were ordered from Pharmacia Biotech. PCR reaction conditions were optimized for the primer pairs, and the markers were tested for levels of polymorphism and ease of scoring. Therefore, genomic DNA from *T. cancriformis* specimens and the plasmid DNA (named 'basis DNA') were amplified under a variety of PCR conditions to specify the optimum, where a clear band of the expected size was visible on a 1.5% agarose gel. The optimization of PCR conditions focused on annealing temperature (test within a range from 50 to 70°C) and magnesium chloride concentration (reaching from 1.0 mM to 2.5 mM). In some cases a touchdown PCR was performed to eliminate unspecific PCR products. The amplification conditions for the latter amplifications are summarized in Table 5-1.

Table 5-1: Touchdown PCR conditions for microsatellite amplification.

Step	Description	
1	94°C 3 minutes	
2	94°C	30 seconds
3	55°C	20 seconds
4	72°C	20 seconds
5	go to step 2 for 2 more times	
6	92°C	30 seconds
7	53°C	20 seconds
8	72°C	20 seconds
9	go to step 6 for 2 more times	
10	92°C	30 seconds
11	51°C	20 seconds
12	72°C	20 seconds
13	go to step 10 for 2 more times	
14	92°C	30 seconds
15	49°C	20 seconds
16	72°C	20 seconds
17	go to step 14 for 2 more times	
18	92°C	30 seconds
19	48°C	20 seconds
20	72°C	20 seconds
21	go to step 18 for 22 more times	
22	72°C 5 minutes	
END		

Several test individuals (*T. cancriformis* from different populations) were investigated in microsatellite amplification to test the degree of polymorphism of the developed microsatellite primers. The amplification was performed in a total volume of 10 µl, containing 1x NH_4 buffer (Bioline), 0.2 mM of each dNTP, 1.5 mM magnesium chloride, 0.2 mM of each microsatellite primer (forward and reverse), 0.03 U Taq Polymerase (Bioline) and 1 µl template DNA (average concentration 1.5±0.8 µg/ml). The reaction mix underwent the following cycling conditions: 4 minutes initial denaturation at 94°C, followed by 39 cycles of 15 seconds denaturation at 94°C, primer-specific annealing temperature (ranging from 50°C to 67°C) for 20 seconds and 72°C for 40 seconds, concluded with 10 minutes final extension at 72°C. The success of the PCR was controlled on 3% agarose gels. The agarose gels were further used to check the amplified fragments for microsatellite DNA via Southern blot (Hillis et al., 1996). The membrane was then hybridized with microsatellite probes and detected by colour precipitation (Eisel *et al.*, 2000). PCR fragments with positive hybridization signals were subjected to further analysis.

5.2.9 Microsatellite separation on Polyacrylamide gels

5.2.9.1 LI-COR System

Gel-apparatus

Genotyping was performed on an automated LI-COR Sequencer (Model 4200 – 11G) with one colour laser detection system (IRDye800) using 41 cm glass plates and trails for polyacrylamide gel preparation (Figure 5-2).

Figure 5-2: Gel-apparatus for genotyping on the automated LI-COR Sequencer (Model 4200 - 11G). Two 41 cm long glass plates, separated by 0.2 mm spacer were overlaid and clamped into two appropriate rails. Finally this cassette was inserted into the sequencer.

Gel composition

A 5.2% polyacrylamide gel was applied to achieve high resolution of the microsatellite fragments. The gel was composed of 12.73 g urea, 16.97 ml distilled water, 3.03 ml 10x Tris-Borate-EDTA buffer (TBE), 45.5 µl Tetramethylethylenediamine (TEMED, National Diagnostics), 212.12 µl 10% ammonium persulfate and of RapidGel XL Sol 40% (Amersham-Pharmacia) to a final polyacrylamid concentration in the mixture of 12%.

Run conditions

For the 41 cm class cassette and the described gel composition the genotyping run was performed under following conditions: scanning speed level 4, voltage 2000 V, current 35 mA, power 45 W, temperature 45°C, run time 4.5 hours. The image produced by the genotyping was exported to appropriate analysis software for fragment analysis (GeneSnap).

Size standards

To determine the fragment size, an external standard (LI-COR 4000-44B) was loaded every fifth lane. The standard fragments covered a size range of 50 to 350 bp.

Software

No specific LI-COR software tools were available for fragment analysis. Therefore TotalLab (phoretix, Version 1.11) and the shareware program image3 (http://www.sanger.ac.uk/Software/Image/) were applied.

5.2.9.2 ALF System

As optimization and analysis of microsatellite markers were not successful the optimization procedure, especially the analysis of the degree of polymorphism of the primer pairs, continued in the University of Hull, where an ALF Express instrument was used.

Gel apparatus and preparation for genotyping

The ALF cassettes were used for preparing the polyacrylamide gel. The glass plates were first washed with distilled water and afterwards rinsed with propanol and wiped dry. Before assembling the cassette the upper 2 cm of the back-side glass were carefully coated with bindsilane solution (25µl gamma-Methacryloxypropyl-trimethoxysilane, 2.5 ml 96% ethanol, 90 µl distilled water, 10 µl 100% acetic acid) to obtain stable wells. Finally the rectangle comb was inserted and thus the cassette was prepared for gel insertion.

Gel composition

The gel preparation was performed with SequaGel XR (EC-842; National Diagnostics). The gel was composed of 28 ml of SequaGel Monomer Solution, 7 ml of SequaGel Complete Buffer and 280 µl of 10% ammoniumpersulfate. The gel was allowed to polymerize for two hours at room conditions or for half an hour placing the cassette for 10 minutes under UV light.

Run-conditions

To screen microsatellite fragments of 100 to 500 bp length, the run conditions on the ALF Express were set as follows: run time 100 minutes, voltage 1900 V, current 65 mA, power 44 W and temperature 55°C.

Size standards

Determination of the fragment size was based on internal size standards, which were added to each sample before loading on the polyacrylamide gel. The size markers were based on plasmid DNA of M13MP18 (provided by the University of Hull). The procedure of producing size markers comprised three steps: First template production for the size markers, second cleaning up the PCR product, and finally production of labelled markers.

The template for size markers was obtained by amplification of M13MP18 template with size-specific M13 primers provided by the Molecular Ecology and Fisheries genetic laboratory of the University of Hull. The master mix of 150 µl for the amplification contained 1x KCl Buffer (Bioline), 0.2 mM of each dNTP, 0.5 mM of unlabelled universal M13 primer, 0.02 U Taq Polymerase (Bioline) and 1.5 µl M13MP18 template DNA. The master mix solution was aliquoted into 24 µl and finally 1.25 µl of the specific size-marker primer were added per tube. These reaction mixes were amplified using the following cycling conditions: 2 minutes initial denaturation at 94°C, followed by 34 cycles of 30 seconds denaturation at 94°C, primer-specific annealing temperature (60°C for size-marker primers 67, 259, 312 and 353; 55°C for size-marker primer 100, 154 and 200) for 30 seconds and 72°C for 75 seconds, finished by a final step of 30°C for 2 minutes.

The PCR products were loaded onto a thick 2% agarose gel, using 1x Tris-Acetate-EDTA buffer (TAE) and large-toothed combs. A 100-base-pair ladder was loaded as well to ensure that the products were of correct size. To obtain a good separation between the product and any non-specific products electrophoresis was set to low voltage (35 V) and the run was performed over maximum distance. After staining the gel with ethidium bromide in TAE buffer, the products of appropriate size were cut out on a UV transilluminator (Figure 5-3). The gel slices containing the PCR product were transferred into the top of a Greiner P20 filter tip, which had been

cut down to the size of a 1.5 ml Eppendorf tube. The products in the filter tubes inside the Eppendorf tubes were centrifuged for 5 minutes at maximum speed. The templates for the size markers were collected at the bottom of the tube leaving the agarose in the filter of the tip. These templates (cleaned first amplification product) were transferred into fresh 0.5 ml tubes and stored at minus 20°C for further application.

At the next and final stage the Cy5 labelling was performed by amplification of the cleaned first amplification product with M13 Cy5 labelled primer (GTAAAACGACGGCCAGT). The mastermix of 400 µl (8x50µl reaction mixes) contained 1x KCl Buffer (Bioline), 0.15 mM of each dNTP, 0.25 mM of labelled universal M13 primer, 0.02 U Taq Polymerase (Bioline) and 4 µl of first amplification product. The master mix solution was aliquoted into 50 µl and specific size-marker primer was added to a final concentration of 0.25 mM per tube. These reaction mixes were amplified using the same cycling conditions as described for the first step of size-marker preparation. The PCR products were finally checked again on a 2% agarose gel in 0.5x Tris-Borate-EDTA buffer (TBE).

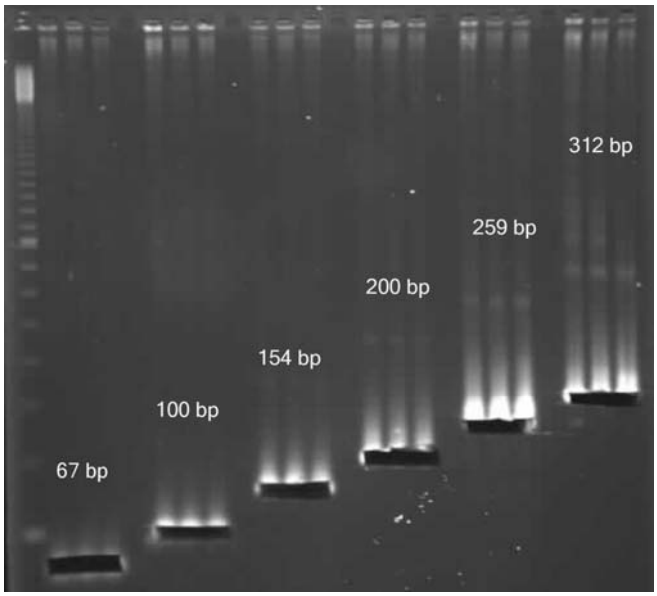

Figure 5-3: Ethidium bromide stained agarose gel after cutting out the size specific PCR fragments.

Software

The fragment analysis was performed with Fragment Analyzer (Pharmacia Biotech version 1.2).

5.3 Results

5.3.1 Sequencing of positive clones

Despite of the enrichment procedure, probing of 215 clones resulted in only 13 positive colonies only. Four of the 'AC' positive clones contained microsatellites longer than 4 repeats (clone T1 to T4). Four of the other prepared plasmids contained no microsatellite motifs, three of them less than four repeat motifs and two of them produced long compound microsatellite that the insert either starts or stops with the microsatellite motif. Thus it was not possible to design primer for those very long microsatellite motifs (Zierold, 2004).

The 383 bp sequence of T1 contained one perfect microsatellite motif of $(GT)_7$ and a very long compound motif $(TG)_4TA(TG)_4TCGG(TG)_{13}TT(GT)_4$ (Figure 5-4).

```
TGGGGGTGCATGCATGCAGCGGGCCGGCATCTCTCTTGGCCAGACTGTCT
TGGTTACGGGTGCAGGGTGTGTGTGTGTGTGTGCGGGGTTGGGGGGGGCATG
TGTGTGTATGTGTGTGTCGGTGTGTGTGTGTGTGTGTGTGTGTGTTGT
GTGTGTATGTGTATATWTGGGGGGGGGGGGGGGTGCATTTTTTTAGTTGG
TTATATGTATGGGTGTACGTTTGGGTGGGGGGKTGGGTGGGGTGGGTGGG
GGGTTTCGGGTATGGTCACGGGGTTGGGTGTGGTGGTTGGGTGGGGATGT
GTGAGTGTTTGTAAGTGCATGTGTTCTGCTTGAACCGGTCTCTGTGGGTG
TAGATGCATGTGGCCTGGTATGTGTCTAGGGC
```

Figure 5-4: Sequence of clone 1 with two microsatellite sequences (highlighted). The template DNA was originated from an individual from Königswartha (Germany) (50 letters per line).

The 885 bp insert of T2 could not be sequenced completely thus subclones were prepared using *Sma*I restriction digest of the basic clone T2. Sequencing and alignment resulted in three microsatellite motifs within clone T2: a long compound and imperfect motif ($(GT)_4(GA)_2GTGAAA(GA)_{10}TTAA(GA)_{17}$), a short perfect microsatellite motif of six AC repeats and a long perfect microsatellite motif of 25 CA repeats (Figure 5-5).

CGGCCGCTCTAGAACTAGTGGATCTGAGAGGGGGGGAGAGGGAGCGAGAG
AGTGTGTGTGAGAGTGAAAGAGAGAGAGAGAGAGAGAGATTAAGAGAGAG
AGAGAGAGAGAGAGAGAGAGAGAGAGAGCAGGAGAGAGGGGGAAAAACGG
ACCCGTCAGTACCCATACCAACAGGACGAGGTTCCTCCCGGGCGGGACTC
ACTACCGGTAACAGTGAGCCCGAAGGAGAGGAGTCACCGGATTCCCTCCT
GATTAACGACCACCACAGCATACCACCAATGCTCGCCCCCCTGACAGACA
CACACACACGAATAGCTACCCTAAACGTCAGAGGGGGGGGATACACAACA
AACGCAGACAGCAAGATACTGCTAGCAGCACACATAGCTAAACGGGAGAG
CATACACGTGCTTATCCTCACCGAAGTTTACACGCACAACCAGTCGTGGA
CACAGCTGGGCCTCACCATCAAACGTGAGACAGGCTATTTCCTAAGAACA
CCCTCCCTGCCAACCACGGCCACYTCACACACACACACACACACACACAC
ACACACACACACACACACACACACACACTCACGGCATCCAACACAACACATC
CCGACACAGATGCACATGCACCACMTYACACCCACACCTACGGCATCTCC
ACACACACCCTCACAAACCCATACATACGGAAGCACATACGAGCACTTAG
TAGGATACACATCGTGGATACCACCACACGCACGACAATACGTCACGTGT
ACGCTMAAGTTGAAAAGCACTCCCTCGTCGTCCCTGCTCAGTACTATAAT
GAGCACCACTACTTTTTCACACACACTAGGGGGCCCGACGTCCTTAAGCT
ATAGTTCGAATAGCTATGGCAGCTGGAGTTTTTCC

Figure 5-5: Three microsatellite sequences detected within the insert of plasmid DNA of clone T2 (highlighted): a long compound and imperfect motif of $(GT)_4(GA)_2GTGAAA(GA)_{10}TTAA(GA)_{17}$, a short perfect microsatellite motif of six AC repeats and a long perfect microsatellite motif of 25 AC repeats (50 letters per line).

The 312 bp insert of clone T3 included an imperfect microsatellite motif of $(CA)_{13}GA(CA)_3$. The microsatellite is placed at the end of the sequence thus the right flanking sequence is restricted to 24 bp (Figure 5-6).

```
ACACACACTTTCTCATCACCACGAGTAATATCATGACTCGTCCCTGCTGC
TCCCTCACGAAAAGTTGAATGCATGTGCACTGCATACGCACGCACACCAC
CATAGGTGCTACACATAGGATGATTCACGAGCATACACGAAGGCATACAT
ACCCAAACACTCCCACACACACCTCTACGGCATCCACACCCACATACACC
ACGTACACGTAGACACAGCCCTACACAACGCAGCCTACGGCACTCACCCA
TATACACACACACACACACACACACACACAGACACACAAAGTTAGAGAGA
CCCACCAGCACC
```

Figure 5-6: Sequencing of clone T3 included a 312 bp long insert with an imperfect microsatellite motif $(CA)_{13}GA(CA)_3$ (50 letters per line).

Sequencing of clone T4 included a 316 bp insert with one perfect microsatellite of 15 AC repeats. The microsatellite sequence was also located at the end of the sequence (Figure 5-7).

```
CCCGGGGGACCACACACACTTTCTCATCACCACGAGTAATATCATGACTC
GTCCCTGCTGCTCCCTCACGAAAAGTTGAATGCATGTGCACTGCATACGC
ACGCACACCACCATAGGTGCTACACATAGGATGATTCACGAGCATACACG
AAGGCATACATACCCAAACACTCCCACACACACCTCTACGGCATCCACAC
CCACATACACCACGTACACGTAGACACAGCCCTACACAACACAACCTACG
GCACTCACCCATATACACACACACACACACACACACACACACACCTTTCT
CCCTCCATATA
```

Figure 5-7: Insert of T4 clone containing a perfect microsatellite of 15 AC repeats.

5.3.2 Primer design and characterization

Primer design was based on the six isolated microsatellite sequences. However, even after magnesium chloride and temperature optimization the amplification of some primers failed or produced unspecific bands. Therefore, various alternative primers for a single repeat unit were designed (compare Table 5-2).

Table 5-2: *Triops cancriformis* microsatellites isolated with selective hybridization using AC repeat motif probe. Microsatellites were defined as sequences containing four or more tandem repeat arrays. Given sequences for repeat unit run in a 5' to 3' direction.

clone origin	microsatellite motif	Primer (v= variant)	Fragment length (bp)	T_A (°C)	5'-3' sequence of forward (fw) and reverse (rv) microsatellite primers
T1	$(TG)_4TA(TG)_4TCGG(TG)_{13}TT(GT)_4$	Tc1-2 v1	202	67	fw: TTG GCC AGA CTG TCT TGG TT rv: CCA ACC CAC CCA CCA C
		Tc1-2 v2	244	55	fw: AGA CTG TCT TGG TTA CGG GTG CAG G rv: CAC CCA ACC CCC TGA CCA CTT A
T2	$(AC)_6$	Tc3-1 v1	190	55	fw: CCT CCT GAT TAA CGA CCA CCA CAG C rv: CGT GTA AAC TTC GGT GAG GAT AAG C
		Tc3-1 v2	215	53	fw: GCC CGA AGG AGA GGA rv: TGT AAA CTT CGG TGA GGA TA
T2	$(GA)_{10}TTAA(GA)_{17}$	Tc3-2 v1	257	60	fw: GGG AGA GGG AGC GAG AG rv: GGG GCG AGC ATT GGT
		Tc3-2 v2	141	55	fw: GGG GAG AGG GAG CGA GAG AGT GTG T rv: GTT GGT ATG GGT ACT GAC GGG TT
		Tc3-4 v1	150	63	fw: AGA GGG AGC GAG AGA GTG TG rv: GGT ACC TCG TCC TGT TGG TA
		Tc3-4 v2	184	58	fw: GGG GGA GAG GGA GCG AGA G rv: CTG TTA CCG GTA GTG AGT CCA
		Tc3-7 v1	197	60	fw: GGG AGA GGG AGC GAG AGA rv: TCT CCT TCG GGC TCA CTG
		Tc3-7 v2	184	60	fw: GGG AGA GGG AGC GAG AGA rv: CTG TTA CCG GTA GTG AGT C
T2	$(CA)_{25}$	Tc3-3	233	64	fw: ACA ACC AGT CGT GGA CAC AG rv: GTT TGT GAG GGT GTG TGT GG
		Tc3-5	245	55	fw: TCC TCA CCG AAG TTT ACA CGCcACA A rv: GGT GTG TGT GGA GAT GCC GTA G
		Tc3-6	288	60	fw: GAC ACA GCT GGG CCT CAC rv: GTC GTG CGT GTG GTG GTA
T3	$(CA)_{13}GA(CA)_3$	Tc4-1	191	55	fw: GAT TCA CGA GCA TAC ACG AAG G rv: GGT GCT GGT GGG TCT CTC TAA CTT T
T4	$(AC)_{15}$	Tc2-3	189	55	fw: ACA TAG GAT GAT TCA CGA GCA TAC A rv: CTA TAT GGA GGG AGA AAG GTG

Primer pair Tc1-2 (alternative 1) did not result in any PCR fragments, whereas the alternative 2 amplified a fragment of the expected size for three out of six samples (see Figure 5-8). Detailed analysis on polyacrylamide gel indicated that almost all samples are homozygote for this locus. Only the sample from Königswartha (Germany) showed a heterozygote-like peak (see Figure 5-9). Furthermore only 2 alleles (226, 230) were found within *T. cancriformis* samples from 7 different European ponds.

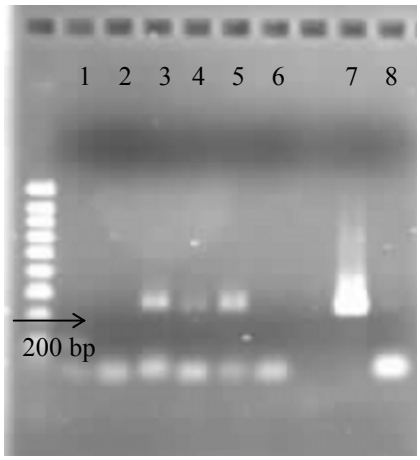

Figure 5-8: Ethidium bromide stained agarose gels loaded with microsatellite PCR fragments amplified with primer Tc1-2 alternative 2. The size marker lane is followed by lane 1 to 6 with *T. cancriformis* samples from different locations (1 = Espolla, Spain; 2 = Neuburg, Germany; 3 = Königswartha Germany; 4 = Godshill, Great Britain; 5 = Neusiedler lake, Austria; 6 = Sicily, Italy), lane 7 represents the PCR product with the 'basis DNA' plasmid and finally lane 8 is the blank control.

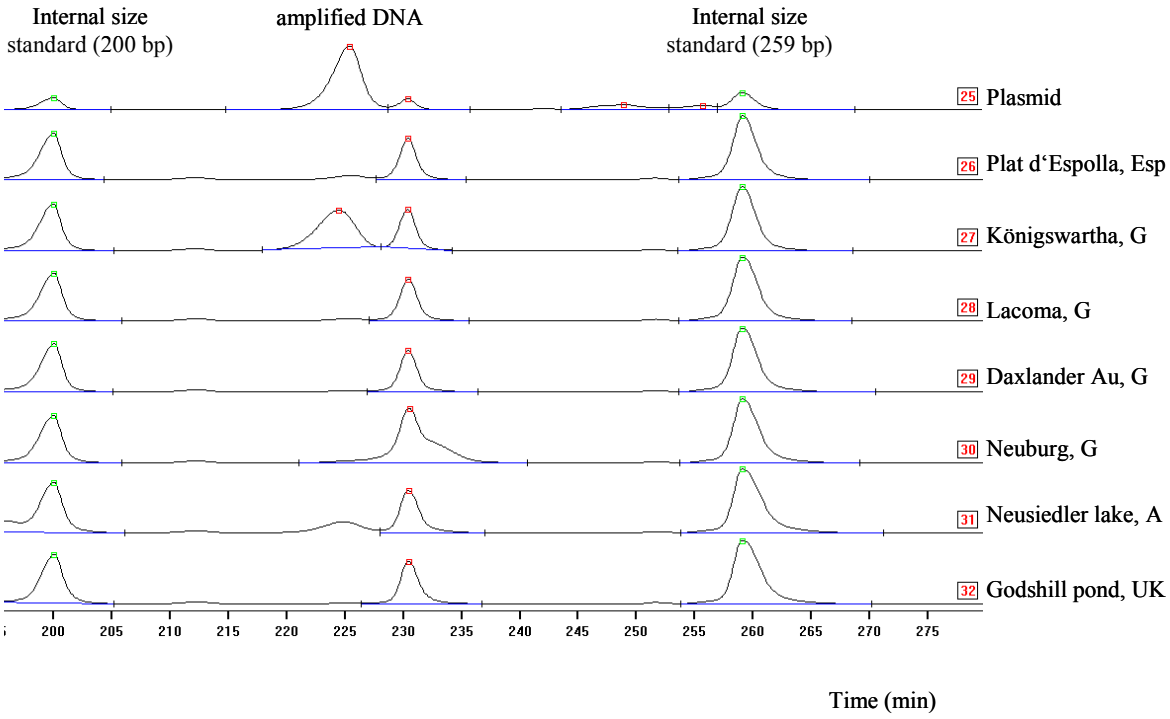

Figure 5-9: Neckline of the locus Tc1-2 analysis on polyacrylamide gel. Red-dotted peaks indicate the amplified fragments; green dotted peaks are internal size standards used for fragment size calibration.

Both variants of primers Tc3-1 resulted in a fragment of the expected size (see Figure 5-10). But analysis of one *T. cancriformis* representative from all sampled locations (compare chapter 6) on polyacrylamide gel (ALF Express^{TM}) resulted in a monomorphic pattern.

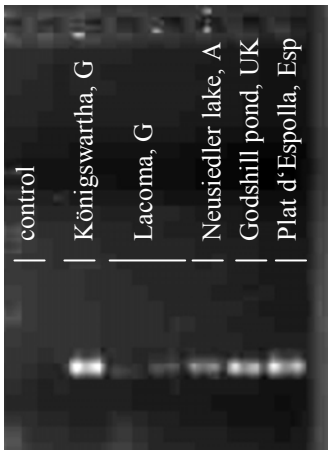

Figure 5-10: Ethidium bromide stained agarose gel loaded with microsatellite PCR fragments amplified with primers Tc3-1 variant 1. All samples resulted in the expected size of 190 bp.

Variant 1 of primer pair Tc3-2 amplified several unspecific fragments. Longer primers (variant 2) resulted in a more stringent amplification product (see Figure 5-11) but the expected size fragment showed only a weak signal compared to the unexpected signal at 400 bp. This longer fragment was not detected in the plasmid amplification product. Neither PCR optimization including increasing temperature and variation of magnesium chloride concentration nor primer-sequence variation resulted in consistent results. Thus this locus was not analysed further.

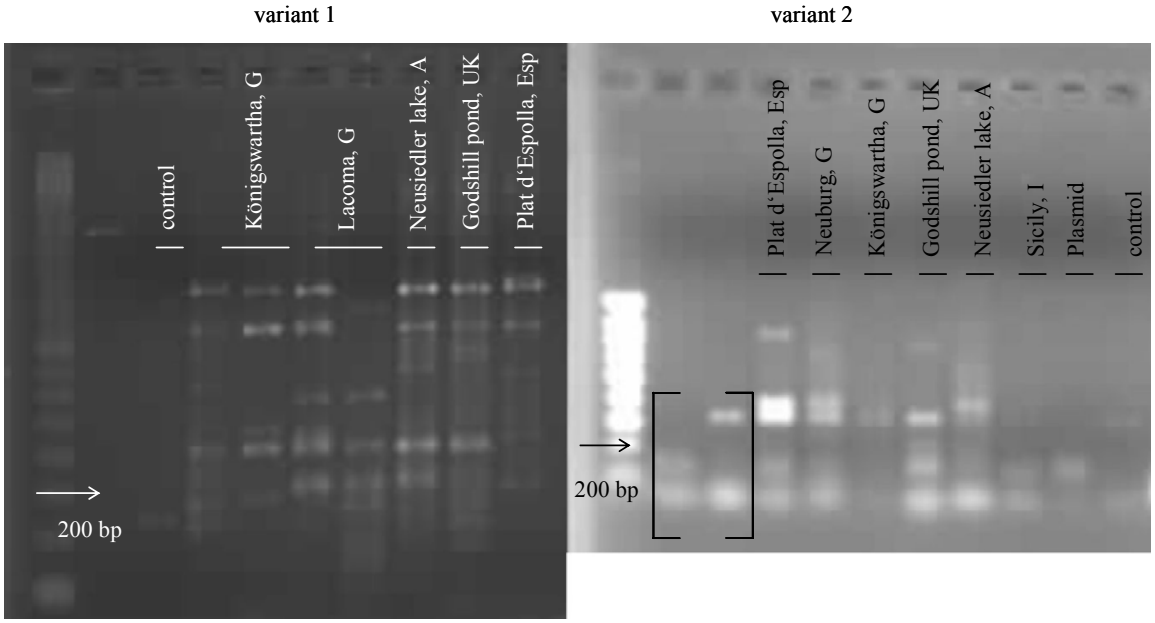

Figure 5-11: Ethidium bromide stained agarose gels loaded with microsatellite PCR fragments amplified with primers Tc3-2 both variants. (Samples in brackets do not belong to loci Tc3-2.)

Both variants of primer pair Tc3-4 amplified a fragment of expected size for the plasmid template, but unspecific fragments for all *T. cancriformis* samples (see Figure 5-12). Magnesium chloride and temperature variation did not result in reduction of unspecific signals. Thus these primers were not appropriate for further analysis.

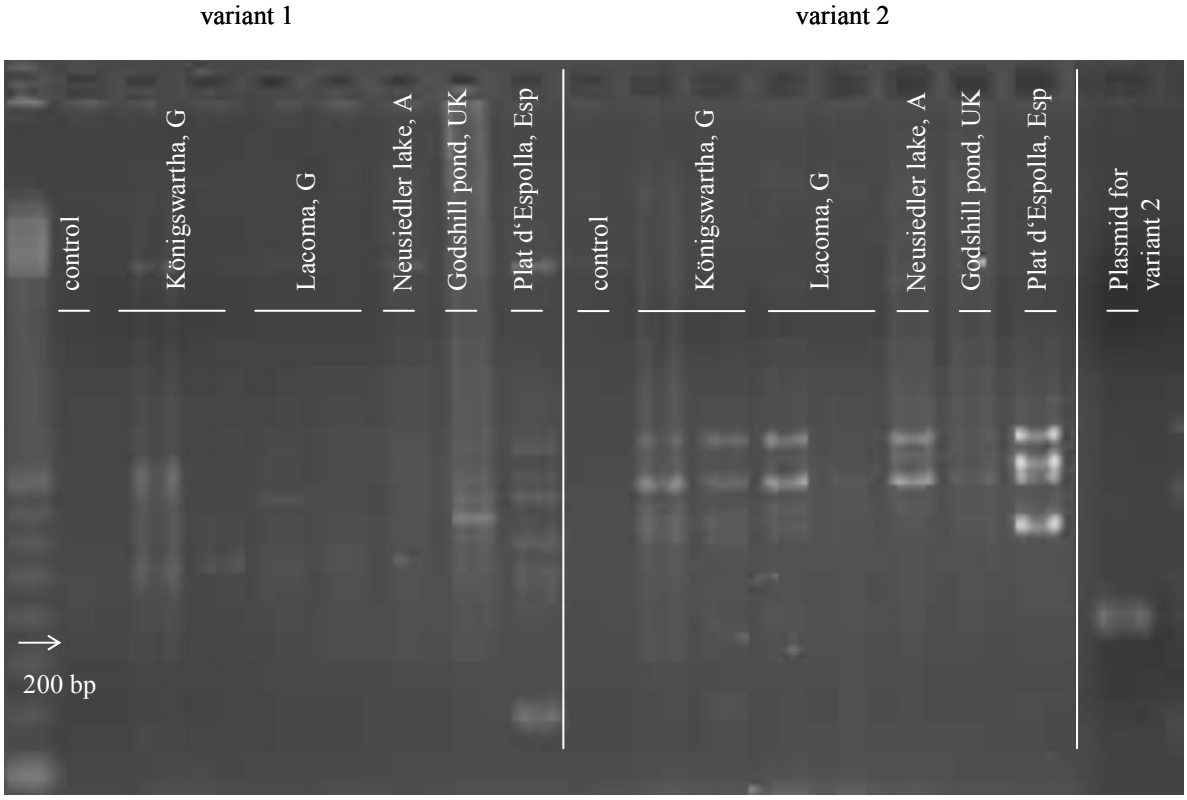

Figure 5-12: Ethidium bromide stained agarose gels loaded with microsatellite PCR fragments amplified with primers Tc3-4 both alternatives.

Both primer variants of Tc3-7 designed to amplify the same microsatellite as Tc3-2 and Tc3-4 produced also unspecific products (see Figure 5-13) and therefore were not applied further.

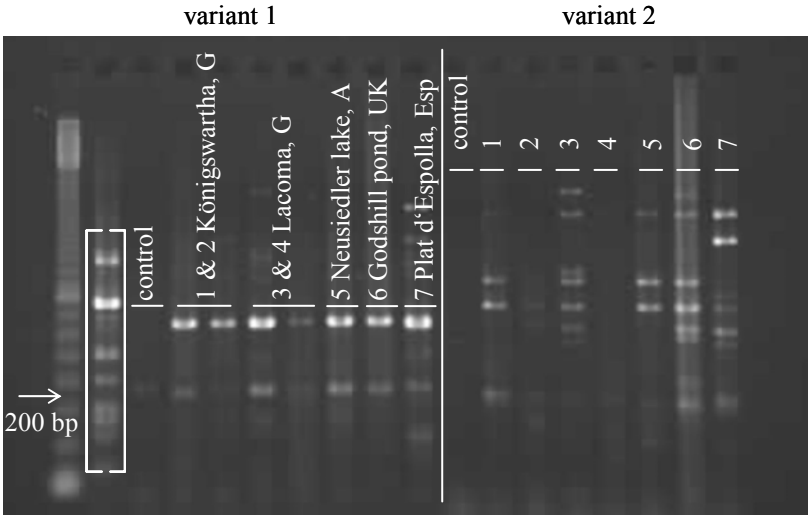

Figure 5-13: Ethidium bromide stained agarose gel loaded with microsatellite PCR fragments amplified with primers Tc3-7 both alternatives. (Sample in brackets does not belong to loci Tc3-7.)

Neither the amplification of *T. cancriformis* samples nor those of the plasmid DNA with microsatellite primers Tc3-3 resulted in a detectable fragment. Unspecific fragments resulted from amplification products with primers Tc3-5 and Tc3-6. Further optimization of PCR conditions did not result in fragments of expected size (see Figure 5-14 for Tc3-6). Thus also these primers amplifying the simple $(CA)_{25}$ microsatellite failed.

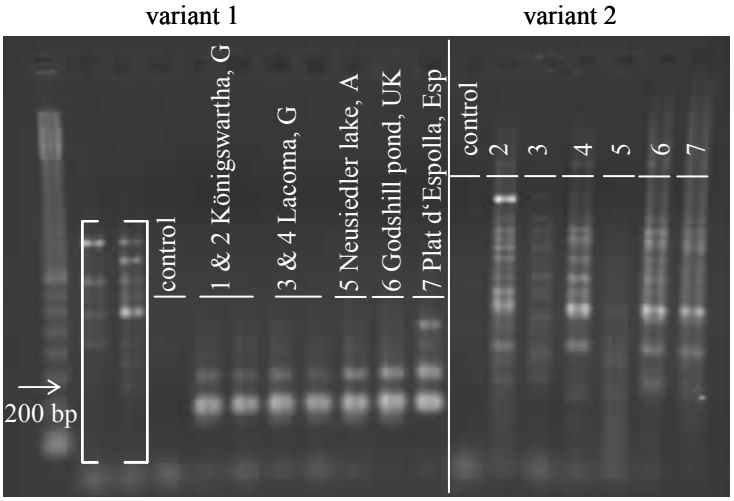

Figure 5-14: Ethidium bromide stained agarose gel loaded with microsatellite PCR fragments amplified with primers Tc3-6 both variants. (Samples in brackets do not belong to loci Tc3-6.)

The amplification reaction with primer pair Tc4-1 produced a fragment of expected size, but also several unspecific bands which could not be reduced by magnesium chloride and temperature optimization (Figure 5-15).

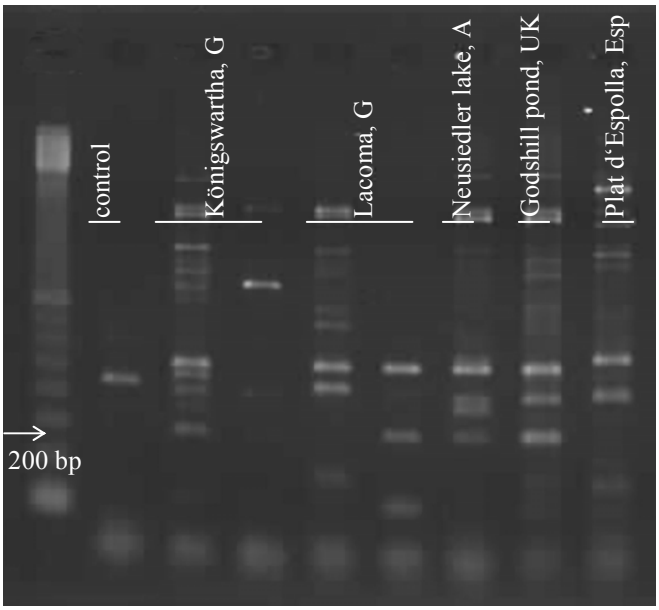

Figure 5-15: Ethidium bromide stained agarose gels loaded with microsatellite PCR fragments amplified with primer Tc4-1.

Amplification with primers Tc2-3 resulted in inconsistency as some samples produced fragments whereas others did not (see Figure 5-16). However, the unsuccessful optimization made this locus also useless for genotyping.

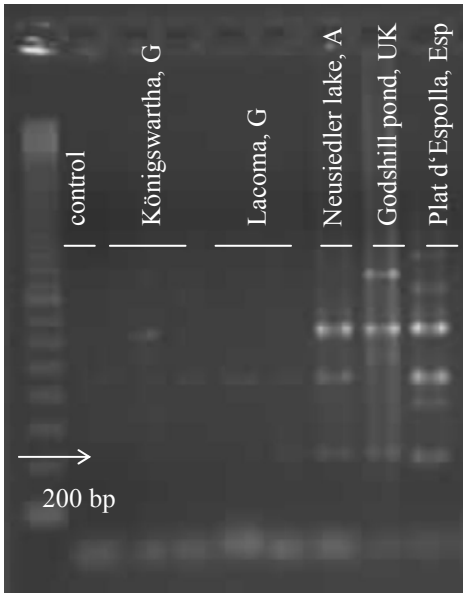

Figure 5-16: Ethidium bromide stained agarose gels loaded with microsatellite PCR fragments amplified with primer Tc2-3

As shown above, microsatellite sequences from *T. cancriformis* could be isolated by the method of selective hybridisation and optimized hybridisation conditions. However, the application of the developed primers did not result in applicable polymorphic microsatellite loci.

5.4 Discussion

The number of clones containing microsatellites was low compared to other studies using hybridisation techniques to enrich microsatellite DNA fragments (Zane et al., 2002; Duff et al., 2004). The loci consistently amplifying fragments of expected size turned out to be monomorphic in the studied *T. cancriformis* populations from Austria, Germany, Great Britain and Spain. This may indicate inbreeding depression as reported for small fragmented populations and also for selfing populations (Havel & Hebert, 1993; Harrison & Hastings, 1996; Frankham et al., 2002; Scanabissi Sabelli & Mondini, 2002)

The presence of several independent microsatellites within the same cloned insert suggests a possible clustering (linkage) as it has been observed elsewhere (Dib *et al.*, 1996; Dietrich *et al.*, 1996; Geist *et al.*, 2003). The flanking of microsatellite loci with *cryptically simplistic* sequences (sequences that contain intermixed repeated motifs, but are devoid of any tandem arrangements) may explain the difficulty experienced in optimizing microsatellites primer for amplification. The interruption of the dinucleotide motif $(GA)_{27}$ by the 'TTAA' (loci Tc3-2, Tc3-4, Tc3-7) sequence nearly in the centre of the microsatellite sequence may be caused by mutation as it has been found in humans (Weber, 1990). The author reports that higher rates of mutation appear to be associated with greater number of tandem repeats in the core sequence of microsatellites.

Recently, dinucleotide microsatellite primer in *T. cancriformis* have been developed by an Italian Group (Cesari *et al.*, 2004). The average allele number (N_A 2.4) for the described five microsatellite loci was only half of that found in that published by Duff et al. (2004) for closely related Spinicaudata (*Eulimnadia texana*). However, the allele number in both large branchiopods groups is low, since in closely related Daphnia average allele numbers of 5-10 are found (Ender et al., 1996; Pálsson, 2000). Nevertheless those recently published *T. cancriformis* primers have been applied for population genetic studies (see chapter 6).

Conclusion

The present reports shows that microsatellite sequences from *T. cancriformis* could be isolated by the method of selective hybridisation and optimized hybridisation conditions. However, the application of the developed primer did not result in applicable polymorphic microsatellite loci.

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Chapter 6

Genetic characterization and analysis of reproductive mode of *Triops cancriformis* populations*

"Sex is the Queen of problems in evolutionary biology"

(G. Bell, 1982)

Abstract

Triops cancriformis (Crustacea: Notostraca) is a small, but ancient crustacean species characterised by a range of reproductive systems. The present paper reports on the genetic structure of European *T. cancriformis* populations based on five microsatellite loci. The paper reports that allelic diversity is present among and within regional populations of *T. cancriformis*. The low, but geographically structured microsatellite variation further supports the subspecies classification for *Triops cancriformis cancriformis* and *Triops cancriformis simplex*. The study also reports about the very first analysis of the reproductive mode in *T. cancriformis* based on microsatellite marker.

* Selected results of this paper have been presented at the Sixth International Crustacean Conference, Glasgow (18 – 22 July 2005) an in Zierold, T., Gómez, A. & Hänfling, B. (2005) Sex in 'living fossils'? Phylogeography and population genetics of *Triops cancriformis*. Proceedings of the Ecological Society **35**: 31.

6.1 Introduction

The tadpole shrimp, *Triops cancriformis* (Bosc 1801) inhabits temporary freshwater pools in inundated floodplains, isolated rainwater pools, rice fields and also fish nursery ponds (Langner, 1985; Brtek & Thiery, 1995; Machado et al., 1999; Umetsu et al., 2002). The distribution of large branchiopods is affected by their drought-resistant cysts, which are efficient agents of passive dispersal including distribution through wind and water (Càceres & Soluk, 2002; Figuerola & Green, 2002; Figuerola et al., 2003; Green & Figuerola, 2005), but also by amphibians or hoofed animals (Frank, 1986; Thiery, 1991; Bohonak & Roderick, 2001). Populations occur even on remote islands, and are apparently found wherever suitable habitats are available (Longhurst, 1955). Thus, the high dispersal abilities afforded by their diapausing cysts and the possibility of unisexual reproduction seemingly account for the wide distribution of *T. cancriformis* including occurrences in Europe (Longhurst, 1955), Japan (Suno-Uchi et al., 1997), North and South Africa (Longhurst, 1955; Brtek & Thiery, 1995; Thiery, 1996) and South Asia (Brtek & Thiery, 1995).

Yet, their distribution over this area is typically patchy which is a consequence of their natural occurrence being restricted to ephemeral freshwater bodies. Despite of their isolation by distance and environmental conditions, *T. cancriformis* populations might be linked. It is very likely that Central European populations distributed very recently (after ice ages, see chapter 4). Thereby cysts developing females able to reproduce without mate had a high potential for founding new populations. In consequence one would suggest that within pure female populations genetic variation is low and selfing may lead to heterozygote deficiencies. The human impact may represent an important role in population structure as cysts of *T. cancriformis* are easily transferred along with fish transports or even through sediment sticking on vehicles (Langner, 1985). As reported by Camargo et al. (2002) brine shrimps (Anostraca) population structure was also influenced through migratory birds. Thus there may be a potential for gene flow among isolated *T. cancriformis* populations.

Molecular analyses of population structure and gene flow between subpopulations have been conducted for a broad species range at a variety of temporal and spatial scales using different molecular markers (Neigel, 1997; Gómez

& Carvalho, 2000; Huber et al., 2001; Johnson et al., 2003; Hänfling et al., 2004). However, population genetic studies on large branchiopods are limited (Sassaman, 1989; Sassaman & Weeks, 1993; Tinti & Scanabissi Sabelli, 1996; Sassaman et al., 1997; Suno-Uchi et al., 1997; Sun et al., 1999b; Abatzopoulos et al., 2002; Weeks & Duff, 2002; Cesari et al., 2004; Weeks, 2004).

Genetic differentiation between *Artemia tibetiana* (Anostraca) populations has been studied by Abatzopoulos et al. (2002) using allozyme analysis and Random Amplified Polymorphic DNA (RAPD) analyses. The results clearly show population substructure influenced by reproductive mode and isolation by distance. Another allozyme study among North American species of *Triops longicaudatus* and *Triops newberryi* showed that among *Triops newberryi* populations of different reproductive types (high male frequencies, balanced male/female ratio and unisexual) no distinct clustering could be found (Sassaman et al., 1997). *Triops longicaudatus*, in contrast, shows a clearer demarcation between populations differing in sex ratio, and reproductive differences are associated with phylogenetic patterns.

A later study by Murugan et al. (2002) addressed again within-species variation and the fact of different reproductive modes using mitochondrial markers. The 16S fragment was not useful to discriminate between the American *Triops* populations, whereas the 12S gene fragments, in agreement with previous allozyme studies, indicated a hierarchical population structure of *Triops longicaudatus* and additionally a mixture of species or at least subspecies. In contrast, the European *Triops cancriformis* seems to be more homogenous, i.e. Italian, Austrian, Hungarian and Russian samples collapsing in a single haplotype (Mantovani et al., 2004).

To study genetic structure of populations, also closely related once, microsatellite loci have become the marker of choice as they are highly polymorphic, codominant genetic markers, abundant and ubiquitous in eukaryote genomes (Jarne & Lagoda, 1996; Goldstein & Schlötterer, 1999).

The present paper will deduce, how single cyst dispersal and geographic isolation may influence the population structure, and further, in which way fish-bred origin influences the genetic variation of *T. cancriformis* in piscicultura. For that *T. cancriformis* populations were typed with five microsatellite loci. Additionally genotyping results will shed light into the reproductive strategy present in females reproducing without mate.

6.2 Material and methods

6.2.1 Sample collection

Nine regions containing water bodies in inundated floodplains, isolated ponds and fish nursery pools in Europe were sampled between 2000 and 2004 (see Figure 6-1). Three regional populations (KW, LA, R) were further distinguished into local populations representing neighboured pools and ponds within that region. Samples included specimens collected using a dip net (5 mm pore diameter) (‘collected live’ see Table 6-1), and sediment, containing resting cysts, from dry ponds where the species was known to occur (‘sediment’, Table 6-1, Figure 6-1). Apart from the individuals from Plat d’Espolla (Spain, PE), which were classified by Alonso (1996) as *T. c. simplex,* all other specimens belong to the nominal subspecies *T. c. cancriformis.*

Table 6-1: Locality information for the *Triops cancriformis* populations included in the population genetic study

Country	Regional population		Local population		n	Geographic location	Material
	ID	Region	ID	Location			
Germany	KW	fish pond area Königswartha, Saxony	Kw11	fish pond 11	3	51°19' N, 14° 21' E	sediment
			Kw12	fish pond 12	19		sediment
			Kw21	fish pond 21	15		sediment
			Kw27	fish pond 27	4		preserved live
			Kw28	fish pond 28	10		preserved live
	LA	fish pond area Lacoma, Brandenburg	La1	fish pond 1	13	51°47' N, 14°23' E	sediment
			La2	fish pond 2	15		sediment
			La3	fish pond 3	4		sediment
	R	Rhine, Rhineland-Palatinate	Da	Daxlander Au	3	48°59' N, 08°17' E	sediment
			Ib	Ibersheim	2	49°43’ N, 08°25’ E	sediment
			Ne	Neuburg	6	48°59’ N, 08°16’ E	sediment
			Ha	Hagenbach	6	49°00’ N, 08°16' E	sediment
	CO	military area, Coburg, Bavaria	-		8	50°15' N, 10°58' E	sediment
Austria	BG	Danube, Lower Austria	-		1	48°10’ N, 16°58’ E	sediment
	LN	near Kaiserlacke,	-		17	47°48’ N, 16°51' E	sediment
Great Brittain	NF	Godshill pond, New Forest	-		18	50°55' N, 01°45' W	sediment
Spain	PE	Plat d'Espolla, Banyoles karstic area	-		16	42°10' N, 02°46' E	preserved live
Italy	SC	Monte Cofano, Sicily	-		3	38°05' N, 12°42' E	preserved live

Live specimens were preserved in ethanol immediately upon collection from the field. However, live specimens could be collected only from the populations KW27 and KW28. Samples and site information from Plat d’Espolla population (PE) were provided by Dani Boix (Institute of Aquatic Ecology and Department of Environmental Sciences, University of Girona Campus Montilivi) and samples from Sicily by Michael Korn (University Konstanz). All other material was obtained from laboratory rearing using sediment containing cysts of *T. cancriformis* (Table 6-1).

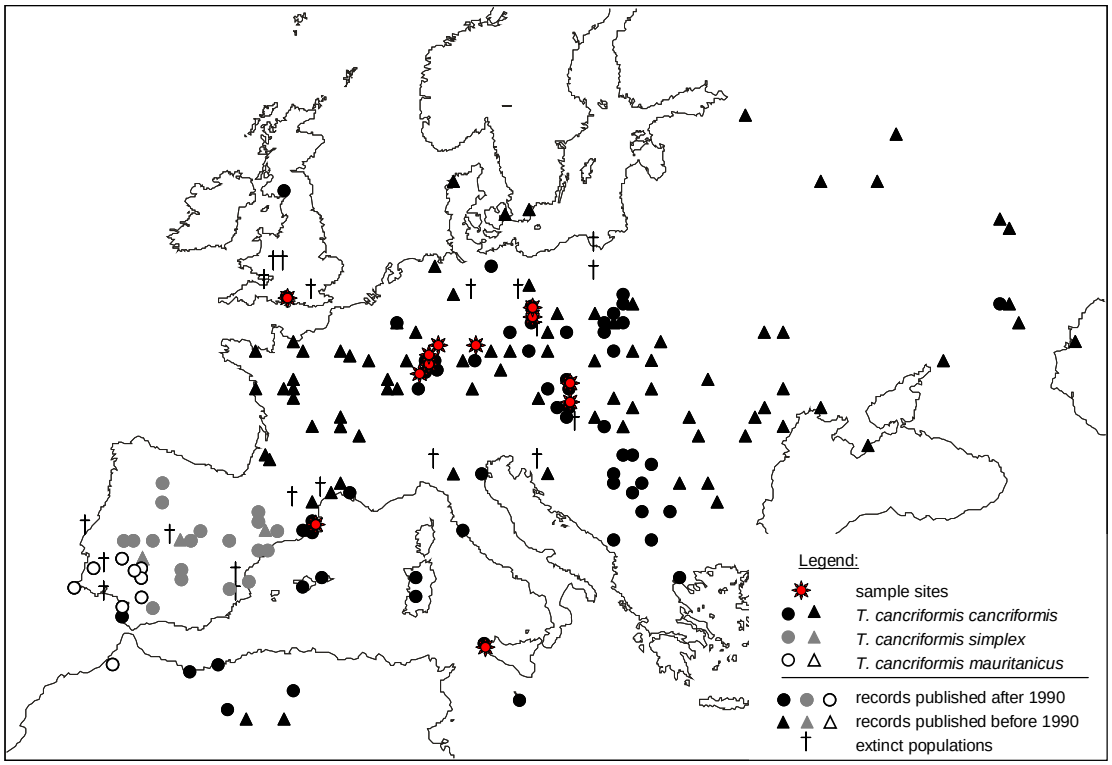


Figure 6-1: *Triops cancriformis* distribution and locations of sample sites for population genetic analyses based on five microsatellite markers.

6.2.2 Laboratory rearing, and resting cyst isolation

Subsamples of sediment (50-100 mg) from sampled populations (Table 6-1) were hydrated in separate 5 l aquaria using 3 l distilled water and placed in an air conditioned room (room temperature 20 °C). The aquaria were exposed to continuous light for the first three days after hydration followed by ambient day-night rhythm. The hatched specimens were fed with dry-fish food (VITA®) and allowed to grow at least until sexual maturity. The specimens were preserved separately in ethanol for further investigations.

Further samples for DNA extraction were obtained from cysts isolated from sampled sediment following procedures described in Gómez & Carvalho (2000). *T. cancriformis* cysts were recognised by their morphology under a stereoscope, and individual cysts were rinsed in distilled water before DNA extraction.

6.2.3 DNA extraction and microsatellite genotyping for population differentiation

About 3 mg of apodous tissue were removed from each ethanol-preserved specimen and allowed to dry from ethanol by placing the tissue on a sterile cellulose paper. The dry tissue was carefully transferred into a 1.5 ml Eppendorf tube and DNA was extracted using commercial DNA extraction kits (Invitek Forensic Kit, PureGene Kit). DNA concentration was quantified using TBS-380 Mini Flourometer (Biocompare) with PicoGreen fluorescence reagent.

Because of the small amount of DNA in *Triops* cysts, microsatellite PCR amplifications were set up in a one-way-flow containment laboratory in which no PCR had previously been carried out. All samples were genotyped for five *T. cancriformis* dinucleotide microsatellite loci, tcAC-8p1, tcAC-9p1, tcAC-10p1, tcAC-14p1, tcAC-10p2. The corresponding microsatellite primers are reported to be species-specific for *T. cancriformis*, and do not amplify any sibling species (Cesari et al., 2004).

Annealing temperature, magnesium chloride concentration and template DNA concentration were optimized for microsatellite amplification and typing with an ALFexpress (Pharmacia, Amersham Pharmacia Biotech, UK). The 10 µl PCR amplification contained: 1 µl DNA (30-50 µg/ml), 1.5mM $MgCl_2$, 200 µM of each nucleotide, 0.2 mM of each primer (forward primer labelled with Cy5), 1x NH_4-PCR Buffer (Bioline), and 0.05 U *Taq* DNA polymerase (Bioline). Microsatellite primer characteristics are summarized in Table 6-2. The resulting PCR products were resolved on 6% polyacrylamide gels using an automated sequencer (ALF*express*, Pharmacia, Amersham Pharmacia Biotech, UK), along with appropriate internal size markers (van Oppen et al., 1997). Allele sizes were scored using the program DNA Fragment Analyzer (Version 1.2, Pharmacia Biotech, UK; 1995).

Table 6-2: Results from Cesari et al. (2004) characterising annealing temperature Ta, allele number and product size range of microsatellite loci developed for *Triops cancriformis*

Primer	Primer sequence (5'-3')	Motif	T_a [°C]	Allele number	Product size range (bp)	Accession Number
tcAC-8p1	F:TGGGTGCGACTTATTCCATT R: GCCAAGTTTGAGGGACTTTG	$(AC)_8$	55	2	148–150	AY568954
tcAC-9p1	F: ACGGTATTGGTGCAAGTGG R: AGTGTACTTGTCGGGGAATCA	$(AC)_9$	55	2	198–200	AY568952
tcAC-10p1	F: GGCGTTTTAGGTAGCTGTGG R: AAGAACGGAGCGGAAGAACT	$(AC)_{10}$	55	3	218–224	AY568953
tcAC-14p1	F: AAGGGGTTGGGAAATGAGAC R: AAAAGCCAACACCAGCAAAT	$(AC)_4AT(AC)_4ATC(AC)_4$	58	2	236–238	AY568955
tcAC-10p2	F: ATTTTCATGGAGGCAACAGG R: ATTCGTGGGAGGACTTCATT	$CATC(CA)_5CT(CA)_2$	50	3	148–190	AY568956

6.2.4 Population genetic variation

Genetic variation can be decomposed into three main components: genetic diversity (the amount of genetic variation), genetic differentiation (the distribution of genetic variation among populations) and genetic distance (the amount of genetic variation between pairs of population). Based on the knowledge of the degree of genetic variation within and among *T. cancriformis*, approaches considering population structure, gene flow between populations, genetic bottlenecks and conservation biology strategies can be deduced.

The genetic diversity has been measured to describe the heritable variation found within and among populations (regional and local). The genetic diversity measures are divided into the determination of variation abundance and its distribution. To determine the variation abundance number of alleles per locus, percentage of polymorphic loci (P) within populations (separated into regional and local) and observed heterozygosity have been recorded using the Excel add-in `MS_tools.xla` (Park, 2001), which contains Microsoft Excel visual basic macros for use with diploid or haploid microsatellite data. The distribution of genetic variation was based on the calculation of average number of alleles per population (regarding to regional and local) and on the assessment of their variance.

To calculate the level of differentiation among *T. cancriformis* populations the significance of genetic differentiation was estimated in GENEPOP (Raymond & Rousset, 1995) by testing the null hypothesis that “the genotypic distribution is

identical across populations" using Fisher's Exact test and Markov chain method (1000 iterations, 100 batches and 1000 iteration per batch). Therefore all surveyed populations, including the local populations for Königswartha, Lacoma and Rhine and the other regional populations without subdivision were included in the analysis. Thus the local populations were considered not to be related. This assumption was necessary to test whether or not populations (including local and regional) show significant genotypic differentiation or if the genotypic distribution is homogenous across them.

To infer population structure the degree of similarity between 'natural' populations, Nei's genetic distance, D, was calculated for each locus (D_N) and over all loci (D_A). Therefore first Nei's index of genetic similarity I_N was calculated after Equation 1

$$I_N = \sum_{i=1}^{m}(p_{ix}\,p_{iy}) \Big/ \left[\left(\sum_{i=1}^{m} p_{ix}^2\right)\left(\sum_{i=1}^{m} p_{iy}^2\right)\right]^{1/2}, \qquad \text{Equation 1}$$

with *p* is the frequency of the *i*th allele of population *x* or *y* and *m* the number of loci. Nei's genetic distance over all loci (D_A) was calculated by combining the probability of choosing two identical alleles at random from populations and the probability of taking the same allele at random from compared populations. Detailed calculation description of Nei's genetic distance over all loci per population are described in Nei (1987). The latter distance was calculated for all possible pairwise combinations of populations. The created distance matrix was used to further generate a dendrogram for the populations using the UPGMA method (Nei & Kumar, 2000) provided by Mega3 software package version 3.1 (Kumar et al., 2004).

6.2.5 Analyses of reproductive strategies

The mating system within *T. cancriformis* has been described to be complex including outcrossing, androdioecy, hermaphroditism and possibly also parthenogenesis. Under the assumption of Hardy-Weinberg equilibrium, differences in the proportion of heterozygotes observed (H_O) and expected (H_E) within a population, can be used to estimate the proportion of inbreeding (selfing). GENEPOP version 3.4 (Raymond & Rousset, 1995) was used to tests for Hardy-Weinberg

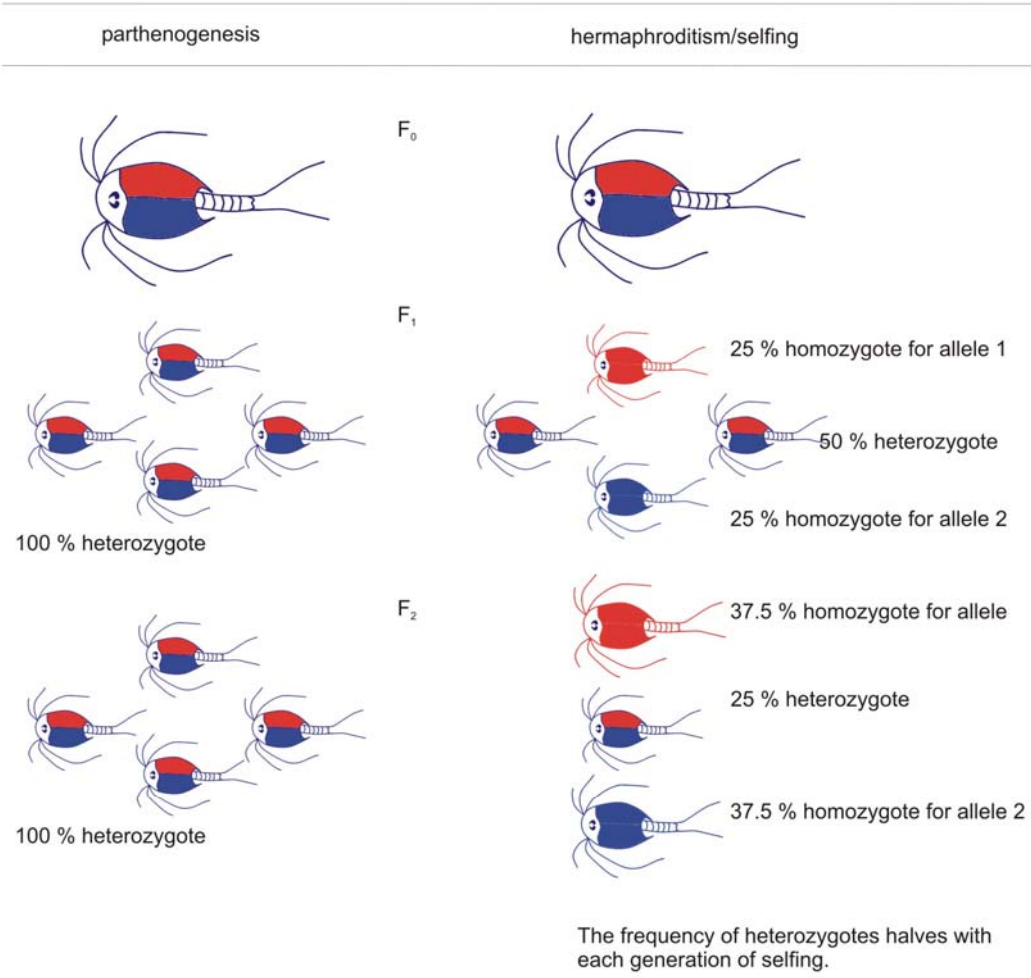
proportions, heterozygote deficiencies, genotypic linkage disequilibrium and genetic heterogeneity among populations.

The recognised inbred populations were further investigated to identify whether they reproduce clonally or by selfing (hermaphroditic or androdioecy population) (see Box 6-1). Therefore, females reared in isolation which resulted to be heterozygotic for locus tcAC-8p1 were selected. These females were checked for resting cysts in their brood pouches. Only individuals with at least 10 resting cysts were chosen for further investigations. The cysts were separated from the females, washed in distilled water and air dried. Afterwards each cyst was transferred into a tube and finally DNA was extracted from the cysts using Chelex extraction as described by Gómez & Carvalho (2000). PCR amplification was performed in a total volume of 10 µl including 1 µl template DNA (5-20 µg/ml), 200 µM of each nucleotide, 0.2 mM of each primer (forward primer labelled with Cy5), 1x 10xHotMaster Taq Buffer (Eppendorf) and 0.25 U HotMasterTaq DNA Polymerase (Eppendorf). For better resolution genotyping was performed on a Beckman sequencer (CEQ8000). The number of homozygote and heterozygote signals from the offspring was counted and a Chi-square test was performed to assess agreement between observed (O) an expected (E) numbers of genotypes according to Equation 2 (df = 1).

$$\chi^2 = \sum (O - E)^2 / E \qquad \text{Equation 2}$$

Box 6-1 Reproductive mode analysis

This analysis is based on the hypotheses that, assuming parthenogenesis, 100 % of the offspring should have the mother genotype as illustrated below. If females reproduce by hermaphroditism three genotypes would be expected: two homozygotes for each allele and one heterozygote. However, in the case of self-fertilization the frequency of heterozygotes is expected to half each generation of selfing. Thus heterozygote deficiency might be observed within hermaphroditically reproducing *Triops cancriformis* populations.



6.3 Results

A total of 163 *Triops cancriformis* resting cysts and individuals, retrieved from 15 different locations (Figure 6-1), were typed for five microsatellite loci Table 6-2).

6.3.1 Genetic characterization of populations

6.3.1.1 Genetic diversity

The results on the amount of allele length variation of five microsatellite loci over the surveyed populations are summarized in Figure 6-2, Table 6-3 and Table 6-4. Within the overall sample a total of 15 alleles were detected. Of these possible alleles, 10 were detected in the Spanish population Plat d'Espolla, whereas only 5 within the Sicilian samples possibly due to small sample size. Middle European populations showed allele numbers over all loci from 5 to 7.

At locus tcAC-8p1 allele 146 was present in the Königswartha population only and allele 144 is a private allele within the population Plat d'Espolla (Spain). The other populations are monomorphic for allele 150.

All populations except Plat d'Espolla are monomorphic for locus tcAC-9p1, where allele 200 is a private allele in the Spanish population.

The most polymorphic locus with five detected alleles is tcAC-10p1. However a maximum of three different alleles was found in a single population. The Spanish population showed highest allele frequencies for allele 214 (tcAC-10p1). Allele 218 was common in all local populations from Königswartha, but almost absent in other populations. In contrast allele 224 (tcAC-10p1) was detected only in populations from Lacoma.

Locus tcAC-14p1 was monomorphic for all local populations belonging to the fishery areas Königswartha and Lacoma as well as for three local populations from Rhine river, and the specimens from Blumengang and Sicily. The Spanish population showed highest allele frequency for allele 238 compared to the other populations.

Locus tcAC-10p2 is almost monomorphic for all analysed populations, thus no genetic diversity was observed. However, within the population Neusiedler Lake a single individual was fixed for allele 188, whereas all other resulted in allele 190.

On the whole there were four alleles found in single populations (private alleles), with average allele frequency of 0.278 (ranging from 0.050 to 0.937).

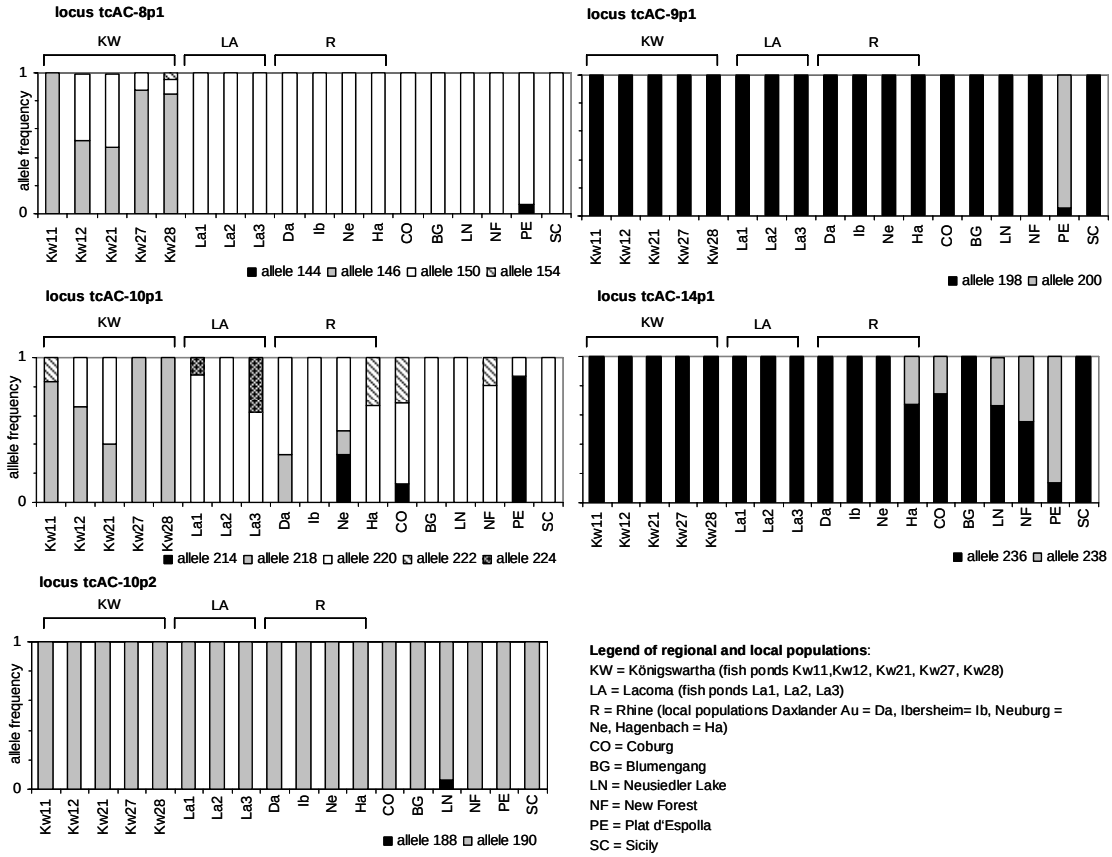


Figure 6-2: Allele length distribution of surveyed microsatellite loci within *Triops cancriformis* populations.

Table 6-3: Observed allele frequencies at five microsatellite loci for *Triops cancriformis* populations, n = number of genotyped individuals per population

		Population*																
		Kw11 n = 3	Kw12 n = 19	Kw21 n = 15	Kw27 n = 4	Kw28 n = 10	La1 n = 13	La2 n = 15	La3 n = 4	Da n = 3	Ib n = 2	Ne n = 6	Ha n = 6	CO n = 8	LN n = 17	NF n = 18	PE n = 16	SC n = 3
Locus	allele																	
tcAC-8p1	144	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000
	146	1.000	0.526	0.533	0.875	0.850	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	150	0.000	0.474	0.467	0.125	0.100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.933	1.000
	154	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
tcAC-9p1	198	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.063	1.000
	200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.937	0.000
tcAC-10p1	214	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.125	0.000	0.000	0.875	0.000
	218	0.833	0.658	0.400	1.000	1.000	0.000	0.000	0.000	0.333	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000
	220	0.000	0.238	0.600	0.000	0.000	0.885	0.967	0.625	0.667	1.000	0.500	0.667	0.562	1.000	0.806	0.125	1.000
	222	0.1667	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.313	0.000	0.194	0.000	0.000
	224	0.000	0.000	0.000	0.000	0.000	0.115	0,033	0.375	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
tcAC-14p1	236	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.667	0.750	0.667	0.556	0.133	1.000
	238	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.250	0.333	0.444	0.867	0.000
tcAC-10p2	188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.059	0.000	0.000	0.000
	190	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.941	1.000	1.000	1.000

*KW = Königswartha, LA = Lacoma, Da = Daxlander Au, Ib = Ibersheim, Ne = Neuburg, Ha = Hagenbach, CO = Coburg, LN = Neusiedler Lake, NF = New Forest, PE = Plat d'Espolla, SC = Sicily, Monte Cofano

In Table 6-4 characteristic indices for genetic diversity are summarized. Comparing the heterozygosities among the three populations Königswartha, Lacoma and Rhine, which were separated further into local populations, the regional population Königswartha showed highest values. Among all regional populations the Spanish population Plat d'Espolla (PE) was the most diverse genetically, indicated by four of five polymorphic loci which results in mean observed heterozygosity of 0.08 and mean expected heterozygosity of 0.14 (Table 6-4). The Sicilian population was observed to be monomorphic for all five loci typed. Within the other populations either one or two polymorphic loci were detected, resulting in P values of 20 and 40. The mean number of alleles per polymorphic locus is low with AP of 2 and 3.

Table 6-4: For regional and local populations of *Triops cancriformis* sample size, expected heterozygosity (H_E) and observed heterozygosities (H_O) given per locus and over all loci additionally allelic richness over all loci is listed in terms of percentage of polymorphic loci (P) and mean number of alleles per polymorphic locus (AP)

Population scale		per locus										over all loci			
		tcAC-8p1		tcAC-9p1		tcAC-10p1		tcAC-14p1		tcAC-10p2					
(reg., loc.)	n	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	P	AP	H_E	H_O
KW	51	0.56	0.25	M	M	0.44	0.25	M	M	M	M	28	2	0.20	0.10
Kw11	3	M	M	M	M	M^1	M^1	M	M	M	M	20	2	0.07	0.07
Kw12	19	0.50	0.42	M	M	0.45	0.37	M	M	M	M	40	2	0.20	0.16
Kw21	15	0.50	0.27	M	M	0.48	0.27	M	M	M	M	40	2	0.20	0.11
Kw27	4	0.22	0.25	M	M	M	M	M	M	M	M	20	2	0.05	0.05
Kw28	10	0.27	0.10	M	M	M	M	M	M	M	M	20	3	0.06	0.02
LA	32	M	M	M	M	0.34	0.24	M	M	M	M	20	2	0.08	0.03
La1	13	M	M	M	M	0.28	0.14	M	M	M	M	20	2	0.04	0.05
La2	15	M	M	M	M	M	M	M	M	M	M	20	2	0.01	0.01
La3	4	M	M	M	M	0.47	0.25	M	M	M	M	20	2	0.11	0.05
R	17	M	M	M	M	0.57	0.36	M	M	M	M	15	2	0.18	0.08
Da	3	M	M	M	M	0.44	0.67	M	M	M	M	20	2	0.11	0.13
Ib	2	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Ne	6	M	M	M	M	0.61	0.33	M	M	M	M	20	3	0.13	0.07
Ha	6	M	M	M	M	0.44	0.67	0.44	0	M	M	20	2	0.19	0.13
CO	8	M	M	M	M	0.57	0.13	0.38	0.13	M	M	40	3	0.22	0.05
LN	17	M	M	M	M	M	M	0.44	0	M	M	40	2	0.12	M
NF	18	M	M	M	M	0.31	0.39	0.49	0	M	M	40	2	0.17	0.08
PE	16	0.12	0.13	0.12	0.13	0.22	0.13	0.23	0	M	M	80	2	0.14	0.08
SC	3	M	M	M	M	M	M	M	M	M	M	M	M	M	M

*KW = Königswartha, LA = Lacoma, Da = Daxlander Au, Ib = Ibersheim, Ne = Neuburg, Ha = Hagenbach, CO = Coburg, Da = Danube, LN = Neusiedler Lake, NF = New Forest, PE = Plat d'Espolla, SC = Sicily, Monte Cofano

M monomorphic allele, M^1 two monomorphic alleles observed only a single time

6.3.1.2 Genetic differentiation

The population differentiation based on allelic distribution resulted for locus tcAC-8p1, tcAC-9p1, tcAC-10p1 and tcAC-14p1 in *p*-values less than 0.0001. Thereby loci tcAC-8p1 and tcAC-10p1 are the most powerful loci for population differentiation. Since for locus tcAC-10p2 *p*-value was 0.767, this locus is not appropriate for population differentiation.

Within the regional population Königswartha highly significant genetic differentiation was found comparing Kw12 *vs.* Kw28 and Kw21 *vs.* Kw28 (Table 6-5). Within the regional populations Lacoma and Rhine no highly significant differentiation could be observed. Among the regional populations highly significant differentiation was observed for comparison with Königswartha, Plat d'Espolla and Lacoma (except the combination with Sicily) (part B in Table 6-5). The Rhine population differs with at least high significance from three populations (Neusiedler Lake, New Forest and Plat d'Espolla). The Sicilian population showed most similarity to all other populations excluding the Spanish (Plat d'Espolla) and the German (Königswartha). On this background six genotypic haplotypes could be identified by collapsing the allele differences over all loci (part C Table 6-5). The most common microsatellite genotype was '4' including populations from the Rhine river, Coburg, Neusiedler Lake and Sicily. Two geographically restricted haplotypes could be identified; 5 occurring in New Forest pool and 6 inhabiting Plat d'Espolla pool.

Table 6-5: Genetic differentiation matrix indicating the significance of pairwise population differentiation over all loci (*** for *p*-value < 0.001 'highly significant', ** for *p*-value < 0.01 'highly significant', * for *p*-value < 0.05 'significant', (*) for *p*-value < 0.1 'poorly significant', n.s. for *p*-value > 0.1 'not significant'; # = only one genotype was present). Population differentiation within regions (KW, LA and R) is highlighted with different colours in **part A** of the table. **Part B** summarizes the differentiation significance levels with all local populations collapsed into one regional population and population BG excluded because of very small sample size. **Part C** illustrates the genotype differentiation resulted from Part A and B.

A General comparison among all typed populations

Reg. pop.	KW					LA					R			CO	BG	LN	NF	PE	SC
Local pop.	Kw 11	Kw 12	Kw 21	Kw 27	Kw 28	La1	La2	La3	Da	Ib	Ne	Ha							
Kw11	-																		
Kw12	(*)	-																	
Kw21	*	n.s.	-																
Kw27	n.s.	(*)	*	-															
Kw28	n.s.	***	***	n.s.	-														
La1	***	***	***	***	***	-													
La2	***	***	***	***	***	n.s.	-												
La3	**	**	**	**	***	n.s.	(*)	-											
Da	(*)	(*)	n.s.	*	***	*	*	n.s.	-										
Ib	(*)	*	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	-									
Ne	*	***	**	***	***	**	**	n.s.	n.s.	n.s.	-								
Ha	**	***	***	***	***	**	**	(*)	n.s.	n.s.	*	-							
CO	**	***	***	***	***	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	-						
BG	n.s.	n.s.	n.s.	n.s.	(*)	n.s.	n.s.	n.s.	n.s.	#	n.s.	n.s.	n.s	-					
LN	***	***	***	***	***	(*)	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	-				
NF	***	***	***	***	***	***	***	*	*	n.s.	***	n.s.	n.s.	n.s.	(*)	-			
PE	***	***	***	***	***	***	***	***	***	*	***	***	***	n.s.	***	***	-		
SC	(*)	**	(*)	*	***	n.s.	n.s.	n.s.	n.s.	#	n.s.	n.s.	n.s.	#	n.s.	n.s.	***	-	

B Regional population comparison

Reg. pop.	KW	LA	R	CO	LN	NF	PE	SC
KW	-							
LA	***	-						
R	***	***	-					
CO	***	***	n.s.	-				
LN	***	***	***	**	-			
NF	***	***	**	(*)	*	-		
PE	***	***	***	***	***	***	-	
SC	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	-

C *Triops cancriformis* microsatellite genotype differentiation

	1	2	3	4	5	6
1	-					
2	(*)	-				
3	***	***	-			
4	(*)	(*)	*	-		
5	***	***	***	*	-	
6	***	***	***	***	***	-

Legend:
1 = Kw11, Kw27, Kw28, BG (BG excluded in further analyses because only one sample available)
2 =Kw12, Kw21
3 = La1, La2, La3
4 = Da, Ib, Ne, Ha, CO, LN, SC
5 = NF
6 = PE

6.3.1.3 Genetic distance and population structure

Genetic distance has been measured for the six microsatellite genotypes defined above (part C Table 6-5). Nei's (1978) matrix of genetic distances is shown in Table 6-6 with its graphical representation in Figure 6-3. As visualized, there are two main branches, one of them is collapsing the Central European populations which have been classified once as *T. c. cancriformis* and the second branch is represented by the Iberian Peninsula population (southern type), classified once as *T. c. simplex.* The Central European group can further be distinguished into the geographically restricted genotypes 1 and 2 representing Königswartha populations (and the single sample from Danube river, BG) and a very common group summarizing genotypes 3 to 5. Interestingly genotype 3, which collapses all specimens from the fish area Lacoma, does not form a single branch with the samples from the nearby fish area Königswartha.

Table 6-6: Matrix of Nei's pairwise unbiased (1978) genetic distance for the identified *Triops cancriformis* microsatellite genotypes (data based on observed allele frequencies summarized in Table 6-3)

	1	2	3	4	5	6	Legend:
1	-						1 = Kw11, Kw27, Kw28, BG (BG excluded in further analyses because only one sample available)
2	0.058	-					2 =Kw12, Kw21
3	0.384	0.124	-				3 = La1, La2, La3
4	0.391	0.135	0.014	-			4 = Da, Ib, Ne, Ha, CO, LN, SC
5	0.466	0.189	0.049	0.013	-		5 = NF
6	1.202	0.898	0.708	0.563	0.509	-	6 = PE

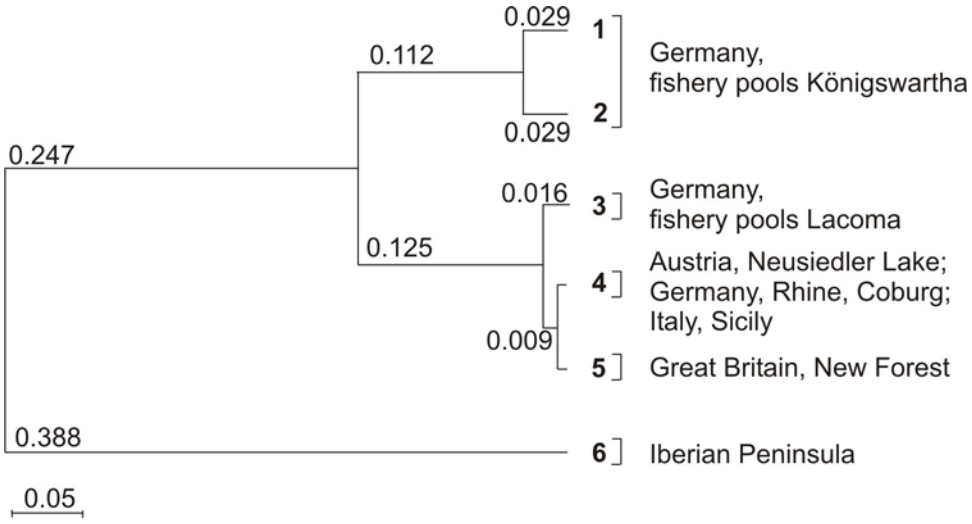


Figure 6-3: UPGMA dendrogram of Nei's unbiased (1978) genetic distance for the for the identified *Triops cancriformis* microsatellite genotypes (branch length is given).

6.3.2 Reproductive strategy

6.3.2.1 Population test

Within the analysed populations with at least 8 typed individuals observed heterozygosities are mostly smaller compared to expected heterozygosities (under Hardy-Weinberg assumptions) (Figure 6-4).

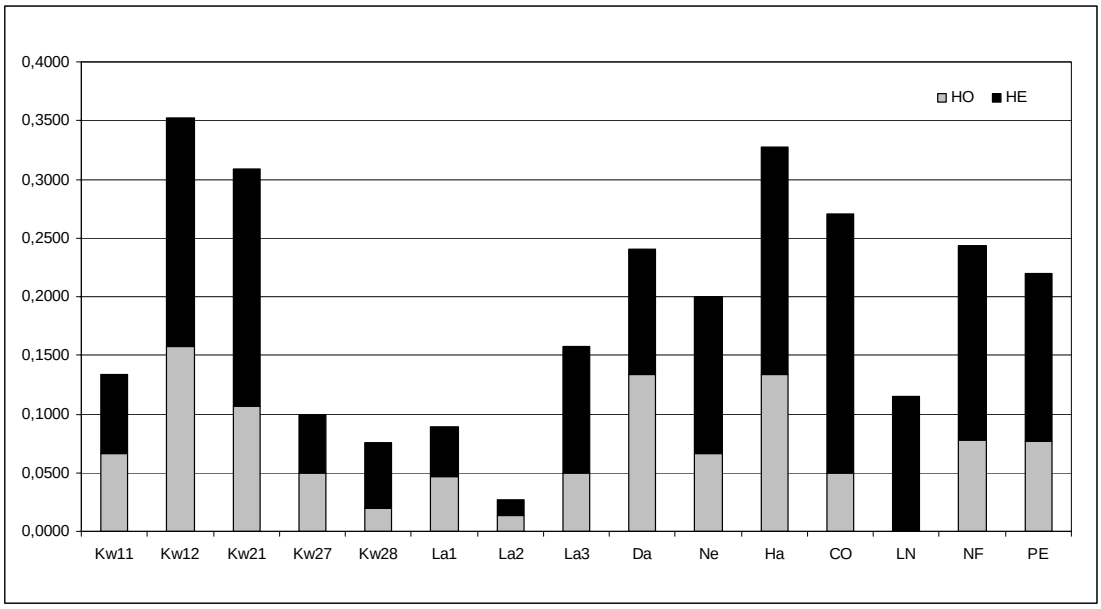


Figure 6-4: Comparison of expected (H_E) and observed (H_O) heterozygosity over all five microsatellite loci for European *Triops cancriformis* populations (n > 7) (KW = Königswartha, LA = Lacoma, CO = Coburg, LN = Neusiedler Lake, NF = New Forest, PE = Plat d'Espolla).

Calculation of the inbreeding coefficient F_{IS} resulted for three of nine populations (with n > 7) in significantly positive multilocus F_{IS} values and thus indicating together with significant deviation from Hardy-Weinberg equilibrium inbreeding in the Austrian population Neusiedler Lake, the German population Coburg and the English population New Forest (Table 6-7). For the Austrian and the English populations no males have been observed among the analysed individuals. Because of direct DNA extractions from cysts no information about sex is available from the Coburg population.

Table 6-7: Genetic variability at five microsatellite loci in 15 *Triops cancriformis* populations. F_{IS} is the multilocus heterozygote deficit (single locus U-score tests, with Markov chain method, *p*-values combined using Fisher's method), when the multilocus test was significant, individual loci showing Hardy-Weinberg deviations marked in bold letters

Populations*		n	Proportion of males	P_{HWE}	F_{IS}
Reg.	Loc.				
KW	Kw11	3	0.33	-	-
	Kw12	19	0.11	0.759	0.194
	Kw21	15	0	0.068	0.481
	Kw27	4	0	-	-
	Kw28	10	0.02	0.053	0.654
LA	La1	13	0.08	1.000	-0.091
	La2	15	0.08	Inf.	0.000
	La3	4	0	-	-
R	Da	3	0	-	-
	Ib	2	0	-	-
	Ne	6	0	-	-
	Ha	6	0	-	-
CO	-	8	n.d.	**0.001**	0.785
LN	-	17	0	**<0.001**	1.000
NF	-	18	0	**<0.001**	0.539
PE	-	16	0.13	0.071	0.472
SC	-	3	n.d.	-	-

* KW = Königswartha, LA = Lacoma, Da = Daxlander Au, Ib = Ibersheim, Ne = Neuburg, Ha = Hagenbach, Co = Coburg, Da = Danube, LN = Neusiedler Lake, NF = New Forest, PE = Plat d'Espolla, SC = Sicily, Monte Cofano

The calculated deviation from Hardy-Weinberg equilibrium (HWE) and the inbreeding coefficient F_{IS} (Table 6-7) indicate that maleless populations are showing significant deviation from HWE and in general higher inbreeding values than populations with males. These higher F_{IS} values can be interpreted by the presence of inbreeding. Additionally tests for linkage disequilibrium (LD) were performed but none of the test was significant (after sequential Bonferroni), possibly due to the lower power of LD tests and to low microsatellite variation. Such an inconsistence between HWE and LD would be extremely unlikely under clonally reproduction, but might be better explained by hermaphroditic reproduction.

6.3.2.2 Genotyping of families

On average 16 cysts (ranging from 5 to 20) were separated from 8 females, heterozygous for locus tcAC-8p1, and applied in offspring analysis. Only cysts from 6 recently preserved females resulted in reliable amplification products, a result which may be caused by cyst damage and DNA destruction during storage. The detected genotype pattern summarized in Table 6-8 demonstrates that all possible three genotypes (allele combination 146/146, 146/150 and 150/159) could be observed in each single offspring group. However, the observed genotype frequency

for the offspring from Kw12Tc1 and Kw12Tc11 did differ significantly from expected under Hardy-Weinberg assumption.

Table 6-8: Overview of paternity analysis of the offspring of six heterogeneous mothers regarding to locus tcAC-8p1. Number of cysts isolated and successfully amplified, observed and expected genotypes within the offspring, Chi-square value and the probability (bold *p*-values indicate that observed genotype frequency differs significantly from expectations)

		genotype 146/146		genotype 146/150		genotype 150/150			
mother	n_{cysts}	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Chi-Square	*p*-value
Kw12Tc1	20	2	5.00	15	9.98	3	5.51	5.08	**0.0243**
Kw12Tc8	19	4	5.26	12	9.47	3	4.26	1.35	0.2451
Kw12Tc11	19	1	4.26	16	9.47	2	5.26	9.02	**0.0027**
Kw12Tc18	5	1	1.67	8	6.67	6	6.67	0.60	0.4386
Kw21Tc1	17	4	4.25	9	8.50	4	4.25	0.06	0.8084
Kw21Tc10	16	3	3.06	8	7.88	5	5.06	<0.01	0.9494

6.4 Discussion

The genetic characterization of species and populations has been a focus of interest during the last fifty years. Factors thought to be responsible for genetic differentiation in other species (Barigozzi, 1982) are observable in *Triops*: ecological isolation and various reproductive strategies. In recent years microsatellite have been mainly used for genetic differentiation (e.g. Ender et al., 1996; Schlötterer, 2002) including population characterization (e.g. Ball et al., 1998; Ciofi & Bruford, 1999; Goldstein et al., 1999; Ciofi et al., 2002; Duff et al., 2004) and paternity tests (e.g. Queller et al., 1993; Blouin et al., 1996; Schlötterer & Pemberton, 1998).

From the presented study using five microsatellite loci to investigate genetic variability for 18 populations six main results obtained. Firstly genetic diversity is highest in the bisexual population, secondly hermaphroditism is more likely to occur in pure female populations of *T. cancriformis* than parthenogenesis, thirdly Plat d'Espolla population on Iberian peninsula differ genetically highly from remaining populations, fourthly the degree of genetic differentiation among regional population is high in contrast to less genetic differentiation among local populations (fifthly) and sixthly the complex reproductive system present in *T. cancriformis* seems to be responsible for species survival over long time.

6.4.1 Genetic diversity and the influence of reproductive strategy

Genetic diversity found overall for the typed *T. cancriformis* populations was low (with AP between 2 and 3) compared to other studies on population genetics of rotifers (Gómez & Carvalho, 2000) and cladocerans (Crease et al., 1990; Ender et al., 1996; Weider & Hobaek, 1997; Colbourne et al., 1998; Pálsson, 2000; Adamowicz et al., 2004). However, differences in genetic diversity could be identified among both regional and local *T. cancriformis* populations. The population Plat d'Espolla resulted in higher genetic diversity than any other population. This is possibly due to the reproductive strategy, since the Spanish population is known to be bisexual (see chapter 4). The lowest diversity was observed in populations where no males have been recorded to date. A medium diversity level is characteristic for Königswartha populations were low male percentages have been reported. It can be concluded from the data that inbreeding will be reduced if males, although at low percentage, occur.

Congruent to this are the observations of diversity found in comparison of bisexual and parthenogenetic *Artemia* species (Sun et al., 1999b). Data provided by Cesari et al. (2004) indicate that within the parthenogenetic *T. cancriformis* population from Oristano (Italy) almost no allelic diversity occurs, whereas the hermaphroditic reference population from Marchegg (Austria) was diverse for all microsatellite loci. These results may suggest that the Sicilian population investigated where no diversity was found is parthenogenetic. However more data are necessary to prove this hypothesis (including increased samples, paternity tests and more polymorphic microsatellite maker).

The observed situation of low allelic diversity and the presence of private alleles in distinct populations might be caused by single cyst dispersal after the glaciations (Weider & Hobaek, 1997; Avise et al., 1998; Coleman et al., 2003), geographic isolation (Brown & Lomolino, 1998) and also by inbreeding (Bijlsma et al., 1999; Keller & Waller, 2002).

6.4.2 Hermaphroditism within *Triops cancriformis* populations

T. cancriformis populations have been described as gonochoric, hermaphroditic or even parthenogenetic (Longhurst, 1954; Wingstrand, 1978; Zaffagnini & Trentini, 1980; Engelmann et al., 1997). The observed inconsistency between HWE and LD would be extremely unlikely under clonally reproduction, but might be better explained by hermaphroditic reproduction (Halkett et al., 2005). Additional hermaphroditic reproduction may be supported by the offspring analysis. However, further research is required including higher sample sizes, breeding experiments and more microsatellite loci.

6.4.3 The status of Iberian Peninsula population

The divergence found among the surveyed populations was sufficient to detect two clusters: one representing the Iberian Peninsula population, which has originally been described as subspecies *T. c. simplex* (Longhurst, 1955; Alonso, 1996) and a second collapsing all other surveyed populations described as the nominal subspecies *T. c. cancriformis*. This subspecies differentiation could not be supported by mitochondrial data (see chapter 4). However, the large mean genetic distance of 0.776 between the *Iberia type* (genotype 6) and the more Central *European type* (collapsing genotype 1-5) may be evidence for species subdivision. Comparable

genetic distances applied for species differentiation in the large branchiopods *Artemia* (Anostraca) have been reported by Abatzopoulos et al. (2002) and Sun et al. (1999a). From the present data for both *types* it could be suggested that genetic differentiation is in an initial stage and the combined forces of complex reproductive strategy, natural selection and genetic drift have started to produce divergent gene pools. Such differentiation processes are reported for other species (Avise & Walker, 1998; Abatzopoulos et al., 2002; Gómez et al., 2002; Bohonak et al., 2004). So far no experiments have been carried out to prove the reproductive isolation between those two *T. cancriformis* types. Future research has to (i) include additional Iberian populations identified once as *T. c. simplex* in genetic analysis, (ii) focus on cross-breeding/fertility tests but also (iii) on a broad morphological survey to prove the taxonomic classification. For further discussion in the present work the terminology of type '*Iberia*' and '*European*' will be further applied.

6.4.4 High level of differentiation among regional populations

Microsatellite markers indicate significant differentiation among *T. cancriformis* from the nine regions studied. The pattern of population difference found among *Iberia* type and *European* type is consistent with differences in reproductive strategies, that is bisexual reproduction in *Iberia type* (Boix, unpublished) *versus* female biased or unisexual reproduction in *European type* (Zaffagnini & Trentini, 1980; Engelmann et al., 1997). The existing level of divergence may closely approximate that it established during population founding. This can be explained by rapid growth in size of a population after founding from few (or single) individuals, as often happens with pond invertebrates. Therefore, the allelic frequency divergence established during colonization history is resistant to decay by migration (Boileau et al., 1992; Gómez et al., 2002).

Within the *European type* the Königswartha populations are separated from others. One hypothesis is that those individuals are immigrants who may have been introduced by fish breed transfer. The close relationship between the Lacoma population (also pisciculture) and other natural occurrences including Rhine river and Neusiedler Lake as well as New Forest could be explained either by anthropogenic immigration through fish breed from Neusiedler Lake (personal communication Langner, 2003) or by natural founding events through passive cysts

dispersal most likely via migratory birds (Bohonak & Whiteman, 1999; Green & Figuerola, 2005). This assumption is congruent with the results shown by Camargo et al. (2002) who demonstrated the influence of birds on the gene flow among isolated large branchiopods. The New Forest population shows low divergence to the mainland populations (Coburg, Rhine, Neusiedler Lake). Theoretically more divergence would be expected due to geographic isolation (Johnson et al., 2003) and inbreeding (Keller & Waller, 2002). Indeed, until 2004 the Godshill pond was the only known *T. cancriformis* occurrence in Great Britain. Very recently a new population has been reported from Southwest Scotland (Morell, 2005). To analyse relationships between those populations inhabiting Great Britain further sampling and genetic studies are necessary.

6.4.5 Low level of differentiation among local population structure

The three regions Königswartha, Lacoma and Rhine have been further subdivided into local populations. Low, but significant genetic differences were only observed among the five Königswartha populations which collapse into two microsatellite genotypes. One hypothesis explaining the differences may be that various lineages have been introduced anthropogenically by fish breed transfer (Langner, 1985) or natural via migratory birds (Bohonak & Jenkins, 2003). An alternative explanation for the observed divergence may be the percentage of males present in the fish ponds. The homogenous populations (with heterozygote deficiencies) Lacoma and Rhine may be the results of inbreeding. But such low polymorphism could also be evidence for parthenogenesis (Lokki, 1976). Since no families from Lacoma and Rhine were genotyped for offspring analysis the question of reproductive mode in those populations can not be resolved yet.

6.4.6 Population persistence in fragmented landscapes

It has been reported by many ecologists that fragmentation of habitats into discontinuous patches disrupts the reproduction, survivorship and movement of flora and fauna (Lande & Barrowclough, 1987; Voigt & Klaus, 2003; Higgins et al., 2005; Leimu & Mutikainen, 2005; Schnitzler et al., 2005). However, ephemeral water bodies like rain pools which are naturally fragmented and highly isolated habitats are not enemy free (Brendonck et al., 2002; Heilmeier et al., 2005). Considering *Triops*

as a characteristic species of ephemeral water bodies (Hödl & Eder, 1996), numerous adaptations are obvious for survival in such exceptional habitats (Carlisle, 1968; Hempel-Zawitkowska, 1969, 1970; Burmeister, 1988; Brendonck, 1996; Booy et al., 2000; Bohonak & Jenkins, 2003). It has been shown that genetic diversity is low within almost all populations of *T. cancriformis* and distinct populations could be separated by differences in allele frequencies (i.e. the presence of allele 220 at locus tcAC-10p1 in New Forest population *vs.* Königswartha population). Thus gene flow seems to be restricted there. Nevertheless monitoring (Hughes, 1997) of single populations does not indicate that populations suffer by reduced fecundity despite inbreeding depressions present in isolated large branchiopod populations (Weeks & Zucker, 1999; Weeks, 2004).

Possibly the complex reproductive system present in *T. cancriformis* is responsible to maintain the minimal genetic diversity required for survival over long term (Bell, 1982; Halkett et al., 2005).

Conclusion

This study reports on population subdivision based on five microsatellite loci of the ephemeral pool invertebrate *T. cancriformis*. Overall the found genetic diversity was low, but sufficient to distinguish several populations. The differentiation is forced by geographic isolation, different reproductive strategy and inbreeding. Low diversity has been found within populations. However, it was shown that population structure is significantly influenced by reproductive strategy and possibly also by immigrants. Offspring analyses from *T. cancriformis* females reared in isolation indicate that hermaphroditism is more likely than parthenogenesis.

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Chapter 7

Final conclusion

„They who would give up an essential liberty for temporary security,
deserve neither liberty or security"
(B. Franklin 1706-1790)

Attempts to protect nature include measures for protection of flora and fauna in their primary habitats. Secondary habitats must also be recognized in practical nature conservation schemes as they may allow for greater population sizes and thus more heterogeneous gene pools. The overwhelming diversity of species present in most communities and ecosystems makes complete species inventories an unrealistic goal of nature protection. Therefore, in many situations in which conservation monitoring is urgently required, single species have acted as the focus for assessment of protection and management of reserves. One category of species used is the large charismatic vertebrate (i.e. tigers or giant pandas) which require substantial reserve areas for long-term survival. Species used in this way are termed 'flagships', because measures taken for their survival and well-being may also benefit the ecosystem as a whole (Pullin, 2002; WWF, 2005). To measure the integrity of an ecosystem, species reacting more sensitively to special threats than other species, and thus responding more rapidly to ecosystem degradation are used. The application of such a 'sentinel species' requires sufficient knowledge about its sensitivity to environmental and

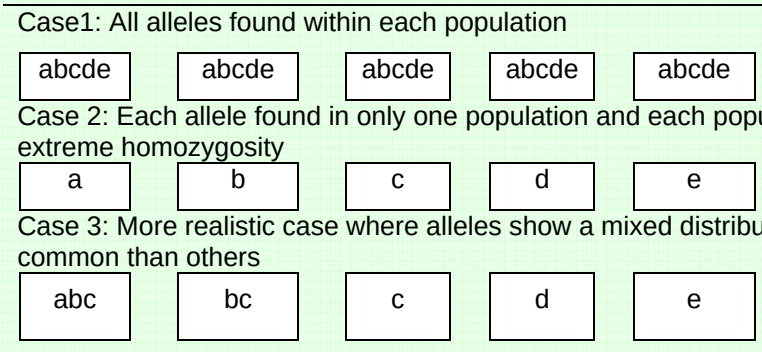
ecosystem change (Pullin, 2002). A third category of species used in management measures are ‘umbrella species’, because monitoring their responses to change safeguards other species (Frankham et al., 2002; Pullin, 2002). In this case management focuses on a single or a few species representing the needs of the majority of other species.

Large branchiopods are locally abundant in specific habitats distributed over a large geographical range. Since large branchiopods (except *Lepidurus arciticus* and *Artemia salina*) are only found in temporary water bodies, species conservation will be successful when such habitats are conserved. Thus, large branchiopods are ‘flagship species’ for ephemeral water bodies both of primary and secondary status. Considering their sensitive response to a permanent waterlogged phase, oxygen reduction and pesticide/herbicide pollutions (Hempel-Zawitkowska & Klekowski, 1968; Simon, 1987; Zierold, 2005), they act as ‘sentinel species’. Thus, monitoring large branchiopods populations will reveal even minor changes in habitat ecology. Furthermore, the protection of those old freshwater crustaceans safeguards the future of other species in ephemeral pools (i.e. *Bombina bombina*, *Lestes sponsa*). Thus large branchiopods represent also ‘umbrella species’.

Conservation activity was and is often directed towards individual species that (have come under threatened) are (considered to be) endangered. However, (dominant) recent conservation action focuses on habitat conservation with the assumption that in doing so one also protects the species and genotypes they contain (Box 7 – 1). This seems logical as one could argue that the only effective way of conserving biodiversity in the long term is to provide appropriate ecosystems (Pullin, 2002; Williams et al., 2003; Williams, 2006).

Box 7–1 **Importance of *in-situ* conservation - habitat loss causes genotype extinction**

Analyses of *Triops cancriformis* showed that distinct populations differ in their morphological and genetic makeup and diversity. The genetic diversity of genes contained within a species is a reflection of that species' adaptation to its ever-changing environment (Pullin, 2002). Since the environment is not constant over time or space, there is no one best combination of genes for a population. The general environmental conditions of *T. cancriformis* habitats remained stable over a long period of time (Carboniferous era) in the sense of going from terrestrial to aquatic and back, but they may have varied considerably in water chemistry or habitat ecology (i.e. water source, sediment composition, presence of vegetation or predators). Consequently, geographically restricted populations which differ genetically from other populations evolve. In fact, genetic data of the present thesis show that genetic diversity among regional *T. cancriformis* populations is higher compared to genetic diversity within populations. Thus, genes or alleles are fixed for populations. The destruction of such a habitat inhabited by geographical 'races' may lead to complete loss of genotype, and possibly also phenotype, as illustrated in the figure below (Box-Figure 1).



Box-Figure 1: Possible distribution of alleles from one gene locus among populations of *T. cancriformis*. At one extreme the genetic diversity is found within each population and the loss of any one population would have minimum genetic impact (case 1). At the other extreme genetic diversity is found only among the populations and the loss of one population would have maximum genetic impact (case 2). The situation found for *T. cancriformis* in the present study is intermediate, with some populations containing more diversity, and some alleles being more frequent and widespread than others (case 3). Thus extinction of population characterized by alleles 'abc' in case 3 will lead to loss of allele 'a' whereas alleles 'b' and 'c' are further represented in other populations (modified after Pullin, 2002).

To prevent decreasing genotype diversity through extinction of key populations, conservation management has to take into account the distribution of genotypes and also the centres of diversity which often are located in ice age refugia (Vogler, 1998; Pullin, 2002).

The occurrence of large branchiopods depends on both extrinsic and intrinsic parameters whereby the ability for production of resting cysts and the change of terrestrial to aquatic phases are essential for the development of large branchiopods (Figure 7-1).

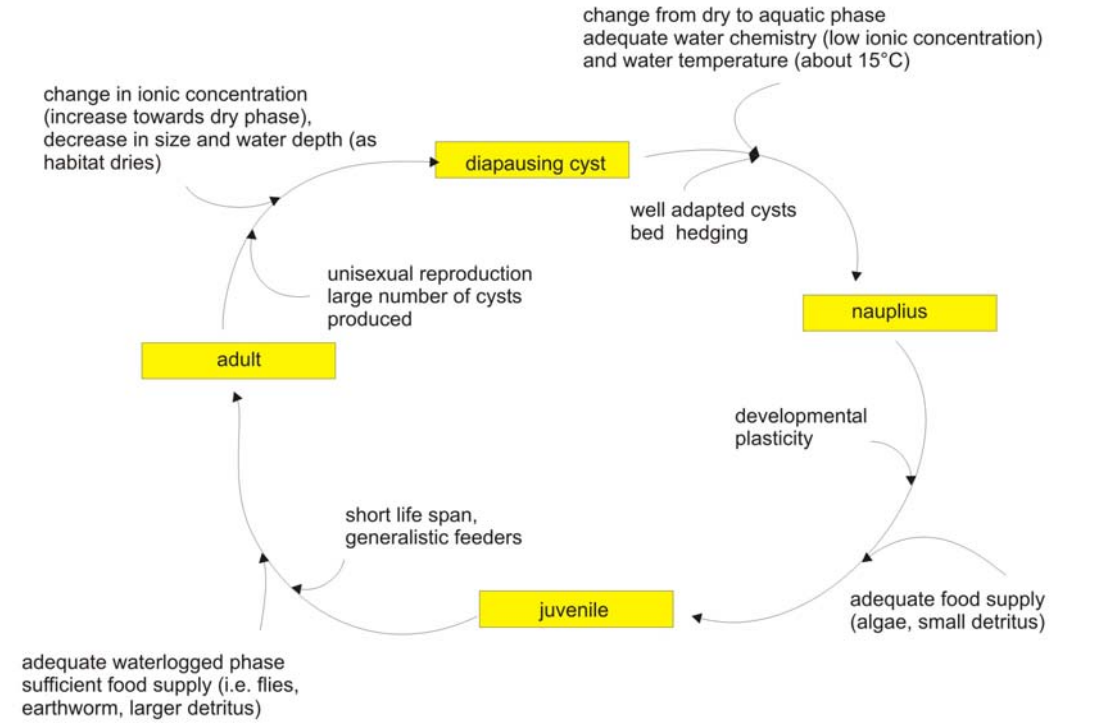


Figure 7-1: Important intrinsic (within circle) and extrinsic (outside circle) agencies of the developmental stages of *T. cancriformis*.

The cysts (also referred to as diapausing cysts) of large branchiopods are the resistant stage which is found in the upper sediment of a temporary water body. After drying, this stage can survive many years (Dexter, 1973). Females of *T. cancriformis* stick their cysts on gravel (Thiéry et al., 1995). As a consequence, the cysts are often found along the periphery of the pool, in the gravel zone, and presumably avoid the central zone of the pool where granularity of the soil is reduced to mud (Thiery, 1997). Own observations of grassy ponds in flood plain meadows showed that *T. cancriformis* females stick their eggs also on blades just above the ground. *T. cancriformis*, as many other inhabitants of temporary water bodies, has employed a strategy such that not all juveniles emerge before the hydroperiod has become properly established (Williams, 2006). Another ‘strategy’ to colonize new niches are

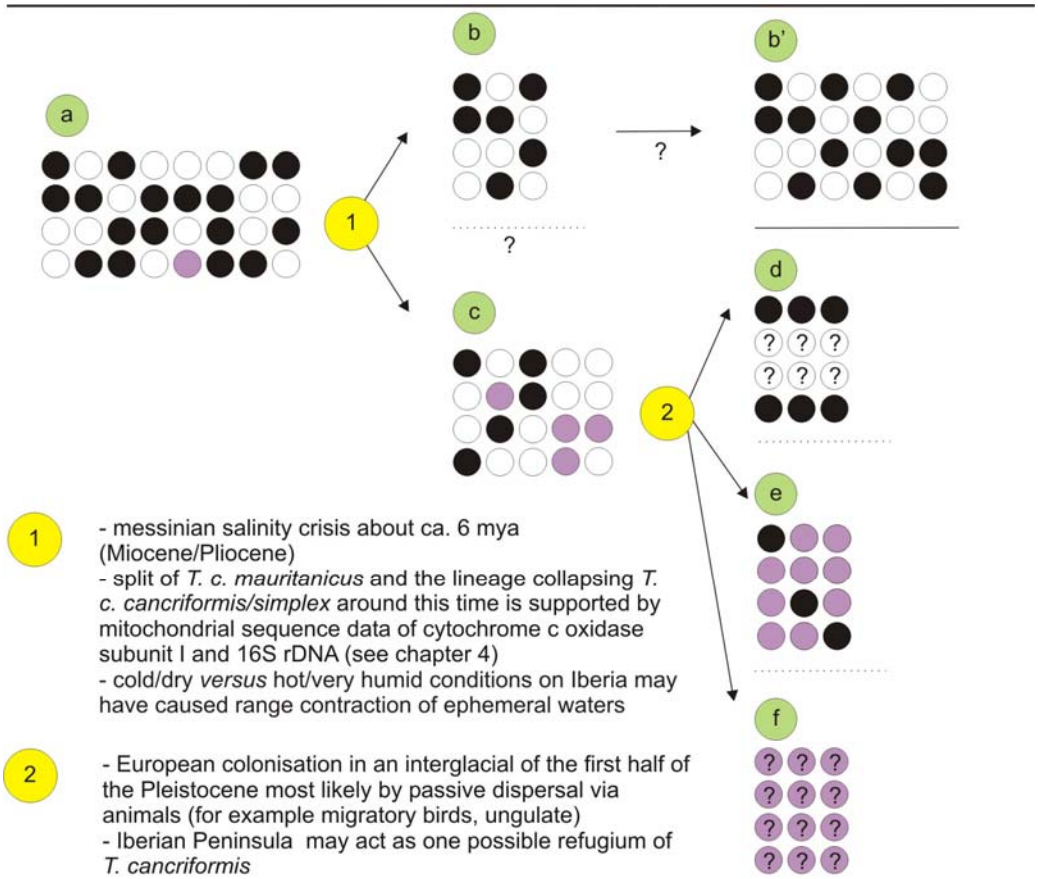
the extremely resistant cysts which are able to pass through the bird's gut unharmed and thus are appropriate for passive distribution via migratory birds (Löffler, 1964; Green & Figuerola, 2005). The cysts are also resistant against high pressure and may therefore also be distributed by strong winds (Tutis, 2002; NRL Monterey Marine Meteorology Division, 2005). Without the diapause cysts, large branchiopods could not have achieved their present eminence as freshwater animals (Fryer, 1996). Notostraca are completely depend on the possession of drought-resistant cysts which enable them to live in predator-free temporary ponds (Fryer, 1996). Fryer (1985) hypothesized that such habitats have changed little for long periods of time and temporary ponds are indeed probably the most permanent of all freshwater habitats under an evolutionary perspective of time.

Indeed large branchiopods are known as ephemeral freshwater organisms existing since the Carboniferous era (Trusheim, 1931; Tröger et al., 1984; Orr & Briggs, 1999). Repeated environmental changes of the ecology of the habitat as well as habitat isolation may have forced the species divergence in *T. cancriformis* (Figure 7-2). This divergence could be recognized in the present work referring to many features including habitat requirements, morphology, genetics and variation in reproductive strategies.

The morphological subspecies *T. c. mauritanicus* was recorded from Mediterranean rain water pools only, whereas the other two morphological subspecies - *T. c. simplex* and *T. c. cancriformis* - show a wide distribution range, including isolated rainwater and floodplain pools. Distinct differences between the Mediterranean subspecies (*T. c. mauritanicus*) and the widely distributed lineage (*T. c. cancriformis/simplex*) were recognized by considering the carina pattern which is tooth-like in *T. c. mauritanicus,* but smooth or rough in the other two currently recognized subspecies.

Based on mitochondrial sequence data of cytochrome *c* oxidase subunit I (COI) and 16S rDNA, timing of the split between *T. (c.) mauritanicus* and *T. cancriformis* (*simplex/cancriformis*) predates the Pleistocene and possibly the Pliocene. Range contraction and subsequent isolation of *Triops* lineages at this time might be most likely due to cold/dry conditions or hot/very humid conditions. Such a deep phylogenetic split within the Iberian Peninsula has been also been observed in other taxa, and indicates either the existence of several independent refugia across

the Iberian Peninsula (Gómez & Lunt, 2006) or colonisation of the southern Iberian Peninsula from North Africa (Martínez-Solano, 2004; Veith et al., 2004). Haplotype diversity and sequence divergence were generally low within *T. c. cancriformis /simplex*, but about 2.5 times higher within the COI data compared to the 16S rDNA data. Such low sequence and haplotype diversity across a wide geographic range suggests that the species has colonised most of Europe very recently. Indeed, according to our molecular clock estimations the age of the *T. cancriformis* clade is consistent with European colonisation in an interglacial of the first half of the Pleistocene. The question of the origin of the species in Europe cannot be resolved until a more comprehensive sampling across the range is obtained.



1 - messinian salinity crisis about ca. 6 mya (Miocene/Pliocene)
- split of *T. c. mauritanicus* and the lineage collapsing *T. c. cancriformis/simplex* around this time is supported by mitochondrial sequence data of cytochrome c oxidase subunit I and 16S rDNA (see chapter 4)
- cold/dry *versus* hot/very humid conditions on Iberia may have caused range contraction of ephemeral waters

2 - European colonisation in an interglacial of the first half of the Pleistocene most likely by passive dispersal via animals (for example migratory birds, ungulate)
- Iberian Peninsula may act as one possible refugium of *T. cancriformis*

a hypothetical ancestral androdioecious population with males (black), pure females (white) and hermaphrodites (purple)

b Isolated rainwater pools almost always without any vegetation are populated by bisexual populations (dioecy) of *T. cancriformis* sp (b). Bisexual population (b') currently recognised as subspecies *T. c. mauritanicus*. High morphological and genetic divergence compared to other *T. cancriformis* subspecies suggest that the original species status given by Ghigi (1921) should be reinstated.

c Refugia within refugia may establish on Iberian Peninsula. Especially cysts from hermaphrodites are advantageous to populate new niches as they benefit from reproducing without mate.

d This pattern can be interpreted as middle and east Iberian bisexual populations which are currently recognised as subspecies *T. c. simplex*. Whether morphological females are pure females or hermaphrodites is an open question.

e Austrian and German populations of *T. c. cancriformis* investigated in this study with low male percentages. Mitochondrial and nuclear DNA data provide no evidence for parthenogenesis. Thus, females are assumed to be hermaphrodites.

f German, Italian and English populations of *T. c. cancriformis* where no males have been reported to date. Studies on nuclear DNA suggest that morphological females reproduce hermaphroditically.

Legend
- Dotted line represents weak barriers between populations. Thus, genetic exchange may still be possible among populations.
- Plain line illustrates strong barrier between populations. Geographically isolated population develops independent from the other.
- Question mark indicate processes or events not resolved so far.

Figure 7-2: Conceptual model about the process of population divergence as they become isolated, illustrated on *Triops cancriformis*.

Considering the reproductive strategy in large branchiopods, one is confronted with a complex situation, including bisexual, female biased and unisexual populations (Brauer, 1872; von Siebold, 1873; Hotovy, 1937; Longhurst, 1954; Zaffagnini & Trentini, 1980; Browne, 1992; Engelmann et al., 1997) (Figure 7-3). This reproductive mode may have developed from dioecy, where reproduction requires the union of gametes produced by two different individuals (pure males and pure females), by so far unclear evolutionary forces to androdioecious (mixture of males and hermaphrodites) (Graham, 1982; Otto et al., 1993; Weeks et al., 2006). The hermaphrodites in *T. cancriformis*, in which genetic evidence implicates the union of two independently derived gametes from the same individual (Sassaman, 1995), are unable to cross with each other. In populations lacking males, hermaphrodites are therefore completely selfing. The conditions, under which males can be maintained in hermaphroditic populations, have been studied on *Eulimnadia texana* (Spinicaudata) in a population genetic model by Otto et al. (1993). There results show that, even if males are rare, they will be maintained in the population due to the male offspring achieved from hermaphroditically reproduction. Additionally parthenogenesis (the transmission of the mother's genotype) in *T. cancriformis* has been discussed by various authors (Hempel-Zawitkowska, 1967; Akita, 1976; Zaffagnini & Trentini, 1980).

Such complex reproductive strategy including androdioecy has also been described for the Spinicaudata (Branchiopoda) (Sassaman, 1989; Weeks & Bernhardt, 2004; Weeks et al., 2006) and is also best studied in the plant species *Mercurialis annua* (Pannel, 1997a, b), *Phillyrea angustifolia* (Lepart & Dommee, 1992; Pannel & Ojeda, 2000; Vassiliadis et al., 2000), and *Datisca glomerata* (Liston et al., 1990; Fritsch & Rieseberg, 1992; Rieseberg et al., 1992). In contrast, the co-occurrence of bisexual and parthenogenetic individuals has been described for the geckos of the genus *Heteronotia* or the grasshopper *Warramaba virgo* (Kearney, 2005).

To date, *T. c. mauritanicus* subspecies have been reported to be bisexual exclusively. The status of the middle and east Iberian populations, currently identified as *T. c. simplex*, are almost always bisexual probably containing hermaphrodites. Populations within the distribution range of *T. c. cancriformis*, as described by Brtek & Thiery (1995), show generally low male percentage or even

none male occurrences at all. Against the background discussed above, it can be hypothesised that *T. cancriformis* derived from an ancestral bisexual population including pure females and also a low rate of hermaphrodites. The hermaphrodite individuals achieved an important role for founder events as it might have happened during the Messinian salinity crisis (about 6 mya) and after ice ages. Consequently the change in environment of the new habitat, both abiotic and biotic, leads to genetic differentiation compared to the ancestral population.

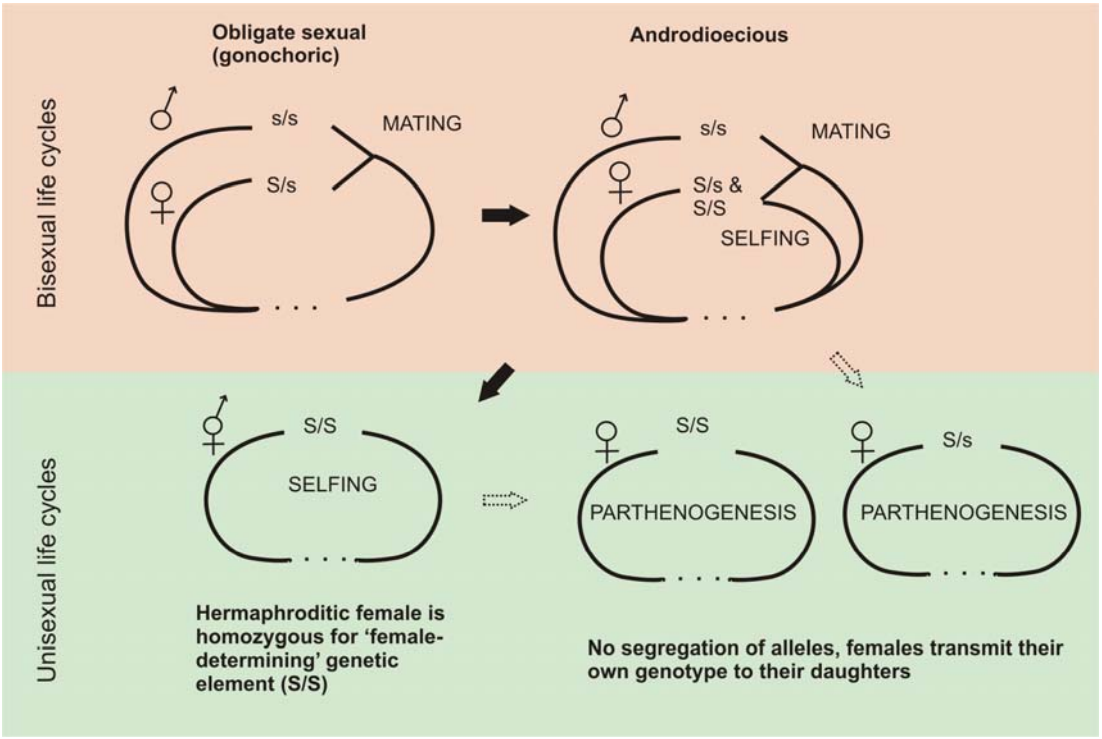


Figure 7-3: Schematic representations of life cycles within *Triops cancriformis*. Genders, genotypes and reproductive modes are indicated for each life cycle. Dots represent the diapause cyst stage and the dotted arrows indicate that the establishment and the occurrence of parthenogenesis are not finally resolved (modified after Sassaman 1995).

The possibility of passive distribution and the potential for populating new habitats by a single cyst, make large branchiopod cysts ~~to~~ an essential tool for conservation management. In fact, large branchiopods are endangered because the environments that support ephemeral waters have been, and continue to be, under threat from human activities. Effective conservation management targets, with benefits for large branchiopod species, should focus on *in situ* management action modules and also consider, additionally, *ex situ* conservation strategies (Figure 7-4).

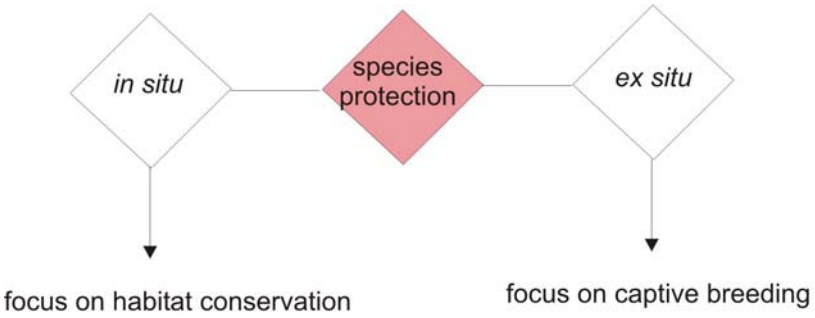


Figure 7-4: Large branchiopod species protection involves in situ activities that is habitat conservation and ex situ activities as captive breeding.

In situ conservation measures must first localize the threats for the habitat that either have an effect on the habitat (on site) or on the ecology of the habitat (on conditions) (Table 7-1).

Whenever "no" threats are observed, as may be the case within protected areas, the habitat development and the species presence have to be monitored and succession has to be controlled to allow the cyclic change of terrestrial and aquatic stag (Table 7-1, action module 1).

Action module 1 (protection):

- Check the current policy and legislation according to the habitat.
- Promote co-operation on the conservation of the large branchiopods throughout its range (i.e. co-operation between neighbouring countries of Danube river)
- Maintenance of the current level of legal protection until the number of populations is large enough that it can no longer be considered (as a threatened) an endangered species.
- Encourage the selection and suitable management of potential sites of local conservation organisations and national agencies.
- Check the current conservation management plan for species requirements.
- Monitor the *T. cancriformis* habitats (and any new sites which may establish) for changes in (i) population status (focus on male and female ratios development), (ii) species co-occurrence and (iii) habitat characteristics.
- Protect habitats from increase in herbaceous vegetation and compaction of soil covering the eggs laid during the previous aquatic phase.

Table 7-1: Overview of threats on ephemeral water bodies, their consequences on habitat location and ecology as well as suggestions for conservation measures and examples.

Threats					
Effect	"no"	on site		on conditions	
Consequences & Threats in detail	Protection • habitat is located in nature protected site • herbaceous vegetation covers the complete habitat and thus depressions are wiped out	Destruction • any kind of construction work leads to complete habitat loss • destruction of habitats results in population extinction and possibly loss in genetic diversity of the species	Fragmentation • split off and decrease in habitat site, small support less species that formerly occurred there • remnant areas will not experience the former frequency of natural disturbance • fragments surrounded by a different and funda-mentally altered ecosystem	Hydrological regime • ephemeral character is disturbed • shortening of waterlogged phase	Water pollution • eutrophication, • herbicide and pesticide load • dung deposits
Conservation measure	**action module 1**	**action module 2**	**action module 3**	**action module 4**	**action module 5**
Aims shortcut	• maintain open character • monitor water regime • monitor species and population abundance	• try to avoid complete habitat destruction • *ex-situ* management strategies (replacement measures and captive breeding)	• connection of fragments to reduce edge effects • *in-situ* management strategies • management of surrounding landscape matrix	• stop melioration • rebuild drainage canals • open/rebuild polder management • stop river straightening	• stop intensive agriculture, herbicide and pesticide load and waste disposal in surrounding landscape matrix and/or catchments area • cutting of surrounding meadows once a year
Examples	monitoring of the large branchiopod habitat "Blumengangsenke" along the Danube river (Austria) (Hödl & Eder, 1996)	destruction of secondary habitats in Lacoma due to opencast mining; sampling of cysts containing sediment performed by Beak Consultants GmbH	construction of dikes, drainage canals within Rhine river floodplain (Simon, 1987)	melioration caused by straightening and deepening of the river and application of artificial pumping station (Simon, 1987; Günther et al., 2005)	intensive agriculture along the Danube river (rotted plants cause anaerobic conditions during inundation, herbicides and pesticides pollute the water) (Eder & Hödl, 1996)

Threats on site, on the location of the habitat (i.e. the river floodplain), may cause complete habitat destruction or fragmentation. Such threats result from construction work within a floodplain (construction of dikes and water power stations, urbanisation). Any conservation management scheme should first raise the question if threats resulting in complete habitat destruction might be avoided. Diminishing a habitat may lead to the deletion of complete genotypes and thus reduce the genetic diversity of the species as shown in more detail in Box 7–1. If habitat destruction can not be avoided, cysts containing sediment should be sampled and applied for translocation (Box 7–2) as well as for captive breeding (Table 7-1, action module 2, Box 7–3). If translocation or reintroducing is necessary to prevent species or key genotype extinction, one has to consider the development of appropriate ephemeral habitats. Detailed descriptions on the establishment of such ecosystems are given by Jeffries (2001) and Biebighäuser (2005). Based on these paper (Jeffries, 2001; Biebighauser, 2005), the main steps in creating an ephemeral pond are (1) finding the appropriate location, (2) checking the infiltration rates of the underground, (3) checking the site hydrology and physicochemical features, (4) recording vegetation and reducing herbaceous vegetation as necessary, (5) reintroducing sediment containing cysts after monitoring the location for at least one waterlogged and one dry phase, (6) monitoring and documenting the hydrological regime, physicochemical water characteristic, large branchiopod presence or absence, the count of males and females, and threats during aquatic and terrestrial phase.

Action module 2 (destruction):

- Sample cysts containing sediment from known and endangered *T. cancriformi*s locations:
    - about 3-5 samples should be taken along the margin of the habitat;
    - gravel deposits or slightly grassy areas should be preferred compared to uncovered and muddy areas within the centre of the location.
- Dry sampled material under room conditions.
- Measure cysts abundance in the sediment by counting eggs in appropriate aliquots of the sediment using a stereo-microscope (large branchiopod species can be determined by cyst morphology (Tommasini & Scanabissi Sabelli, 1989; Thiéry & Gasc, 1991; Thiéry et al., 1995)).
- Store dried sediment in plastic bags under cool, dry and dark environment; all samples should be labelled with name of person collecting samples, sampling date, locations, number of sample from those location, mean number of *T. cancriformis* cysts found in selected aliquots and notes about other large branchiopods cysts.
- Perform breeding experiments:
    - to control hatchability of cysts and species composition and
    - to study influence of change in environmental features.
- Promote research into selected aspects of the autecology of *T. cancriformis* to refine habitat modelling and site management.
- Select a series of suitable nearby sites for potential reintroduction/transloation experiments and study abiotic parameter over at least one dry and one waterlogged phase. The assessment of suitability may be aided by comparing the conditions at alternative sites to those with known occurrences of *T. cancriformis*.
- Implement protocol on new sites after reintroduction or translocation.
- Reintroduction/translocation activities have to be performed following IUCN guidelines.

Box 7–2 | ***Ex situ* conservation - translocation of *Triops cancriformis* cysts**

Translocation includes both the artificial movement of individuals/resting eggs between natural populations and the release of (captive reared) material (often resting eggs) to reintroduce or augment a population. Translocations have been widely utilized in conservation programs to alleviate the detrimental effects of inbreeding depression (Haig, 1998; Storfer, 1999). Considering *T. cancriformis,* translocation has been applied to reintroduce populations both in former inhabited locations and in new environments. Several criteria have been associated with successful translocation of *T. cancriformis*. These include habitat quality and when and where species material is released relative to the extent of the habitat. Many habitat creations schemes specify that biological material of local source should be used in reintroductions. A major reason for the use of a local source is the claimed importance of conserving locally adapted genotypes which are assumed to show high fitness. However the importance of local source has been discussed controversially by Wilkinson (2001) and Sackville Hamilton (Sackville Hamilton, 2001). Wilkinson's (2001) key message is that the ever-changing nature of the environment means that a strong emphasis on the importance of local source is often hard to justify. Sackville Hamillton (2001) disputes Wilkinson's conclusion that local sources are unimportant for the fitness of long-lived individuals and states that use of locally sourced material should be standard practice, except where the reintroduction of non-local genotypes is specifically justified in terms of conservation genetics. Working with *T. cancriformis* one should recognize that regional phenotypes and also geographical-restricted genotypes occur. Therefore reintroduction measures should be performed using material of local source. Translocation of *T. cancriformis* reported so far refer to artificial movement of cyst containing sediment (Braasch et al., 1993; Maitland, 1995). Successful translocation of adult *T. cancriformis* individuals from one pond to another pond in nature has not been reported. Nevertheless own observations showed rearing captured adult individuals in laboratory resulted to be successful. The latter method can be used to receive resting eggs which further may be applied for captive breeding or reintroduction. Resting eggs can also be obtained by mud sampling from habitats with known *T. cancriformis* occurrences. The sediment should be sampled from the upper 1-10 cm during the terrestrial phase of the habitat and air dried before storage under dry and dark condition until their application. The eggs appear to be capable of persisting in the soil for decade or more (Moore, 1967; Dexter, 1973; Thiéry & Gasc, 1991; Brendonck, 1996).

Box 7–3 | *Ex situ* conservation - captive breeding of *Triops cancriformis*

When a species reaches very low numbers or its habitat becomes critically endangered, the decision may be taken to remove some individuals from the wild and attempt to conserve them in captivity (Pullin, 2002). The removal of individuals, or their resting eggs, from their natural habitat into captivity, either to breed or to maintain a genetic stock, is an *ex-situ* conservation measure. Captive breeding can result in detrimental genetic effects in captive populations, such as inbreeding or the loss of genetic variability (Frankham et al., 2002). However the data provided in this study suggest that inbreeding depression and low genetic variability seems to be common but not disadvantageous in *T. cancriformis*. An official captive breeding project on *T. cancriformis* has been established in United Kingdom (Chester Zoo, Dudley Zoo, Wildfowl &Wetlands Trust Martin Mere) first to maintain a captive population of native *T. cancriformis* as an 'insurance' against their extinction in the wild, and second, to produce a 'Species Action Plan' (SAP) for English Nature as a protocol for the future *in-situ* and *ex-situ* conservation of *T. cancriformis* (Hughes, 1997a). The developed SAP (Maitland, 1995) lays down the following objectives and targets for *ex-situ* conservation:

(1) Establishment of breeding populations of *T. cancriformis* in captivity which can then be used as sources of stock for research and reintroductions.
(2) Investigation of environmental factors affecting cyst production, development and survival of adults leading to recruitment in order to achieve a better understanding of the reproductive strategy in relation to environmental variables.
(3) Promotion of research into selected aspects of the autecology of *T. cancriformis*, including relevant physiology, to help refine habitat and site management and captive breeding requirements.
(4) Promotion of research on wild and captive-bred populations to determine their genetic structure and suitability as founders for introductions.
(5) Maintenance and expansion of the captive breeding programme until the British populations are considered to be beyond threat.

Similar projects may be possible in Germany where cysts containing sediment has been taken from Lacoma, a location which is destroyed now by opencast mining. The sediment is stored at the Museum of Natural History in Freiberg and further projects may be planned in cooperation with the company BEAK Consultants GmbH (Freiberg, Saxony).

Ephemeral water bodies are often fragmented naturally as for example isolated Mediterranean rainwater pools. But the occurrence of temporary waters in floodplains is more and more limited by fragmentation processes due to agricultural land use and urbanization, which disturb the natural water regime of the floodplain

(Figure 7-5). In this sense, fragmentation is characterized by four main points (i) the total area remaining is smaller, (ii) the proportion of edge in relation to the total area is greater, (iii) any given point within the fragment is on average closer to the edge than before and (iv) on average each fragment is more isolated from other fragments than before (New, 1995; Pullin, 2002). Consequently the ephemeral habitats may dry out faster and become covered by herbaceous vegetation which in the long term leads to population extinction. Conservation measures should therefore focus on the connection of the fragmented habitats or at least on their preservation (Table 7-1, action module 3).

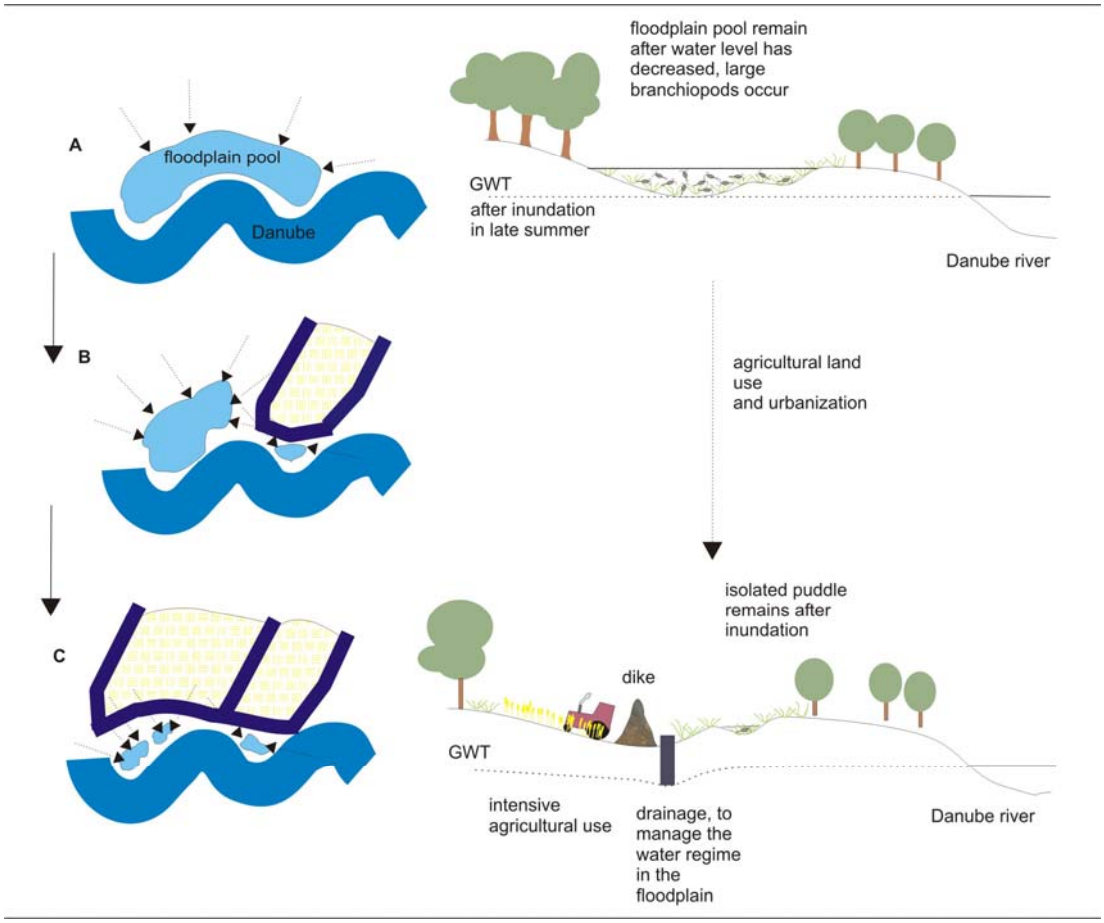


Figure 7-5: Schematic outline of the fragmentation of floodplain pools exemplary on Danube river floodplain east of Vienna. The natural floodplain regime (A) is disturbed by drainage and intensive agricultural use (B, C). In consequence the floodplain pools are becoming fragmented and edge effects (small dotted arrows) increase (influence of pesticide, nitrate and drainage) (picture Zierold, 2006).

Action module 3 (fragmentation):

- Maintain the natural process of flooding (hydrological regime) for the establishment of ephemeral water bodies to avoid ongoing fragmentation.
- Manage the activities in the neighbourhood of the reserve (maintain disturbance regime, reduce edge influence, limit pollution, cooperation with landowner).
- Monitor habitat quality and species development.
- In areas where there is significant human pressure, efforts should be made to encourage the use of reserve for educational and perhaps recreational purposes to raise (community awareness).

Threats affecting the ecological conditions of the ephemeral habitat (during the waterlogged phase) could result in changed hydrological regime and also in water pollution. The natural hydrological regime of a floodplain, characterized by regular flooding, will be disturbed by drainage canals, melioration measures, polder systems and other constructions preventing the water inflow into the floodplain or promoting artificial drainage after floods. These activities either prevent the establishment of ephemeral habitats, and thus the development of large branchiopods, or shorten the waterlogged phase. If the latter effects dry up habitats before large branchiopods reach reproductive stage, the resting egg bank will be decreased and this will affect the population size and genetic diversity of future generations. Conservation management actions should target benefits on the reestablishment of a natural floodplain character which includes the rebuilding of drainage canals and melioration constructions (Table 7-1, action module 4).

Action module 4 (hydrological regime):

- Rebuild river course of a river to reduce river bed deepening and to increase groundwater level and inundation in the floodplain
- Stop artificial drainage and melioration activities.
- Apply also action module 3.

Since *Triops cancriformis* responds very sensitively to oxygen reduction, threats like eutrophication or dung disposal cause the death of the organisms and thus the production of resting eggs is reduced. The application of herbicides and

pesticides, often related to intensive agriculture in drained floodplains, also leads to death. Whether those threats damage cysts deposits(?) has not been the object of scientific research to date. However, pesticide application in the fishery ponds and rice fields, and the presence of ivermectin[1] in the water, leads to decreased population sizes with respect to nonthreatened generations. Long-term effects are not clear (Takahashi et al., 1984; Langner, 1985; Hughes, 1997b). To reduce the impact of threats affecting the water quality, intensive agriculture has to be converted into extensive forms. Examples from the Danube and Rhine river floodplains prove that *T. cancriformis* occurs in extensively managed corn fields. Further activities should focus on the control of waste disposal (organic matter) in small floodplain depressions (Table 7-1, action module 5).

Action module 5 (water pollution):

- Control the management in the surrounding area (stop waste disposal in surrounding landscape matrix and catchments area.
- Intensive agriculture should be replaced by extensive land use. Wherever agricultural use is given up, meadows should be cut once a year or grazed by sheep or cattle.

To summarize this, the protection of large branchiopods inhabiting ponds in floodplain areas can only be achieved by maintaining the natural process and interaction present in the river ecosystem.

[1] In the mid-1980's ivermectin was introduced as probably the most broad-spectrum anti-parasite medication ever. It is effective against most common intestinal worms, most mites and some lice. Mar Vista Animal Medical Center (2004) Ivermectin. www.marvistavet.com/html/body_ivermectin.html.

Final Statements

(1) Most of the local/regional extinction of *T. cancriformis* could be attributed to change in land use which consequently leads to habitat destruction.

(2) *T. cancriformis* is well adapted to survive in isolated ephemeral water bodies and therefore the protection of both primary and secondary habitats of *T. cancriformis* is of higher priority than reintroduction measures.

(3) To prevent decreasing genotype diversity through extinction of key populations, conservation management schemes have to take into account the distribution of genotypes and also the centres of diversity.

(4) Opportunities among the public for the appreciation and conservation of temporary ponds and their important flora and fauna have to be promoted.

(5) Single site studies have to be combined in order to estimate threats on ephemeral habitats world wide and to establish conservation management strategies for long term preservation of biodiversity.

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