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RESEARCH ARTICLE **(Open Access)**

Molecular Evolution of P-glycoproteins (P-gp) in Teleost Fishes

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Abstract:

Multixenobiotic resistance (MXR) in aquatic organisms exposed to natural toxins or anthropogenic contaminants is a phenomenon analogous to multidrug resistance (MDR) in mammalian tumor cell lines tolerant of anti-cancer drugs. Multidrug resistance is commonly due to the elevated expression of transmembrane P-gps which actively transport a wide variety of structurally and functionally diverse compounds. The effect of xenobiotic exposures on P-gp activity and protein titer has been examined in wild and captive populations of aquatic invertebrates and vertebrates. Molecular evolution of P-gp was investigated in teleost fishes, which exhibit remarkable diversity in morphology, behavior, and adaptations. Several phylogenetic analysis were performed using all teleosts P-gp amino acid and complementary DNA (cDNA) sequences present in GenBank. P-gp molecular evolution in teleosts seemed to follow identical evolution pathways to teleost fishes speciation.
Keywords: MXR; phylogenetic analysis; molecular clock.

1. Introduction

Many aquatic species are able to survive in environments which contain high levels of multiple anthropogenic pollutants or natural product toxins. This MXR phenomenon is similar to MDR first observed in tumor cell lines resistant to anti-cancer drugs [1]. Overexpression of a 170 kDatransmembrane P-gps was found to prevent the accumulation of cytotoxic drugs in resistant cells. MDR is of clinical importance because many human tumors have inherent or acquired P-gp-mediated drugresistance and do not respond to chemotherapy. P-gp is found endogenously in specialized epithelial tissues involved in secretion and excretion such as the mammalian gut, liver, and kidney, as well as on endothelial cells of capillary blood vessels at the blood-brain barrier. P-gp acts as an energy-dependent pump to translocate a wide variety of structurally and functionally diverse substrates. These compounds tend to be moderately hydrophobic, planar, natural products which are often substrates for or metabolites of detoxification enzymes such as cytochromes P450 (CYPs) [1]. P-gps prevent the cellular accumulation of endogenous metabolites, phospholipids, and xenobiotics in exposed animals and cell cultures. P-gp-like proteins have been described in a variety of aquatic organisms including sponges, mussels, oysters, clams, worms, and fish. As a specific transmembrane efflux transporter that pumps structurally different xenobiotics out of the cell, in Pgp (Abcb1) appeared to be the key mediator of the so-called MXR defence system [2,3] in aquatic organisms. It has been shown that the MXR phenomenon is constitutive to aquatic organisms, inducible in response to pollution [4-6], and sensitive to specific environmental pollutants/chemicals commonly called MXR inhibitors or chemosensitisers [7,8]. Both natural products and anthropogenic contaminants found in the aquatic environment appear to be substrates and inducers of the multixenobiotic resistance transporter in aquatic organisms. These observations suggest that in addition to normal cell function, P-gp activity may contribute to the relative hardiness of some aquatic species exposed to xenobiotics. In addition to well characterized detoxification systems (phase I, II, III enzymes, heat shock proteins, etc.), the induction of a multixenobiotic defense mechanism in organisms living in polluted environments may explain why contaminant spills cause more severe adverse effects at pristine sites than in already polluted areas [1]. P-gp, member of the adenosine triphosphate (ATP)–binding cassette (ABC) superfamily [9], has unusually broad poly-specificity, recognizing hundreds of compounds as small as 330 daltons up to 4000 daltons [10, 11]. Most P-gp substrates are hydrophobic and partition into the lipid bilayer [12, 13]. Thus, P-gp has been likened to a molecular "hydrophobic vacuum cleaner" [14], pulling substrates from the membrane and expelling them to promote MDR and MXR. The X-ray structure of apo P-gp at a resolution of 3.8 Åwas recently obtained [15] (Figure 1). It revealed distinct drugbinding sites in the internal cavity capable of stereoselectivity, which is based on hydrophobic and aromatic interactions. Apo P-gp

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structures have an inverted "V" shape, inward-facing conformation, for drug entry, whereas the outward-facing conformation releases the substrate to the extracellular medium [15].

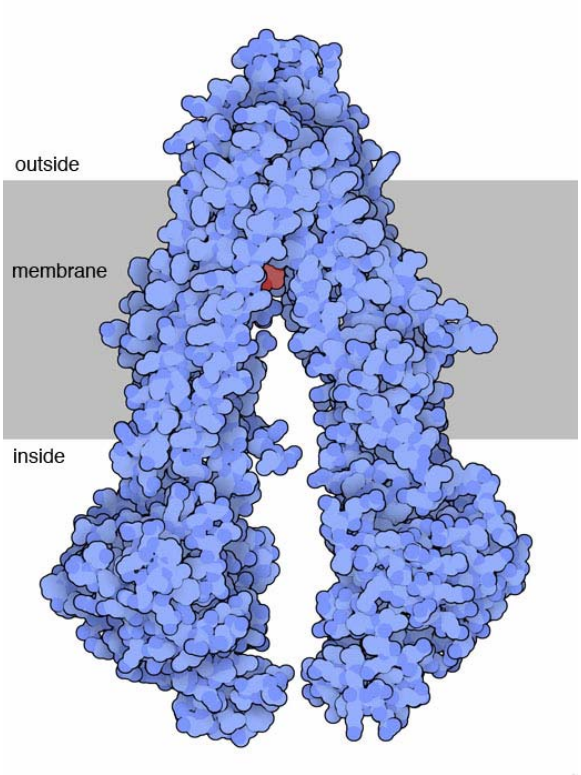

Figure 1. Mouse P-gp 3dimensional structure (PDBID: 3G60).

Expression of MXR proteins may be an ecotoxicologically important characteristic, as it could critically influence the susceptibility of aquatic organisms to pollutants [16, 17, 1]. The tissue distribution of P-gps is indicative of their function in accelerating excretion or preventing the uptake of xenobiotics. In addition to their occurrence in tumor cells [18, 19], expression of P-gps associated with an mdr1-like mechanism have been detected in normal teleost tissues that are involved in a secretory, absorption or barrier function such as the liver, kidneys, gills and intestine [20, 21, 22, 4].

Teleost fishes, with about 27 000 species [23], are the largest and most diverse group of vertebrates. Teleosts account for more than 99% of ray-finned fishes (Actinopterygians) which diverged from lobe-finned fishes (Sarcopterygians) about 420 million years ago (Mya). They exhibit remarkable diversity in their morphology, behavior, and adaptations [24]. These evidences prompted me to investigate the molecular evolution of P-gp in such a high number of candidate species taxonomy group. For this purpose, several phylogenetic analysis were performed using teleost amino acid and cDNA sequences.

2. Material and Methods

P-gp amino acid and coding cDNA sequences of Oncorhynchusmykiss, Oreochromisniloticus, Xiphophorushellerii, Poeciliopsislucida, Trematomusbernacchii, Dicentrarchuslabrax, Pseudopleuronectesamericanus, Platichthysflesus, Carassiusgibelio, Poeciliopsislucida, Barbusbarbus, Cyprinuscarpio and Chondrostomanasus, were found in GenBank (www.ncbi.nlm.nih.gov/genbank/). All respective sequences were aligned using T-Coffee multiple sequence alignment software package [25]. jModelTest [26] was used to carry out statistical selection of best-fit models of nucleotide substitution to analyzed organisms P-gp molecular evolution. Analyses were performed using 88 candidate models and two types of information criterion (Akaike Information Criterion-AIC and Corrected Akaike Information Criterion-cAIC). For selection of the best-fit model of analyzed protein evolution was used ProtTest3 [27]. 122 candidate models and three types of criterion (Akaike Information Criterion-AIC, Corrected Akaike Information Criterion-cAIC and Bayesian Information Criterion-BIC) were used in these statistical analyses. The P-gpcDNA and amino acid sequences phylogenetic trees were build using the Bayesian inference (BI) method implemented in Mr. Bayes 3.2 [28]. Four independent runs, each one with four simultaneous Markov Chain Monte Carlo (MCMC) chains, were performed for 1,000,000 generations sampled every 1000 generations. FigTree v1.3.1 software was used to display the annotated phylogenetic trees.

3. Results and Discussion

3.1. Molecular Clock Tests

The molecular clock has become an indispensable tool within evolutionary biology, enabling independent timescales to be placed on evolutionary events. Despite these valuable contributions, date estimates derived from molecular data have not been without controversy. In particular, when molecular clocks have been employed to estimate the timing of recent events already tentatively dated on the basis of palaeontological, archaeological or biogeographic sources, conflicting dates are frequently obtained [29]. In its most extreme form, the molecular clock hypothesis postulates that homologous stretches of DNA evolve at essentially the same rate along all evolutionary lineages for as long as they maintain their original function. It was

shown that the substitution rate of mitochondrially encoded proteins has increased in the order of fishes, amphibians, birds, and mammals and that the rate in mammals is at least six times, probably an order of magnitude, higher than that in fishes. The higher evolutionary rate in birds and mammals than in amphibians and fishes was attributed to relaxation of selective constraints operating on proteins in warm-blooded vertebrates and to high mutation rate of bird and mammalian mitochondrial DNAs [30]. Since the assumption of rate constancy is violated even within Mammalians, a truly universal molecular clock that applies to all organisms cannot be assumed to exist [31].

In order to know which was the best-fit model to analyzed P-gp protein sequence evolution a Bayes factor comparison (Mr. Bayes 3.2) was performed to test the strict clock model against the non-clock model using P-gp amino acid sequences. I used an accurate assessment of the marginal model likelihoods using the stepping-stone method. It estimates the model likelihood by sampling a series of distributions that represent different mixtures of the posterior distribution and the prior distribution [32]. The stepping-stone method was applied to the P-gp dataset using 510000 generations with a diagnostic frequency of 2500 in 2 independent runs for each of the tested models. The marginal likelihood values are shown in table 1.

Table 1. The marginal likelihood values in each of the 2 independent runs and the resulting mean values for each of the tested models using the stepping-stone method.

Run	Unconstrained	Strict Clock	Relaxed Clock (CPP)	Relaxed Clock (TK02)	Relaxed Clock (IGR)
1	-13819,83	-12878,03	-12858,76	-12872,65	-12863,56
2	-13820,73	-12878,03	-12858,75	-12863,93	-12865,77
Mean of Marginal Likelihood	-13820,18	-12878,28	-12858,75	-12864,62	-12864,15

The strict-clock model (-12878,28) is almost 942 log likelihood units better than the non-clock model (-13820,18). A difference exceeding 5 log likelihood units is usually considered very strong evidence in favor of the better model [33]. However, in phylogenetics the unrooted model of phylogeny and the strict molecular clock model are two extremes of a continuum. Despite their dominance in phylogenetic inference, it is evident that both are biologically unrealistic and that the real evolutionary process lies between these two extremes [34].

Local molecular clocks are another alternative to the global molecular clock. A local molecular clock permits different regions in the tree to have different rates, but within each region the rate must be the same [35]. This new method conveniently allows a comparison of the strict molecular clock against a large array of alternative local molecular clock models [35]. As it is shown in table 1, CPP model (-12858,75) is 20 log likelihood units better than the strict-clock model (-12878,28) and nearly 6 log likelihood units better than the other two local molecular clocks, TK02 (-12864,62) and IGR (-12864,15) model. Thus, the analyzed P-gp molecular evolution is based on a local molecular clock model (CPP). Phylogenetic analyses performed on Cu,Zn SOD amino acid sequences by Santovito [36] and colleagues showed an erratic differentiation of these proteins in antarcticteleosts and concurred with the theory of the "unclock-like" behaviourof Cu,Zn SOD evolution [37]. My empirical results demonstrated that Cu,Zn SOD evolution isn't the only example of "unclock-like" behavior, but P-gp evolution in the analyzed teleost fishes, seemed to behave "unclock-like", too. I used the CPP molecular clock model in the phylogenetic tree.

3.2. Phylogenetic Tree Constructions

Recently, several isoforms of non-Pgp efflux transporters were discovered in various mammalian tissues, which implied that MXR in aquatic biota might also be a multi-transporter mechanism. Among non-Pgp ABC proteins, members of a multidrug resistance-associated protein (MRP) subfamily ABCC have been shown to be toxicologically relevant [38-40]. Earlier studies [41-43] demonstrated the expression of MRP-related genes in a number of fish and invertebrate species [44-46]. Thus, it was indispensable to include in our analysis the complete amino acid and cDNA sequence of O. mykiss MRP2 (gi 185134790). P-gp amino acid sequences of teleost fishes and mammals share a high degree of homology between them (76 % to 96 %) [47]. Full and/or partial P-gp and O. mykiss MRP2 amino acid sequences were aligned using T-Coffee in combined libraries of local and multiple alignments, which are known to

induce high accuracy and performance in sequence alignments [25]. Generally, the P-gp amino acid sequences alignment showed good alignment quality values foreach of the analyzed sequences. The figure 2 shows the most conserved region between different taxonomic groups

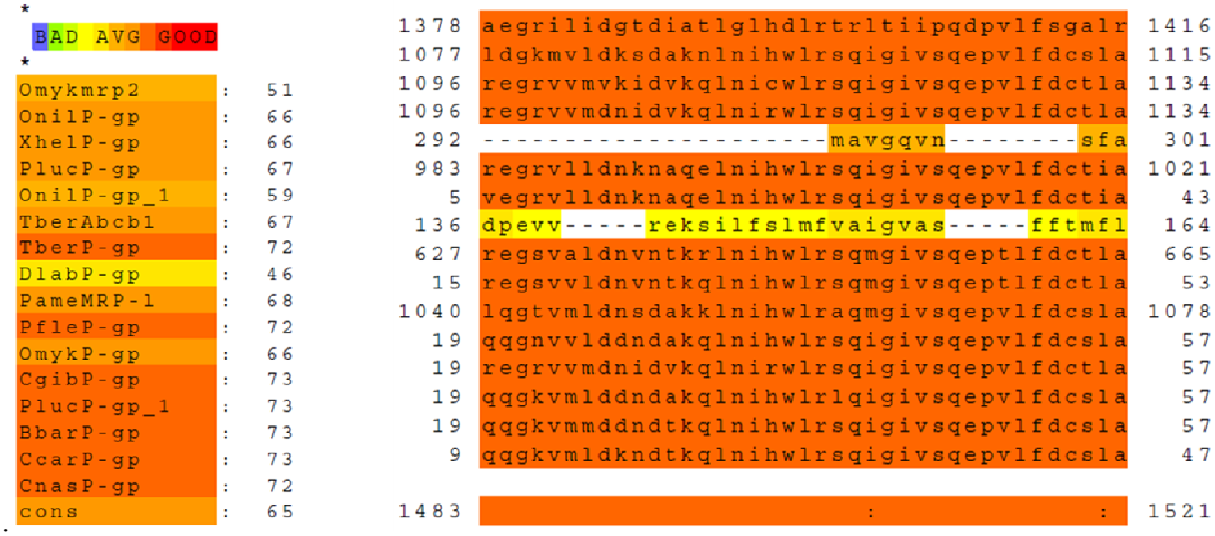

Figure 2. The alignment quality values generated by T-Coffee for each P-gp amino acid sequences (left) and the best similarity region of analyzed teleosts P-gp sequences alignment (right).

ProtTest3 [27] was used for amino acid sequence evolution best-fit model determination. I decided to use a mixed model, because each of the used statistical criterion (AIC, cAIC and BIC) yielded a different result from the others. Phylogenetic relationships of all these amino acid sequences were determined using the most powerful statistical method of BI. The best phylogeny generated by the BI method is depicted in figure 3.

jModelTest 0.1.1 software [26] determined the GTR+G model as being the best-fit model of P-gp and non-P-gpcDNA sequence evolution with a gamma shape value (four rate categories) of 1.0920 using AIC and cAIC statistical criterion (-lnL= 23836.5935). T-Coffee program was used for the alignment of cDNA sequences of the analyzed P-gp and O. mykiss MRP2, too. In figure 4 is shown the best phylogeny generated by the application of BI method into the all these different organisms P-gp and non-P-gpcDNA sequences.

The comparison of the phylogenetic trees showed that the best resolved phylogenetic tree was the cDNA sequences based phylogenetic tree (Figure 4). Its nodes were supported by higher bayesian posterior probabilities values than those of the amino acid sequences based phylogenetic tree (Figure 3). The *O. mykiss* MRP2 sequence wasn't grouped together with any P-gp sequence in each of the generated phylogenetic tree, showing that its evolution had followed a different pathway from that of P-gps in teleosts. Another interesting observation was that one of the P-gp sequences of *P. lucida* (gi 311977219) wasn't positioned as a sister group with the other P-gp sequence of the same teleost fish (gi 67866957). It was grouped together with the P-gp sequence of *X. hellerii*. *P. lucida* and *X. helleri* are members of *Cyprinodontiformes* order. It seemed that a gene duplication event happened in *P. lucida*, which resulted in 2 orthologues genes. Probably, each of them encodes for a specific P-gp protein. Previous studies on ABC gene family evolution in vertebrates revealed that all ABC protein subfamilies found in *Ciona* and zebrafish correspond to the human subfamilies, with the exception of a single ABCH subfamily gene found only in zebrafish [46]. Our results and earlier studies results suggested that *P. lucida* might have several P-gp proteins, like the zebrafish ABC proteins.

From the observation of the best resolved phylogenetic tree, the cDNA based phylogenetic tree (Figure 4), it was evidenced that the P-gps were grouped in two big clusters. In one of them were included P-gps of salmoniformes (*O. mykiss*) and cypriniformes (*B. barbus*, *C. gibelio*, *C. carpio* and *C. nasus*). Though the phylogenetic relationships between cypriniformes P-gps weren't determined, this teleost order P-gps were present in a specific clade suggesting a common origin of the cypriniformes P-gps. In the remaining big cluster were present all perciformes P-gps and the European flounder (*P. flesus*) P-gp. *P. flesus* is a member of the *Pleuronectiformes* order. It was relevant to note that though all the latter big cluster species were members of *Acanthopterygii* superorder, *P.*

americanus(*Perciformes*order) P-gp was closer to *P. flesus* (*Pleuronectifomes* order) P-gp than to *O. niloticus* P-gp sequences (perciformescDNA sequences). The phylogenetic relationships of *P. flesus* and analyzed perciformes P-gps suggested that European flounder P-gp gene could have gone under convergent evolution.

In order to know if the teleosts P-gp phylogeny (Figure 4) reflect the teleost phylogeny we compared the topology of the cDNA based phylogenetic tree with the topology of actinopterygian phylogenetic tree build by Near [48] and colleagues. From the comparison of the analyzed species P-gps with the actinopterygian time-calibrated phylogeny based on nine nuclear genes and 36 fossil age constraints [48], emerged a good correspondence between the phylogenies. In the actinopterygian phylogeny,

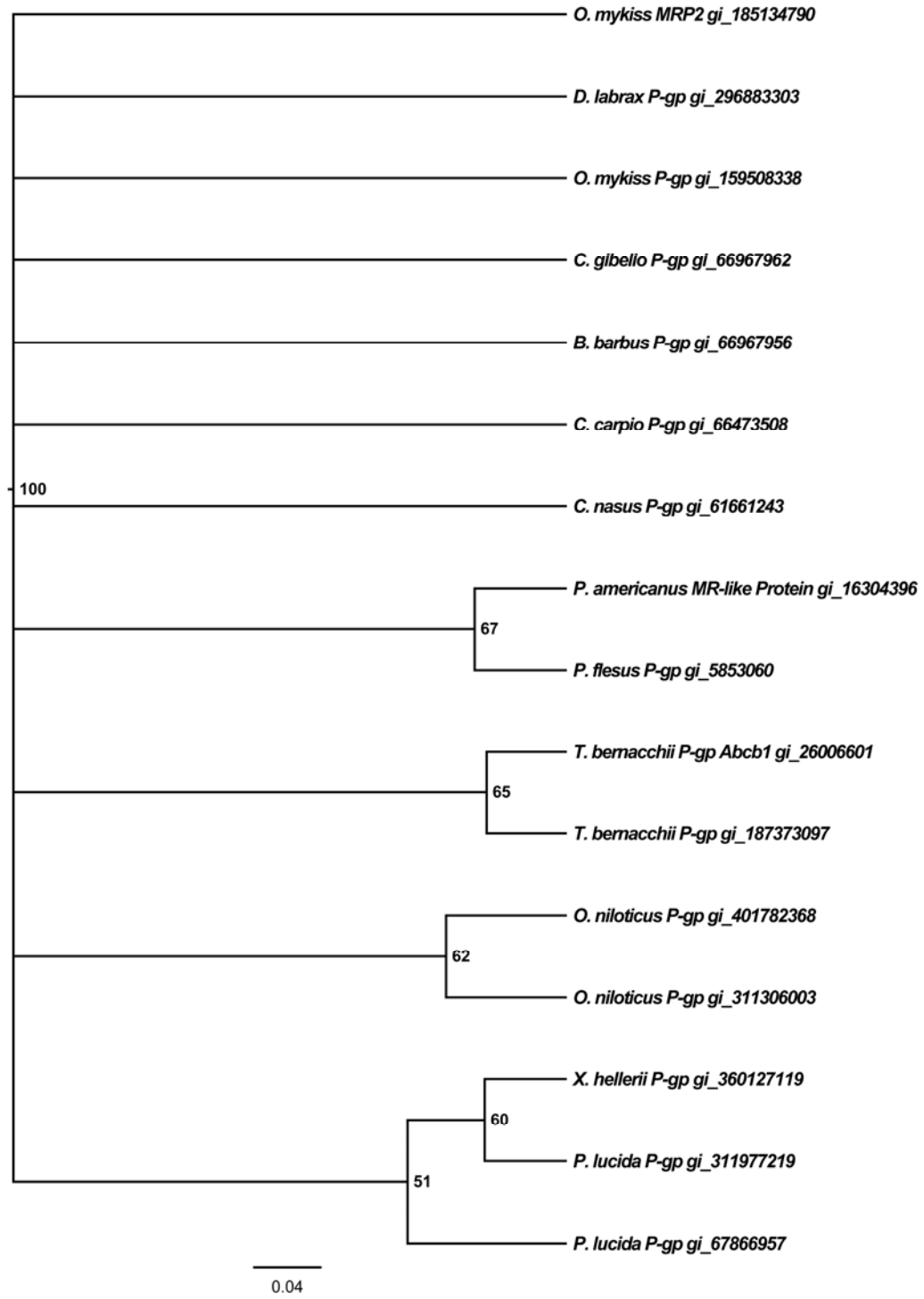

Figure 3. Phylogenetic relationships among P-gp amino acid sequences using BI method (arithmetic mean = -12793.698; harmonic mean = -20083.673). Posterior probability values higher than 50% are indicated on each node. The scale for branch length (0.04 substitution/site) is shown below the tree.

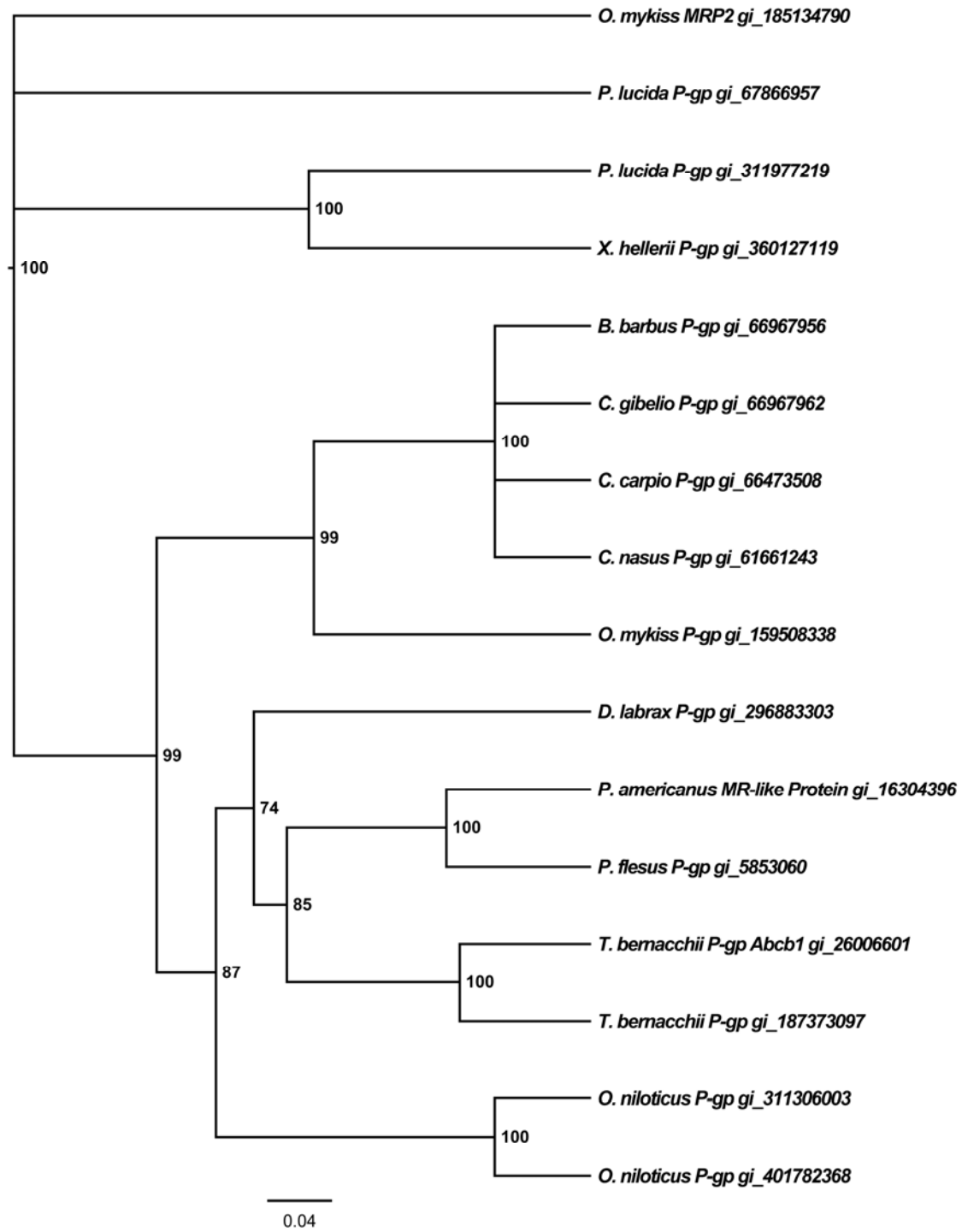

Figure 4. Phylogenetic relationships among P-gpcDNA sequences using BI method (arithmetic mean = - 21757.714; harmonic mean = - 21777.237). Posterior probability values higher than 50% are indicated on each node. The scale for branch length (0.04 substitution/site) is shown below the tree.

cypriniformes and salmoniformes were positioned close to each other and far from perciformes and other *Acanthopterygii* superorder members. Teleosts P-gp phylogeny (Figure 4) showed an identical topology to actinopterygian phylogeny except for *P. flesus* P-gp. Salmoniformes and cypriniformes P-gps were positioned closer to each other than to *Acanthopterygii* superorder members. The good correspondence between P-gp and teleosts phylogeny suggested that P-gp molecular evolution had followed the same evolution pathway as the speciation of the analyzed teleost species.

4. Conclusions

P. lucida and P. flesus P-gp genes could be good examples in teleosts demonstrating active process that included adjacent duplications, presumably by unequal crossovers; nonadjacent duplications believed to occur by chromosome or genome duplication; and the loss of genes through deletions and sequence

degeneration, observed in D. rerio ABC genes [46]. However, further analyses are needed to confirm previous hypothesis. Generally, P-gp molecular evolution in teleosts seemed to follow identical evolution pathways to teleost fishes speciation. Other teleosts P-gpcDNA and amino acid sequences would be extremely useful in P-gp molecular evolution studies. Evolution studies could give further helpful information for fish P-gp structural and functional studies.

5. References

1. Bard SM: **Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms.***Aquatic Toxicology 2000*, **48**: 357–389.
2. Kurelec B: **The multixenobiotic resistance mechanism in aquatic organisms.***Critical Reviews in Toxicology 1992*, **22**: 23-43.
3. Kurelec B, Smital T, Britvić S, Pivčević B, Krča S, Jelaska D, Balen S, Sauerborn R, Mustajbegović S: **Multixenobioticdefence mechanism in aquatic organisms.***PeriodicumBiologurum 1997*, **99**: 319-328.
4. Smital T, Sauerborn R: **Measurement of the activity of multixenobiotic resistance mechanism in the common carp *Cyprinuscarpio*.***Marine Environmental Research 2002*, **54**: 449-453.
5. Smital T, Sauerborn R, Hackenberger KB: **Inducibility of the P-glycoprotein transport activity in the marine mussel *Mytilusgalloprovincialis* and the freshwater mussel *Dreissenapolymorpha*.***Aquatic Toxicology 2003*, **65**: 443-465.
6. Xu C, Yong-Tao LC, Kong AT: **Induction of Phase I, II and III drug metabolism/transport by xenobiotics.***Archives of Pharmacal Research 2005*, **28**: 249-268.
7. Kurelec B: **A new type of hazardous chemicals: The chemosensitizers of multixenobiotic resistance.***Environmental Health Perspectives 1997*, **105**: 855-860.
8. Smital T, Luckenbach T, Sauerborn R, Hamdoun AM, Vega RL, Epel D: **Emerging contaminats-pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms.***Mutation Research 2004*, **552**: 101-117.
9. Sharom FJ: **ABC multidrug transporters: structure, function and role in chemoresistance.***Pharmacogenomics 2008*, **9**(1): 105-127.
10. Ramachandra M, Ambudkar SV, Chen D, Hrycyna CA, Dey S, Gottesman MM, Pastan I: **Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state.***Biochemistry 1998*, **37**(14): 5010-5019.
11. Lam FC, Liu R, Lu P, Shapiro AB, Renoir JM, Sharom FJ, Reiner PB: **beta-Amyloid efflux mediated by p-glycoprotein**. *Journal of Neurochemistry 2001*, **76**(4): 1121-1128.
12. Gottesman MM, Pastan I: **Biochemistry of multidrug resistance mediated by the multidrug transporter.** *Annual Review of Biochemistry 1993*, **62**: 385–427.
13. Gatlik-Landwojtowicz E, Aänismaa P, Seelig A: **Quantification and characterization of P-glycoprotein-substrate interactions.***Biochemistry 2006*, **45**(9): 3020-3032.
14. Raviv Y, Pollard HB, Bruggemann EP, Pastan I, Gottesman MM: **Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells.***Journal of Biological Chemistry 1990*, **265**(7): 3975-3980.
15. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G: **Structure of Pglycoprotein reveals a molecular basis for poly-specific drug binding.***Science 2009*; **323**: 1718-1722.
16. Smital T, Kurelec B: **The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants**. *Mutation Research 1998*, **399**: 43–53.
17. Minier C, Eufemia N, Epel D: **The multi-xenobiotic resistance phenotype as a tool to biomonitor the environment.***Biomarkers 1999*, **4**(6): 442–454.
18. Köhler A, Lauritzen B, Bahns S, George SG, Förlin L, Van Noorden CJF: **Clonal adaptation of cancer cells in flatfish liver to environmental contamination by changes in expression in P-gp related MXR, CYP450 GST-A and G6PDH activity.***Marine Environment Research 1998*, **46**: 191–195.
19. Cooper PS, Vogelbein WK, Van Veld PA: **Altered expression of the xenobiotic transporter P-glycoprotein in liver and liver tumours of mummichog (*Fundulusheteroclitus*)**

from a creosotecontaminated environment.*Biomarkers 1999*, **4**(1): 48–58.

20. Hemmer MJ, Courtney LA, Ortego LS: **Immunohistochemical detection of P-glycoprotein in teleost tissues using mammalian polyclonal and monoclonal antibodies.***Journal of Experimental Zoology 1995*, **272**: 69–77.
21. Schramm U, Fricker G, Wenger R, Miller DS: **Pglycoprotein-mediated secretion of a fluorescent cyclosporine analogue by teleost renal proximal tubules.** American Journal of Physiology 1995, **268**: 46–52.
22. Bard SM, Woodin B, Stegeman JJ: **Induction of the multixenobiotic resistance transporter and cytochrome P450 1A in intertidal fish exposed to environmental contaminants.***Toxicological Sciences 1998*, **42**: 16.
23. Nelson JS: **Fishes of the World. edn 4. New York**: John Wiley & Sons; 2006.
24. Ravia V, Venkatesha B: **Rapidly evolving fish genomes and teleost diversity.***Current Opinion in Genetics & Development 2008*, **18**: 544–550.
25. Notredame C, Higgins DG, Heringa J: **T-Coffee: A novel method for fast and accurate multiple sequence alignment.***Journal of Molecular Biology 2000*, **302**: 205-217.
26. Posada D: **jModelTest: phylogenetic model averaging.***Molecular Biology and Evolution 2008,* **25**: 1253-1256.
27. Darriba D, Taboada GL, Doallo R, Posada D: **ProtTest 3: fast selection of best-fit models of protein evolution.***Bioinformatics 2011*, 27: 1164-1165.
28. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP: **MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space.***Systematic Biology 2012*, **61**(3): 539-542.
29. Loewe L, Scherer S: **Mitochondrial Eve: the plot thickens.***Trends in Ecology & Evolution. 1997*, **12**: 422–423.
30. Adachi J, Cao Y, Hasegawa M: **Tempo and mode of mitochondrial DNA evolution in vertebrates at the amino acid sequence level: rapid evolution in warm-blooded vertebrates.***Journal of Molecular Evolution 1993*, **36**: 270-281.
31. Powell JR, Caccone A: **Intraspecific and interspecific genetic variation in *Drosophila*.***Genome 1998*, **31**: 233-238.
32. Xie W, Lewis PO, Fan Y, Kuo L, Chen MH: **Improving marginal likelihood estimation for Bayesian phylogenetic model selection.***Systematic Biology 2011*, **60**: 150-160.
33. Kass RE, Raftery AE: **Bayes factors.***Journal of the American Statistical Association 1995*,**90**: 773-795.
34. Drummond AJ, Ho SY, Phillips MJ, Rambaut A: **Relaxed phylogenetics and dating with confidence.***PLoS Biology 2006*, **4**: 88.
35. Drummond AJ, Suchard MA: **Bayesian random local clocks, or one rate to rule them all.***BMC Biology 2010*, **8**: 114.
36. Santovito G, Cassini A, Piccinni E: **Cu,Zn superoxide dismutase from *Trematomusbernacchii*: functional conservation and erratic molecular evolution in Antarctic teleosts.***Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 2006*, **143**(4): 444-454.
37. Ayala FJ, Barrio E, Kwiatowski J: **Molecular clock or erratic evolution? A tale of two genes.***Proceedings of the National Academy of Sciences USA 1996*, **93**: 11729–11734.
38. Leslie EM, Deeley RG, Cole SPC: **Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters.***Toxicology 2001*, **167**: 3-23.
39. Chan LMS, Lowes S, Hirst B: **The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability.***European Journal of Pharmaceutical Sciences 2004*, **21**: 25-51.
40. Dean M, Hamon Y, Chimini G: **The human ATP-binding cassette (ABC) transporter superfamily.***Journal of Lipid Research 2001*, **42**: 1007-1017.
41. Sauerborn R, StupinPolanèec D, Žaja R, Smital T: **Identification of the multidrug resistance-associated protein (mrp) related gene in red mullet (*Mullusbarbatus*).***Marine Environment Research 2004*, **58**: 199-204.
42. Žaja R, SauerbornKlobuèar R, Smital T: **Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepatoma cell line.***Aquatic Toxicology 2007*, **81**: 365-376.
43. Žaja R, Munic V, SauerbornKlobuèar R, Ambriovic-Ristov A, Smital T: **Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchusmykiss*)**

hepatocytes.*Aquatic Toxicology 2008*, **90**:322-332.

44. Cai S-Y, Soroka CJ, Ballatory N, Boyer JL: **Molecular characterization of a multidrug resistance-associated protein, Mrp2, from the little skate**. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 2003*, **284**: 125-130.

45. Miller DS, Graeff C, Droulle L, Fricker S, Fricker G: **Xenobiotic efflux pumps in isolated fish brain capillaries.***American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 2002*, **282**: 191-198.

46. Annilo T, Chen ZQ, Shulenin S, Costantino J, Thomas L, Lou H, Stefanov S, Dean M: **Evolution of the vertebrate ABC gene family: analysis of gene birth and death.***Genomics 2006*, **88**: 1-11.

47. SauerbornKlobucar R, Zaja R, Franjević D, Brozović A, Smital T: **Presence of ecotoxicologically relevant Pgp and MRP transcripts and proteins in Cyprinid fish.***ArhivzaHigijenuRadaiToksikologiju 2010*, **61**(2): 175-182.

48. Near TJ, Eytan RI, Dornburg A, Kuhn KL, Moore JA, Davis MP, Wainwright PC, Friedman M, Smith WL: **Resolution of ray-finned fish phylogeny and timing of diversification.***Proceedings of the National Academy of Sciences USA 2012*, **109**(34): 13698-13703.