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The invasive potential of Prussian carp in Finland under the light of a novel semi-clonal reproductive mechanism

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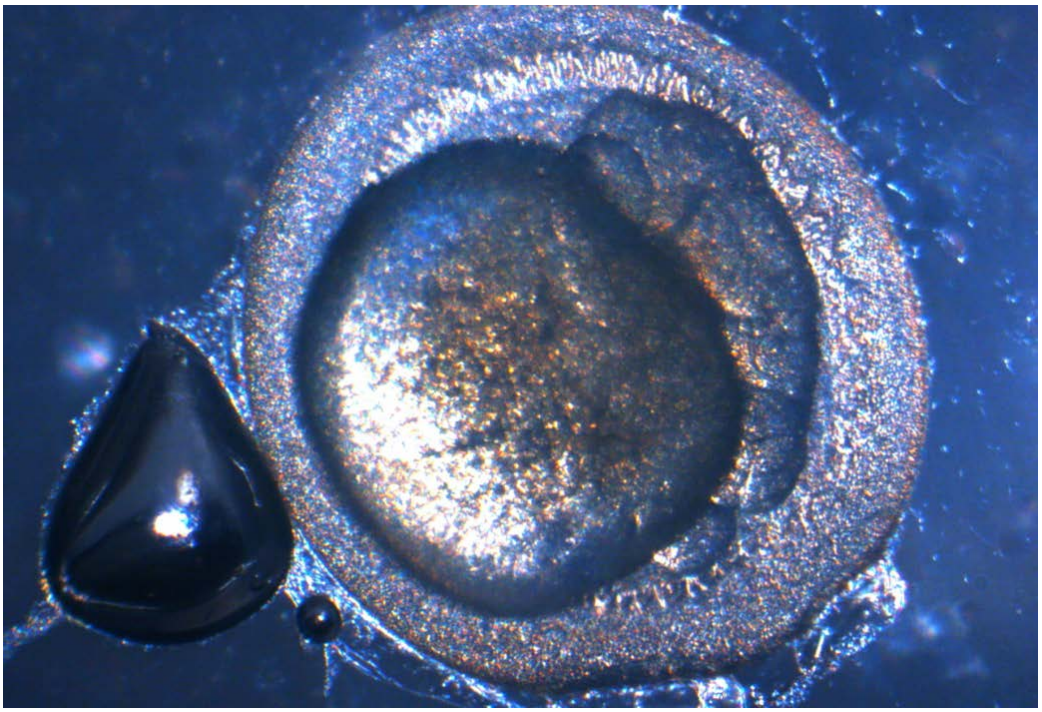

Diese Arbeit ist all jenen gewidmet, die das Leben verstehen wollen, hinterfragend, zweifelnd, auch gegen Glauben und Dogmen - jedoch ohne die Achtung vor diesem Leben zu vergessen, dessen Teil wir sind!

Tämä työ on omistettu kaikille niille, jotka pyrkivät ymmärtämään elämää, kyseenalaistaen, epäillen, myös vasten uskomuksia ja oppilauseita - kuitenkin unohtamatta kunnioitusta tätä elämää kohtaan, jonka osa olemme!

This work is dedicated to all those who want to understand life, questioning, doubting, also against believes and dogmas - but never forgetting the respect towards this life which we are a part of!

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ABSTRACT

A group of closely related East Asian fish of the genus *Carassius* has invaded Europe mainly during the last century. As this group consists of sexual and asexual complexes with different ploidies it is very difficult to apply the traditional species concept. The polyploid gynogenetic forms have been mostly summarized under the synonym *Carassius* (*auratus*) *gibelio*, commonly known as Prussian or gibel carp. Diploid, sexual forms are often classified as conspecific. When invading European waters, Prussian carp had a strong impact on the ecosystems, but especially on the only European carassiid, the crucian carp. In a case study, the Prussian carp complex currently invading Finland was analyzed ecologically and genetically. In a breeding experiment the potential sexual hosts of asexual Prussian carp in Finland and their genetic interactions were assessed. All tested hosts were found to be suiting. The studied complex was found to be highly diverse and consisting of a sexual and a gynogenetic lineage that spread independently. The latter is capable of receiving introgressions from sexual hosts regardless of genus. The degree of genetic introgression into the complex remains unresolved, but there are enough hints to propose an evolutionary effective reproductive system of introgressive gynogenesis, which will explain the Prussian carp's extreme success. Based on the results and literature the invasive potential and threat to the European crucian carp was evaluated. It was found probable that gynogenetic Prussian carp will invade Southern Finland and eradicate crucian carp resulting in a high Finnish and Nordic responsibility for the conservation of European crucian carp in uninvaded areas.

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TIIVISTELMÄ

Ryhmä samaan *Carassius* -sukuun kuuluvia kaloja on kotiutunut Eurooppaan pääasiassa viime vuosisadalla. Tämän ryhmän koostuessa suvullisesti ja suvuttomasti lisääntyvistä sekä eri ploidioita omaavista komplekseista perinteisen lajikäsitteen soveltaminen on erittäin hankala. Polyploidiset, suvuttomasti lisääntyvät muodot on useimmiten yhdistetty synonyymin *Carassius (auratus) gibelio* alla, joka on yleisesti tunnettu hopearuutanana. Diploidit, suvullisesti lisääntyvät muodot luokitellaan usein samaan lajiin kuuluviksi. Kun laji levisi Euroopassa, on havaittu lajin vahvoja vaikutuksia ekosyysteemeihin ja erityisesti sukunsa ainoaan eurooppalaiseen lajiin, ruutanaan. Tässä tutkimuksessa Suomeen levinneitä hopearuutanoita tutkittiin perimällisistä ja ekologisista ominaisuuksistaan. Lisäksi gynogeneettisen eli muiden lajien sukutuotteista loisivan hopearuutanan mahdolliset maiti-isännät Suomen oloissa sekä geneettiset vuorovaikutukset näiden kanssa on selvitetty kasvatuskokeessa. Kaikki tutkitut isännät todettiin sopiviksi. Tässä tutkittu kompleksi osoittautui erittäin monimuotoiseksi sekä koostuvan yhdestä suvullisesti sekä yhdestä suvuttomasti lisääntyvästä linjasta, jotka leviävät toisistaan riippumatta. Jälkimmäinen kykenee vastaanottamaan perimää maiti-isänniltään näiden suvusta riippumatta. Muilta lajeilta tulevan geenivirran laajuus jäi selvittämättä, mutta on perusteltua esittää hopearuutanan omaksi evolutiiviseksi strategiaksi introgressiivinen gynogeneesi. Tämä selittäisi hopearuutanan menestyksen. Näiden selvitysten tuloksiin ja kirjallisuuteen perustuen arvioitiin hopearuutanan leviämismahdollisuudet Suomessa ja kotoperäiseen ruutanaan kohdistuva uhka. Arvioitiin todennäköiseksi, että gynogeneettinen hopearuutana tulee valtaamaan Etelä-Suomen ja hävittämään ruutanan. Tästä seuraa että Suomi ja Pohjoismaat kantavat suuren vastuun Euroopan ruutanan säilymisessä hopearuutanan valloittamattomilla alueillaan.

Contents

1. INTRODUCTION

1.1. Motivational background

The Prussian carp is one of the big riddles in European fish biology. Probably a wrong identified, polyphyletic species complex (see point 2.1.1.) of partly hybrid origin (see point 2.3.) it does not fit into the species concept usually used for vertebrates. Additionally its clonal reproduction mode and possible interspecies crosses contradict the species definition as a reproductively isolated, internally mixing gene pool. As such, it is a very interesting study object for evolutionary biology as it is probably succeeding for tens or hundreds of generations – spreading successfully as well as remaining competitive in its original area.

This naturally arisen, hybrid-origin, partly polyploid and ploidy-changing system of evolutionarily developing clones, which can reproduce without conspecific or closely related mates, is far less studied than many similar systems, which are either invertebrates or much less diverse and less successful. This must change in the face of biotechnology and bioengineering trying to offer humankind solutions to sustainably increase fish protein production and also reducing its environmental impacts. At the face of billions of polyploid, monosex and hybrid fish being released into fish farms and open waters every year, at the face of dozens of transgenic fish species being under development, some of them already approved by authorities, we might have a closer glimpse of what is there in nature - showing alternatives and warnings. Hybrids, polyploid and/or unisex fish stocks are used because they are stated to be sterile and would not genetically harm wild stocks or displace them. Prussian carp are all of this – naturally. Transgenic fish are assumed to have lower chances in the wild. Fish with technically doubled maternal genome will have higher survival when bearing a non-close-relative paternal genome than do diploid outcomes of natural hybridization. As we will see there are reasons to argue, that the same is true for natural polyploid being fertilized by other species and that these fish even evolutionarily profit from this – or at least do not suffer. There might be lots of aspects we can learn from these fish when considering use of techniques in aquaculture – not excluding mechanisms we might technically utilize. For that we must understand these exceptional fish, which is one goal of the present study.

Next to these abstract and large scopes to which this study can contribute only small pieces of the puzzle, there is a very concrete question to be solved with this work. The introduction and invasion of Prussian carps in Europe seems to have environmental impacts, especially on the endemic crucian carp. For this, we need to know the potential of Prussian carp to invade the last large areas in Europe it has not yet colonized – the water-rich Fennoscandian area – and its possible impact here. As the species was recognized in Finland only as late as 2005 (Lauri Urho and Jussi Pennanen, pers. comm.), and Sweden in 2010 (own observations, Jussi Pennanen, pers. comm.) or 2011 by DNA analysis (Wouters et al. 2012), respectively, there is a strong need of information to estimate the impact and need for counter measures in time. This study aimed to contribute for this need.

1.2. Scope and realization

Practically, this study aims to investigate the invasive and evolutionary potential of the Prussian carp complex at the example of the populations invading Finland. The invasive potential is studied by checking all local species for their potential to serve as sexual hosts. This has not been done before. Further, this study tries to give an insight into the reproduction modes actually found in Finland – at the current outer limit of this

spreading species – and comparing it with knowledge from other areas, where the species is already established. This includes a short description of observed stock developments and ecological effects and a placing of the Finnish populations within the European populations which the conclusions are drawn from. To get an idea of the evolutionary potential, this study tries to understand the complicated system of combined reproductive modes. Due to small resources this Master's thesis is only a small scratch on the surface of a system studied far less than many comparable systems, but it shows that it is worth being studied much more.

These goals are achieved by two distinct practical investigations: A) a genetic comparison of fish in wild populations in the light of existing knowledge about Prussian carp and B) a breeding experiment and a genetic comparison between mothers and offspring of the experiment. These two investigations will be treated separately in description of methods, results and their discussion, but thematically connected as a conclusive synthesis at the end of this thesis.

1.2.1. A) - Genetic comparison of wild populations

To find differences in genetic structure and reproductive mode between populations, but also to identify genetic structure within populations, individuals from different Finnish and Central European populations were analyzed with respect to individual ploidy (using flow cytometry) and evolutionarily neutral molecular markers. For the latter purpose microsatellites were found most suitable reflecting relatively young changes in individual and population genetics. The genetic data were compared to the sex and habitus-based taxonomic identity of the individuals and data about sex ratios and hybridization of the according populations.

1.2.2. B) - Breeding experiment

The first goal was to test, which of those Finnish fish species that might potentially spawn together with Prussian carp, could induce egg development in Prussian carp, and to which extent. Considering spawning times and -behaviour, only cyprinid species came into consideration: the Cyprininae common (*Cyprinus carpio*) and crucian carp (*Carassius carassius*) and tench (*Tinca tinca*, Tincinae), and the Leuciscinae bream (*Abramis brama*), bleak (*Alburnus alburnus*), white bream (*Blicca bjoerkna*), roach (*Rutilus rutilus*), rudd (*Scardinius erythrophtalmus)* and zope (*Ballerus ballerus*). Common carp has been proved numerous times to induce egg development in Prussian carp (Stein & Geldhauser 1992, Lieder 1955) and was therefore excluded from the experiments. Of the remaining species only rudd has been proved once as potential host (Stein & Geldhauser 1992). The close relative of roach, a Balkan roach, *Rutilus ylikiensis* has been stated to be an important host, but the authors give no experimental proof for their assumption (Paschos et al. 2004). In addition, a male of a possibly sexual lineage was added to the experiments.

To enable simultaneous treatment of the eggs of one batch with multiple species' milt, cryopreservation was used to effectively gain and store high quality sperm from wild sperm donors at the natural spawning time of each species. Thus milt of all potential hosts could be applied to the eggs of any Prussian carp whenever it was ready to spawn. The simultaneous multihost treatment of a single batch was necessary to have comparative data. Some basic genotype analyses were possible from hatched and unhatched larvae, but to get phenotype data as morphometry and survival, each treatment had to be raised separately to a stage that made analyses possible. Phenotype data were collected to answer

the question about possible fertilization, mother-offspring inheritance and mechanisms of gynogenesis in Prussian carps.

To assess the ploidy of mothers and their offspring their genome size was analyzed using flow cytometry.

2. BACKGROUND INFORMATION

2.1. The “Prussian carp” in Europe

2.1.1. Nomenclature - definitions

The taxonomy of carassiids can be currently viewed as being unresolved. During the last years scientists tended to use own species names for each known Asian form. Nonetheless there is still a vast amount of scientists using the old system of treating basically all Asian carassiids as subspecies of the goldfish *C. auratus*. The naturally coloured Asian carassiids introduced to Europe are usually referred to as *C. gibelio* or *C. auratus gibelio*. However, molecular data suggest that there are different groups existing which are quite distinct (Kalous et al. 2007, Rylková et al. 2009, Kalous et al. 2012, Takada et al. 2010), finding strong distinctiveness even within these groups (Kalous et al. 2007, Rylková et al. 2009, Hänfling et al. 2005, Brykov et al. 2005, Takada et al. 2010). A big problem is the continuous gene flow and hybridizations between different lineages (Takada et al. 2010). This is further complicated by hybrid origin (Xiao et al. 2011, Lamatsch & Stöck 2009, Zhou & Gui 2002, Chun et al. 2001, Murakami & Fujitami 1997, see also point 2.3.) and gynogenesis (for explanation see 2.2.) of triploid carassiid lineages,

as well as the introgression of genetic material from other species or even clades (Papoušek et al. 2008, Tóth 2004, Liu et al. 2007).

Thus, fish referred to as *C. auratus ssp.* or *C. gibelio* found in Europe are obviously polyphyletic according to morphological and molecular data (Kalous et al. 2007, Kalous et al. 2012, Lukáš Kalous, pers. comm., Hänfling et al. 2005). Still, the problem remains mainly unresolved, and up to date only one species other than *C. gibelio* and *C. auratus auratus* - *C. langsdorfii* (Kalous et al. 2007) has been identified. This is by far not the whole truth, and findings of Takada et al. (2010) show already four different mitochondrial haplotypes from only 20 European samples from only three countries that might be interpreted as distinct species by a conservative point of view. Based on analyses of 43 carassiid specimen Kalous et al. (2012) also suggest a yet undescribed species of *Carassius* from Mongolia that has been misidentified as *C. gibelio* earlier. Misidentifying undescribed species as known ones must be assumed common due to the lack of taxonomic alternatives for identifiers.

Also the name used for the Asian carassiids other than goldfish is debatable. *C. gibelio* Bloch, 1782, was derived from *Cyprinus gibelio* described by Marcus Elieser Bloch (Bloch 1784). The species Bloch described was the stunted ecomorph of the crucian carp *C. carassius* (Jörg Freyhof pers. comm., Bade 1901, Grote et al. 1909, Heckel & Kner 1858) the only carassiid native to Europe. Kalous et al. (2012) describe the only existing syntype being replaced by a specimen of crucian carp according to Paepke (1999). Being originally the name of a morph of another species - nowadays an invalid synonym of that species - *C. gibelio* should not be used for the species (-group) considered here. Although Heuschmann (1939) tried to explain the native origin of a species group he considered to be *C. gibelio*, it seems very probable that the taxa considered have not been in Europe at

Bloch's times and he cannot have described them (see point 2.1.2.). Rather unscientific, old descriptions of fish called "giebel" or similarly as e.g. in Richter (1754) or Birkholz (1770) and Heckel & Kner (1858) can be either clearly identified as crucian carp, or there is no clear identification possible. In the late 19th century, Bloch´s description was already recognized as crucian carps (Siebold 1863, Bade 1901, Grote et al. 1909). Confusion arose again after the introduction of Asian carassiids, mainly in the 20th century (see point 2.1.2.). Kalous et al. (2012) tried to solve this problem by redescribing *C. gibelio* on a new neotype. The acceptance of this solution has to be observed in future. Also, it offers no solution for the taxonomical status of polyploid, asexual carassiids as described here. The neotype is a diploid male with morphometric traits rather untypical for polyploid groups typical for Northern Central and Northern Europe.

To avoid any confusion I will use common names as follows: **Crucian carp = *C. carassius***, **goldfish = *C. auratus***, **Prussian carp** = **the Asian carassiid complex observed in Europe**, excluding taxonomically clear, diploid, sexual goldfish. I will avoid the use of imprecise scientific names for this complex or parts of it. As an exception I will mention the species *C. langsdorfii* (often referred as "ginbuna") separately although I assume that unidentified "lineages" of that species form a part of the Prussian carp complex in Central Europe (see also: Kalous et al. 2007, Kalous et al. 2012, Vetešník et al. 2008). As **species** I will carefully treat well acknowledged species as those above. I will mention **subspecies** referring to literature were this term is intensively used without commenting on the actual taxonomic state of the taxon in question. For dividing different genetic groups of Prussian carp that clearly differ from the other groups in terms of genetics, reproductive modes, habitus and, for clones, allele patterns, I will use the term **lineage**. This term assumes that there is no large genetic exchange between these lineages without commenting on the physical possibilities of such or the taxonomic state of the lineage. In clones, lineage will concern several individuals with a common allele pattern.

In ecological sense, as environmental impact and competition with native species, Prussian carp and Goldfish can be seen as functionally equal, and studies made on the impact of either group will be used to discuss the impact of Prussian carp.

2.1.2. The invasion of Asian carassiids

Different, closely related carassiids were introduced to Europe from East Asia since the 17th century (Grote et al. 1909, Pelz 1987, Bade 1901). This started with the import of sexually reproducing goldfish as ornamental fish. These established wild populations in the warmer southern and western regions of the continent (Hänfling et al. 2005, Bade 1901), and also hybridized with the endemic crucian carp (Hänfling 2005, Wheeler 2000). Many older findings of "*C. gibelio*" not identical with crucian carp have to be considered as naturalized goldfish (Heckel & Kner 1885). For example Heuschmann (1939) describes clearly two different morphs of "*C. gibelio*" in Germany, one of which (the only one he has obviously seen!) can be assigned as goldfish based on the well described colour and fin patterns and the occurrence of blood parasites against which Prussian carp seem to be resistant (Deinhardt, unpublished data). In southern Europe and England goldfish are nowadays widespread and a danger to crucian carp (Hänfling 2005, Wheeler 2000).

The first trustable records for Prussian carp date back to an introduction of "Japanese carp" around 1900 to Poland (Gasowska 1938). Bănărescu (1964) mentions a first appearance in the lower Danube for the 1920's without giving references. These fish and newer descriptions from Germany (Heuschmann 1939) do not mention any abnormal sex ratios. Noticing the high scientific standard and large amounts of used base data of the

considered publications, there must be assumed, that these and other fish described before the late 1940's were bisexual (Lieder 1955). First mentions of a gynogenetic mode of reproduction of European Prussian carp come from Soviet publications (Drjagin 1949, Berg 1949), and for Central Europe in 1955 (Lieder 1955). However, the ploidy of these fish was still 2n (n=94) according to analyses done by Lieder (1955) in Germany. Hints on triploidy accumulate since the 1960's from Eastern Europe (Cherfas 1966). Hence either a series of invasions must be assumed - possibly including several species or lineages - as can be proved by the mentioned Polish introductions and several Soviet propagations (Vetemaa 2005, Pihu et al. 2003, Mezhzherin & Lisetskij 2004), or a shift in ploidy and reproductive mode. The technical possibility of the latter can be proved by vast evidence (Zhou & Gui 2002, Takada et al. 2010) including findings made in this research (see point 4.2.2.). Following the given evidence, several lineages or even species of Prussian carp have been introduced to Europe, spread and evolved there. Nowadays there are found fully and partly sexual populations, different ploidies in males and females and high morphologic diversity. There is also evidence of hybridization with the endemic crucian carp (Wouters et al. 2012, Papoušek 2008, Mezhzherin & Lisetskij 2004, Tóth 2005, Ratschan et al. 2009) with 2n hybrids having partly normally developed gonads (Mezhzherin & Lisetskij 2004, Deinhardt, unpublished data). These facts show a highly diverse, dynamic system of closely related complexes, effectively invading European waters and rapidly threatening natural ecosystems, especially the existence of the endemic crucian carp (Mezhzherin & Lisetskij 2004). To Finland the Prussian carp spread in the late 1990's, probably via the Gulf of Finland from Estonia or Russia (Lauri Urho and Jussi Pennanen, Finnish Game and Fishery Research Institute, Helsinki, pers. comm.).

Prussian carps seem to be superior to crucian carp, especially in small water bodies free of piscivorous fish and in waters with heavy human impact: In such waters they have been commonly suspected and observed to displace crucian carps partly or even totally (Mezhzherin et al. 2004, Deinhardt 2008, Stein & Geldhauser 1992, Josef Wanzenböck, Research Institute for Limnology – Mondsee, University of Innsbruck, pers. comm.) although the existing literature and data is sparse. This success is due to many ecological and life history advantages, especially the ability to reproduce gynogenetically (Deinhardt 2008, see also point 2.2.). The gynogenetic reproduction is often dependent on the ecologically similar crucian carp as sperm host (Reshetnikov 2003, Pihu et al. 2003, Vetemaa et al. 2005), but does not totally rely on it. It can be assumed that any cyprinid is a potential sperm donor and several species have been proved or suspected (Pihu et al. 2003, Zhou & Gui 2002, Paschos et al. 2004). The yet unclear extent of hybridization and gene flow between crucian and Prussian carp is a potential threat to the genetic integrity of this endemic species. Due to displacement from certain parts of its natural niche the total genetic variation of crucian carp can be expected to decline and thus the species will change ecologically-genetically throughout almost all its natural area.

2.2. Asexual reproduction and gynogenesis in fish

Among fish there is a wide range of asexual reproduction modes, sometimes paired with partly sexual reproduction and gene flow (Lamatsch & Stöck 2009, Stenberg & Saura 2009, Pandian & Koteeswaran 1998). The development of unfertilized eggs is called **parthenogenesis** (Stenberg & Saura 2009, Tirri et al. 1993). Other than in some parthenogenic reptiles (Kearney et al. 2009) asexual reproduction in fish and other oviparous vertebrates (amphibians) is always sperm-dependent (Kearney et al. 2009, Lamatsch & Stöck 2009) which is called **gynogenesis** (Lamatsch & Stöck 2009) or sperm-dependent parthenogenesis. This makes asexual fish **sexual parasites** that utilize other

individuals' gametes for their own reproduction, excluding the host genomes from further reproduction. The explanation for this obligatory sperm dependence is that the fish egg needs the shell reaction caused by the penetrating sperm to start its development. It is also speculated that occasional fertilization events might be of high importance for clonal populations, whereas those without this advantage (or true parthenogenesis) are commonly seen as evolutionary dead ends (Schartl et al. 1995, Green & Noakes 1995). Another, very effective, form of asexual reproduction is **hybridogenesis** (for details see Lamatsch & Stöck, 2009 or Goddard & Schultz, 1993), where the haploid paternal genome is integrated into the nucleus together with the maternal haploid (sometimes diploid) genome as in a normal fertilization. It is part of the developing individual and expressed in somatic cells. In the germ line, on the other hand, it groups with the other chromosome sets during metaphase of the first division, but chromosomes are not mixed as normal in meiosis, but in the telophase all paternal chromosomes are separated into a polar body and disposed (Goddard & Schultz 1993). This means that the genome inherited is always the unchanged maternal one. This is called a **hemiclone** (Vrijenhoek et al. 1977, Vorburger et al. 2009).

In gynogenesis, a female fish produces unreduced egg cells. In theory the mechanisms can be mitotic division, fusion of divided oocytes after the first or second meiotic division (enables crossing over) and inhibition of the second meiotic division, which enables chromosome shuffling (but also produces total homozygotes). In the Prussian carp studied here, the mechanism was assumed to be an endomitotic division before the first meiotic division (Stenberg & Saura 2009) or a mitotic division (Cherfas 1966). This means genetically a perfect copy of the maternal genome (Lamatsch & Stöck 2009, Stenberg & Saura, 2009). Phenotypically, this does not necessarily hold true in polyploids due to differential expression of the different alleles (see Pala et al. 2010). The paternal genome of the penetrating sperm is usually not integrated, but the penetration only activates the developing of the egg into an individual (Lamatsch & Stöck 2009, Pihu et al. 2003).

This reproductive strategy does not always totally exclude paternal genes from contributing to the next generation - there might happen **paternal introgression** (=paternal leakage): The whole chromosome set or single chromosomes or parts of them might fuse into the offspring genome and even continue in the next generation. Ploidy elevation means factual hybridization, but often in an uneven relation as a result of di- or polyploid egg cells. For the Amazon molly *Poecilia formosa* (Lamatsch 2001) and Prussian carp (Zhou & Gui 2002) paternal leakage in the form of so-called microchromosomes is proved. These are rudimental chromosomes of the disintegrated paternal genome. Some alleles of microchromosomes were proven to be inherited and even expressed in *P. formosa* (Nanda et al. 2007, Schartl et al. 1995, Lamatsch 2001). Nonetheless, these genetic "leaks" in sperm-dependent asexual reproduction are seen as a rare exception, although they are discussed to have massive impact on evolutionary fitness of clonal populations or genomes (Beukeboom & Vrijenhoek 1998, Schartl et al. 1995, Green & Noakes 1995). The introgression of smaller amounts of foreign DNA into a clonal genome might not change most of the individuals' properties or the way of reproducing, but is breaking with the traditional definition of a clone as an identical genetic copy. Lacking a proper term, I will refer to this kind of clone as a **semiclone**, describing a basic, unchangedly gynogenetic, polyploid clonal genome with introgressed foreign DNA, which can vary between individuals or lineages. Other than in a hemiclone the introgressions can partly or wholly inhere.

Gynogenesis as described here is not to be confused with the biotechnical term for producing offspring from only the maternal genome by maternal chromosome doubling and deactivated sperm (Beaumont & Hoare 2003, Tave 2001, Pandian & Koteeswaran 1998).

2.3. Hybridization and polyploidy in fish

Natural polyploidy occurs in many fish families and species (Lamatsch & Stöck 2009, Pandian & Koteeswaran 1998). Well known are Asian carassiids (Lamatsch & Stöck 2009), the Amazon molly (Lamatsch & Stöck 2009,), *Poeciliopsis monacha-lucida* (Lively et al. 1990) and loach hybrids of the families *Cobitis* and *Sabanajewa* (Lamatsch & Stöck 2009, Ritterbusch & Bohlen 2000). Most of these fish seem to exist in quickly and irregularly changing habitat mosaics exhibiting rapid changes in metapopulation structure. Typical habitats are dry-falling desert rivers and large lowland river flood plains of temperate areas. Some have very successfully colonized anthropogenic, highly disturbed areas as ponds, rice fields and drained marshlands.

Asexual reproduction in vertebrates is considered to be always a product of hybridization (Lamatsch & Stöck 2009) and is often paired with polyploidy. This pairing might have three reasons: first, triple chromosome sets impair meiosis and will produce aneuploid germ cells or cells with varying ploidy (Tave 2001, Pandian & Koteeswaran 1998, Flajšhans et al. 2008). This prevents successful reproduction of the resulting hybrids with their parents or among each other. The only way such a hybrid might successfully reproduce and also avoid hybrid breakdown in the F2 (Verspoor et al. 2007) is the appearance of ameiotic reproduction - coincidence or not is another question. Secondly, in diploid species having evolved asexual reproduction - as a result of hybridization (Kearney et al 2009) as described before - newly appearing triploids are not excluded from reproduction but the inherited traits for gynogenesis tend to work in them as well (Stenberg & Saura 2009). Such triploids can be triple hybrids or have a double chromosome set from either parent, which prevents hemizygosity of harmful alleles. Both kinds of triploid hybrids can be equally fit or superior to their diploid counterparts in many cases which in turn facilitates their chance of becoming common within the clonal population. Thirdly, polyploidy is incompatible with chromosomal sex determination (Stenberg & Saura 2009, Pandian & Koteeswaran 1998). So, asexual reproduction is not automatically a consequence of tri- or polyploidy, but the only reproductive refuge for most triploids produced by reproductive (meiotic/fertilization/gynogenetic) malfunction. This is in spite of the fact, that hybridizing and odd polyploidy do facilitate asexual reproduction via inhibiting normal meiosis and thus resulting in eggs which are - if developing - basically viable without a paternal chromosome set (Kearney et al. 2009). Cloning is also the only way to maintain the not inheritable superiority (hybrid vigor) of a hybrid genome (Kearney et al. 2009, Tave 2001). This might be an explanation why clonal reproduction, commonly seen as "dead end of evolution", is successful in many animals, but also, why all asexual vertebrates are of hybrid origin (see also point 2.4.).

As mentioned above, di- and polyploid asexuals and potentially other polyploids can produce di- or polyploid gametes (Pandian & Koteeswaran 1998, Flajšhans 2008). These can fuse with each other or haploid gametes from sexual species producing new hybrids and new ploidies. This is also used in biotechnical fish breeding (Tave 2001, Pandian & Koteeswaran 1998) combining e.g. diploid sperm (from tetraploid males) with haploid eggs to produce triploids of a certain sex. Alternatively, the fusion of a single chromosome set of a haploid sperm with the three chromosome sets of a gynogenetic individual producing a tetraploid hybrid can be a stepping stone towards new sexual reproduction:

Even numbers of chromosome sets enable meiotic division to occur based on the principle, that the first meiotic division or reductional division needs homologous chromosomes to be aligned pairwise. There are also observations of Prussian carp with a chromosome number indicating triploidy but the total chromosome number being halved during meiosis (Flajšhans 2008). Fan & Shen (1990) and Zhou & Gui (2002) explain this with functional diploidy of these fish. This diploidization in odd polyploids is not the rule and even questionable, because in this case nonhomologous chromosomes would be aligned during metaphase. On the other hand diploidization is what enables gonochoristic reproduction in tetra- and other even polyploids as mentioned above. This process, used in fish breeding biotechnics (Tave 2001, Pandian & Koteeswaran 1998), occurs in nature and was proved to have happened during the evolution of salmonids (Verspoor et al. 2007, Kottelat & Freyhof 2007), the polyphyletic barbels (Kottelat & Freyhof 2007), the tench *Tinca tinca* and the cyprininae (Tave 2001, Kottelat & Freyhof 2007) including economically important fish as carp, goldfish and all other carassiids. This has happened millions of years ago and the genomes are largely rediploidized, preventing the genome of these species from breakdown into other than two chromosome sets (Verspoor et al. 2007, Tave 2001). But there still exist many loci that code for the same phenotypes, complicating the understanding of the genetics of such species (Verspoor et al. 2007). Tetraploid Prussian carp observed quite frequently (Flajšhans et al. 2008, Mezhzherin 2004, Xiao et al. 2011) might be a first step towards a similar diploidization doubling the originally tetraploid genome one more time on the way back to sexual reproduction.

Polyploidy can be divided by the origin of the involved genetic material into auto- (genes from the same species) and allopolyploidy (genes from hybridization). This separation is artificial and imprecise as is separation into species, and genomes from individuals defined as conspecific can still be distinct enough to have an influence on e.g. meiotic processes or offspring survival. Both forms of polyploidy can be produced by the same mechanisms. The mechanisms enabling polyploidy are either disturbed meiosis resulting in di- or polyploid gamete (mostly egg) nuclei. Such a gamete will fuse with a haploid one to further elevate the ploidy, but - theoretically - in parthenogenetic organisms a ploidy elevation within the genome as an extreme case of autopolyploidy can be possible. A third possibility is the incomplete first mitotic division resulting in chromosome doubling. The first meiotic division or reduction division happens in most fish in the gonads, the second usually happens after egg fertilization (Beaumont & Hoare 2003, Tave 2001, Pandian & Koteeswaran 1998), which is, in most fish, in the free water. In principle, both divisions can be interrupted, resulting each in a doubling of the expected chromosome set of the gamete and producing odd ploidy after fertilization. Interruption during the first division will produce identical copies of the maternal genome, because the homologous chromosomes will not be separated and shuffled and crossing over will be inhibited. However, this is rather improbable, since disturbances are rare to cause this in the gonads without inhibiting spawning. Possible disturbances may be genetic inhibition of gene expression for tubulin or other proteins important for a working spindle apparatus. This might be a mechanism enabling natural gynogenesis. Another possibility is a chemical inhibition as caused by colchicine or other spindle poisons. Much more probable is the disturbance of the second division of the oocyte, which allows shuffling of chromosomes and loci, but produces completely homozygous gametes (Tave 2001, Pandian & Koteeswaran 1998). The second division happens after fertilization and during egg swelling and hardening, and can be easily disturbed by environmental factors, namely temperature (warm or cold), chemical or physical (pressure change) shock (Tave 2001). In nature this can be upwelling cold groundwater, vegetation with eggs sticking on pressed

into cold bottoms by a fisher's boot or eggs getting squeezed on substrate. The natural occurrence of autotriploids proves for the sensitivity of fish eggs to meiosis disturbance. There might be also an influence of chromosome incompatibility that might explain the high number of only triploid hybrids found between some species as e.g. *Salmo salar x trutta* (Verspoor et al. 2007). Another explanation is that diploid hybrids are not viable and triploids created by chance (as described above) just reflect the high rate of natural polyploidization. In many invertebrate parthenogenetic clones, meiosis is usually disabled in the first division and is practically a mitotic division resulting in the total reproduction of the parental genome. The externally caused doubling of chromosome sets in eggs described here is normally combined with the fusion with the penetrating sperm nucleus (1n), producing odd chromosome sets in the offspring, usually triploids. The mechanisms described here for oocytes are theoretically also possible for spermatocytes, but less probable since spermatogenesis is completed within the gonads. Also their success might be lower for di- or polyploid spermatozoa being less motile and less fit (Flajšhans et al. 2008). The shocks listed for disturbing the second division can also inhibit the first mitotic division (first cleavage) of the diploid blastula (Tave 2001, Pandian & Koteeswaran 1998), which will result in tetraploid fish. Disturbances at later cleavages might even produce mosaic individuals (Pandian & Koteeswaran 1998) with cells of different ploidy. In nature we usually find polyploids as a result of hybridization of mostly closely related species, so that the chromosome number of each set does not differ a lot. Tóth et al. (2005) produced under experimental conditions viable triploid hybrids of Prussian carp and *Pethia conchonius* with the paternal set having just half the chromosome number than one of the two maternal sets.

The existing triploid gynogenetic species seem to be always hybrids i.e. allotriploid (Lamatsch & Stöck 2009). The reasons are not known yet, but the advantages and disadvantages of hybrids per se are well known: heterozygosis and diverse alleles contributing to hybrid vigor (Tave 2001) and, on the other hand, incompatible interactions of alleles at different loci (epistasis, cumulative effects) may reduce viability and fitness (Tave 2001, Stenberg & Saura 2009). Combination of two or more differently selected genomes can cause maladaptation of the hybrid (Verspoor et al. 2007). The advantages and disadvantages of polyploids are related to the origin of the different chromosome sets and depending on the genes involved. Totally homozygous polyploids - as diploids - suffer from their homozygosis, but also gain advantage from expression of advantageous recessive alleles. Clones as produced in gynogenesis will have an evolutional disadvantage described in point 2.4. Heterozygous polyploids have the advantage of combining a wide variety of different alleles and reduced probability of expression of dysfunctional or deleterious alleles. A higher growth rate and maximum size predicted from the bigger cell size of polyploids (Pandian & Koteeswaran 1998) is usually not true due to a reduced cell number and/or reduced growth in vertebrates (Tave 2001, Beaumont & Hoare 2003, Pandian & Koteeswaran 1998). A big problem of polyploids is often their physiological or functional infertility (Tave 2001, Verspoor et al. 2007) and the prevalence of only one sex, at least in triploids. The dominance of one sex is explained by only cloning of the maternal genome, or by Haldane's rule (´when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous (heterogametic) sex', Haldane 1922, Schilthuizen et al. 2011. Hybrids have no evolved mate preferences and in fish usually try to spawn with parental or related species. In several fish species reproductive discrimination of pure species against hybrids can be observed (Castillo et al. 2007, Verspoor et al. 2007). In the Japanese carassiids *C. langsdorfii* and *C. sp.* this is not found (Hakoyama et al. 2001), but in Prussian carp this is not studied yet.

2.3.1. Ploidy definition for Prussian carp

As already mentioned, the subfamily of cyprininae has gone through a tetraploid stage more than 10Mya (Yuan et al. 2010). This led to rediploidization of the whole genome i.e. the whole tetraploid genome was ordered such that during meiosis there were grouped only two homologous chromosomes together. Although there can still be found double loci within one set, many have disappeared or changed into different direction such that there are no homologous chromosomes in a set anymore. Hence it is impossible to halve one set into two sized as the original chromosome set before the initial polyploidization. A good example is the normally working sex determination via an XY-sex-chromosome system in bisexual Cyprininae producing equal sex ratios in e.g. carp, goldfish and crucian carp. Many traits have been proved to be determined in a normal Mendelian pattern in goldfish and carp (Tave 2001), which are among the most studied models of fish geneticists. Thus bisexual Cyprininae are functional diploids, and it is debatable to count their ploidy according to the usual 1n chromosome number of the most other members of the family of Cyprinidae, which is around 25 chromosomes. A 1n chromosome set of Cyprininae-species contains about 50 chromosomes.

For simplicity, in this thesis the term **ploidy** and the sign "**n**" are used synonymically (what they are not, in reality) and genome size is converted into ploidy using as a factor the theoretical size of one chromosome set of an average triploid Prussian carp for haploid or 1n. E.g. a fish with three carassiid chromosome sets and one Leuciscine set will have a genome size of approx. 3,5n. The fish is genetically a full tetraploid, but its ploidy will be indicated as 3,5n as long as the origin of the genes cannot be identified.

2.4. Theoretical problems of asexual organisms

In theory, clonal or asexual organisms reproduce twice as fast as sexual ones, because there is no "unproductive" sex, as males. According to this "two-fold cost of sex", the existence of sexual organisms seems a paradox. The existence of sex is explained by the possibility of rapid genetic change and exchange i.e. recombination. These enable a far quicker evolution and thus adaption to changing environmental constraints than asexual reproduction. The means of recombination maximize the probability of beneficial mutations to A) be combined and thus maximizing their use to the individual and B) the probability of any mutation to be brought into a useful context in combination with other alleles. Recombination, in combination with epistasis also enables the "storage" of alleles being "useless" at a certain time but being available after conditions changed and then might be useful. The evolution of clones depends solely on mutation rate and usefulness of a certain allele in its current combination at the time of its appearance in the genome. The adaptational ability of a clonal organism is thus far lower than of a sexual organism. In other words, the probability that a clone evolves a certain fitness improvement in a given time is far lower than for a sexual complex (=gene pool) of organisms owing recombination and allele diversity. A clone is always only its own gene pool. This means that sexual organisms have a higher long-term fitness in a changing environment. Parasite-host- and predator-prey-relationships are recognized as one of the most rapidly changing factors. Parasites and hosts are co-evolving such that the evolution of one side forces the other to evolutionarily adapt to maintain its fitness. Among others this arms race between parasites and hosts is described in the Red-Queen Theory (Van Valen 1973) and is seen as one of the strongest driving forces in evolution and maintenance of sex enabling quick evolution and adaptation (Neiman & Koskella 2008, Lively et al. 1990).

This theory was also underpinned with studies on populations of gynogenetic fish, their sperm donors (=sexual hosts) and their parasites, where a population balance between gynogenetic sperm parasites and their sexual hosts was explained with a higher parasite load of the non-adaptive clones (Lively et al. 1990, Hakoyama et al. 2001). A certain problem in these studies is that the gynogenetic clones were always dependent on the availability of certain sperm hosts, so there was not only a competitive, but also a dependence relationship between the species. In Prussian carp, this is only partly the case, since it seems not to rely on only one sexual host occupying the same ecological niche, but can switch its host on a broader taxonomic scale. As mentioned above (point 2.1.2.), gynogenetic Prussian carps seem to totally displace the sexual species. This pattern is connected with three special features, seeming to be the explanation of this exception from the Red-Queen theory. First, the clones are non-native invaders, using the delay in evolutionary time to fully use their reproductive advantage before parasites get adapted to them (Deinhardt 2008). Secondly, as mentioned under point 2.1.2., Prussian carps are not always pure clones. Thirdly, Prussian carp might profit from hybrid vigor, especially when including genes or genomes of their host. Prussian carp seems to be a successful, opportunistic model of an organism complex using different modes of reproduction in combination with ecological and evolutional factors favoring them (Deinhardt 2008). It is not a paradox to the Red-Queen-theory, but a proof thereof: The Prussian carp system profits from a short term “immediate fitness” combined with enormous dispersal and adaptational capacity. It can be assumed that the opportunistic way of Prussian carp to spread and get established via gynogenesis and to withstand evolutional constraints works mainly in disturbed and dynamic, fractured environments, where it can effectively use the time delay of parasite evolution to exert overwhelming competitive pressure on adapted species. A reversible sexual phase guarantees continuous “cycling” - in other words: the Prussian carp system is participating in the evolutionary race with other means than its competitors (Deinhardt 2008). This is still a theoretical analysis demanding for further examination and experimental proof. This study aims to help elucidate a fundament for this theory.

3. MATERIALS AND METHODS

3.1. Wild fish

Samples of altogether 104 Prussian carp, 11 crucian carp, 17 presumed hybrids (Prussian x crucian carp) and of 4 goldfish and 2 known hybrids (goldfish x crucian carp) were acquired from different populations in Finland, Germany and breeders (for specific numbers, see Table 1.). All Prussian carps used as spawners in the breeding experiment were also sampled for this examination and are included in the given numbers. The fish were caught and identified 2008-2010. The fish were caught by angling, hand netting, traps, and hand. Samples of seven hybrids were provided by Jussi Pennanen (RKTL/Finnish game and fisheries research institute). These fish were not available for identification; suggestive identification was based on morphometric traits and size data provided together with the samples. For identification of sex ratio in the Finnish population, in addition to own data there were also data collected by Jussi Pennanen and Lauri Urho (both RKTL, Finnish Game and Fisheries Research Institute) between the years 2005 and 2009). Before analyses, all fish were grouped into taxonomic groups (species, hybrids, types of Prussian carp) according to their habitus, a subjective method that is only partly based on countable or measurable traits. Further groupings (lineages, clones) were done according to microsatellite patterns.

Table 1. Numbers of adult carassiid fish used for genetic analyses (ploidy, microsatellites) and as breeders, sorted by population and species.

Population	Prussian carp	crucian carp	gold fish	assumed hybrids	spawners (♀,♂)
Pond 1, Helsinki, FIN	50	4	0	0	5*
Pond 2, Helsinki, FIN	8	0	0	3	4**
Pond, Salo, FIN	31	0	0	0	1
Baltic Sea, Helsinki, FIN	2	2	0	12	0
Pond, Bad Freienwalde, D	12	3	0	0	0
Ditch, Bad Freienwalde, D	0	0	0	2	0
Fish farm, Brandenburg, D	0	2	0	2	0
Commercial fish breeders, ISR	0	0	4	0	0
Sum	104	11	4	19	10

*including one crucian carp male not genetically analyzed
**including one Prussian carp male included also in the genetically analyzed fish

3.2. Breeding experiment

3.2.1. Experimental design

The eggs of several females per population (if available) had to be treated with milt of every potential sperm host, to enable comparisons of egg susceptibility to foreign sperm and reproductive modes between and within populations. The treatments had to be done with every species simultaneously to equal sized egg portions of the same batch to enable comparison of treatment success among potential host species. The egg amount per treatment had to be reasonably large to gain reliable data. Based on experiences from preceding experiments the needed egg number per treatment was approx. 500-1000 (assuming minimal egg development rates of 10% and minimal larval survival of 30%) to gain at least 15-30 offspring for genetic and morphometric examinations. From batches too small to get enough eggs for all treatments, the portion size was not compromised, but treatment number reduced. For the same reason treatments were prioritized in the following order: 1) fresh milt (Prussian and crucian carp for maximum data breadth to study the mode of innerspecific/innergeneric reproduction), 2) negative control (to exclude true parthenogenesis), 3) any leuciscine cryopreserved milt (as for the specific question a “works/does not work” was sufficient and broader data gain from many families less important than for the preceding treatments) and 4) cryopreserved milt of those species that have been applied as fresh milt (to compare the effectiveness and effects of fresh and cryopreserved milt).

Each treatment at each egg batch was treated as a "family". Rates of egg development, hatching and larval and juvenile survival were collected. From each family about 100 juveniles were isolated and raised to an average size above 3cm when morphologic and morphometric traits can be assessed and there is enough tissue available for repeated genetic measurements.

3.2.2. Species used as sperm-donors

All species listed in point 1.2.2. could be obtained except for zope. Of these milt could be obtained from all but tench. The obtained milt was cryopreserved and applied as described below.

3.2.3. Milt collection and storing

Due to the different spawning times in different species, the technical impossibility of preserving egg cells, and the necessity of treating the same egg batch with milt of all species, the milt was collected at occurrence of spawning of each species. It was then cryopreserved to be applied simultaneously at the induced spawning of the laboratory kept female Prussian carp. To have comparable results from fresh milt (and to improve the spawning readiness of the Prussian carp), crucian carp males were kept with the assumed Prussian carp females and brought to spawn to use their fresh milt. At spawning, one Prussian carp was found to be male, and milt of this and one crucian carp male was freshly stripped and applied as positive control at every stripping of females. All other treatments were done with cryopreserved milt.

Males of the potential Leuciscine host species and crucian carp were collected from the interconnected lakes Leppävesi, Jyväsjärvi and Päijänne in the city of Jyväskylä, Central Finland during spring and summer 2010 by angling, hand netting, gill netting, hand catching and fish traps. They were transferred alive to the laboratory facilities in Jyväskylä University. There they were killed by a sharp blow on the head and stripped immediately. For this, the fish was dried, wrapped into paper and turned onto its back (except large bream which were kept lying on their flanks). Then the anal area was dried thoroughly and covered with paper. Only the immediate anal area was freed from paper and milt gently sucked with a syringe with a needle (diameter 0,6mm) shortened to 1cm. The fish was stripped carefully to keep the milt running. Stripping was stopped, when the milt contained blood spills. Contaminations by blood or urea were avoided, contaminated milt discarded. The milt was collected straight into an Eppendorf research tube stored on ice. The procedure lasted 1-5min/fish. The milt of 2-11 individuals was pooled (see also Table 3.) to guarantee functional sperm in the preserved sample. The milt was stored on ice between 5 and 30 minutes before start of the cryopreservation protocol.

The protocol for cryopreservation was developed following Urbányi et al. (2006) with modifications: pooled milt was gently mixed with nine parts cryomedium (350mM glucose, 30mM Tris-HCl, pH 8,0) and immediately added 1,1 parts methanol under gentle stirring. The milt-cryomedium mixture was then pipetted as 50µl drops onto a stainless steel plate that has been stored at -80°C before use. After max. two minutes the plate with the droplets was returned to -80°C. After 10-15 minutes the frozen drops (=pellets) were collected within one minute into a cryotube and stored in liquid nitrogen until use. Storing time for cryopreserved milt used in this experiment was 10-11 months.

After preserving the milt, the left-overs of pure milt and milt-cryomedium mixture were checked for sperm motility under a microscope (200 x magnification) by adding water. This happened 1-2h after stripping. If tested for a species, only motile sperm were used for the breeding experiments. Some milt samples were tested for fertilization ability by applying them to just stripped roe of Leuciscine fish within 24h, following the same protocol as given for the breeding experiments in 3.2.6. Hatching of normal larvae was assumed a positive fertilization test for sperm (Tables 7. and 12.).

The milt of carassiid males (one crucian and one Prussian carp) was stripped before stripping of female spawners and applied directly within a time span of 10 min to 6 h. Stripping followed the above procedure except for killing. After stripping the males were returned to their tanks. This procedure was repeated at every day a female was to be stripped. The gained milt was stored on ice for use for the rest of the day and then tested for motility and discarded.

3.2.4. Wild spawners for the breeding experiments

Wild Prussian carp, their hybrids and crucian carp were caught in May-June 2010 by angling and hand netting from two ponds in Helsinki (Ponds 1 and 2) and one pond in Salo, Finland, and individuals brought to Jyväskylä in isolated boxes. The numbers of individuals per population used for genetic analyses and breeding experiments are listed in Table 1.

3.2.5. Pre-treatment of female spawners and stripping

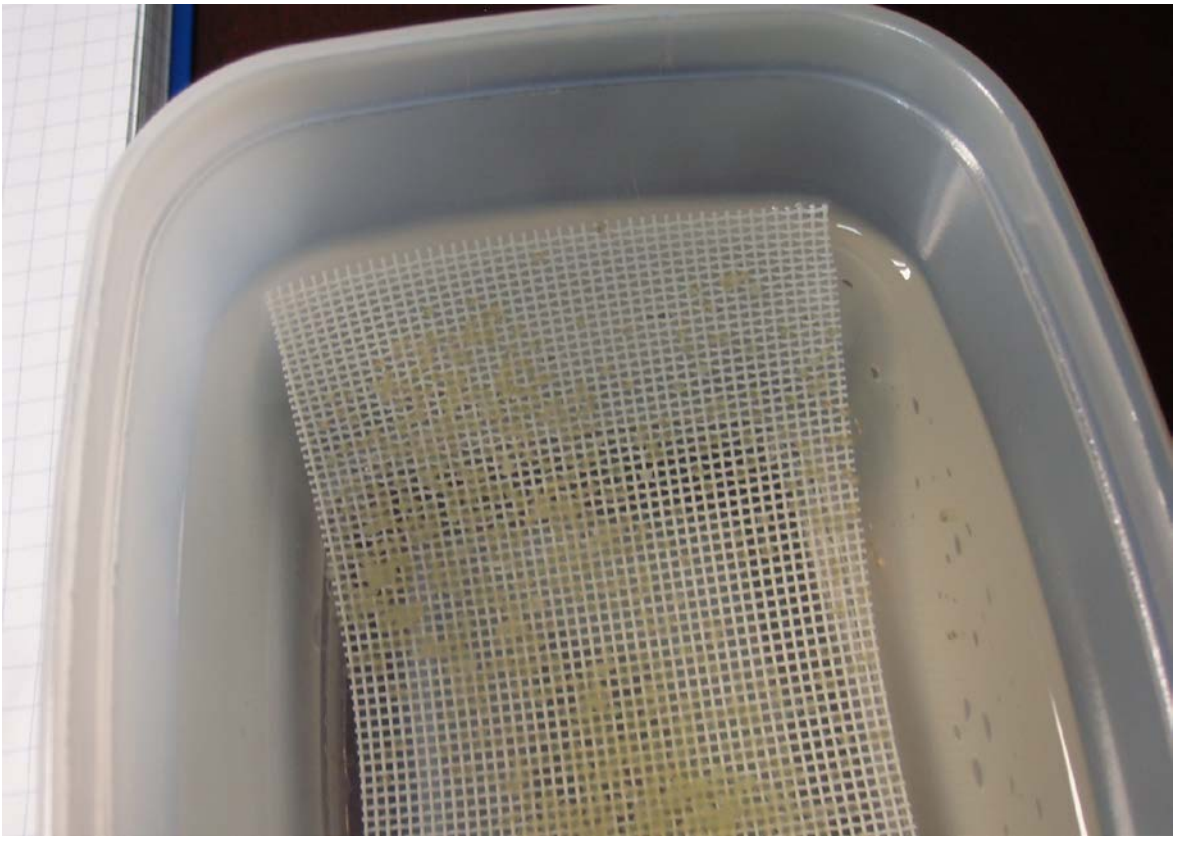
At arrival to the lab facilities the fish meant for breeding were immediately put into prepared 300 l tanks of suited temperatures and a natural-like light cycle at time of arrival. After omitting weak individuals the remaining fish were treated for 2h with 0,3 mg malachite green/100 l to prevent water mold and protist infections. After this the fish were allowed to acclimatize for 3 days, after which feeding started. The Helsinki fish had already spawned and were left alone to develop new gonads. The fish from Salo were caught at spawning and partly stripped at arrival. These fish were also allowed to recover and start a new cycle of oogenesis regardless if already spawned or not. The temperature was 20^{o}C and the light cycle unchanged from June until end of October, when temperature was declined to 4^{o}C overwintering temperature and light declined to 6h/d within one month. The fish were fed with standard rainbow trout pellets (3, 4 and 6mm, Rehuraisio Oy, Raisio, Finland) ad libitum 3-5 times/week. The wintering period had constant day length of 6h and temperatures varied between 4 and 8^{o}C. From beginning of February 2011 to mid-March the temperature was raised to 15^{o}C and kept at this level until mid-April, when it was raised to 20^{o}C. Light cycle was raised at the same time continuously to reach 18h day length in mid-April. The fish from the two Helsinki populations were kept together with carassiid males to ensure gonad ripening. For the same purposes two tench males and one female were added to the fish from Salo. After the fish from Helsinki had reached immediate spawning condition (softening gonads, extruding anus), chosen spawners were treated with a gonadotropin-releasing hormone agonist (GnRH–a, Ovopel, Interfish, Hungary) at 500mg/kg and stripped after 12h. The fish of the Salo Pond population did not seem to approach spawning without any carassiid male in the tank, so there was added one crucian carp male and after one week hormone treatment at the same dose as above was possible to be applied to one individual. The next day this fish could be stripped as well. All presumed hybrids were kept with the other fish of their population (Pond 1) and treated with the same hormone treatment 3 times (1 week interval) repeatedly after the previous treatment showed no effect.

For stripping, females were wrapped into paper, the anal area freed from paper and dried, including anal fin. During this, the genital opening was closed by pressing a finger on it to inhibit roe loss due to pressure or fish movements. The fish was held over a dry plastic bowl and the roe stripped into this.

3.2.6. Fertilization, incubation and hatching

Each roe batch (=all stripped roe from one female) was fertilized within 30 minutes and until then stored at room temperature. It was aliquoted into dry, clean 50ml reaction tubes in portions of about 700-1000 eggs. Each portion (=later called family, if treatment was successful) was to get an own, independent treatment to induce egg development. If using fresh milt (carassiids) there was added 1µl milt to the pure eggs and mixed by gently shaking the tube. After one minute 5ml clean, temperated well water from the supply of the spawner's tank was added, and the tube gently shaken. Then it was let al.one for about one

minute to fertilize. After this, the treated eggs were poured onto a fly net (plastic, mesh size 1mm) surface hanging horizontally in temperate well water in isolated containers of one liter (Picture 1.). Possible egg accumulations were spread within some seconds by a gentle water stream from a pipette, if possible. Then the eggs were let al.one for at least 12 hours to avoid accidental polyploidization during the first cleavages. The same treatment was given to the controls, adding 1µl of temperate well water instead of milt. The sperm density in the milt was not assessed, however, the portioning aimed on an oversupply to assure full fertilization success. For fertilizing with cryopreserved milt, the above protocol was used with minor changes: A pellet of the respective Leuciscine species milt was taken from the nitrogen storage with sterile metal forceps and placed immediately at the wall of the horizontally held fertilization tube right next the roe. Then 5ml slightly warmed (22-25°C) well water was added and the tube shaken at the same time to allow the sperm escaping the pellet mucus at melting. Then the tube was let al.one for one minute to fertilize as for the above treatment. The sperm density from pellets was aimed on full supply but could not be controlled because the degree of mucus development was very variable and could not be controlled nor could it be quantified.



Picture 1. Just stripped and milt-treated Prussian carp eggs on fly net, resting in water to swell.

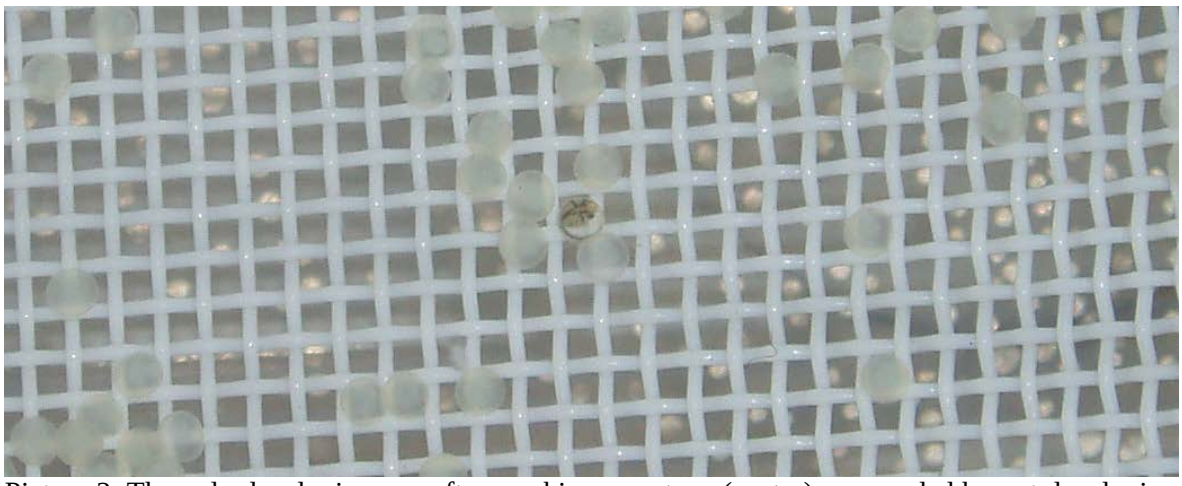
3.2.7. Rearing

After the first 12 hours post fertilization, the water of the egg containers was changed twice a day. After counting at eye stage after 2-3 days, the nets with the eggs stuck to it were put in a troughflow system to avoid metabolite accumulation. In the system each family was in a 1l cage, where the fish could hatch and start feeding. Hatching was monitored every 4-8 hours, and the nets with shells and dead eggs were removed immediately after all normal larvae had hatched to avoid molding. At the same time feeding started with low amounts of commercial larval feed (Gemma Micro 150, Skretting,

Norway). The amount was raised when feeding was observed. After large scale successful feeding was observed, live artemia were fed additionally. Due to technical problems the temperature in the egg containers and trough-flow varied between 20 and 25°C. Detritus and dead larvae were removed. When fish were large enough for moving (after 12-14d), 100 normal seeming individuals per family were moved into 15l aquaria with small water exchange (2-5l/h). The families were fed for 2 further months ad libitum with artemia, and additionally with Gemma Micro 150. Then, at a size of about 15-20mm, the food was switched to commercial rainbow trout starter feed pellets (Rehuraisio Oy, Finland) that were step-wise size-adapted to the growing fish. The families were fed ad libidum several times a day and kept at 20-25°C until investigation. Occurring water mould in the detritus was treated by cleaning and application of 10g NaCl/l under continued flow. Occurring diseases were prevented from spreading by elimination of the whole family and disinfection of the aquarium and surroundings.

3.2.8. Counting

The eggs were counted when the eyespots became visible (24-40h after fertilization, Picture 2.). Depending on the percentage, either the developing or the non-developing eggs were counted. In some cases with unfavourable visibility the percentage of developing eggs was assessed by counting only a section of approx. 100 eggs. The number of non-hatched eggs and unviable larvae and was determined after 7-8d when clearly all normal larvae had hatched and filled their swim bladder. In the most cases the number of hatched, viable larvae was assessed by subtracting these numbers from the earlier determined number of developing eggs. Only in small families, a true count of viable larvae was possible. For some families, the number of feeding larvae was also assessed by counting the larvae when definitely all normal larvae have fully started feeding. This is approx. one week after hatching.



Picture 2. The only developing egg after reaching eye stage (centre) surrounded by not developing eggs on a fly net after 30h.

3.2.9. Eggs, larvae and fish produced during the breeding experiments

From the 8 female breeders of all used populations there were produced about 34881 eggs in 42 different treatments. Of these hatched approx. 9382 viable larvae (27%) of which 1188 fish in 22 families could be reared up to an age at which determination of morphological traits was possible. Out of these juveniles there were sampled 384 fish. Additionally there were sampled 739 hatched and unhatched larvae. Of those there were

genetically analyzed 61 larvae and 330 juvenile fish. For morphometric analyses there were used 360 juvenile fish, which include almost all genetically analyzed juveniles.

3.3. Sampling and measuring

The bred juvenile fish were sampled during November 2011. Of each family one of the largest individuals was set aside to survive as backup. From this a fin clip and all non-invasive measures were taken. Then at least 30 individuals were chosen semi-randomly (choosing only the largest and smallest three individuals, randomizing the other) for morphometric measurements and ploidy analyses. Total length (TL) and weight (g) were analyzed before the individual was terminated by a sharp blow on the head. No other measurable traits were taken, because of the strong deformations due to low feeding intervals and high density during the larval stage. Then the number of lateral line scales was counted, referring to unsure scales as half numbers. The counts were conducted at the left side of the fish if possible. Soft rays of dorsal and anal fin were counted, indicating the last ray as 1½ if diverging below the skin. Dark pigmentation intensity of the fully spread anal fin was evaluated on white background on a scale from 1 to 3 (1=low background pigmentation, no visible pigment spots, 2=visible dark pigment spots causing a darker appearance of the fin tissue, 3=high density of pigment spots giving the fin a clear black appearance). From the largest individuals blood samples were taken from the caudal vein. 0,3ml blood was immediately injected into 1ml 100% ethanol p.A. at 4°C, shaken to prevent clumping and stored at 2-4°C. The blood was shaken again after 24h and 1 week. From all fish fin clips from the caudal and dorsal fins were taken and stored in 80% ethanol p.A. at 2-4°C (from smaller individual all fins or alternatively the whole caudal tissue was used). As backup, eyes and brain were sampled into another tube with 80% ethanol at 2-4°C. Then the fish were opened to assess sex and sexual maturity. Fish with not developed gonads can be classified as sterile, unless they are smaller than 3cm. Male gonads wider than 1,5mm and female gonads with greenish colouration were defined as mature. Doubtful gonads were put to 80% ethanol to assure sex. After some seconds female gonads develop a clear transversely folded surface structure, while testes stay plain (see Picture 3.).

At the same time the pigmentation of the peritoneum was determined from 0 (no pigmentation) to 6 (dark black) and according to the occurrence or dark spots or macromelanophores (0=no spotting, 1=some small spotting, 2=large and/or many spots). At the beginning of sampling, all fish were classified by occurrence of reddishness of eye and fin pigmentation (0=no red pigment visible, 1=red pigment visible). This classification was assessed for only a part of the fish, as the pigmentation was only noticed during sampling.

Wild (=adult) individuals including spawners were all sampled for blood by drying an area above the anal fin, removing two scales and taking 0,3-1ml blood via an 0,6mm injection needle from the caudal vein and treating it as above. Additionally there was often taken fin clips from the outmost parts of the dorsal fin. These sampling methods are not lethal and can be repeated. The spawners were sampled immediately after stripping.

Picture 3. Gonads after 5 min in 80% ethanol. Left male, right female gonads.

3.3.1. Ploidy analysis

Ploidy was estimated by measuring the intensity of light reflection of the stained DNA of single nuclei using a flow cytometer with mercury lamp (PAII, Partec, Germany), and directly comparing this to the intensity of a standard of known DNA content (chicken red blood cells). The internal standard allows calculating the DNA content or genome size of cells of the investigated individuals and their individual variation (coefficient of variation, CV). Comparison to a known diploid, closely-related species (e.g. crucian carp) gives a straightforward estimate of the measured individual's ploidy. The measurements were conducted on ethanol fixed samples of blood, fin clips or brain tissue, respectively, depending on the size of the fish. For adult fish, measurements from blood were taken for calculations of ploidy, for young fish, fin or brain tissue was used. Comparative measurements of different tissues of the same individuals were performed to correct for possible differences between tissues

Staining and measurements were performed according to Lamatsch et al. (2000). Per sample between 5000 and 50 000 nuclei were measured, mostly around 20 000. If the measurement of an individual produced two or more independent peaks, it was assumed that the individual has cells of different ploidy within the measured tissue i.e. the individual is a mosaic. The ploidy for wild fish was roughly estimated by comparison to the diploid standard crucian carp. For more exact ploidy estimations the average of all measured triploid Prussian carps was divided by three as a standard haploid genome.

For mosaic individuals an average was calculated of the different peaks observed to have a single value for statistic processing. This simple average is the same as assigning each ploidy of an individual to an own statistic individual that must be weighed in every calculation. The average was not weighed by the observed frequency in the measured cells, as multi-tissue measurements showed that frequencies can change between different tissues (Lamatsch 2001).

3.3.2. Microsatellites

The microsatellites were tested by Dunja Lamatsch and Maria Pichler (both University Innsbruck, Institute for Limnology, Mondsee) from fin clips. The used Microsatellites were GF17 and GF29 (Zheng et al. 1995), MFW7 and MFW17 (Crooijmans et al. 1997) and J1 and J62 according to Yue & Orban (2002).

The data were analyzed with the program GeneMapper4.0. and checked optically. Fish were grouped according to their allele patterns. Exceptions from allele patterns were analyzed for introgression possibilities. Relatedness was inferred based on shared alleles. Differences between similar clonal lineages were seen as introgression when they were rather explainable by allele gain from another lineage/species than mutation. Meiotic sexuality as reason for introgression was rejected, when the main part of the allele pattern was unchanged.

3.4. Statistics

All measured morphological, colour and sexual traits were compared with each other and with DNA content and DNA content difference (mother-offspring) using IBM SPSS 20. To investigate if a trait differed within groups, if and how it is inherited, it was grouped by family, mother or father, respectively, and analyzed using Univariate ANOVA. To investigate, if a physical (=phenotypic) trait depends on DNA content and DNA content difference (offspring - mother), they were tested for correlation. To investigate any interrelations between two traits they were also tested for correlation. Pearson correlation was used if both variables were continuous and normally distributed; Kendall's correlation factor was used in all cases were at least one variable was not continuous or unevenly distributed.

To test if haploid genome size of different species differs, the measured DNA content was divided by the individual's ploidy and the gained values grouped by species and tested with ANOVA, Tukey's b -test).

3.5. Permits

Catching, transport, husbandry of fish in Finland as described above as well as conduction of the experiments was based on an animal test permit No. ESLH-2009-02780/Ym-23. Transport of fish from the sea to the inland-based laboratories in Finland and keeping in these required a permit from the veterinary authority EVIRA, which was granted in 2009 (No. 1967/0515/2009) and 2010 (No. 2684/0515/2010).

4. RESULTS

4.1. Wild fish – genetics and phenotypes

The examined populations were found to be different from each other with respect to their ploidy, genetics, sex ratio, morphology and the intrapopulation variation of these traits. Also, there could be identified several clones and lineages within populations.

4.1.1. Phenotypes

Besides clear crucian carp, goldfish and their hybrids, two main types of Prussian carp could be identified by habitus. These existed partly sympatrically. One representing the most common Prussian carp form found in inland populations (in the following called Prussian proper, Picture 4.) being almost entirely female, and one shallower, bisexual form

resembling the descriptions of *C. langsdorfii*. In addition there were found three types of hybrids, a clear hybrid in Pond 2, Helsinki (Picture 5.), a polyform hybrid from the Baltic Sea with very variable traits as morphometrics and expression of body depth, resembling sometimes more either ancestral species (Picture 6.), and finally a Prussian carp from Germany with slightly crucian carp-like habitus and well expressed deep body – usually found only in crucian carp. The latter one appeared to be an Asian carassiid with introgression from crucian carp. All these phenotypes were confirmed by genotype analysis, and clonal Prussian carp could be further subdivided into clones and lineages. From all Finnish populations one or several adult males of the "proper" type were identified from mature gonads.

Picture 4. Clonal ("proper") Prussian carp, 3n, pond 2, Helsinki.


Picture 5. Hybrid of Prussian carp and crucian carp, 2n, Pond 2, Helsinki.

Picture 6. Two Prussian carp hybrids (around 1kg) from the Finnish Baltic Sea showing strong (left) and no expression (right) of body depth as predation defense, a mechanism only typical for crucian carp. The fish at the left is also closer to crucian carp by habitus, but not meristic traits. Picture by Lauri Urho (RKTL/Finnish Game and Fisheries Research).

4.1.2. Ploidy

Comparative measurements from blood and fin tissue of the same individuals showed that blood and fin differed significantly (paired sample T-test, P=0,03) by 3,8% ±4,1% (see Table 2.). The conversion factor for fin measurements to resemble blood is thus 1,038. All fin measurements were converted for further statistical measurements.

Table 2. Differences in DNA content estimates between measurements of fin and blood samples of the same individuals. Measurements conducted for two 3n Prussian carp, one 2n Prussian carp (-hybrid?), one goldfish and two crucian carp.

species	ploidy	DNA content pg/nucleus			
		blood	fin clip	difference	%
Prussian carp	3	6,70	6,33	0,38	5,9
Prussian carp	3	6,80	6,28	0,53	8,4
Prussian carp	2	4,20	4,30	-0,10	-2,3
goldfish	2	4,28	4,23	0,05	1,2
crucian carp	2	4,73	4,50	0,23	5,0
crucian carp	2	4,55	4,35	0,20	4,6
average				0,21	3,8
SD					4,1

In the three Finnish pond populations of Prussian proper habitus, two were found to consist of all-triploid females, one population in Helsinki Pond 1 consisted of 50% 3n and 50% 4n individuals (N=34). One of the two proper type males from Pond 1 was measured and found tetraploid. A further fish of that population with male attributes (tubercles) but no running milt was triploid. In Helsinki Pond 2 one morphologically distinct, *C. langsdorfii*-like 2n Prussian carp male was found, as well as three morphological hybrids also showing a diploid genome size. The comparative German population of morphologically clear Prussian carp was all triploid (N=12), whilst the German Prussian carp with some crucian carp habitus traits (suspected introgressions) were diploid (N=2). From the Baltic Sea there was caught only one morphologically clear Prussian carp (proper) which was triploid. One female of *C. langsdorfii*-like habitus was diploid. Four out of five morphologically identified and five out of seven inferred hybrids were diploid, one identified hybrid was triploid and one inferred hybrid was aneuploid (<2n) and two

inferred hybrids were mosaics of 2n and additional aneuploid cells. Of the five identified hybrids from the Baltic Sea two were male, two female and one sterile (i.e. no gonads were found in a 1,3kg fish). One of the males was triploid, the other diploid. Of seven inferred hybrids from the Baltic Sea three were female, two unsure females with testicle-like tumors in the ovaries, and two of unknown sex (data from J. Pennanen, RKTL, Helsinki). All crucian carp measured from different populations were diploid (FIN N=7, D N=5). The same is true for the four goldfish and their two hybrids.

The genomes of different species and hybrids had different sizes if reduced to haploid chromosome sets (see Table 3.). The different species Prussian carp (proper), crucian carp and goldfish differ significantly from each other (ANOVA, Tukey's b, alfa<0,05) with goldfish-crucian hybrids and Prussian-crucian hybrids falling into the same genome size range as Prussian proper. The *langsdorfii*-like fish were very different from each other in genome size. The male had an even larger 1n genome (2,4 pg) than crucian carp and the female (2,24pg) grouped between goldfish and Prussian proper.

Table 3. Average genome size in terms of DNA content calculated relative to DNA content of chicken red blood cells and theoretical size of its haploid fraction for different groups of carassiids analyzed (DNA content/2,3pg). Groups are ordered by size of haploid genome.

Carassiid group	sampling locality	N	DNA content (pg)	SD	interpreted ploidy	theoretical 1n (pg)	SD
goldfish	breeder, ISR	4	4,37	0,04	2n	2,18	0,02
langsdorfii-like Prussian, female	Baltic, FIN	1	4,48	-	2n	2,24	-
Prussian (X crucian?)	carp ponds, D	2	4,57	0,05	2n	2,28	0,02
Prussian proper	Baltic, FIN	1	6,87	-	3n	2,29	-
Prussian proper	Pond, D	27	6,90	0,09	3n	2,30	0,03
crucian X goldfish	garden pond, D	2	4,59	0,03	2n	2,30	0,02
crucian X Prussian	Baltic, FIN	4	4,59	0,04	2n	2,30	0,02
Prussian proper	Pond 1, FIN	20	6,90	0,12	3n	2,30	0,04
Prussian proper	Pond 1, FIN	23	9,20	0,08	4n	2,30	0,02
Prussian proper	Pond 2, FIN	4	6,89	0,01	3n	2,30	0,00
crucian X Prussian	Baltic, FIN	1	6,96	-	3n	2,32	-
Crucian carp	different pops, FIN, D	11	4,74	0,04	2n	2,37	0,02
langsdorfii-like Prussian, male	Pond 2	1	4,80	-	2n	2,40	-

4.1.3. Microsatellite data - genetic variability and relationship

All six microsatellites suited to discriminate clonal and semiclonal lineages of Prussian carp from each other, to infer relatedness and to identify possible introgression. Also they suited for inferring relatedness of the clones with single diploids and an isolated polyploid individual and the hybrids.

Three microsatellites were found diagnostic in this study to identify crucian carp, but two of these (J1 and J62) were not found from hybrids, whereas the very distinct crucian alleles in GF29 were present in all/one of two (FIN/D) assumed hybrids and most tetraploids (N=18/20). The specific but very polyform and not very distinct alleles of MF17 were not seen directly diagnostic, but confirm the patterns inferred by GF29 for most hybrids (N=6/9) and all tetraploids identified by GF29 (N=18). One allele of GF17 is shared with crucian carp and two clones (Salo pond and pond 2, Helsinki) and is thus not

diagnostic. Nonetheless it also resembles the crucian share of the most hybrids (9/10) and all tetraploids identified by GF29 (N=18) (and not in the triploids sharing the otherwise same allele patterns – see example in Table 6.) and thus confirms the findings made with GF29.

In J1 there was a very distinct allele that was found in three of five goldfish and their hybrids. As it was not present in all fish, it is not fully diagnostic. There were two alleles of GF17 found only in goldfish and its hybrids, but another allele (found in three fish) is only two base pairs (bp) different to an allele found in 2n Prussian carp and Prussian hybrids, and the other is found in only four fish and differs by two bp from one Prussian (proper) allele and one bp from a 2n Prussian allele. All markers have alleles that discriminate goldfish and Prussian carp as one group from crucian carp as the other.

Based on these markers there were identified three 3n clones and one 3n/4n semiclonal system (for definition see point 2.2.) showing additional introgression from crucian carp to an unreduced Prussian carp genome on most markers and many irregularities in the allele patterns of the clonal Prussian carp genome. The irregularities found were doubling, replacement and/or deletion of alleles and introgression (via replacement) of foreign alleles, in two cases from crucian carp and in several cases possibly from another Prussian carp lineage. Individuals of each (semi-) clone had almost identical allele patterns (for clones and exceptions from the patterns see Table 4.). In the semiclonal population (pond 2, Helsinki) three individuals were found that are almost identical with another clone from Salo pond but carry one alternative allele which is also found in the sympatric semiclone. In most markers each clone and semiclone shared one or more alleles with the most or all other (semi-)clones and many of these alleles were also shared with some diploid and hybrid Prussian carps. Still, each (semi)clone had its own, unique allele pattern. In pure clones each individual had the same allele pattern within a marker. In semiclonal lineages certain alleles within a marker differed in presence or number between lineages, but other alleles were always present - a pattern that is not explained by sexual recombination. With the exception of one marker (for two clones), the allele patterns in every marker differed between all clones. Common patterns (allele frequencies, common alleles) between two clones were not continuous between markers. All possible allele frequencies were observed from total heterozygosis (3n and 4n) to total homozygosis (only 3n). Generally, in clones there was a high rate of heterozygosis and in many markers the different alleles were very different in size (other than in crucian carp).

The 4n male from Pond 2, Helsinki, fitted without any exception into the semiclone as a 4n individual with crucian carp introgression. The other male from that population were not examined as was not the male from Salo pond. The *langsdorfii*-like, 2n male from Pond 1, Helsinki, was genetically totally different from the clonal rest of its population. It exclusively shared several alleles in four markers with the hybrids and the 2n *C. langsdorfii*-like female from the sea and German diploids. It also shared most of these distinct alleles with the hybrids from pond 1, Helsinki, but only for two markers (of five working) it shared common Prussian carp alleles with the hybrids. These hybrids also had alleles in two markers that they did not share with the male. The male shared one allele with crucian carp and two of its sympatric hybrids.

The bisexual hybrids from the Baltic Sea shared alleles with crucian carps in four markers. They also shared some common Prussian carp alleles, but most non-crucian-carp alleles were shared with 2n Prussian carp and the Prussian X crucian carp hybrids from Pond 2, Helsinki, and with gold fish. As well they had several exclusive alleles. Only 2 markers showed a uniform allele for all measured individuals.

The German diploids, though being sympatric and morphologically similar, did not have a single allele in common.

The crucian carps originating from two populations in Germany (N=2 and 3) and two in Finland (Baltic Sea, N=2, Pond 2, N=5) were very diverse and the only totally uniform alleles were the two null-alleles in J1.

The four goldfish showed only three uniform alleles in two markers, which were all common markers with most Prussian carps. This is despite the fish came from the same transport of the same variety from the same breeder in Israel. The two goldfish hybrids were of different ancestry and did not share all alleles with the gold fish. Many loci being homozygous for alleles characteristic for either ancestor the fish were F2 hybrids or backcrosses. In some markers (JE62, MF7, MF17) there were certain alleles that goldfish shared mainly with 2n Prussian carps or Prussian hybrids. In all markers were alleles that were only found in 2n Prussian carp or Prussian carp hybrids or that were shared only with these and/or goldfish. One allele of MFW7 was shared by all goldfish, one Prussian hybrid and two tetraploid Prussian carp.

Table 4. The different Prussian carp lineages from all populations as discriminated using microsatellites. For each population examined there are indicated the lineages found and the observed properties thereof: relative ploidy (n), Number of individuals examined with microsatellites (N), habitus type (as determined before genetic investigations), within-clone genetic variation (if exception: N individuals/total N alleles/N markers), existence of diagnostic allele patterns (considering all markers used to ensure accuracy) shared by all individuals, existence of any alleles considered diagnostic for crucian carp in any individual of the lineage and remarks considering relatedness of the lineage.

	population	lineage and clonality	n	N	habitus type	within-clone genetic variation	diagnostic allele patterns	diagnostic crucian alleles	relatedness remarks
D	pond	clone	3	26	Prussian proper	allele deletion (3/2/3), allele change of 2bp (1/1/1), introgression from crucian carp (1/4/2)	yes	in 1 fish	
FIN	pond, Salo	clone	3	31	Prussian proper	allele deletion (3/5/3),	yes	unclear	
FIN	pond 1, Helsinki	semiclonal group	3/4	49	Prussian proper	doubling/deletion of numerous alleles in 5 markers, more alleles than ploidy allows for, many different alleles at one locus, irregular Introgression from crucian carp at some markers	yes	yes	
		clonal? (N too small)	3	3	Prussian proper	identical with Salo except one allele in one marker, which is common with sympatric semiclone	shared with Salo		identical with clone from Salo, except one marker allele and one supernumerous allele
FIN	pond 2, Helsinki	1 clonal	3	8	Prussian proper	allele deletion (4/2/2), 2different allele patterns in 1 marker	yes	unclear	
		sexual lineage	2	1+ 3	shallow Prussian (*C.langsdorfii*-like)			yes	hybrids related with male (share several alleles), but not (all) its offspring (2 individuals exhibit also foreign Prussian/goldfish alleles)
		bisexual hybrids	2	3	hybrids F1			yes	at 2 markers no crucian alleles
FIN	Baltic Sea, Helsinki	see remarks	3	1	Prussian proper	identical with 1 lineage from Helsinki 1 except 1 totally unique allele	shared with Pond 1		almost identical with 1 semiclonal lineage from pond 1, Helsinki
		sexual? (1♀)	2	1	shallow Prussian (*C.langsdorfii*-like)				shares exlusive alleles with clone from pond 2
		bisexual hybrids	2/3	6	hybrid F2 or further			yes	share exclusive alleles with male from pond 2 and diploid shallow Prussian carp from Baltic Sea
D	carp breeder	bisexual hybrids	2	2	Prussian backcrossing/ introgression			yes	

Table 5. Alleles of all used markers found in this study in a certain species in comparison to findings of other studies. For Prussian carp, alleles found from only one clone (or single individuals) and being present in another species (=resembling introgressions) group are in brackets. Alleles found diagnostic in this study are bold. 4n Prussian carp are excluded from the data.

Marker	species	Hänfling et al. 2005	Vetesnik et al. 2007	Papousek et al. 2008	this study
GF 17	goldfish	184-212			194,196,219
	crucian	182		180	187,189
	Prussian	186-220	188,190,192	188	187,196,198,200,(206,208),212*
	*C.langsdorfii***		190,192		197,**213,217**,206,208
GF29	goldfish	189-207			197,206
	crucian	210-**228**		212,214	**228,230**
	Prussian	193	199,207	195,199,201	197,205,207,213,214,(228,230)
	*C.langsdorfii***		191,199		197,207
MFW7	goldfish	157-191			**167**,185
	crucian	178-196		158	190,192,195,198,199,**202**,**203**,**206,207**,210
	Prussian	175-199	179,199	175	(165,169,)176,(185),188,190,192,193,194,195,199,210
	*C.langsdorfii***		175,199		190,192,207
MFW17	goldfish	216-256			235,251,263
	crucian	242-250			**249**,251,**253,256**
	Prussian	222-232	222,226		223,230,235,(249)264
	*C.langsdorfii***		212,216		**206**,**219**,230,259
J1	goldfish				**137**,171
	crucian				**0**
	Prussian		147,163		152,156,167,171
	*C.langsdorfii***		127,134		**165**,167
J62	goldfish				189,191
	crucian				**171,175,179,180**
	Prussian		176,178		0,187,189,(191)
	*C.langsdorfii***		124,178		187,189,**190,**191

*fourth allele in 3n clone, microchromosome?

**includes shallow 2n Prussian carp from this study

Table 6. Example of microsatellites revealing different lineages in one population (T) and relationships to other populations (S, O). There are two main lineages (1 and 1b) of one clone with some inner diversity in population T and one different clone (2b) closely related to the clone of population S (2). Individual O-242 differs by only one allele from some of one lineage of population T. Rows=individuals, columns=loci (3-4/marker), cells=alleles, allele numbers=base pairs. Null-alleles are expressed by a 0. Introgressed crucian carp alleles are marked bold (both, diagnostic and common). Alleles differing from the main patterns are marked grey. Alleles found otherwise only in goldfish or 2n Prussian carp are marked with an asterisk*.

code		Lineage	n	J1				J62				GF17				GF29				MF7				MF17			
O	242	1	3	156	156		167	187	0	189			196	196	198	197	0	205		199	192		205	235	235	235	
T	141	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	151	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	154	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	159	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	175	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	176	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	177	1	3	156	156		167	187	0	189			196	196	198	197	0	205		190	192		205	235	235	235	
T	226	1	3	156	156		167	187	0	189			198	198	198	197	0	205		190	192		206	235	235	235	
T	250	1	3	156	156		167	187	0	189			196	196	198	197		205	**228**	190	192		205	235	235	235	
T	283	1	3	156	156		167	187	0	189			196	196	196	197	0	205		190	192		205	235	235	235	
T	158	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**230**	190	192	185*	205	235	235	235	**253**
T	171	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**194**	205	235	235	235	**249**
T	178	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**202**	205	235	235	235	**249**
T	225	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**202**	205	235	235	235	**249**
T	227	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**194**	205	235	235	235	**253**
T	251	1b	**4**	156	156	**0**	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**194**	205	235	235	235	**253**
T	146	1b	**4**	156	156	156	167	187	187	189	189	**187**	196	196	198	197	0	205	**228**	190	192	**196**	205	235	235	235	**249**
T	280	1b	**4**	156	156	156	167	187	187	189	191*	**187**	196	196	198	197	0	205	**228**	190	192	**202**	205	235	235	235	**249**
T	150	2b	3	152		171	167	187	0	189		187	196		198	197	0	214		176	193	194		223	230	264	
T	170	2b	3	152		171	167	187	0	189		187	196		198	197	0	214		176	192	194		223	230	264	
T	173	2b	3	152		171	167	187	0	189		187	196		198	197	0	214		176	193	194		223	230	264	
S	410	2	3	152		171	167	187	0	189		187	196	200	212	197	0	214		176	193	195		223	230	264	
S	411	2	3	152		171	167	187	0	189		187	196	200	212	197	0	214		176	193	195		223	230	264	
S	412	2	3	152		171	167	187	0	189		187	196	200	212	197	0	214		176	193	195		223	230	264	

4.2. Breeding experiments

During the experiment there were produced 334881 eggs and 9382 viable larvae (27%) from eight female breeders in 42 treatments. 1188 fish in 22 families could be reared. Of these around 600 in 16 families were brought to an age at which determination of morphological traits was possible.

4.2.1. Host compatibility and survival

Milt of all applied hosts induced egg development in Prussian carp resulting in viable, feeding offspring. Some hosts seem to be able to truly fertilize eggs of some individual Prussian carp (see point 4.2.2.). Unfortunately, there was not enough data to compare the efficiency of carassiid with that of Leuciscine milt in inducing egg development, since most used milt of the former was fresh, that of the latter all cryopreserved. Within these two types of treatment, however, there were large differences in efficiency between different host taxa and mothers as seen in Table 8. The most effective cryopreserved milt in inducing egg development was that of rudd. The induction of development was truly caused by sperm, since the control, conducted with water for every egg batch, was always negative.

Cryopreserved sperm induced egg development despite the fact that the sperm never showed any motility when tested at thawing, and most of them had broken flagella. In addition, thawed milt pellets turned into insolvable mucus that obviously holds all sperm within it. Only such areas thawing at water contact dissolve and release sperm.

Table 7. Results of artificial insemination experiments. In the control, well water was used instead of fresh sperm.

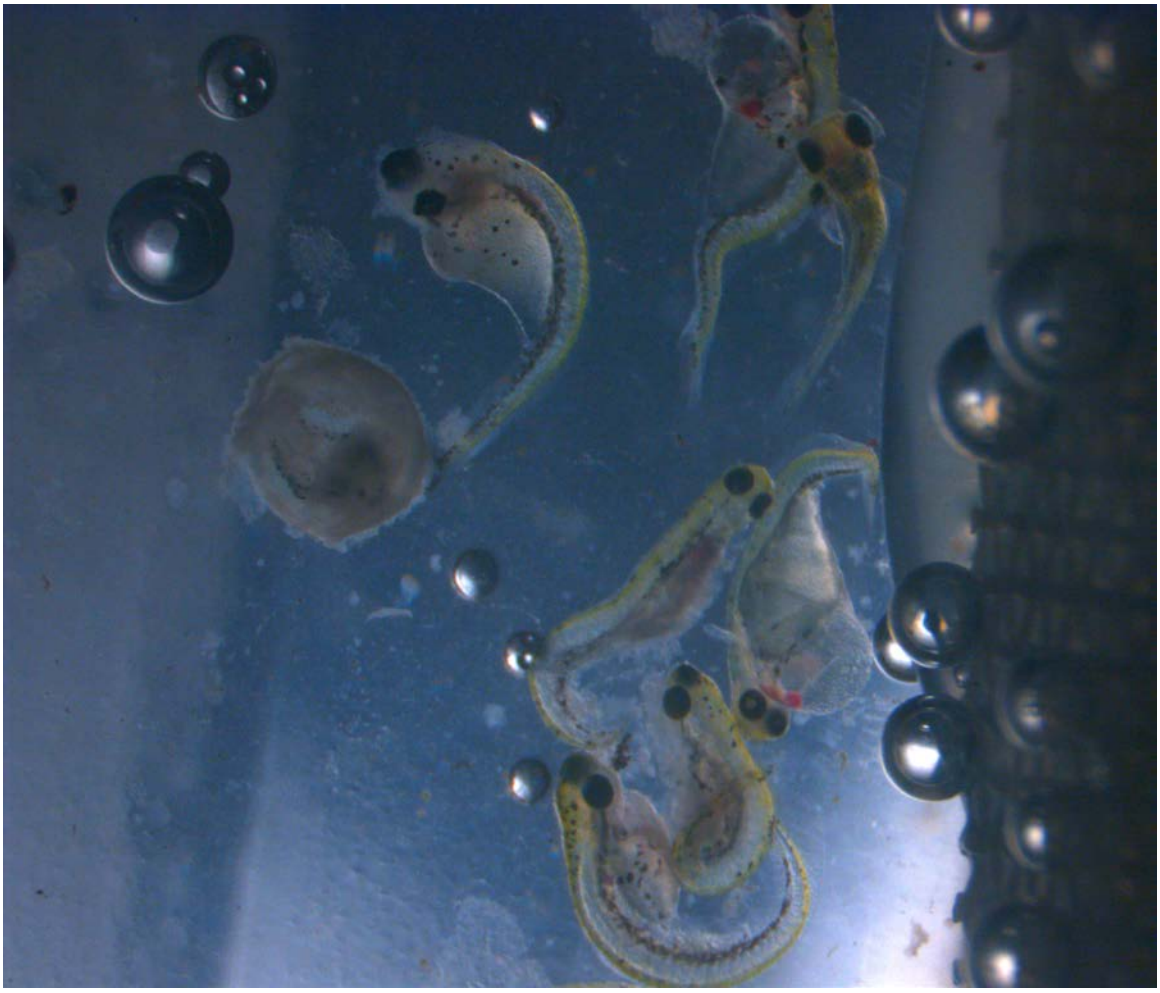
Host species	fresh / cryo-preserved	No. males pooled	motility tested	fertilization ability tested	developing eggs?	viable larvae?
Prussian carp, 2n	f	1	+	*	+	+
crucian carp	f	1	+	no	+	+
crucian carp	cryo	3	+	no	+	+
bleak	cryo	11	+	+	+	+
bream	cryo	1	no	no	+	+
white bream	cryo	3	no	no	+	+
roach	cryo	3	no	no	+	+
rudd	cryo	4	+	+**	+	+
Control	*	*	*	*	-	-

*impossible

**only cryopreserved milt tested, 64% of (bleak) eggs fertilized in test

Developing eggs survived in all cases without bigger problems to hatching (hatching rate family average 67% of developing eggs, see also Table 8.), but in the eggs of all 4n mothers the hatching rate was lower (family average 46%) despite good or moderate developing rates. Hatching was delayed in comparison to the batches from 3n mothers by about two days and many larvae died in the egg or during hatching. The hatched larvae were mainly deformed (Picture 7.). The few normally shaped larvae of these families still did not fill their swim bladder, did not start feeding and died within eight days after insemination or one to three days after hatching. The only surviving individuals developed, hatched and behaved totally normally: Of 4n mother T-1010 the high numbers of developing eggs (around 80% of treated eggs) treated with carassiid milt did not result in

any survivor, but one of two developing eggs inseminated with bleak milt survived. From mother T-1012 both families gained by treatment with carassiid sperm showed lower, but acceptable development rates over 50%, but only 23% and 8% of these survived to feeding, respectively. The eggs of the 4n females were treated the same way and with the same materials, water and chemicals and stored in the same water circulation as the preceding and subsequent batches of 3n mothers, which resulted in viable offspring (Picture 8.).



Picture 7. Inviable larvae of T-1012, 2d after hatching. Deformations, open blood circulation (red spots below heart) enlarged yolk sacs.

Picture 8. Feeding, all healthy larvae of different 3n mothers produced under identical conditions as larvae in Picture 7 above.

4.2.2. Genome size

For genome size analyses 61 hatched and unhatched larvae and 330 juvenile fish were used. It was found to vary within batches and families (Tables 8. and 9.). The offspring of females from Helsinki Pond 2 had almost no variation in genome size and resembled their triploid mothers, but DNA content was in average slightly higher. A few exceptions were mosaic fish with a part of the measured cells being aneuploid (<3n). Representational pictures of mosaic individuals' measurements can be seen in Figure 1.e) and f). Other the also 3n mothers from Salo and Helsinki Pond 2, which produced in all families a large variation of aneuploid (>3n), triploid, tetraploid and mosaic (mainly average ploidy >3n) offspring. In these cases almost all ploidy changes observed raised the genome size. Of the tetraploid mothers one fish (T-1010) also produced offspring with a wide range of genome size ranging from aneuploid mosaics (< 3n) over 3n up to larger aneuploids and 4n. The other two females produced mainly 4n offspring, with some exceptions ranging from 3n to 5n. The few surviving offspring were tetraploid except one triploid. A good overview of generational changes in ploidy and variation within families can be taken from Table 8.

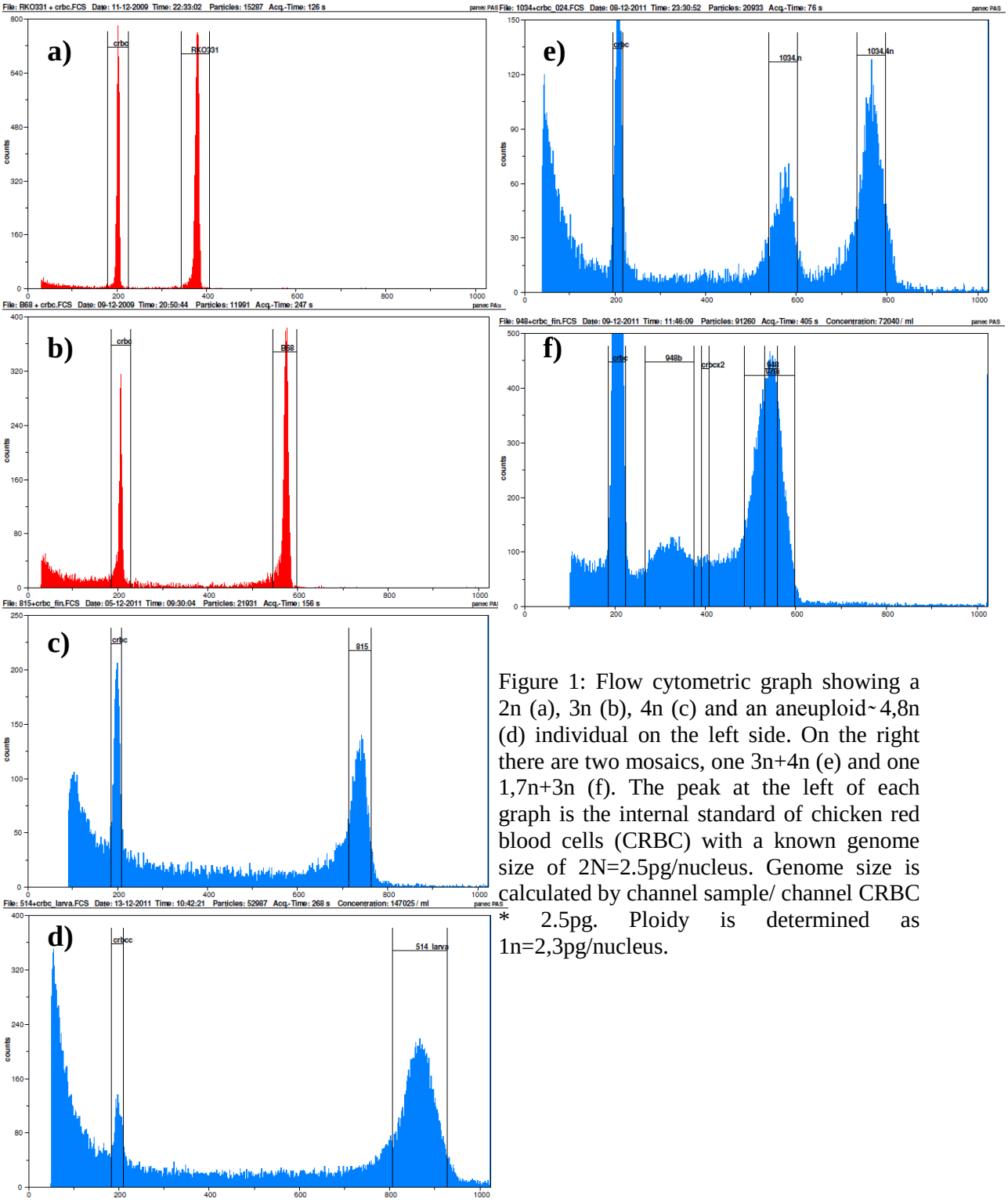


Figure 1: Flow cytometric graph showing a 2n (a), 3n (b), 4n (c) and an aneuploid~4,8n (d) individual on the left side. On the right there are two mosaics, one 3n+4n (e) and one 1,7n+3n (f). The peak at the left of each graph is the internal standard of chicken red blood cells (CRBC) with a known genome size of 2N=2.5pg/nucleus. Genome size is calculated by channel sample/ channel CRBC * 2.5pg. Ploidy is determined as 1n=2,3pg/nucleus.

Table 8. Results of the breeding experiments. Origin of the mother: V=Pond 2, Helsinki, T=Pond 1, Helsinki, S=pond, Salo. Sperm donor species: C.c.=*Carassius carassius*, PRC=Prussian carp, A.a.=*Alburnus alburnus*, A.b.=*Abramis brama*, B.b.=*Blicca bjoerkna*, R.r.=*Rutilus rutilus*, S.e.=*Scardinius erythrophtalmus*. Ploidies: MOS=mosaic individuals (the different ploidies observed in the mosaics are not indicated separately), AN=aneuploid individuals. Ploidy is calculated as the multiple of a theoretical haploid chromosome set of Prussian carp. Egg survival is living offspring at hatching and total survival at start of food intake, both in % of developing eggs. *=cryopreserved milt.

mother	n	sperm donor	developing eggs (%)	egg survival	total survival	ploidies observed	mean DNA difference	SD	No. ind. measured for DNA content
V-1005	3	PRC	99,8	98,7	>0	3	0,15	0,04	32
		C.c.	97,6	99,6	>0	3	0,12	0,15	33
		S.e.*	20,0	95,8	>0	3;MOS	0,12	0,32	27
V-1007	3	PRC	98,9	99,5	>0	3;MOS	0,06	0,81	32
		C.c.	91,1	98,3	>0	3;MOS	0,18	0,19	30
		A.b.*	0,1	100	>0				
V-1008	3	PRC	93,4	98,6	>0	3	0,10	0,43	16
		C.c.	95,8	98,8	>0	3	0,37	0,27	31
T-1009	3	PRC	63,1	80,2	>0	3;4;MOS;AN	0,83	1,06	53
		C.c.	67,0	78,6	>0	3;4;MOS	0,73	0,88	31
		S.e.*	2,0	16,7	0				not measured
T-1010	4	PRC	81,0	78,5	0	3;4;MOS	-1,30	1,79	5
		C.c.	78,0	78,5	0	3;4;MOS	-0,32	0,71	5
		A.a.*	0,3	50,0	50,0	3	-2,52	0,16	2
		B.b.*	0,2	0	-				not measured
T-1011	4	PRC	66,0	69,7	0	4;5	-	-	5, data not analyzed
		C.c.	45,0	69,8	0	4;MOS	-0,16	-	5, 1 analyzed
		A.a.*	0,7	0	-				not measured
		B.b.*	0,0	-	-				not measured
		S.e.*	2,3	45,0	0				not measured
		R.r.*	0,1	0	-				not measured
		C.c.*	2,9	0	-				not measured
T-1012	4	PRC	64,0	62,8	14,2	3;4	0,50	0,15	6
		C.c.	53,2	81,3	6,7	4	0,35	-	3, 1analyzed
S-1015	3	PRC	32,0	62,8	19,5	3;4;MOS;AN	1,44	1,29	14
		C.c.	88,0	79,5	22,7	4	2,85	-	1
		A.a.*	7,0	95,8	88,7				not measured
		A.b.*	0,0	-	-				not measured
		B.b.*	10,0	29,1	27,7	4	1,04	0,96	3
		R.r.*	6,0	100	61,9	3	0,27	0,14	2
		S.e.*	47,0	95,0	57,0	3;4;6;MOS;AN	0,92	1,33	47
		C.c.*	2,0	80,0	33,3	3	0,27	-	1
total									**384 measured, 371 analyzed**

4.2.3. Phenotypic variation

For morphometric analyses 360 juvenile fish were used, which include almost all genetically measured juveniles (N=383).

Unfortunately, almost all families of the mother S-1015 from the Salo population had to be killed when they were infected by an unidentified bacterial disease. Of the Helsinki Pond 1 population (T), only a low number of offspring of two 4n mothers survived to

morphometric measurements. So there is no reliable morphological data for these groups, but only for the Helsinki Pond 2 population (V), which was almost uniform in terms of ploidy.

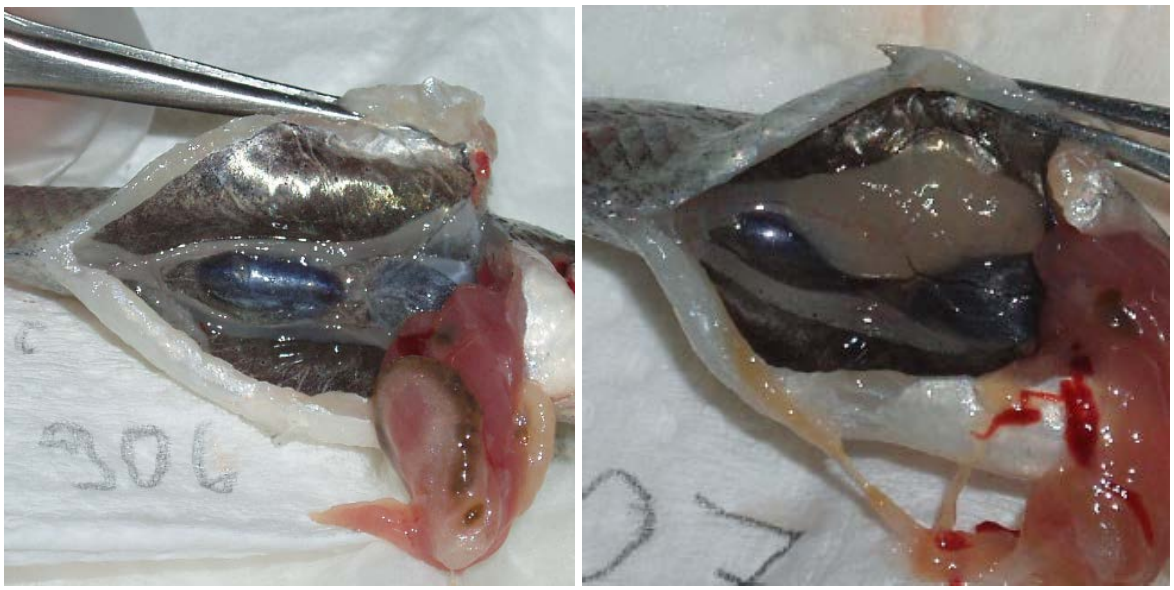
Contrary to expectations, however, the phenotypic variation was eminent. There was large variation within families, but also between (for data, see appendix, Table 13.). The countable traits (lateral line scales, fin rays) exhibited within family variation in almost all cases (for exact data see appendix, Table 13.).

During sampling, more than one habitus type of fish could clearly be identified within almost all families. They could, however, not be classified because they were formed by a mixture of continuous traits that were only partly linked, and single traits also sometimes appeared in relatively "untypical" fish. One example is the reddish pigmentation listed among the traits. This appeared in a continuum and was mostly combined with a sharp snouted "mopshead" while it was often absent from fish with protruding eyes, round snout, underdeveloped operculum and sometimes lower body. Two good examples from the same family can be seen in the appendix on Picture 9. Cases of strong reddish pigmentation were often coupled with a yellowish liver.

Besides differences in colouration and habitus, one clear difference in metabolism could be observed between the three Finnish populations: The edges of the tank containing the fish from Salo was always covered with an oily substance clearly identifiable as unpalated residues of the trout pellets the fish were fed with. These rose from the feces. This phenomenon was not observed in the tanks with fish from Helsinki. It cannot be said, if all fish from Salo exhibited this trait, but at least a large percentage of the 30 fish was necessary to produce the amount of oily leftover observed. In the offspring of the only fish of that population that has reproduced, the phenomenon was not observed. But these offspring had to be culled before they might have expressed the trait. This can be assumed because the fish of one tank in which two batches had been mixed (the Salo and a Helsinki mother) expressed the same trait, but only one month after the pure Salo offspring have been culled.

Picture 9. Two sibs of the same family (T-1007 X crucian carp), both 3n. The upper one expresses reddish pigmentation and a sharp snouted "mopshead", the lower one has no red pigment visible and a rounded, short snout and protruding eyes. also, it is slightly lower bodied. Both individuals represent the opposite extremes of a trait continuum.

The sex of most offspring could be determined. In almost all families one or even several males were found. All determined offspring of 4n females (N=5) were males. Other than the males of 3n-female-derived families (which were all at juvenile stage), they had fully developed, semi-adult gonads (see Picture 10.).



Picture 10. Gonads of a semi-adult male (left) and female (right). The female exhibits a single-side development of the gonads.

4.2.4. Statistic comparisons

The difference between batches (offspring of one mother) was statistically significant (Univariate ANOVA, Table 9.) in all traits except number of lateral line scales, number of anal fin rays and irregular scales. The difference between treatments (sperm host species) was significant only for DNA content difference (mother-offspring), peritoneum colour, anal fin pigmentation, peritoneum flecking and occurrence of reddish pigmentation. The combined grouping of mother and father (=families) shows significant differences only for DNA difference, length, peritoneum colour and reddish pigmentation.

DNA content and DNA content difference (mother – offspring) autocorrelated and were thus rejected from further analysis. DNA content did not correlate significantly with any morphologic trait except sex. DNA content difference in turn correlated with back fin rays, anal fin pigmentation and adolescence. There were many correlations among different countable traits and among pigmentation traits, but less of a countable with a pigmentation trait. The only trait that did not correlate significantly with any other trait was the existence of irregular scales. Length and anal fin pigmentation were correlating with almost all morphologic traits (Table 10.)

Table 9. Univariate ANOVA significance of between-families-differences in DNA-content difference (mother-offspring) and phenotypic traits as grouped by mother, father or family (motherXfather). 1-30 individuals and one treatment per family. *=ordinal variable.

	DNAcontent difference	TL	lateral scales	anal fin rays	dorsal fin rays	peritoneum colour*	anal fin pigmentation*	sex	adoles cence*	flecking of peritoneum*	red pigmentation*	irregular scales*
mother	0,00	0,00	0,67	0,32	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,63
father	0,00	0,45	0,92	0,59	0,99	0,00	0,07	0,53	0,25	0,00	0,00	0,43
mother X father	0,02	0,00	0,40	0,13	0,81	0,00	0,31	0,67	0,42	0,27	0,03	0,55

Table 10. Correlations of offspring DNA content (pg/nucleus) and DNA content difference between offspring and mother with physical traits of the offspring. Significant correlations marked bold are rejected as being autocorrelations. ** significant at 0,01 level (2-tailed). * significant at 0,05 level (2-tailed).

Pearson Correlation

		DNA content (pg/nucleus)	Lateral line scales (N)	DNA difference (pg/nucleus)	TL (cm)	Anal fin soft rays (N)	Back fin soft rays (N)	Peritoneum colour (1...5)	Anal fin pigmentation (0…2)	Sex (f=1, m=2)	Sex (f=1, m=2)	Peritoneum flecking (0...2)	Reddish pigmentation (0...2)
Lateral line scales (N)	Pearson Correlation	,043											
	Sig. (2-tailed)	,486											
	N	264											
DNA difference (pg/nucleus)	Pearson Correlation	**,790****	,003										
	Sig. (2-tailed)	,000	,961										
	N	383	256										
L (cm)	Pearson Correlation	,000	,278**	-,034									
	Sig. (2-tailed)	,996	,000	,570									
	N	295	281	287									
Anal fin soft rays (N)	Pearson Correlation	-,049	,856**	-,067	,325**								
	Sig. (2-tailed)	,397	,000	,255	,000								
	N	297	284	289	317								
Back fin soft rays (N)	Pearson Correlation	,031	,780**	,151*	,301**	,714**							
	Sig. (2-tailed)	,596	,000	,012	,000	,000							
	N	287	278	279	307	312							

Kendall's tau_b

		DNA content (pg/nucleus)	Lateral line scales	DNA difference (pg/nucleus)	TL (cm)	Anal fin soft rays (N)	Back fin soft rays (N)	Peritoneum colour (1...5)	Anal fin pigmentation (0…2)	Sex (f=1, m=2)	Sex (f=1, m=2)	Peritoneum flecking (0...2)	Reddish pigmentation (0...2)

			(N)										
Peritoneum colour (1...5)	Correlation Coefficient	,036	,068	,029	,235**	,138*	,044						
	Sig. (2-tailed)	,531	,300	,648	,000	,037	,482						
	N	170	163	167	192	193	186						
Anal fin pigmentation (0…2)	Correlation Coefficient	,048	,040	,142**	,294**	,117*	,209**	,151*					
	Sig. (2-tailed)	,296	,444	,004	,000	,028	,000	,016					
	N	293	279	285	318	317	308	193					
Sex (f=1, m=2)	Correlation Coefficient	-,127*	-,075	-,124	,053	,188**	,024	-,079	,043				
	Sig. (2-tailed)	,044	,300	,068	,380	,009	,725	,244	,541				
	N	168	161	165	186	187	180	183	185				
Adolescence (0/1)	Correlation Coefficient	,110	,099	,147*	,224**	,066	,126	,133	,107	-,200**			
	Sig. (2-tailed)	,083	,170	,031	,000	,369	,066	,051	,128	,006			
	N	168	161	165	186	187	180	183	185	190			
Peritoneum flecking (0...2)	Correlation Coefficient	,114	,133	-,008	,224**	,120	-,072	,392**	,191**	-,205**	,122		
	Sig. (2-tailed)	,069	,060	,909	,000	,092	,278	,000	,005	,005	,096		
	N	168	163	165	192	192	184	190	193	183	183		
Reddish pigmentation (0...2)	Correlation Coefficient	-,060	,068	-,079	-,034	-,029	-,006	-,220*	-,237**	-,199	-,036	-,050	
	Sig. (2-tailed)	,313	,348	,211	,572	,683	,933	,041	,001	,077	,749	,661	
	N	193	161	187	195	195	190	77	195	78	78	76	
Unregular scales (0/1)	Correlation Coefficient	-,048	-,066	-,029	,009	-,067	-,047	-,050	,012	-,005	-,003	-,047	,052
	Sig. (2-tailed)	,306	,223	,564	,846	,226	,375	,453	,818	,949	,964	,510	,473
	N	299	285	291	321	321	310	193	319	190	190	193	195

5. DISCUSSION

5.1. Genetics of wild fish

In Asian carassiids a great genetic diversity has been found concerning mitochondrial lineages, nuclear DNA, ploidy and sex ratio (see point 2.1.2.). Also in preliminary studies for the present one were found different ploidies, habita and sex ratios. In the literature there are some hints that fish of different ploidy (Mezhzherin & Lisetskij 2004) or habitus (Vetešnik et al. 2008) belong to different lineages or even (sub-) species. To investigate the genetic and reproductive state of the Finnish populations they were analyzed for clonality, relatedness, habitus and possible introgressions from other carassiids. For this they were also compared with hybrids, goldfish and sexual and asexual Prussian carp from Germany.

5.1.1. Species discrimination and genetic identity

Other than with crucian carp, Prussian carp and goldfish shared many (7) of their alleles with each other, at least among some individuals/clones. Of the not shared alleles of goldfish (7) only three are more distinct than two bp difference. Based on the narrow gold fish data set used here, populations of both species can be probably discriminated from each other but not necessarily diploid individuals. To distinguish the two species clearly, other markers must be searched for. Also the use of genome size might be a good tool, although 2n Prussian carp and their hybrids had a very large range of sizes (1n=2,24-2,33pg) and one individual was very close in genome size (1n=2,24pg) to goldfish (1n=2,18pg). As shown by Rylková et al. (2010) and Kalous et al. (2012) both species can be distinguished with the help of mitochondrial DNA, but this is not necessarily true for nuclear DNA. Interbreeding and/or a recently shared ancestral genepool would make species identification difficult or even impossible, as morphologic traits are not suitable for identification of individuals with natural colouration. Nonetheless, differing genome sizes in both species give hope that they are genetically distinct enough to also find working markers (microsatellites or other) to discriminate them, as divergence of genome size needs evolutionary time in which also genes will diverge. A close relatedness of the 2n Prussian carp and the ancestors of the hybrids to gold fish, but also relatedness to the clonal lineages can be inferred.

To which extent (semi-) clonal and diploid lineages exchange genetic material is difficult to say, but the high number of alleles found only in diploids and hybrids lets assume that these markers are either linked to sexuality or there is even a strong barrier between these different lineages. Such a barrier might even be on species level, although introgression to a clone was found. The (semi-) clonal Prussian carp from Germany (3n) and Finland (3n, 3+4n) are sharing the most alleles in all markers and are, by habitus, extremely similar to each other. Based on shared and not shared alleles we can group diploid and polyploid Prussian carp into two different lineages, as mentioned above. In Sweden three different lineages from China, Japan and Russia, respectively, have been reported (Wouters et al. 2012). Different lineages were also found in Europe (Kalous et al. 2012, Takada 2010). Thus we can assume that the fish in this study belong to two of these or even further mitochondrial lineages. It is, however, possible that the two or more groups identified here with microsatellites do not fit at all into the separation made with mitochondrial markers if sexual reproduction or introgression has mixed nuclear and mitochondrial lineages and nuclear identity has totally changed, even into different directions for the same mitochondrial lineage, as argued by Beukeboom and Vrijenhoek

(1998). This remains to be tested. The most essential results are that, based on the finding of unshared alleles in both lineages here, none of the both lineages can be stated to be part or product of the other and, based on the inner relatedness of the lineages, both have spread independently to Finland and Germany.

The taxonomic and reproductive state of the Prussian X crucian carp hybrids remains unclear, but because of the changing allele patterns in the six markers we can conclude that the Finnish hybrids as a group are not the F1 product of a straight hybridization of pure Prussian carp or goldfish with crucian carp, but rather an outcome of backcrossing events or F2 hybrids. Wouters et al. (2012) found for Swedish hybrids that the mitochondrial DNA was always that of Prussian carp. The findings of Wouters et al. (2012) cannot be automatically assumed for the populations studied here. The only diploid (male) from Helsinki Pond 1 cannot have been the father of two of the three sympatric diploid hybrids and its diploid sperm infer that it is probably not the father of any. Thus it is possible that a diploid female in the same population is the mother of the hybrids. Whether some of the hybrids are fertile, interbreed with each other or with either ancestral species (as inferred by Mezhzherin et al. 2004) or whether there is also clonal or semi-clonal reproduction, remains unresolved. Still, finding of structurally normal gonads of either sex in larger hybrids leave space for such assumptions. Any fertile hybrids, however, would enable or accelerate genetic introgressions into ancestral species and hence breakdown of species borders.

The contribution from crucian carp is clear in the examined phenotypical hybrids as well as the tetraploid semiclones, but the share of one allele (187bp in GF17) between crucian carp and some clones remains unclear. Are these common alleles signs of earlier introgression into Prussian carp or shared, undiagnostic alleles? As the Finnish crucian carps and probably also the German ones have had no possibility to gain any introgression from Prussian carp (which invaded the ponds and in Finland the region after the sampled crucian carps have hatched) and also the inheritance from goldfish is extremely improbable (goldfish introductions are rare in both regions and have low survival chances), a possible introgression must have happened from crucian carp into the Prussian carp bearing the alleles in question. However, comparison with data from other studies (see point 5.1.3.) shows that the origin of the observed alleles can probably not be proven. This problem asks for a screening of fish from East Asia e.g. Amur River or more southern drainages, where crucian carp does not occur (Reshetnikov 2003).

5.1.2. Clonal genotypes

The polyploid Prussian carps classified here as "proper" consist clearly of different clones as depicted in Tables 4. and 6. that share much more alleles with each other (also across Finnish and German populations) than with sympatric/geographically close sexual 2n Prussian carp or their hybrids. The clones are mainly consistent with populations, which can be explained by small starting stocks, mainly one or two individuals that rapidly reproduced and formed large single clone populations of hundreds (Helsinki, Pond 1 and 2) or even hundreds of thousands of individuals (Salo Pond). Some clones show some small differences between individuals in single alleles that can be only explained by point mutations or measurement errors, autorecombination (change of allele frequency/allele doubling and replacement, e.g. change from 187/192/195 to 187/195/195) or paternal introgression (total change of single alleles, partly alleles found only from other species or clones). These changes were found mainly in single individuals, but there were also identical copies, which indicate that an allele change had been passed on to the next generation (as identical changes of single alleles in different individuals are extremely

unlikely). Only one of the observed clones (Pond 1, Helsinki, semiclone, see Table 6.) shows more variation where allele patterns in several markers have changed. The clone consists of different lineages with only slightly changed allele patterns. In two of these clonal lineages a whole additional genome was found making them tetraploid. This can be traced back to crucian carp and is thus introgressed. The introgression does not change the allele patterns in the Prussian carp part of the genome. The Prussian carp genome varies between lineages of the semiclone in a way that is not explainable by normal sexual recombination, as patterns in most markers remain unchanged. These changes are most likely caused by allele doubling or deletions. The large genetic variability caused by such autorecombination and paternal introgression in this clone will not change the clonal identity, but probably have an effect on phenotypical diversity and thus long term (>>2 generations) fitness of the clone "founder" and any individual having genetically diverse offspring. As the large introgressions (whole genome) are almost not visible in the habitus and might be inherited to an even smaller extent (see points 5.2.2. to 5.2.4.), and other introgressions observed were minor (single alleles) I consider all individuals with the same basic pattern in the Prussian carp (=clonal) part of the genome as members of a semiclone.

The different clones identified here, however, are so different in their allele pattern (with one exception, in none of the markers allele patterns were shared between any clone.) that they must have been developing independently from each other over many generations, which means that they might have come together or independently from Asia to Europe. Another explanation would be a younger sexual origin either from sexual ancestors or via occasional sexual reproduction between clonal lineages or repeated sexual introgression into such lineages. The possible sexual ancestor (or at least one of them), however, is not known (as stated above, the sympatric sexual Prussian carp are too different to be a close ancestor) and might be found as far as from Asia, if the clones have been introduced to Europe as such. The existence of clonal reproduction before the origin of a clonal lineage cannot be proved or disproved. The slightly changed allele pattern between the semiclone lineages and the small genetic differences of some individuals to geographically distinct members of almost identical clones proves also origins of different clones via asexual reproduction after introgression events.

The introgressed crucian carp genome cannot be identified in two markers: the fish show four Prussian alleles instead. A backcross-hybrid identity of the introgressed crucian carp genome is possible, though unlikely, because in the four other markers the fourth allele is from crucian carp in every individual. For one marker the introgressed alleles were even highly diverse. This indicates that the introgressions came from more than one crucian carp individual.

5.1.3. Comparison of microsatellite data to literature

As mentioned above, the microsatellites used here were suitable to distinguish clones from each other, but they were not diagnostic enough to assign individuals to (sub-) species of Asian carassiids. As we chose microsatellites that were used in other studies, too, it enables a comparison with these studies. Here, some of the alleles (J1, MFW17) which look distinct for *C. langsdorfii* in the data set of Vetesník et al. (2007) to distinguish it from Prussian carp are suiting well into the scale shown for Prussian carp or gold fish in this study (indeed, the authors use allele patterns, not diagnostic alleles). Also the marker GF29 found diagnostic for crucian carp by Hänfling et al. (2005) and Papousek et al. (2008) as well as in this study shows a range overlap, when data of all three studies is combined. The range of this marker found from crucian carps in this study, however, was still at the not overlapping i.e. diagnostic end of the range.

Table 5. shows how strongly the data differs between studies (i.e. studied populations) and how carefully introgression, hybridization or species assignment must be interpreted. The comparison, however, does not change the main patterns observed in this study – mainly due to the sufficient number of markers used to enable the combination of approximate outcomes to form a clearly significant probability.

The very different allele ranges for *C. langsdorfii* in Vetesník et al. (2007) shows that the similar-looking diploid Finnish fish probably belong to a different group. Compared with data from Hänfling et al. (2005), the allele 187 of marker GF17 seems to fall into the ranges of all carassiids, which makes it probable, that this allele was not introgressed from crucian carp into two 3n clones (as discussed in 5.1.1.).

5.1.4. Origin and state of Finnish Prussian carp within the Asian carassiid complex in Europe

Based on the many shared microsatellite alleles Finnish Prussian carps are obviously closely related to Prussian carp from Germany. A clearer separation line than between geographic areas can be drawn between bisexual diploids and polyploid clones. Morphologically determined hybrids with crucian carp seem to be more related to diploid Prussian carp. The two forms coexist and probably there is – at least unidirectional (from 2n to polyploids) – gene flow. Both forms are closely related to the Asian goldfish, but the diploids are – also by habitus – closer. Recent interbreeding as well as even belonging of goldfish and diploid Prussian carp to the same species cannot be excluded. Crucian carp spawns with both lineages of Prussian carp, introgresses into polyploids and forms hybrids with diploids. Diploids and polyploids are capable of producing males and receiving gene flow from other species or lineages. Only the polyploid form was proven (by identical copies of heterozygotic allele patterns in several individuals) to reproduce gynogenetically, but some of the hybrids (carrying typical alleles of the diploid form) are polyploid themselves and their capability of gynogenesis cannot be excluded (see also points 2.2. and 2.3.).

Prussian carp or at least some lineages or (sub-) species (depending on definition and interpretation of the authors) are sometimes argued – also due to the misinterpreted species description by Bloch from 1784 (see point 2.1.) – to be native for some areas of Central Europe (see also point 2.1.1.). For the fish considered here as Prussian carp this cannot be true, because a species with such effective reproduction as the gynogenetic form must be expected to have spread much earlier than in the 20th century (Point 2.1.2.) all over Central, Baltic and Eastern Europe from the argued areas at the headwaters of Elbe, Rhine and tributes of the Baltic Sea. This is especially true as the area is traditionally carp breeding area since medieval times (Van Damme et al. 2007), Bloch's "*Cyprinus gibelio*" associated with carp ponds (Bloch, 1784), and the spread of Asian carassiids (Gasowka 1938, Tóth et al. 2005) and other invasive cyprinids (Van Damme et al. 2007) in the same area along carp trade is proved. The previous existence of pure crucian carp and their current existence and that of hybrids in the areas of Europe that are now invaded by hybridizing Prussian carp in waters now dominated by these makes it extremely improbable that any of the two forms described here has lived there sympatrically over centuries and millennia without wiping out crucian carp by outcompeting and hybridization or without the latter developing prezygotic barriers to prevent hybridization or sperm parasitism. I thus conclude that, as argued throughout this thesis, Prussian carp found from Finland originate from East Asia as do all other obviously East Asian carassiids found from Europe – the only European carassiid being crucian carp.

The existence of genetically very distinct lineages and clones of Prussian carp within two of the examined pond populations can be interpreted as a sign for introduction by anglers into these ponds. The ponds in question are almost inaccessible for natural migration except for large autumn floods during which Prussian carps can be assumed not to rise into shallow water and even ditches which are the only access ways to these ponds (Carassiids are warm water species that will retreat to sheltered deeper waters at low temperatures and avoid currents (=energy loss) and shallow waters (=high predation risk for slow warm water organisms)). The ponds are situated in an urban environment and for both I met at least one person who has introduced fish into them and also persons who actively utilize the Prussian carps as a source of food. All these circumstances make a deliberate or unintended introduction the most plausible explanation for the observed genetic distinctiveness within the populations. The origin of the stocking material remains unclear as an almost identical individual from the Baltic Sea was caught close by the pond (5 km coast line) inhabited by the related clone. A downstream dispersal of this individual towards the sea is possible and even highly probable through a small ditch. Stocking straight from the nearby sea is as possible as transport from Estonia (via ferry without strong border controls) or Russia. Transport and release of living bait fish from Moscow to Lake-Finland has occurred according to Finnish fishermen (O-P. Tervo, Mäntyharju, pers. comm.), and fishermen with East-European background met in Finland during sampling told to have propagated crucian and Prussian carp to small ponds in their home countries as a hobby and stressed the superiority of Prussian to crucian carp. The natural arrival of Prussian carp to the coast of Finland can be assumed to have happened via the Baltic Sea from the dense populations of Estonia and Russia. This would be a logic consequence of the massive area extensions of Prussian carp in Estonia in the nineties as described by Vetemaa et al. (2005). The simultaneous invasion of different (clonal and gonochoristic) lineages at the Finnish coast (as well as in Sweden) might be coincidence, but is suspicious. It would be interesting to investigate a possible (cross-) dependency of clonal and diploid lineages or human propagation activities over the Baltic Sea.

5.2. Breeding experiment

All sperm-dependent parthenogenetic fish are shown to be of hybrid origin. Usually they are dependent on sperm of males of closely related (in most cases paternal) host species. The invasive Prussian carp, however, seems to outcompete its primary host in Europe, crucian carp, from several habitats. According to literature, it seems to be capable of host switch, i.e. using sperm of other species than crucian carp. In our breeding experiment we wanted to show which co-occurring species might serve as sperm donors for Prussian carp in Finnish waters. The gynogenetic mode of reproduction, however, had to be proven, because polyploid hybrids can be viable. For this sake the ploidy of the offspring was compared to the mothers. In addition, phenotypes of offspring of different sperm donors were compared to reveal possible introgressions via differences in expression.

5.2.1. Sexual hosts

The experiment showed that milt of all species used can induce the development of at least some eggs of Prussian carp. The negative control using water instead of sperm confirmed that egg development could not be caused without sperm via parthenogenesis i.e. that Prussian carp is dependent on sperm hosts. It can be assumed, that the low development rates (0-47%, average 6,2%) with Leuciscine milt were mainly due to the method of cryopreservation used. For cyprinid species development rates of eggs fertilized

with conspecific cryopreserved milt ranged in literature around 60% (Babiak & Glogowski, 1998, Horváth et al. 2003, Urbányi et al. 2006, Horváth et al. 2007). Such experiments were conducted under better conditions allowing optimal freezing and melting of milt. The experimental conditions might be the main factor for failure when using cryopreserved sperm (Jorma Piironen, pers. comm.). The main reason for low development rates must be seen in the reduced motility of cryopreserved sperm. Melted under the microscope or in a medium, the sperm never showed any motility when tested, and most of them had broken flagella. In addition, thawed milt pellets turn into insoluble mucus that obviously retains all sperm within it. Only such areas thawing at water contact dissolve and the sperm inside get freed. The large variation of induction rates with rudd milt shows, that there is probably a much larger potential when the method is correct and adapted to each species. At best (47%), rudd milt achieved similar development rates as when treating congeneric bleak eggs with the same cryopreserved milt batch (64%), both ranging within the scale of literature data. In addition, the very low development rates (average 1,5%) achieved when applying cryopreserved crucian carp and Prussian carp milt show that the rates achieved with preserved milt do not reflect rates when applying fresh milt (average 76%). In other words, in nature we can expect substantially higher development rates when Prussian carp can place its roe into the milt cloud of spawning Leuciscines. The quantitative outcome of this experiment does not tell anything about the relative efficiency of different host species for inducing Prussian carp egg development in nature. Nonetheless, as a fundamental qualitative result, the physiological possibility of Leuciscine sperm to induce Prussian carp egg development was proven for the first time for four European Leuciscine species in addition to the only one proven before (rudd).

5.2.2. Phenotype heritability

The clearest difference in offspring phenotypes was the difference between offspring of different mothers indicating a clear heritability of almost all physical traits analyzed. The differences between families were mainly insignificant, indicating that the data is usable and not too biased by random family (=tank) differences. Also the difference between offspring originating from eggs treated with the same species' milt regardless of their mother might be an indicator for inheritance from the sperm host to the offspring. However, clear changes in countable traits of the offspring towards numbers of the paternal species (e.g. more anal fin rays, lateral line scales or less back fin rays) were not found. In addition, the low amount of offspring from other sperm treatments than carassiid hosts might bias the outcomes of the tests. Comparing only between the two carassiid hosts, no significant differences or correlations between treatments can be found. Interestingly, all observed significances are traits linked to pigmentation which also correlate among each other and thus suggest a true observation of inheritance. Colouration has also been the only visible expression of introgressed genes in livebearers (Lamatsch, 2001, Schartl, 1995).

The correlation analyses must be interpreted carefully, as the data is strongly grouped, also for many continuous factors. Nonetheless, the correlations show certain directions, e.g. the low influence of DNA content on morphological traits shows a low meaning of ploidy for phenotype, while the change of DNA content from mother to offspring has significant influence on expressed traits. This indicates that DNA is not added or lost at random. Also, the data correlations suggest a genetic coupling of scale and fin ray numbers among each other as well as among different pigmentation traits, but not between these two trait groups.

All in all, the phenotypes analyzed here showed too much variation within families and were too close to the mothers' traits and were thus a weak tool. The offspring analyzed

were overall very similar to their mothers and no sperm-host -specific expression was observed in morphometry or habitus. The only signs showing a clear direction of paternal influence was the flecking of the peritoneum in all families (higher occurrence and/or density with other sperm donors than Prussian carp) and, among the offspring of one tetraploid mother (more lateral scales and darker pigmentation with crucian carp as sperm donor in comparison to Prussian carp as donor). However, all offspring of that mother had a little more crucian carp –like appearance from their second month on. This makes doubt, if the observed differences were caused as an interaction of paternal genes and those of the maternally inherited crucian carp genome that was not found expressed in the mother.

Due to the assumed deactivation or silencing mechanism (see 5.2.3.) phenotypic traits are no good measurement for paternal introgression in the first generation, but should be investigated for predictive power in the second generation after an assumed introgression. However, it must be underlain with molecular methods.

On the other hand, phenotypic variation in offspring expressed almost exclusively from the clonal genome of a single (fore-) mother shows very well the enormous phenotypic potential that a polyploid clone bears in its genome. At least a major part of the observed intraclonal phenotypic variation can be expected to be caused by individual differences in expression of the same genes or by random expression of different genes or by changes in allele patterns within the germ line. An explanation for this genetic variation can be expected to be the probably hybrid origin of polyploid gynogenetic Prussian carp (see points 2.2. and 2.3.), such that within the clonal genome there is, even without any new introgression, a wide range of alleles. This can also be concluded from the often very different allele sizes within the same marker and high heterozygosis in almost all markers (Table 6.) in clones.

5.2.3. Ploidy and heritability

Large individual variation between offspring, and both, elevation and reduction of offspring DNA content in comparison to their mother could be observed in many families. Overall, a clear elevation of family average DNA content for triploids (+9,2%) and a clear drop for tetraploids (-6,2%) was observed. The difference found between mother and offspring might be partly explained by the use of different tissues: The mother's ploidy was assessed from blood and the offspring's mainly from fin tissue. This, however, would only explain for an error of <0,09pg/nucleus or +3,8% of the offspring genome (=used conversion factor), while the differences found in all except one family exceed this value (see Table 8.). Also, mothers and offspring were measured with the same machine, method and in alternate measurements to exclude bias. Thus, the observed trend is true and means an average elevation of DNA content for offspring from 3n mothers regardless of host species or treatment of sperm.

Therefore, based on the data it must be assumed that the paternal genome or a part of it was included into the genome of some offspring of all females, i.e. paternal introgression happens regularly. Important is the shown fact of possible transgeneral introgression of Leuciscine DNA into carassiid gynogenetic offspring. In some cases offspring of 4n mothers showed a large reduction in genome size. This does not necessarily exclude paternal introgression into the reduced genome, as the observed genome sizes vary strongly and deviate from clear ploidies. However, the only survivors of these offspring showed a clear elevation (0,5pg) of DNA content. Unfortunately, the origin of the offspring genomes was not analyzed. For treatments with carassiid milt the observed elevation of DNA content by 1n to a 3n maternal genome can be as well interpreted as

doubling of one maternal chromosome set (or, alternatively, of doubling of a meiotically reduced, diploid maternal genome). For treatments with Leuciscine milt, however, the most plausible explanation for genome elevation smaller than 1n is paternal introgression sized about one haploid chromosome set (which is, depending on species, between half and two third the DNA content of a carassiid, see Table 11.). However, a functional fertilization in the form of equally expressed paternal genes could not be observed from habitus (all families except the offspring of T-1012 showed a typical Prussian carp habitus) and only small hints for such expression were found from statistical analysis of meristic traits (5.2.2.).

This assumed introgression of paternal genomes must be still verified with suited molecular markers, but it is the most probable explanation. But how to explain the varying sizes of the additional DNA observed in such families, where introgression from Leuciscine sperm could be inferred? Considering the genome introgression from cryopreserved sperm into eggs a probably high rate of damaged DNA must be taken into account. Babiak & Glogowksi (1997) and Horváth et al. (2007) found many haploid and aneuploid offspring when applying cryopreserved milt in carp. In the present study, when testing the method there was found that cold-shocked bleak roe of low quality partly developed to normal offspring when applying fresh milt, but resulted in almost completely unviable offspring when applying cryopreserved milt. Here, the intact fresh sperms might have probably outbalanced cold-shock damages of the maternal DNA, while the preserved sperms might have been damaged themselves giving a fatal combination. Significant DNA damage of cryopreserved sperm in the present breeding experiment (as the preceding facts suggest) and their introgression might explain the huge genome size variation observed especially in families from 3n mother S-1015. Such a mechanism of (cryo-) damaged DNA that is disturbing and inhibiting the rejecting mechanism in gynogenesis might also explain the high frequency of especially incomplete introgression in the breeding experiments. This is underlined by the fact that no incomplete additive introgression was observed in any of the three well investigated (7-50 individuals/population) wild origin populations of the mothers. Also complete introgression was found in only one population. Damaged DNA might be even an explanation for the often incomplete rejection of the foreign genome seen as mosaic individuals. But it does not explain the obvious introgression of whole or partial genomes from fresh milt. Next to factors related to the DNA itself, genome introgression might be facilitated by the temperature drop in the eggs when mixing them with the frozen pellet, but rather there should be expected a disturbance of meiosis II of the egg: Mitosis of a fused chromosome set would be technically impossible at this short time after sperm contact thus a shock-disturbed mitosis is no explanation for the happened introgression. This would cause a doubling of the maternal genome size. This in turn would result with a triploid mother in genome sizes of 6n (or approx. 6,5n with Leuciscine sperm, respectively) which was not observed. Thus we can exclude an influence of temperature shock on introgression in the experiment.

The method of cryopreservation might also heavily damage sperm surface proteins of sperm used for species discrimination. This might give reason to argue that fresh sperm might be rejected by the egg and exclude Leuciscines as sperm hosts in nature. However, the induction of development by Leuciscines (*Scardinius erythrophthalmus*) has been shown earlier (Stein & Geldhauser 1992). Thus the qualitative outcomes from this insemination experiment can be applied to nature.

There is also reason to doubt if there are any processes of genome size elevation either within the maternal genome or even the whole offspring genome, as there were

elevations in offspring DNA content considerably larger than the paternal 1n genome, reaching almost 6n for (unviable) offspring of 3n mothers with Leuciscine milt treatment. These phenomena, however cannot be explained without identifying the origin of the amplified DNA.

Table 11. Haploid DNA contents of several Leuciscine species and crucian carp as determined in numerous studies. Data collected from internetsource 1.

species	2n chromosome number	1n DNA-content (pg)	1n DNA-content (pg), determined with standard *Gallus domesticus*
Abramis brama	50-52	1,30-1,37	1,30
Alburnus alburnus	50-52	1,28-2,08	1,28-2,09
Blicca bjoerkna	50	1,26	1,26
Carassius carassius	100	2,14	
Rutilus rutilus	50	1,21-1,32	1,21
Scardinius erythrophthalmus	50	1,41	1,41

The present study showed survival of tetra- and aneuploid offspring with presumed paternal introgression over several months. This was expected as in fish breeding laboratories even transgeneral crosses resulted in totally viable offspring via artificial polyploidization (Pandian & Koteeswaran, 1998). The more interesting fact was that there was no large difference in habitus observed among the different families. In laboratories, already small portions of paternal DNA were observed to be expressed in artificial (Liu et al. 2007) gynogens. Also in crossing experiments with natural gynogens there were earlier observed differences in colouration (Lamatsch 2001, Schartl 1995). Whether a mechanism exists, which largely deactivates paternal DNA, needs confirmation with expression data (Pala et al. 2010). This was not possible as a disease made a culling necessary before the juveniles in question had reached an adequate size for collecting these data. This was probably the biggest loss in this study.

The death of almost all offspring (3n, 4n and aneuploids) of 4n females gives reason to assume that there is a deactivating mechanism for paternal introgressions as these mothers had 1n crucian carp introgression. Otherwise there is no explanation for the mothers being viable but their offspring, regardless of ploidy, not. A logical explanation would be that introgressed lethal alleles are not expressed in the mothers which can thus be expected to be the first generation after introgression or unchanged clones of such (functional F1). If not silenced, the paternal genome is then expressed and can be lethal in the next generation (functional F2) seen as offspring in the experiment. In polyploids supernumerous genes are silenced at random, but what enables silencing of one specific chromosome set remains an open question for future studies. The deactivating (=silencing) mechanism, however, must lose its power in the germ line of the mothers. This might be associated with the even number of homologous chromosomes in tetraploids, which might enable meiotic pathways and thus recombination, although reduction was not directly observed. Meiotic mechanisms in evenly numbered chromosome sets, however, are in opposition to the observed triploid offspring. The suspected silencing mechanism is not perfect, as differences in peritoneum colour between 3n and 4n Prussian carps have been found within the same populations (Deinhardt 2008).

An escape from lethal expression would be the exclusion of the paternal genome in a kind of hybridogenesis. One reason to assume reduced 3n eggs at least in some cases is the fact that the only surviving offspring of one 4n mother was a 3n female. The loss of lethal

alleles, however, could also be the outcome of random reduction. On the other hand all survivors of another mother were 4n males. This could be explained by crucian carp Y-chromosomes. One Y could come from the mother's deactivated, now expressed paternal genome, a second from a possible 1n introgression into a 3n egg. One or even two Y-chromosomes from crucian carp would cause automatically development of the male sex. By Mendelian genetics, however there should be still 50% or at least 25% females among the survivors. For their absence there seems no explanation, especially as predicted by Haldane's rule the surviving sex should be the homogametous one, which should be female for any Cyprinid. Anyway, the influence of gonosomes is quite unpredictable in polyploids and there was no marker available for gonosomes. Also the existence of the XY-system in carassiids can only be assumed as there is no other system known from cyprinids, and goldfish were proven to be XY (Tave, 2001). Therefore more information is needed on sex determination in Prussian carp and on origin of the offspring's genome.

Based on the combined results of breeding experiment and wild fish genetics, it is possible to draw conclusions about pathways and possibilities of reproduction used by Prussian carp, their meaning to the species, European nature and evolutionary fish biology as described in the following points.

5.2.4. Observed reproductive modes in the light of literature

Based on ploidy and microsatellite data, for polyploid Prussian carp females there can be inferred the three following reproductive pathways that might exist parallel within the same populations, probably even within the same individuals:

1. Gynogenesis, allowing for frequent paternal introgressions in the form of fragments of the paternal genome. This can explain the observed minimal changes in phenotypes in offspring with unchanged ploidy or slightly elevated DNA content.
2. "Asymmetric hybridization", i.e. introgression of a whole haploid paternal genome into the larger, polyploid maternal genome. This was observed, but still needs for experimental and molecular confirmation over generations. Here, whole chromosome sets are introgressed, but the species phenotype remains almost the same due to the genetic overweight of the maternal genome or a possible deactivation mechanism as discussed under point 5.2.3. If inherited, the paternal genes might be partly expressed in later generations. There will be individual differences in expression due to loss of different parts of the paternal genome or even changes in allele patterns with different active genes in different individuals. This would be a highly effective system to gain foreign genes and to test them "individually" under natural selection maximizing the fitness of the clonal individuals susceptive to introgression. Also a stepwise hybridization or genetic "swamping" via repeated introgressions can be imagined reducing the risk of a whole generation hybrid breakdown and giving the invasive species the chance to freely utilize the genepool of the local species.
3. Hybridogenesis. This is rather improbable, but rare hybridogenesis – or a reductional division process resembling it - might cause survival of some individuals in the case of lethal introgression being a "loophole" strategy. In the literature there is no hint on hybridogenesis in *Carassius* or any genus of *Cyprininae*. Still, one case in this study gives reason to argue this possibility: The only surviving offspring of one 4n mother was triploid with no divergent habitus or morphometry. All other surviving offspring of another 4n mother were tetraploid and exhibited some habitus traits of crucian carp (the paternal species introgressed

into the mother) - other than their mother itself. Ploidy reduction can also be caused by incomplete meiotic reduction of the egg. For this, however, there is no proof either, and the clean Prussian carp habitus of the offspring makes this even more improbable.

For polyploid males, hybridization and sexual reproduction must be inferred when reproducing with gonochoristic, diploid carassiid females.

For 2n, bisexual Prussian carp, sexual reproduction could be inferred, especially in the light of hybrids obviously descending from this lineage. On the other hand, the 2n sperm of the 2n male from Pond 2 give a riddle, if these fish would also be able to produce polyploid offspring and clonal lineages. This might be the case in the observed 3n hybrids. However, these were sterile. Still, a reproductive 2n hybrid population seems possible, as hybrids are more common than either parental species in the Baltic Sea (12 of the sampled 16 fish were hybrids) and most individuals seem not to be F1. Microsatellites as well as the wide range of morphologic differences between individuals let assume that under the regime of certain selection criteria, fertile hybrids might be favored and fertile ones will continue reproduction as own taxonomic groups. However, the continuity of this pathway can be doubted seeing only infertile hybrids in this experiment. On the other hand, the functional infertility (no gametes produced despite hormone treatment and spawning tubercles) of hybrids of both sexes in the laboratory could be explained by delayed sexual maturity causing this seemingly infertility of smaller hybrids. Taking into account fully developed gonads found in some of the larger hybrids (over 1 kg), delayed maturity seems more plausible than true sterility. These hybrids should be studied to find out if there is a reproducing hybrid population independent of the paternal species, or if these fish are only a non-evolving byproduct of the Prussian carp invasion, as observed with goldfish in England (Hänfling, 2005) and hybrid zones of other invasive Cyprinids (Hayden et al. 2010).

5.2.5. Technical problems

While mixing of sperm to wrong treatments could be easily prevented and also equal quality of eggs across the treatments of a batch was assured, an obvious problem was the arrangement of the experiments, where open tanks were situated side by side. This might have enabled fish jumping into a neighbour tank. During the whole experiment there were found five fish outside their tank, one of them being in a neighbour tank. It is possible, though improbable, that more fish might have joined another family and falsified the genetic and morphometric traits of that. Only 5-10% of all fish, however, were large enough to jump out of their tanks, and a jump over the gap into the next tank was much more improbable than just leaving the own one. Also, the observed heteromorphy in pheno- or genotype within families are often 10-50% of all fish (up to 100), and exceptional fish were mostly of the lowest size range, much too small for being able to jump off their tank. So, unnoticed mixing between families might have happened, but its influence on the outcome of this experiment can be assumed as marginal if not meaningless.

6. CONCLUSIONS

6.1. Potential hosts and invasive potential of gynogenetic Prussian carp in Fennoscandia

In the literature crucian carp is mainly mentioned as sexual host of Prussian carp in Central (Lieder 1955) and Eastern Europe (Pihu et al. 2003, Reshetnikov 2003, Mezhzherin & Lisetskij 2003). Crucian carp has very similar spawning habits and low discrimination of spawning partners as can be seen from readily mixing populations of goldfish and crucian carp (Wheeler 2000, Hänfling et al. 2005). Joined spawning of male crucian with female Prussian carps in the wild was observed during fishing of material for the present study. There is no doubt, that crucian carp is the main or only host in the Finnish ponds of the present study, although e.g. tench and roach occur in some of these, too. Although crucian carp is quickly replaced by Prussian carp in those waters, longevity (max. 32 years in Finnish waters, Raitaniemi et al. 2000) and long spawning time of male crucian carps (up to one month/individual in experimental tanks) enable Prussian carp to establish strong populations to disperse from and develop within, long before disappearance of the sexual host. Large population size and production of many generations (maybe also accompanied by introgressions from hosts) are good conditions for developing males by the sheer number of individuals, because there will always be a chance to produce functional males – as seen from the demonstrated polyploid males from Helsinki Pond 1 and the one male found even from the very homogenic clone in the Salo pond. Males in turn are prerequisite for an "independent" reproduction of the mainly gynogenetic population. The commonness of crucian carp in almost all of Northern Europe promises good conditions for Prussian carp to establish here. On the other hand, crucian carp is almost totally missing from larger lakes, running waters, and clear, oligotrophic waters prevailing in almost all Fennoscandia. The more north, the more crucian carp is restricted to small, often anoxic ponds and hypertrophic lakes or bays, which are all usually well isolated from each other by unsuitable habitat. For this reason, there is only a low chance for Prussian carp to ever reach those waters they might establish in. Thus, for its own establishment, Prussian carp needs a step-stone system through unsuitable waters to reach water bodies suiting for its main host. The chance to reproduce with more common Leuciscine species might enhance its spread through less suitable waters and getting over barriers in time and space. Given this opportunity to spread, the ultimate limit of Prussian carp must be assumed close to the limit of crucian carp. This limit will be more discussed below.

As shown in this study, Prussian carp can potentially use all Leuciscines with overlapping spawning time and spawning habits as sexual hosts. Further cyprinids in question are carp and tench, the former being verified (Stein & Geldhauser, 1992), the latter suspected to be sexual host (Reshetnikov 2003). It is highly probable that both species can and will function as sexual hosts in Northern Europe. Nonetheless, for spreading of Prussian carp both might be of low importance, because of their low occurrence in northern waters, and a limit far south of the assumed limit of Prussian carp and carp being rarely stocked, not self-sustaining. The rather species-specific and individual spawning habits of tench make it a quite improbable target for spawning attempts. Nonetheless, especially stocked carp might be of high importance to maintain high densities in some source populations with low occurrence of crucian carp, especially at the Baltic coast and some larger, shallow lakes. Among the Leuciscines, bleak and bream might be rather improbable candidates due to their discriminating partner choice, although their spawning time would be very suiting for Prussian carps, and surely the huge

spawning shoals will attract those. Roach, on the other hand, spawn very early at water temperatures (12-15°C) the Finnish Prussian carp seemingly do not spawn, because their gonads are not ripe yet (own observations). However, according to own observations in Central Europe they do spawn together, and spawning together with Balkan roach at 12-14°C is shown by Paschos et al. (2004), which lets us assume a small possibility that also roach can serve as sexual host for Prussian carp specially adapted to a quicker gonad development in northern environment. The most probable host candidates among Leuciscines are thus rudd and white bream, both widely spread in the southern third of Finland (roughly, south of 63°N) in warmer, eutrophic, and often turbid lakes. Both are spawning at times (May - June) and day water temperatures (18-22°C) suiting for Prussian carp. According to own observations from hybrids, both species seem to be frequently involved in Leuciscine hybridizations in Finnish waters, the occurrence of which seems higher in turbid waters. A possibly smaller inducing rate when applying milt of Leuciscines should not be seen as an obstacle for spreading of Prussian carp, with total numbers of hundreds of developing eggs per spawner. But it might influence the establishment in an area, where Prussian carp is dependent on these species as hosts. A more important factor will be the species specific spawning behavior and mate discrimination that occurs among Leuciscines and might be very important for inhibiting interLeuciscine hybridization (Brian Hayden, pers. comm.). The possibility that turbidity might weaken mate choice (Engström-Öst & Candolin 2006) and species discrimination (Seehausen et al. 1997) mechanisms and the commonly found (although not noticed in literature) observation that Prussian carp seem to thrive especially in turbid waters infers that the importance of Leuciscines to Prussian carp rises with turbidity. High densities of Leuciscines might further increase the probability that Prussian carp will find a mate. These high densities will probably not have a large effect on survival of Prussian carp juveniles since these seem, according to observations during the breeding experiments, to be specialized on benthic food such as protists and larger macrozoobenthos, other than Leuciscines that are mainly planktivorous. Thus competition with these hosts cannot be expected for juveniles. Due to the mate discrimination in Leuciscines, lower optimum habitat overlaps with these and the high optimum habitat overlap between crucian and Prussian carp, a large scale host switch to Leuciscines resulting in dense Prussian carp populations as observed in Greece (Paschos et al. 2004) and even a spread outside the range of crucian carp is rather improbable in Finland. However, the meaning of Leuciscines, especially rudd and white bream for the spread of Prussian carp can be expected to be eminent.

Environmental factors mainly influencing the regional spread of Prussian carp will be eutrophication (habitat improvement) and turbidity (protection from predation, possibly easier host switch) of main water courses, availability of disturbed habitats (Pelz 1987), especially smaller ponds and lakes, the state of larger piscivore populations, especially pike, migration possibilities in running waters and anthropogenic propagation. At the moment, Finland sees rather an era of oligotrophication in many waters, partly also a reduction in turbidity, with the exception of peat production areas. On the other hand, construction of artificial wetlands for rain water retention, as biotopes and park elements or for nutrient and sediment retention creates a high number of nutrient rich small habitats with extreme conditions. Together with attempts to improve migration ways for fish, especially in South Finland, this might open step-stone pathways for Prussian carp to invade urban as well as rural end even forest areas. Also, the effect of improved environmental conditions might be negated by climate change effects, which would raise productivity and sediment load of many waters, enhance population development of many

host species, and, via quicker growth, enhance survival chances of Prussian carp. Widespread gill netting of predatory fish in Finland is not expected to improve the biological defense against spreading Prussian carp in main water courses. On the other hand, cyprinid species as crucian carp are not valued in Finland and the Nordic Countries, and their value is still decreasing, especially in rural areas. This reduces the chance that Prussian carp will be propagated intentionally or unintentionally in the mainly rural areas of Northern Europe. The rather new phenomenon of "specimen angling" on one hand and the continuing immigration of Central and Eastern Europeans to cities in the Nordic countries on the other hand will raise the risk of introductions of Prussian carp and other economically worthless species especially in urban areas: Hobbyists can be interested in unvalued species and start propagate them for own fishing purposes and East and some Central European cultures traditionally value cyprinids, and fishing and propagation of carassiids are common. As a warning: the introductions of two of the three pond populations studied here can be seen as a result of stocking by “hobbyists” (see point 5.1.4.).

As stated above, whatever factor enables a local establishment of the invasive species increases also its chance to spread. Spreading, in turn, enables regional establishment and adaptation. Population sizes and time of existence are driving factors in evolutional development of this complex, but also physical and ecological isolation. Isolation in turn is enforced by the mainly asexual reproduction of this fish. Given the natural occurrence of cyprinid species and its relative stability in Finland, there can be expected following scenario for Finland: 1. The Prussian carp will inevitably spread into all suitable habitats along the Baltic coast until about the Finnish city of Vaasa, north of which the main host, crucian carp, is not common anymore in coastal waters. It will also rise into all passable rivers, ditches and channels, and invade the warm and productive waters of the heavily agricultural coastal South Finland until roughly 62°N, and establish populations in all suitable habitats as ponds, wetlands and creeks, if it finds crucian carp there. It will be probably introduced successfully into many urban and suburban ponds by anglers in that area. Sporadically it might be found from its spreading pathways as rivers, lakes and open coast, but it will mainly be observable in its established populations in small or heavily disturbed waters. This development is probably not to be stopped. 2. Also quite probable will be the invasion via some introductions and maybe also rivers and channels into Southern Finnish inland until about 63°N, where Prussian carp will spread slower due to the vast areas of unsuitable large lakes and the resulting lack of crucian carp. Very sensitive areas for establishment of Prussian carp will be agricultural areas with many interconnected, productive, small, shallow lakes as e.g. around the city of Hämeenlinna. Also urban areas are under a higher threat because most potential introducers live there, and are interested in getting “their” special fish established close to their home. Natural spread will probably depend on the occurrence of white bream and rudd and on turbidity of the connecting waters. This step, however, is not very threatening, though probable. 3. If Prussian carp establish populations or clonal lines that are adapted to utilize the early spawning time of roach and bream and to produce more fertile males these might spread into all reachable habitats of South Finland and along the coast and via introductions much further north. This, however, will need a strong influence of climate change and introductions. Both seem improbable to cause changes strong enough for being useful to Prussian carp. Anyway, this possibility should be kept in mind when monitoring the spread of this fish.

For the gonochoristic diploid Prussian carp the same invasive potential cannot be assumed, because they cannot utilize other species for spreading, but always need a conspecific mate.

6.1.1. Establishment via gynogenesis and genotypic and phenotypic diversity

As discussed in point 2.3., polyploid clones can be equal or superior in fitness to their gonochoristic counterparts. An almost twofold potential reproductive rate (probably a little lower due to larger egg size in Prussian carp) and the sexual parasitism will quickly lead to replacement of crucian carp by gynogenetic Prussian carp, which can be total in cases of host switch as discussed under 6.2. The situation with gonochoristic lineages might be different, but the closely related goldfish seem to ecologically displace and/or genetically "swamp" crucian carp even without any reproductive advantage in Western and Southern Europe (Wheeler 2000). The phenotypic and partly genotypic variation within offspring of the same polyploid mother and whole clones as found in this study and also the large variety of different clones raise the chance that for any new area and/or niche there will be a suiting individual or clone available to succeed under natural selection, get established and start a further development, maybe under sex-like pathways as described under the next point.

6.1.2. Adaptation via introgression and natural selection

Prussian carp, once locally established, can be expected to gain locally adapted genes and genomes from its hosts via paternal introgression (see also next point) – mainly crucian carp. An expression of these genes, despite the assumed silencing mechanism in the 4n females (functional F1, See point 5.2.3.) can be assumed to some extent (see points 5.2.2. and 5.2.3.). This will enable them to adapt to local and regional niches and become persistent and get better chances for spreading further in space and across ecological niches. An important gain to a clonal line, of course, can be the higher susceptibility to introgression and production of males in polyploid lineages after introgression of small amounts of foreign DNA. This would speed up the rate of introgression, possibly allow for intraclonal recombination (via introgression from clonal males) and thus facilitate evolution and adaptation under natural selection. Another important feature to circumvent harmful influences of gonochoristic hybridization (hybrid breakdown, maladaptation) is the asymmetric hybridization (=introgression of a whole 1n paternal chromosome set) with the obvious paternal deactivation in F1 (discussed in 5.2.3.). Here the gained genes would be exposed to natural selection in a larger group of offspring (in the invasive stage) and after a possible reduction (see point 5.2.4.) or random changes in allele frequency. This minimizes the risk of a possibly lethal or harmful allele interaction that could eradicate the entire F1 and thus inhibit a gain of only useful genes to any offspring. The random expression (not all alleles of a locus are expressed in polyploids) of randomly preserved paternal local genes only after a reshuffling with a majority of maternal alleles guarantees for a wide range of phenotypes. These will be subject to an effective selection already in the first “round” of selection, making an introgression of useful genes into the invasive species more probable than in gonochoristic species. The same is true for smaller introgressions as single genes or microchromosomes, although it can be expected to be a slower process due to smaller amounts of introgressed DNA.

Mezhzherin & Lisetskij (2004) show that in large reservoirs of the Ukraine crucian carp is already totally swamped and assimilated into, i.e. it exists only as introgression in, the genepool of Asian carassiids. A good example for a similar development of adaptation via introgression in Finland is the different expression of body depth, an adaptation of

crucian carp to predation, in hybrids of crucian and sexual Prussian carp in the Baltic Sea. This feature does not exist in Asian carassiids and might, if introgressed into sexual or clonal lineages, mean a new gained adaptation to clear water systems of Europe dominated by optically hunting predators. The expression of this trait varies between individuals and future studies will show, which state will be selected for and if it will appear also in gynogenetic populations. An answer might be found from some decades-old Prussian carp populations from Austria that possess one crucian carp allele (Lamatsch & Deinhardt, unpublished data) and are seemingly deeper in their neck (personal observations).

6.2. Threat to existence and genetic integrity of crucian carp in invaded water bodies and protection of the species

The spread of Prussian carp has been observed to be accompanied by replacement of and hybridization with crucian carp everywhere in Europe as described in point 2.1.2. Also all four ponds studied here were populated only recently, but Prussian carp made up already the largest part of the fish biomass there and juvenile Crucian carps were absent in three of the ponds, and rare in the fourth (Deinhardt, unpublished data).

It can be expected that any clonal Prussian carp will totally replace crucian carp in waterbodies without piscivores only by its higher reproductive potential and its superior life history traits (larger egg and larva size, faster growth, younger maturation age, bigger size at maturation, no predator-avoiding and thus feeding-inhibiting behavior; arguments from Deinhardt 2008) and optional parasitizing on crucian carp milt. Hybrids or inviable offspring from crucian carp eggs fertilized by occurring Prussian carp males could even speed this up. This development will be further ensured, if the clones are capable of host switch and/or production of functional males. This seems probable as discussed below. In waters with predators the crucian carp's defense strategy (see Holopainen et al. 1996, Pettersson et al. 2003) will counterweigh the traits of the defenseless Prussian carp, but flooding from predator-free source populations and possible hybrids (either, yet unregistered hybrids e.g. from male polyploids, or via additionally invading 2n fish) might be here the key to total loss of crucian carp as described by Mezhzherin et al. (2004). The latter can be inferred based on the fact that possibly fertile hybrids from the Baltic Sea show the defense trait body depth to different extents, which would make them more competitive to crucian carp. The fact that normal gynogenetic sexual parasites usually develop a (cyclical) population equilibrium with their sperm hosts (McKay 1972) or can be expected to go extinct with the outcompeted host, does probably not apply for Prussian carp, as they are capable of host switch and of producing rare males. The latter ones were found to produce milt in this study, and findings from self-sustaining single-species populations in Estonia (Pihu et al. 2003) might be applicable for the Finnish populations, too. Even if the alternative hosts might not be as effective, a shrunk crucian carp population will not persist in an established vast majority of Prussian carp. Thus, crucian carp will be probably lost wherever Prussian carp appears. As in inland Fennoscandia there are the last large-scale areas without Prussian carp or similar Asian carassiids in the EU, maybe whole Europe, the protection of these last refugia must be considered.

Crucian carp is sometimes argued to have naturally reached only southern Finland and the coastal areas of the Bothnian Bay. Thus populations of north Scandinavian and Finnish inland areas might be non-native and low in genetic variation (as a result of being spread from pond to pond with low amount of individuals in a step-stone system with repeated bottlenecks). Nonetheless, these populations might be the last refugia of crucian carp from being ecologically replaced and genetically assimilated by its stronger competitor. These populations deserve thus a higher protection status – even higher than

e.g. isolated populations within the native range which are surrounded by waters already populated by Prussian carp or gold fish as they are under continuous threat of being invaded and replaced or losing their genetic integrity.

The spread of Prussian carp as depicted in 6.1. seems unstoppable. Also the protection of isolated crucian carp populations within the Prussian carp's range makes no sense, because it is a question of time until a Prussian carp from nearby reaches the population. Larger, expensive programs to stop the invasion are also not feasible as they must be very extensive, will be of low efficiency and sustainability and receive low acceptance due to crucian carp's low socio-economic significance. There are practically only two realistic measures: 1. Sensibilization of anglers for the problem to stop the spread of the species to uncolonized areas, and to encourage local actions to eradicate established populations and 2. Designation of a reasonably defendable zone where environment authorities have to - and must be able to - eradicate any established population of Prussian carp. Following the scenario in 6.1. a reasonable southern limit of a Prussian carp-free North-East Finland would be the southern limits of the Oulujoki drainage. Successful invasion to this area is very improbable and introductions would probably be only successful in small ponds. These are easy to poison or drain and in that area are of no or low economic value. A protection of southernmore crucian populations cannot be assured without causing disproportionate costs caused by eradications and reintroductions with pure crucian carp.

6.3. Introgressive gynogenesis and paternal leakage in clones as a successful strategy in evolution

Since the system studied here includes elements of sexual and asexual reproduction, but mainly guarantees the continuity of a goldfish-like taxon that is able to reproduce without conspecific males, and additionally hardly produces them – and at the same time uses other species for reproduction without reducing its long-term (>>2 generations) fitness, we can talk about gynogenesis. This is not remarkably reduced by the fact, that obviously the organism can include the host species' genome, since the result will be no hybrid by definition, but rather a member of the maternal taxon with some introgression. This system is not hybridogenesis. This is for two reasons: The genome acquired to the offspring can be - assumedly - clonally transmitted to the next generation, but is not or almost not expressed in the F1. Second, the sexual parasite is not dependent on its close relative host.

This system also cannot be seen as contagious gynogenesis - or infectious apomixis (=parthenogenesis) as used by botanists - which means the transmission of asexual genes from asexual into sexual lineages or species via hybridization (Hörandl, 2009). Here, this was not the case as asexuality was not transmitted to a sexual lineage. In addition, the offspring of the inferred interspecific introgressions happened from a sexual into an asexual species which remained asexual and also mainly retained the maternal species' attributes, i.e. are no true hybrids. Thus, no attribute of contagious gynogenesis were found. Of course, the contageousity of this case is theoretically only possible via males, which might have access to gonochoristic reproduction i.e. fertilize the eggs of gonochoristic carassiid females. This can even be inferred to be the reason why gynogenesis can be found in different, carassiid taxa and mitochondrial lineages with mainly gonochoristic populations (Takada et al. 2010). For this pattern Takada et al. (2010) infer independent evolution of gynogenetic lineages from gonochoristic populations, but their data allows the interpretation of infectious gynogenesis as well. But infectiousness, if existing, would be only a part or side-product of the system, while the gain of small

introgressions might be a much larger and more meaningful aspect of reproduction and evolution in this system.

As a result of these facts, I suggest to name this yet undescribed system **introgressive gynogenesis**. It has the following characteristics: 1. Individuals of this system are normally unisexual. Males are exceptional and of very low meaning to the fitness of their mother unless they are fertile and the only males for their female sibs to spawn with in a population. Males, often products of introgression, will provide more accessible genetic material to different lineages via introgression. 2. Females produce gametes with a viable genome (2n or preferably larger). 3. Conspecific or allospecific sperm are able to start egg development independent of if the egg is fertilized or not. The mechanism inhibiting fertilization is imperfect, leading to occasional introgression of the paternal genome or parts of it which are included into the developing offspring. 4. The introgressed paternal genome is not or to a small extent expressed in the F1 offspring. 5. The foreign genome might or might not be retained in the germ line. 6. Points 2. and 4. infer that the females are polyploid and of allopolyploid origin. 7. Clonal lineages superior in reproductive power will spread to and colonize new areas as described for Prussian carp. The continuing introgressions and varying retention will lead to populations with a high diversity of genotypes, phenotypes and adaptations. The proportion of males will rise in time because of introgressions. But, even if sexuals would appear, due to their utilization via introgression, the gynogenetic reproduction will not disappear unless there are no sexual hosts at all and non-host competitors with always higher fitness. 8. As a result of these properties, the system concerned will be conserved and develop as a multi-semiclonal species. It will continuously gain allospecific adaptations via introgression. The genetic diversity will be maintained by interspecific and interclonal introgression, allele pattern changes and natural selection. Speciation will be possible and favored by segregation of different lineages by spatial and ecological barriers or adaptation to different hosts. Due to its extremely high capability to spread and to gain new adaptations, such a system should have a high potential for diversification. At the example of Asian carassiids we can see a high adaptive radiation in East Asia (see e.g. Takada 2010), but also in the European populations there is a wide range of geno- and phenotypes already. Problematic is the distinction and survival of species, as closely related (sub-) species with the same system of introgressive gynogenesis can be expected to melt to one complex or system if they inhabit the same area and spawn together. Thus a division into different species needs a proof that there is no greater gene flow between the different lineages observed. The only European carassiid, the crucian carp can be expected to be outside such a gynogenetic melting pot as it is strictly gonochoristic and hybrids seem to be either sterile or the gene flow is rather one-directional into gynogenetic lineages (see point 6.2.).

Introgressive gynogenesis must not be seen as an exception or even an incomplete developmental step from sexual to asexual reproduction. Rather it is an evolutionary strategy of its own, connecting the ecological advantages of asexual reproduction with the evolutionary necessity of gaining new genes, taking the high fitness risk of crossing species barriers to gain the advantages of “tapping” unrelated gene pools. This and mainly clonal reproduction make the individual independent of conspecifics and large gene pools without discarding the access to other individuals' beneficial genes. This system has been successful for a long time (as polyploid carassiids isolated over 1Mya are spread over all East Asia, even into isolated areas - see Takada et al. 2010) and is probably competitive in rapidly changing environmental step-stone mosaics with high resource density, which offer refugia and spreading paths for some hardy individuals. Introgressive gynogenesis in a "typical" invasive species will reduce the common genetic problems of such as inbreeding,

bottle necks, founder effect or simply finding a partner. It must be seen as a "rising star" in a world more and more turned upside down by man, who promotes spread along distance, destroys grown environments and creates new ones giving chances for opportunistic, hardy, quickly adapting species to get to new areas and get naturalized there with no considerable competition or predation.

A similar system with smaller introgressions into polyploid eggs has been described in flatworms (D'Souza et al. 2006), but these are allosperm-dependent hermaphrodites. In these flatworms sperm are reciprocally exchanged between conspecific gynogenetic individuals and fitness advantages and introgression can be bidirectional i.e. they are not sexual parasites *per se*. Thus the system clearly differs from introgressive gynogenesis as the clone is not parasitizing on sexual species nor receiving foreign genes from locally adapted sexuals that it can outcompete with its quantitatively more effective reproduction. It is rather the reduction of sex and recombination to a necessary, smaller level in a species that does not depend on a separate unproductive sex which is competing to achieve equilibrium. The authors talk about "occasional paternal inheritance" and "sex-like processes".

6.4. Sex and recombination in clonal lineages - is gynogenesis asexual?

The reproductive mode observed here allows for different scale introgressions into a continuous clonal genome - up to the size of a whole haploid genome. The latter one can be also seen as fertilization - though an asymmetric one.

As discussed in point 6.3., the system described here would regularly allow for introgression, which is not a malfunction of the system. Also gynogenesis in general, other than true parthenogenesis, physically allows for paternal introgression via small scale leakage (Lamatsch 2001) and even fertilization and in many species this was observed (e.g. Schartl et al. 1995, D'Souza et al. 2002, this study). Parthenogenesis allows for changes in allele frequencies, but not for introgression, while gynogenesis allows for both and thus - in theory - for recombination between the genomes of different lineages and taxa. For this reason it could be theoretically seen as a form of sexual reproduction, although the offspring can be often considered as clones and introgression was not observed in all practical cases. It only reduces the amount of sex and the energetic investment into production of males to a minimum with the help of sexual parasitism.

However, as long as there is no obligate mechanism of gene flow between clones demonstrable (e.g. obligate production of small proportions of males, hermaphroditism), the most fundamental characteristic of sexual species, recombination between conspecific individuals, does not apply. The finding of characteristic alleles from one clone in some individual of another do not prove this, neither does the obviously unsystematic occurrence of clonal males. Introgressive gynogenesis is thus an asexual system, other than the system described by D'Souza et al. (2006, see 6.3. for discussion).

For future research it remains to investigate, if introgressive gynogenesis is maybe more common among gynogenetic animals, especially vertebrates. Also the possible occurrence of true sexuality in such systems or possible cyclic sexual-asexual systems or contagiousity should be intensively looked for, as both might explain the polyphyletic occurrence of gynogenesis observed in literature and the clonal diversity found in this study.

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INTERNETSOURCES

1. http://www.genomesize.com/, 26.11.2012

APPENDIX

Table 12. Overview on performance of all families in the breeding experiment. Each row=1 family, indicating mother, species state of milt, development-hatching- and survival rates and total numbers. Empty fields mean that the treatment or count was not done in the specific case. *=The egg number had to be estimated. **=survival estimated 2 weeks after feeding started Underlined numbers of developing eggs were estimated from partial counts and total numbers. Sperm donor species: C.c.=*Carassius carassius*, PRC=Prussian carp, A.a. *Alburnus alburnus*, A.b.=*Abramis brama*, B.b.=B*licca bjoerkna*, R.r.=*Rutilus rutilus*, S.e.=*Scardinius erythrophtalmus*. 0=negative controll with water instead of milt.

mother	sperm donor	milt				eggs	developing at eye stage			viable at hatching		viable at feeding			Swarms established
code	species	cryopres.	No. males	mobility	fertiliz.	N	N	%	unhatched or inviable	No.	% of dev eggs	N	% of hatched	% of all eggs	No. fish
V-1005	PRC		1	+		1050	1048	100	14	1034	98,7				102
V-1005	C.c.		1	+		1200	1171	98	5	1166	99,6				100
V-1005	S.e.	x	4	+	+	950	190	19,2	8	182	95,8				100
V-1005	0	-	-			1660	0	0		0	0				
V-1007	PRC		1	+		1000	989	99	5	984	99,5				102
V-1007	C.c.		1	+		980	893	91	15	878	98,3				100
V-1007	A.b.	x	1			1280	1	0,1	0	1	100,0			0,1	1
V-1007	0	-	-			1200	0	0		0	0				
V-1008	PRC		1	+		990	925	93	13	912	98,6				100
V-1008	C.c.		1	+		622	596	96	7	589	98,8				101
V-1008	0	-	-			1040	0	0		0	0,0				
T-1009	PRC		1	+		553	349	63	69	280	80,2			1,8**	10
T-1009	C.c.		1	+		740	496	67	106	390	78,6				102
T-1009	S.e.	x	4	+	+	295	6	2	5	1	16,7			0,3	1
T-1009	0	-	-			139	0	0							
T-1010	PRC		1	+		633	513	81	110	403	78,5	0	0	0	
T-1010	C.c.		1	+		750	585	78	126	459	78,5	0	0	0	
T-1010	B.b.	x	3		+	600	1	0,2	1	0	0				
T-1010	A.a.	x	11	+	+	630	2	0,3	1	1	50,0	1	100	0,2	1
T-1010	0	-	-			360	0	0							

T-1011	PRC		1	+		770	508	66	154	354	69,7	0	0	0	
T-1011	C.c.		1	+		720	324	45	98	226	69,8	0	0	0	
T-1011	S.e.	x	4	+	+	700	20	2,9	11	9	45,0	0	0	0	
T-1011	A.b.	x	1			642	0	0		0	0				
T-1011	B.b.	x	3		+	735	17	2,3	10	7	41,2	0	0	0	
T-1011	A.a.	x	11	+	+	440	3	0,7	3	0	0				
T-1011	R.r.	x	3		+	1340	2	0,1	2	0	0				
T-1011	PRC	x	1	+		700	15	2,1	6	9	60,0	0	0	0	
T-1011	C.c.	x	3			600	5	0,8	5	0	0				
T-1011	0	-	-			910	0	0							
T-1012	PRC		1	+		231	148	64	55	93	62,8	21	22,6	9	6
T-1012	C.c.		1	+		141	75	53	14	61	81,3	5	8,2	3,5	3
S-1015	C.c.		1	+	+	1000*	880	88,0	190	700	79,5	200	28,6	20	101
S-1015	PRC	x	1	+		1700	544	32,0	47	250	46,0	106	42,4	6,2	105
S-1015	C.c.	x	3			1000	15	2,0	0	12	80,0	5	41,7	0,5	5
S-1015	S.e.	x	4	+	+	560	263	47,0	29	250	95,0	150	60,0	26,8	100
S-1015	A.a.	x	11	+	+	1100	71	7,0	10	68	95,8	63	92,6	5,7	36
S-1015	B.b.	x	3		+	1580	141	10,0	13	41	29,1	39	95,1	2,4	3
S-1015	A.b.	x	1			1340	0	0							
S-1015	R.r.	x	3		+	1300	21	6,0	1	22	104,8	13	59,1	1	9
S-1015	0	-	-			700	0	0							
total						**34881**	**10817**		**1133**	**9382**					**1188**

Table 13. (next two pages) Difference in DNA content (mother-offspring) and physical traits of the offspring grouped by batches (mothers' codes, **bold**) and families (sperm donor species codes below mothers). Family-specific data clearly differing form other batches or within a batch are marked grey. Single families of mothers T-1010 and T-1011 are not shown due to low amount of data. These data can be found from Table 8.

	DNA difference			length			lateral scales			peritoneum colour			anal fin rays			dorsal fin rays		
	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD
V-1005	**92**	**0,13**	**0,20**	**110**	**4,7**	**1,17**	**112**	**30,1**	**3,07**	**86**	**2,9**	**0,90**	**110**	**5,4**	**0,46**	**104**	**15,6**	**1,58**
C.c.	33	0,12	0,15	33	4,6	1,30	34	29,3	5,85	12	2,2	0,66	34	5,3	0,78	33	15,5	2,65
S.e.	27	0,12	0,32	27	4,9	1,11	28	30,5	0,73	25	3,2	1,00	27	5,4	0,21	25	15,6	0,60
PRC	32	0,15	0,04	50	4,7	1,12	50	30,4	0,67	49	2,9	0,81	49	5,4	0,17	46	15,8	0,68
V-1007	**63**	**0,12**	**0,59**	**62**	**4,8**	**1,26**	**63**	**30,5**	**0,59**	**23**	**2,8**	**0,75**	**62**	**5,4**	**0,18**	**60**	**16,1**	**0,56**
C.c.	31	0,18	0,19	31	5,0	1,05	31	30,5	0,60	11	3,3	0,64	31	5,5	0,09	31	16,2	0,50
PRC	32	0,06	0,81	31	4,7	1,43	32	30,5	0,59	12	2,3	0,49	31	5,4	0,22	29	16,0	0,61
V-1008	**56**	**0,24**	**0,38**	**62**	**4,9**	**0,96**	**62**	**30,6**	**0,64**	**20**	**2,9**	**0,79**	**61**	**5,5**	**0,06**	**60**	**16,3**	**0,60**
C.c.	30	0,37	0,27	31	4,9	0,97	31	30,4	0,75	11	2,5	0,69	31	5,5	0,09	31	16,3	0,61
PRC	26	0,10	0,43	31	4,9	0,96	31	30,7	0,50	9	3,3	0,71	30	5,5	0,00	29	16,3	0,60
T-1009	**84**	**0,80**	**0,99**	**75**	**5,6**	**1,69**	**72**	**30,6**	**0,66**	**54**	**2,8**	**1,05**	**75**	**5,4**	**0,17**	**74**	**16,4**	**0,69**
C.c.	31	0,73	0,88	31	4,9	1,30	29	30,6	0,71	13	3,5	0,78	31	5,5	0,15	31	16,4	0,79
PRC	53	0,83	1,06	44	6,1	1,76	43	30,6	0,63	41	2,6	1,05	44	5,4	0,18	43	16,4	0,62
T-1010	**12**	**-1,10**	**1,42**															
T-1011	**1**	**-0,16**	**-**															
T-1012	**7**	**0,48**	**0,15**	**9**	**7,9**	**1,39**	**10**	**31,4**	**0,57**	**8**	**4,9**	**1,46**	**10**	**5,5**	**0,00**	**10**	**16,3**	**0,59**
C.c.	1	0,35	-	3	9,1	0,29	3	32,0	0,00	2	6,0	0,00	3	5,5	0,00	3	16,3	0,76
PRC	6	0,50	0,15	6	7,3	1,36	7	31,1	0,48	6	4,5	1,52	7	5,5	0,00	7	16,3	0,57
T-1015	**68**	**1,03**	**1,30**	**1**	**4,3**	**-**	**3**	**29,7**	**1,15**	**1**	**5,0**	**-**	**3**	**5,3**	**0,29**	**2**	**15,8**	**0,35**
B.b.	3	1,04	0,96															
C.c.	1	0,27	-															
C.c.	1	2,85	-															
R.r.	2	0,27	0,14	1	4,3	-	2	29,0	0,00	1	5,0	-	2	5,3	0,35	1	16,0	-
S.e.	47	0,92	1,33															
PRC	14	1,44	1,29				1	31,0	-				1	5,5	-	1	15,5	-
Grand Total	**383**	**0,42**	**0,93**	**320**	**5,1**	**1,42**	**322**	**30,4**	**1,88**	**192**	**3,0**	**1,03**	**321**	**5,4**	**0,30**	**310**	**16,1**	**1,09**

	pigmentation anal fin			sex			adolescence			peritoneum flecking			reddish pigmentation			unregular scales		
	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD	cases	average	SD
V-1005	**111**	**1,8**	**0,70**	**83**	**1,96**	**0,19**	**83**	**0,0**	**0,11**	**87**	**0,36**	**0,76**	**40**	**0,9**	**0,33**	**111**	**0,10**	**0,30**
C.c.	34	1,9	0,65	13	1,92	0,28	13	0,1	0,28	12	0,42	0,79	28	1,0	0,00	34	0,18	0,39
S.e.	27	1,9	0,70	25	1,96	0,20	25	0,0	0,00	25	0,88	1,01	12	0,6	0,51	27	0,11	0,32
PRC	50	1,7	0,74	45	1,98	0,15	45	0,0	0,00	50	0,08	0,40				50	0,04	0,20
V-1007	**61**	**2,1**	**0,62**	**24**	**1,92**	**0,28**	**24**	**0,0**	**0,00**	**22**	**0,14**	**0,35**	**49**	**0,2**	**0,41**	**63**	**0,06**	**0,25**
C.c.	31	2,3	0,51	10	1,90	0,32	10	0,0	0,00	10	0,20	0,42	24	0,3	0,44	31	0,13	0,34
PRC	30	2,0	0,69	14	1,93	0,27	14	0,0	0,00	12	0,08	0,29	25	0,2	0,37	32	0,00	0,00
V-1008	**61**	**2,2**	**0,53**	**20**	**2,00**	**0,00**	**20**	**0,0**	**0,00**	**20**	**0,10**	**0,45**	**49**	**0,8**	**0,43**	**62**	**0,03**	**0,18**
C.c.	31	2,1	0,56	10	2,00	0,00	10	0,0	0,00	11	0,00	0,00	24	0,7	0,48	31	0,06	0,25
PRC	30	2,2	0,50	10	2,00	0,00	10	0,0	0,00	9	0,22	0,67	25	0,8	0,37	31	0,00	0,00
T-1009	**74**	**2,2**	**0,68**	**53**	**1,91**	**0,40**	**53**	**0,2**	**0,43**	**54**	**0,04**	**0,19**	**57**	**0,5**	**0,50**	**75**	**0,05**	**0,23**
C.c.	31	2,4	0,55	13	2,00	0,41	13	0,4	0,51	13	0,00	0,00	26	0,3	0,47	31	0,06	0,25
PRC	43	2,0	0,74	40	1,88	0,40	40	0,2	0,41	41	0,05	0,22	31	0,6	0,49	44	0,05	0,21
T-1010																		
T-1011																		
T-1012	**9**	**2,7**	**0,50**	**5**	**1,00**	**0,00**	**5**	**0,8**	**0,45**	**7**	**1,71**	**0,49**				**9**	**0,00**	**0,00**
C.c.	3	3,0	0,00	2	1,00	0,00	2	1,0	0,00	2	2,00	0,00				3	0,00	0,00
PRC	6	2,5	0,55	3	1,00	0,00	3	0,7	0,58	5	1,60	0,55				6	0,00	0,00
T-1015	**1**	**2,0**	**-**	**3**	**1,67**	**0,58**	**3**	**0,0**	**0,00**	**1**	**0,00**	**-**				**3**	**0,00**	**0,00**
B.b.																		
C.c.																		
C.c.																		
R.r.	1	2,0	-	2	1,50	0,71	2	0,0	0,00	1	0,00	-				2	0,00	0,00
S.e.																		
PRC				1	2,00	-	1	0,0	-							1	0,00	-
Grand Total	**317**	**2,1**	**0,67**	**188**	**1,91**	**0,32**	**188**	**0,1**	**0,30**	**191**	**0,26**	**0,64**	**196**	**0,6**	**0,50**	**323**	**0,07**	**0,25**