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Genetic basis of resistance to stress in fishes. Molecular and classical investigations in a few model organisms

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Abstract. The paper focuses on the recent scientific results from genetics of thermal stress tolerance and discusses about heat shock genes in several model fish organisms, such as: the guppy (*Poecilia reticulata*), the Antarctic fish (*Trematomus bernacchii*), the desert fish (*Poeciliopsis lucida*), the zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), and others.

Key Words: HSP, thermal stress, coldwater, heat, temperature, RAPD, model, fish.

Tartalom. A jelenlevő dolgozatunkban bemutatjuk genetikai kutatásaink eredmenyeit, amelyek a hőmersekleti-stressz iránti állóképeseget, illetve a hőmérsékleti sokkal felelos géneket vizsgálták különböző halfajok esetében: *Poecilia reticulata*, *Trematomus bernacchii*, *Poeciliopsis lucida*, *Danio rerio*, *Takifugu rubripes* és más halfajok.

Kulcsszavak: HSP, hőmérsékleti stressz, hidek víz, meleg, hőmérséklet, RAPD, model, hal.

Rezumat. Lucrarea prezintă rezultate ştiinţifice recente din domeniul geneticii rezistenţei la stres termic şi abordează genele de şoc termic la câteva specii de peşti model, ca: guppy (*Poecilia reticulata*), peştele de Antarctica (*Trematomus bernacchii*), peştele de deşert (*Poeciliopsis lucida*), zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*) şi altele.

Cuvinte cheie: HSP, stres termic, apă rece, căldură, temperatură, RAPD, model, peşte.

Introduction. Fish are exposed to biotic and abiotic stress factors in the native environment, and especially in captivity (Iwama et al 1999a). Chemicals, disease, physical factors, crowding and exposure to predators are most important stressors in fishes (Iwama et al 1992, 1998, 1999b). Like other animals (Rabindran et al 1991; Lo et al 2004; Rinehart et al 2006), stressed fish exhibit a generalized stress response, which is characterized by an increase in stress hormones and the consequent changes at the physiological, organismal and population levels (Wendelaar-Bonga 1997; Barton 1997; Iwama et al 1999a). There is also a generalized stress response in animals at the cellular level (Hightower 1991; Kondo et al 2004). This cellular stress response has its origin in the DNA sequence of a few genes encoding heat shock proteins (HSPs). HSPs are molecular chaperones, antigens of immune responses in autoimmunity. So far, HSP27, HSP30, HSP47, HSP60, HSP70, HSP90 have been studied in many fish species (Norris et al 1997; Iwama et al 1999a; Molina et al 2001; Kondo et al 2004).

The scope of the present study was to analyze in an integrated manner the most important results obtained in the field of tolerance to thermal stress in fish and to draw several common conclusions regarding its genetic bases.

The Guppyfish (*Poecilia reticulata* Peters). There are, in aquaria, coldwater fish species and tropical fishes, the guppy being one of the latter (Bud 2002). However, the guppy has the ability to survive in cooler waters that would certainly kill most tropical fishes (Mag & Bud 2006; Mag et al 2006b; Păsărin et al 2007). In their paper "Detection of a low temperature-resistant gene in guppy (*Poecilia reticulata*), with reference to sex-linked inheritance", Fujio et al (1990) reported an X-linked gene, responsible of low water-temperature resistance in guppy. Sixteen years later, in the paper: "Effect of temperature on sex ratio in guppy *Poecilia reticulata* (Peters 1860)", Karayücel et al (2006) emitted the hypothesis according to which the same gene could be involved in high water-temperature resistance of guppy. In that moment, the above mentioned gene became a very important one, especially for guppyculture and generally for aquaculture, because of extrapolation possibility of these studies to many cases of economical and commercial important species.

In a CEEX-Biotech project (Research of Excelence Program, no 140/2006) our team tested a number of 150 random primers (Biosearch Technologies Inc.) in order to identify RAPD markers which could be associated to low temperature resistance in guppy. However, 71 random primers have not amplified DNA at all, 35 primers have not generated DNA polymorphisms, and 44 of them generated (more or less) polymorphisms. Only one random primer (no 77) was associated with cold tolerance (5'd(CCAACGACCA)3'; see Figure 1) in the Red Blond variety (Plate 1).

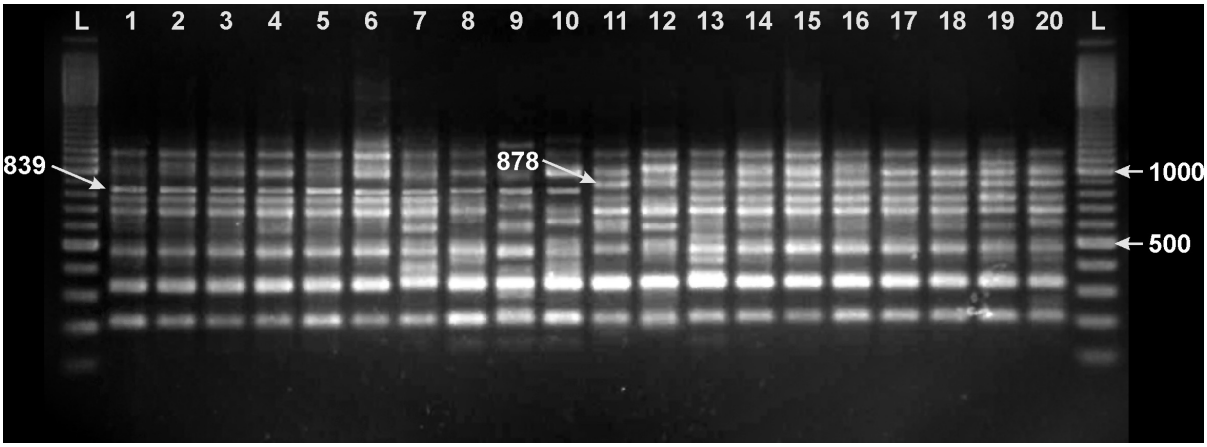

Figure 1. Polymorphism, induced by the primer 77, associated with cold tolerance in the Red Blond guppy. The supplementary DNA fragment of 878 bp is associated to coldwater resistance (11-20), and the supplementary fragment of 839 bp is associated to coldwater sensitivity (1-10).

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used to illustrate the two distinct fish clades: coldwater resistant group and coldwater sensitive group (see Figure 2). However, using the same primer (77), our repeated RAPD analyses failed to discriminate between coldwater resistant and sensitive individuals in other guppy strains. The primer was useless even for identification of coldwater resistant fishes from an F_B generation whose father and both grandfathers were Red Blond males (see Figure 3).

After the identification of coldwater resistant individuals, the next step forward of the research in the project CEEX 140/2006 (Mag et al 2006a) was obtaining of a homozygote guppy strain for coldwater resistance gene, and this was possible due to the X-linkage of *Nigrocaudatus* II (*Ni*) which was used as color marker gene. The breeding program of the research was based on a ♂Red Blond X ♀Half-Black Black cross, followed by a backcross of F_1 females with the initial Red Blond male (see Figure 3; Petrescu-Mag et al 2007).

One hundred of individuals, homozygote for the coldwater resistance gene, were further tested for resistance to heat. The results indicated a significant higher tolerance to heat of these ones compared to control lot (data not shown). These data supported the hypothesis of Karayücel et al (2006) and indicated the common identity of coldwater

resistance gene and heat tollerance gene. This X-linked gene (for resistance) seems to encode a protein with multiple role, a molecular chaperone, a protein formally included in HSPs group. We concluded at least some of the HSPs were involved in both coldwater resistance and heat tollerance, modulating animal metabolism under different environmental conditions.

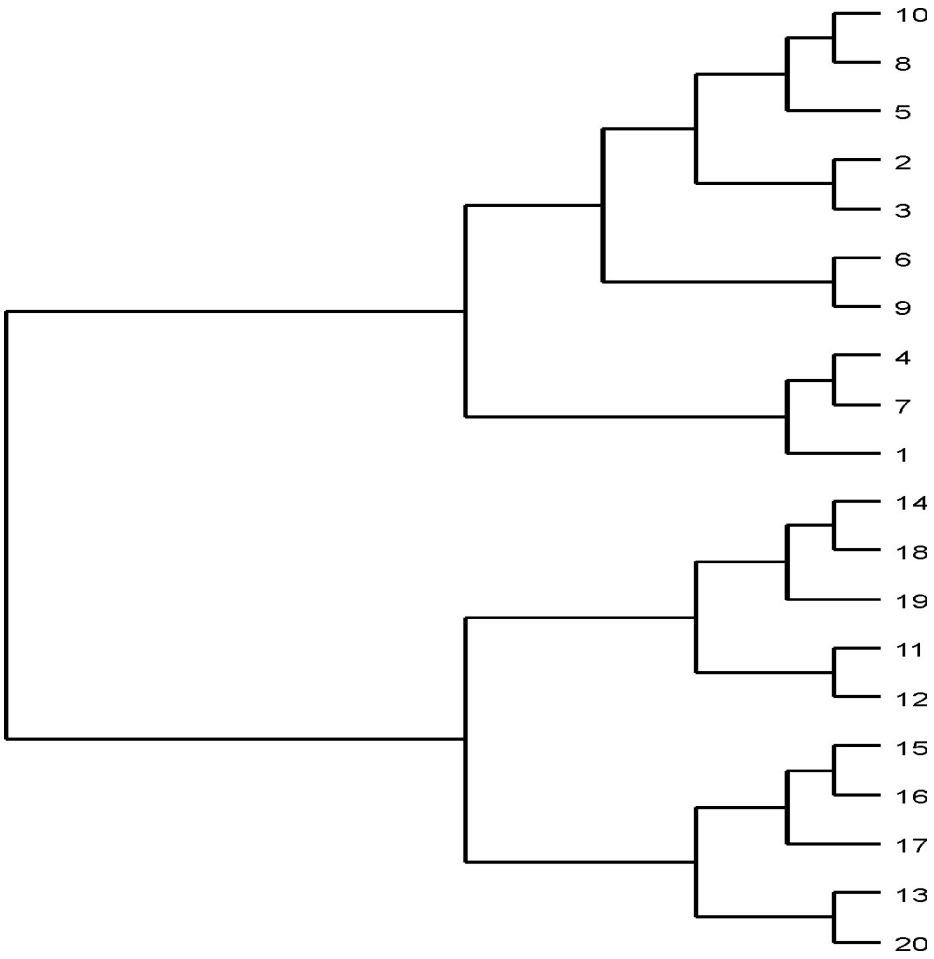

Figure 2. The two phenotypic distinct groups illustrated by UPGMA (when genomic DNA was amplified with the primer no 77).

So far, RAPD technics showed to be useful for estimation the population variability or organismal variability (Brunell & Whitkus 1997), in taxonomy (Adams & Demeke 1993; Esselman et al 2000), or sometimes RAPD markers can be associated with the presence of some sequences in a genome (e.g. Y-specific RAPD markers) and with a trait (Bardakci 2000). In any case, RAPD technics shows low repetability, and that is why we further orientated our attention mainly to sequencing the DNA fragments of interest. In our project PN II 51-056/2007 (Bud et al 2007), the main goal of the research is sequencing the most important HSP genes from three species: the guppy (*P. reticulata*), the common carp (*Cyprinus carpio* Linnaeus), and the rainbow trout (*Oncorhynchus mykiss* (Walbaum)).

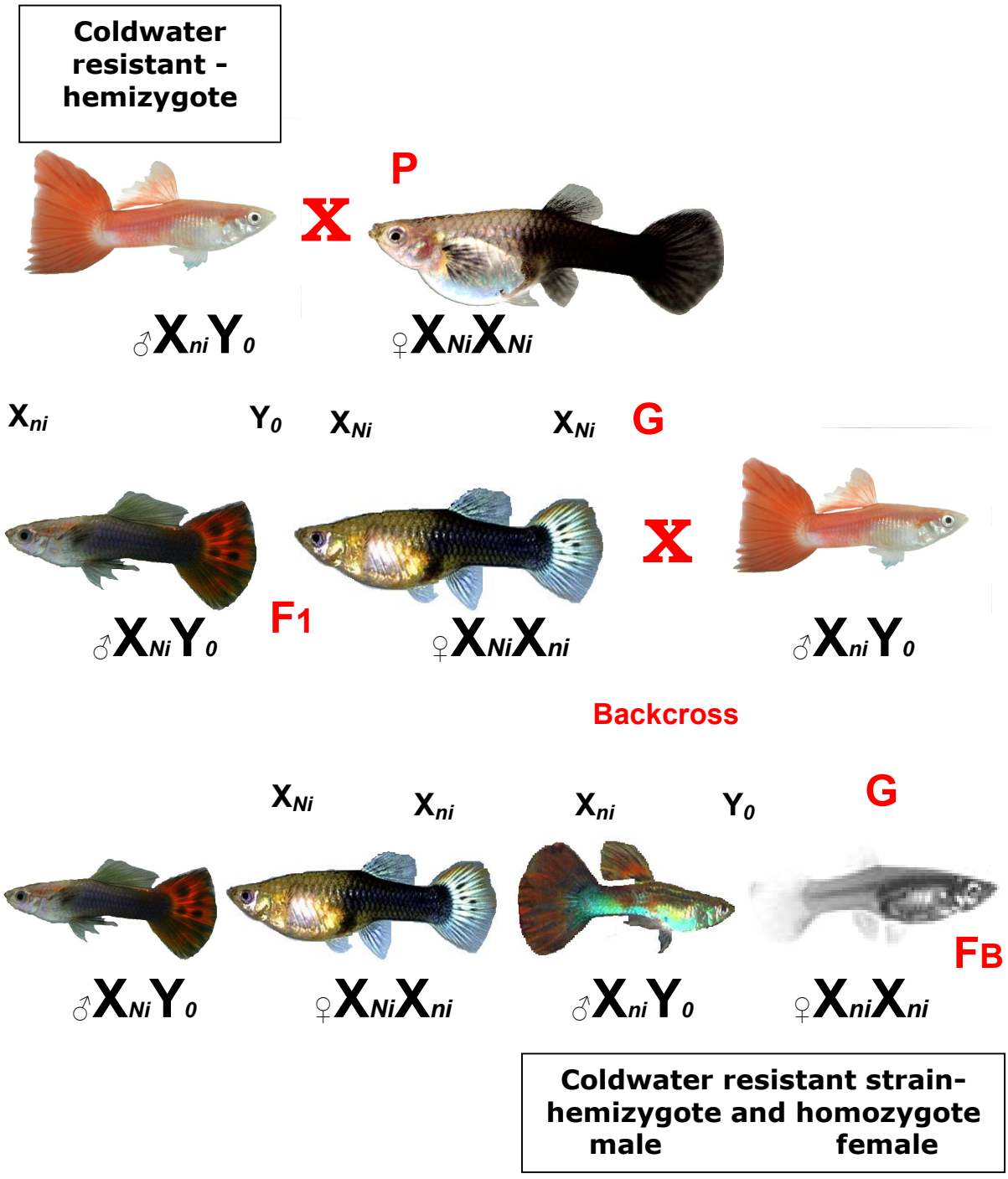

Figure 3. The breeding program in the project CEEX 140/2006 (P – parents; G – gametes; F1 – first generation after the cross of P; F_B – first generation after the backcross; X and Y – the sex chromosomes; Ni – a mutant and dominant gene; *ni* – the wild allele at the *Ni* locus).

The Antarctic Fish (*Trematomus bernacchii* Boulenger). The emerald rockcod, *T. Bernacchii* (a notothenioid fish species, see Plate 2), is an ideal model for studying the process of cold adaptation (Buckley et al 2004). Hofmann et al (2000) examined the occurrence of the heat-shock response in the highly stenothermal Antarctic fish (*Trematomus bernacchii*), to determine whether this response has persisted in a lineage that has encountered very low and stable temperatures for at least the past 14-25 million

years. The patterns of protein synthesis observed in *in vivo* metabolic labelling experiments that involved injection of (35)S-labelled aminoacids (methionine and cysteine) into whole fish previously subjected to a heat stress of 10°C yielded no evidence for synthesis of any size class of heat-shock protein (Hofmann et al 2000).

Parallel in vivo labelling experiments with isolated hepatocytes similarly showed significant amounts of protein synthesis, but no indication of enhanced expression of any class of HSP (Hofmann et al 2000; Buckley et al 2004). The heavy metal cadmium, which is known to induce abundent synthesis of HSPs, also failed to alter the pattern of proteins synthesized in hepatic cells (Hofmann et al 2000). Although stress-induced chaperones could not be detected under any of the experimental condition used by the same collective, solid-phase antibody analysis revealed that a constitutively expressed 70 kDa chaperone was present in *T. bernacchii*, and amounts of this chaperone increased in brain, but not in gill, during 22 days of acclimation to 5°C. The apparent absence of a heat-shock response in this highly stenothermal species was interpreted by above mentioned authors as an indication that a physiological capacity observed in almost all other organisms has been lost as a result of the lack of positive selection during the millions of years at stable sub-zero values of the Southern Ocean temperature.

To elucidate the mechanism responsible for the absence of HSP induction, Buckley et al (2004) examined several stages of the HSP gene expression pathway, including transcription factor activity, HSP70 mRNA production and protein synthesis patterns, in hepatic cells from the Antarctic fish. HSP70 mRNA was detected, as was heat shock factor 1 with DNA-binding activity. However, exposure to high temperature and to chemical inducers of the heat shock response failed to increase HSP70 mRNA levels, HSF1 activity or the concentration of any size class of HSPs. These results suggest that HSPs, inducible in nearly every other species, are expressed constitutively in the cold-adapted *T. Bernacchii* (Buckley et al 2004). The cold and generally non-polutted waters of the nearshore Atlantic ecosystem may have rendered the inability to induce HSPs above a constant level selectively neutral over evolutionary periods (Buckley et al 2004).

The Desert Fish (*Poeciliopsis lucida* Miller). The clearfin livebearer is a desert minnow (Wischnath 1993), presents a particular interest because of the highly stressful nature of its environment (White et al 1994).

The small heat shock proteins (sHSPs) are a diverse group of stress-inducible proteins characterized minimally by a molecular ranking between 15 and 30 kDa and a conserved region of approximately 90 aminoacids in the C-terminal region of the protein. They can be found in animals (Jakob et al 1993), plants (Waters et al 1996) and bacteria (Wilhelm et al 2003). One of the *P. lucida* sHSPs is closely related to avian HSPs and mammalian HSP27, while the other is homologous to salmon and *Xenopus* HSP30 (de Jong et al 1993). This indicates a common origin, and Norris et al (1997) found that the gene duplication which generated the HSP27 and HSP30 lineages occured prior to the divergence of fish and tetrapods.

The Zebrafish and Pufferfishes. *Danio rerio* (Hamilton) or the zebrafish, *Takifugu rubripes* (Temminck & Schlegel) the pufferfish (or fugu), and *Tetraodon nigroviridis* (Marion de Procé) or the spotted green pufferfish (see Plates 3-5) are three fish species whose genomes are completely sequenced and annotated (Jaillon et al 2004). These are reference points for the molecular comparative analyses (Hedges 2002; Marchler-Bauer et al 2007). After sequencing, each HSP or related gene is aligned and compared against these genomes or specific DNA sequences from databases. Bioinformatic tools and statistic methods are used for drawing phylogenetic trees, and then phylogenetic distances between species or genes are estimated in million of years (Myr) (Friedman & Hughes 2001; Hughes et al 2001). Because they are essential for species existence, HSPs are a group of highly conserved proteins (Marchler-Bauer et al 2007). Their comparative analyses help us to understand better the origin of species, and to find hypothetical common ancestors (Hedges 2002; Jaillon et al 2004).

Regeneration of fins in *Danio rerio* requires the formation and maintenance of blastema cells. These ones are not derived from stem cells but behave as such, because

they are slow-cycling and are thought to provide rapidly proliferating daughter cells that drive regenerative outgrowth. Makino et al (2005) indicated that HSP60 was required for blastema formation and maintenance. They used a chemical mutagenesis screen to identify *nbl* (a zebrafish mutant with an early fin regeneration defect). Fin regeneration failed in *nbl* due to defective blastema formation (Makino et al 2005). The same authors reported also that *nbl* failed to regenerate hearts. Positional cloning and mutational analyses revealed that *nbl* resulted from a V324E missense mutation in HSP60. This mutation reduced HSP60 function in binding and refolding denatured proteins. HSP60 expression was increased during formation of blastema cells, and dysfunction produced mitochondrial defects and apoptosis in that cells (Makino et al 2005). These data indicated that HSP60 was required for the formation and maintenance of regenerating tissue.

Conclusions. Looking to the studied animals and also to their multiple stressors: coldwater, heat, tissue regeneration, detoxification and so forth, we may conclude and underline HSPs have a wide range of functions, processes and components.

The guppyfish (Osteichthyes: Cyprinodontiformes) is a good model for studies such as sex-dependent thermal resistance. A thermoresistant guppy strain was created using exclusive the classical genetic mothods: crosses between different strains, backcrosses, sex-linkage, phenotype analyses, and thermal resistance tests.

The emerald rockcod, *T. Bernacchii*, a notothenioid fish species (Osteichthyes: Perciformes), is an ideal model for studying the process of cold adaptation.

The clearfin livebearer *Poeciliopsis lucida* (Osteichthyes: Cyprinodontiformes), a desert minnow, presents a particular interest because of the highly stressful nature of its environment. One of the *P. lucida* sHSPs is closely related to avian HSPs and mammalian HSP27, while the other is homologous to salmon and *Xenopus* HSP30. This indicates a common origin, and Norris et al (1997) found that the gene duplication which generated the HSP27 and HSP30 lineages occured prior to the divergence of fish and tetrapods.

Danio rerio - the zebrafish, *Takifugu rubripes* - the pufferfish (or fugu), and *Tetraodon nigroviridis* - the spotted green pufferfish are three fish species whose genomes are completely sequenced and annotated. These are reference points for the molecular comparative analyses. After sequencing, each HSP or related gene is aligned and compared against these genomes or specific DNA sequences from databases. Bioinformatic tools and statistic methods are used for drawing phylogenetic trees, and then phylogenetic distances between species or genes are estimated in million of years (Myr). Because they are essential for species existence, HSPs are a group of highly conserved proteins. Their comparative analyses help us to understand better the origin of species, and to find hypothetical common ancestors.

Knowing the various HSPs, knowing their complete nucleotidic sequences, and also their aminoacid sequences, development of different molecular markers becomes possible, and these ones are excellent tools for animal and vegetal organisms improvement.

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Plate 1. Red Blond variety, used as genitor in the breeding program and production of the cold resistant guppy strain (the first is a male, the second is a female).

Plate 2. *Trematomus bernacchii* (photo: Zimmermann C.; source: www.fishbase.org)

Plate 3. *Tetraodon nigroviridis* - (photo: Liu K. H.; source: www.fishbase.org)

Plate 4. *Takifugu rubripes* – the pufferfish (photo: Shao K.-T.; source: www.fishbase.org)

Plate 5. *Danio rerio* – male and female (photo: Noren M.; source: www.fishbase.org)

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