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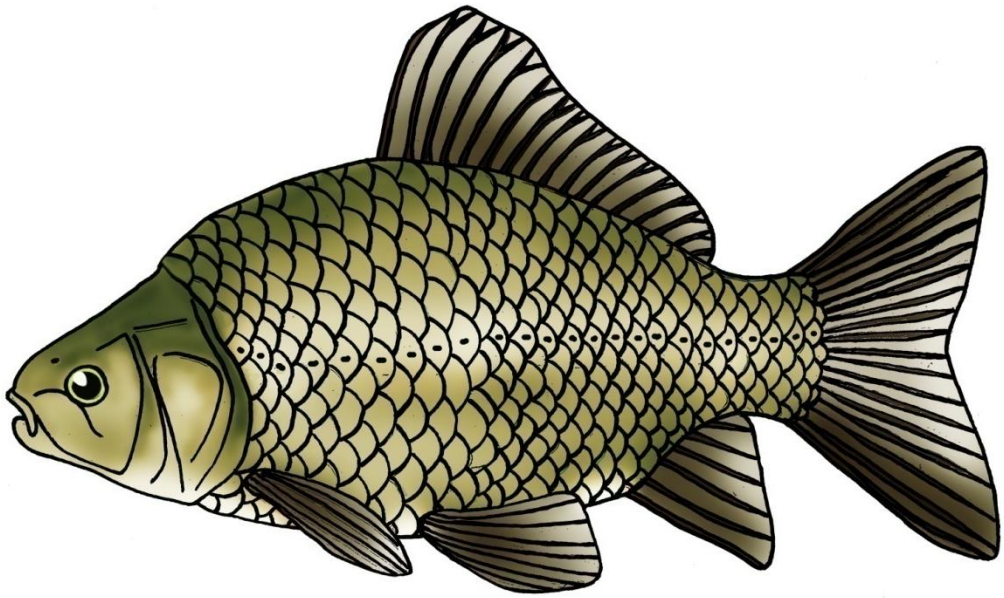
Fylogenetická struktura a zoogeografie rodu karas (*Carassius*)

doktorská dizertační práce
(soubor vědeckých prací s komentářem)



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V Praze dne

Poděkování

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Nelze nepoděkovat i rodině a prátelům, kteří mě po celou dobu studia podporovali.

Práci bych ráda věnovala in memoriam svému otci, který nikdy nechápal, co na těch rybách mám a svému dědovi, který poté, co se dozvěděl, že budu studovat na České zemědělské univerzitě, málem dostal infarkt.

Obsah

1. Úvod

Na tomto místě bych ráda vysvětlila, jak došlo k tomu, že se předmětem mého studia staly právě ryby rodu karas. Podle očekávání by možná měla následovat pasáž pojednávající o tom, že jsem se o karásky zajímala již od útlého dětství a v akváriu mi závojnatky plavaly dříve, než jsem začala chodit…. Ne, tak to nebylo. V dětství jsem měla jasno. Nejprve jsem se chtěla stát kosmonautem, postupem času jsem uvažovala o dráze prezidentky. Následně přišlo období vzdoru, kdy jsem chtěla být popelářem. To naštěstí rychle odeznělo a vysnila jsem si dráhu veterinářky. S nástupem puberty a s tím spojené značné hormonální nerovnováhy jsem si usmyslela, že budu žokejem. Toto krizové období jsem díky rodičovské péči ustála bez jakékoliv újmy a nastoupila na všeobecné gymnázium. Po jeho dokončení jsem šla na „zemědělku“. To jsem v plánu neměla, ale nevzali mě na „Karlovku“, takže jsem vzala, co bylo. Mladická nerozvážnost mi říkala, že pracovat v zoo a uklízet tam exkrementy po velkých afrických kopytnících je také fajn. Jenže hned v prvním semestru jsem narazila na Lukáše Kalouse, který mě intenzivně přesvědčoval o tom, že nejzajímavější věcí na světě jsou karasi a že bych u něj mohla dělat bakalářku na toto téma. Nejprve jsem příliš nechápala, co po mně vlasně chce, ale nakonec jsem souhlasila. V tu chvíli zmizely mé dětské sny v propadlišti dějin a začala éra karasologie.

Tato dizertační práce volně navazuje na bakalářskou práci „Příbuznost domestikovaných forem karase zlatého (*Carassius auratus*) k ostatním zástupcům rodu karas na základě porovnání sekvencí mitochondriálního genu cyt*b*“ a diplomovou práci „Fylogenetická analýza a geografické rozšíření rodu karas (*Carassius*)“. Otázky kladené v těchto pracích byly většinou zodpovězeny, nicméně mnohonásobně více jich na základě zjištěných výsledků vyvstalo. Předkládaná dizertační práce na téma Fylogenetická struktura a zoogeografie rodu karas (*Carassius*) si dala za cíl, jich co nejvíce objasnit.

2. Přehled o současném stavu poznání

2.1. Taxonomie rodu *Carassius*

Rod karas (*Carassius*) patří do čeledi kaprovitých (Cyprinidae); řádu máloostní (Cypriniformes). Máloostní představují vůbec největší a pravděpodobně i evolučně nejúspěšnější řád primárně sladkovodních ryb. Čeleď Cyprinidae je po čeledi Gobiidae druhou největší čeledí obratlovců. Nejstarší fosilní nálezy těchto ryb pocházejí již z období Eocénu (Zardoya *et* Doadrio, 1999; Nelson, 2006).

Vzhledem k problematické taxonomii rodu karas považuji za nutné vysvětlit pojmy, které budou v následujícím textu použity. Termín taxon je druhové jméno, které je v této práci přiřazeno k samostatné mitochondriální linii v rámci fylogenetických vztahů rodu karas a zahrnuje ryby, které svým geografickým původem a morfologií odpovídají původnímu popisu taxonu. Za biotyp se považují různě ploidní a různým způsobem se rozmnožující formy, které lze rozlišovat v rámci jednotlivých mitochondriálních taxonů. Komplexem se rozumí skupina blízce příbuzných taxonů, všech jejich biotypů, vzájemných hybridů i doposud nespecifikovaných genotypů, které jsou k nim vzhledem k podobné morfologii řazeny.

Rod karas Jarocki (1822) zahrnuje jen několik málo taxonů. Jako první byli Linném (1758) popsáni karas obecný (*Carassius carassius*) a karas zlatý (*Carassius auratus*). Následně Bloch (1782) popsal karase stříbřitého (*Carassius gibelio*). Další čtyři zástupce, vyskytující se na japonských ostrovech, popsali Temminck a Schlegel (1842) jako karase gengorobunu (*Carassius cuvieri*), karase ginbunu (*Carassius langsdorfii*), karase nagabunu (*Carassius buergerii*) a karase nigorobunu (*Carassius grandoculis*). Do skupiny japonských karasů označovaných jako „buna“ jsou řazeni ještě další dva zástupci, kteří nemají vědecká druhová jména a označují se jako *Carassius auratus subsp.*1 a *Carassius auratus subsp.*2. Posledním popsaným karasem je *Carassius (auratus) argentheapthalmus* (Nguyen a Ngo, 2001).

Vlastní struktura a fylogenetické vztahy uvnitř rodu jsou nejasné. V současnosti existuje několik různých pojetí, která se dají zobecnit a uspořádat do dvou základních verzí. Jeden přístup rozlišuje pouze dva druhy rodu karas – karase obecného (*C. carassius*) a karase zlatého (*C. auratus*). V případě karase zlatého je pak rozlišováno několik geografických forem

a biotypů, které jsou uváděny jako poddruhy (*C. auratus auratus, C. a. gibelio, C. a. cuvieri,C. a. langsdorfii, C.a. buergeri, C.a. grandoculis, C.a. subsp.*1 a *C.a. subsp.*2). Takto nahlíží na strukturu rodu například Hensel (1971), Baruš *et* Oliva (1995), Hosoya (2000) nebo Szczerbowski (2002) a to většinou na základě morfologických šetření. Dvoudruhové pojetí rodu je stále zastáváno i přes skutečnost, že např. karas gengorobuna byl ze systému poddruhů vyčleněn a potvrzen na úrovni samostatného druhu *C. cuvieri* (např. Murakami *et al*., 2001; Takada *et al.,* 2010). Druhá skupina autorů (např. Kottelat, 1997; Kottelat *et* Freyhof, 2007; Kalous 2005; Kalous *et al.*, 2007) naopak považují taxony *gibelio*, *auratus*, *langsdorfii* a *cuvieri* za samostatné druhy, přičemž se opírají o genetická data a údaje o biologii jednotlivých druhů. Status ostatních taxonů by měl být teprve vyjasněn. Molekulární analýzy (Papoušek *et al.,* 2008; Kalous *et* Šlechtová, 2004; Rylková *et al*., 2010; Takada *et al*., 2010; Yamamoto *et al.,* 2010; Kalous *et al*., 2012; Gao *et al*., 2012; Rylková *et al*., 2013; Wang *et al*., 2013; Luo *et al*., 2014) navíc ukazují, že druhová diverzita rodu karas je větší, než se doposud uvádělo.

Problematika taxonomie rodu vychází již ze základu, kdy bez ohledu na přístup autorů, není jasné, k čemu jednotlivá jména taxonů vztáhnout.

Karas zlatý byl popsán na základě jedince, o jehož pokročilé domestikaci a vlivu záměrného šlechtění není pochyb. Linné popisoval rybu nejen barevně ale i morfologicky značně vzdálenou původnímu divokému fenotypu. Nejnovější genetické studie analyzující početný vzorkový materiál pocházející z mnoha různých lokalit ukázaly, že označení *C. auratus* pokrývá rozsáhlý genetický soubor karasů (Gao *et al*., 2012; Takada *et al*., 2010; Rylková *et al*., 2013). Luo *et al*. (2014) navíc identifikovali několik linií, které jsou z pohledu genotypů u geografie poměrně samostatné. Naproti tomu studie zaměřená na domestikované a ferální formy *C. auratus* prokázala, že genetická diverzita těchto ryb je přes morfologickou variabilitu velmi nízká (Komiyama *et al*., 2009; Rylková *et al*., 2010; Wang *et al.,* 2013). Vyvstává tak otázka, co je pravý *C. auratus*, kde jsou hranice vnitrodruhové diverzity a ke kterému genotypu vztáhnout Linného popis.

V případě *C. gibelio* zjevně došlo k záměně či ztrátě typového jedince a nebylo tak jasné, k jakému morfotypu se popis vztahoval. K dispozici je pouze kresba, na jejímž základě nelze provést revizi a jedinec, který popisu ani zmíněnému vyobrazení neodpovídá (Paepke, 1999; Kalous, 2005; Kalous *et al*., 2012). Bylo tak nutné fixovat vědecké jméno k určitému

genotypu a morfotypu. V případě *C. gibelio* byl jako neotyp vybrán diploidní samec pocházející z oblasti popsané Blochem (Kalous *et al*., 2012).

Vzhledem k různému značení, kdy nelze jednoznačně říci, o jakou genetickou entitu se jedná (především starší literatura založená na morfologických znacích), jistou biologickou provázaností a genetickou blízkostí, která neodpovídá klasickému pojetí druhu, se v současnosti používá označení *Carassius auratus* komplex. Do této skupiny spadají taxony *auratus*, *gibelio* a *langsdorfii*. Tendence zahrnovat do komplexu i taxon *langsdorfii* ale začala procházet změnou, jelikož genetický klastr *C. langsdorfii* je od komplexu *C. auratus* jednoznačně a výrazně oddělen. Navíc je sám o sobě velmi diverzifikovaný. Vytváří tři oddělené sublinie, jejichž vzájemné genetické vzdálenosti jsou příliš velké, aby se daly vysvětlit vnitrodruhovou variabilitu. Vychází tak najevo, že se ve skutečnosti jedná o druhou nevyřešenou skupinu, kterou lze analogicky označit jako *Carassius langsdorfii* komplex (např. Takada *et al*., 2010; Rylková *et* Kalous, 2013). Následující studie pak pravděpodobně zodpoví otázku existence tří sublinií v rámci skupiny *C. langsdorfii* komplex. V úvahu připadají taxony: ginbuna (*C. langsdorfii*), nagabuna (*C. buergerii*), nigorobuna (*C. grandoculis*), *C. auratus subsp.*1 a *C. auratus subsp.*2. Velkou genetickou diverzitu *C. langsdorfii* komplex zmiňují i Murakami *et al.* (2001) a Yamamoto *et al.* (2010) s tím, že *C. buergeri* a *C. grandoculis* jsou diploidní biotypy *C. langsdorfii*.

Fylogenetické analýzy poslední doby identifikovaly genotypy, které nenáleží k žádnému doposud popsanému taxonu. Nejvýraznější je mitochondriální linie pracovně označovaná jako „*M*“ podle Mongolska, kde byla poprvézaznamenána (Kalous *et* Šlechtová, 2004). Peňáz *et* Dulmaa (1987) na tento taxon pravděpodobně narazili, ale na základě morfologického šetření dospěli k závěru, že se jedná o místní populaci *C. gibelio*. Příslušnost k taxonu *C. gibelio* byla ale vyloučena; mitochondriální linie stojí mimo celý *C. auratus* komplex (Papoušek *et al.,* 2008; Kalous *et al.,* 2012; Rylková *et al.,* 2013).

Řešení problematické taxonomie rodu komplikují především biologické vlastnosti jeho zástupců. Je to hlavně gynogenetický způsob rozmnožování, existence polyploidních biotypů a s tím spojený vznik klonálních linií, kterými je charakteristický *C. gibelio* (např. Kottelat *et* Freyhof, 2007) a *C. langsdorfii* (např. Murakami *et al*., 2002). Taxonomie těchto linií se vymyká klasickému přístupu a je třeba na ni pohlížet odlišným způsobem (Dowley, 1989;

Dubois, 1990; Echelle, 1990). Dále je třeba brát v úvahu skutečnost, že karasi se plodně kříží jak mezi sebou, tak i s jinými druhy ryb (např. Liu *et al*., 2001; Hänfling *et al*., 2005).

Ploidní úroveň dříve patřila mezi taxonomické znaky, kdy byli diploidní (2n=100) jedinci považováni za *C. auratus*, kdežto morfologicky podobní jedinci s triploidní sestavou (3n=150) automaticky za *C. gibelio* (Arai *et* Fujiki, 1977; Vasil'eva *et* Vasil'ev, 2000; Sczczerbowski, 2002). *C. gibelio* se ale vyskytuje jak v triploidní, tak i v diploidní formě (Abramenko *et al*., 2004; Lusková *et al*., 2004; Apalikova *et al*., 2008). Současné studie navíc prokázali triploidní sestavu u *C. auratus* (Takada *et al*., 2010; Xiao *et al*., 2011; Rylková *et al*., 2013); U *C. langsdorfii* komplex jsou taktéž známé diploidní i triploidní biotypy (Hosoya, 2000; Iguchi *et al*., 2003; Takada *et al.,* 2010). Mongolské populace podle dosavadních šetření také vykazují různou ploidní úroveň (Apalikova *et al.*, 2011; Rylková *et al*., 2013). Taxony, u kterých lze ploidii považovat za taxonomický znak jsou karas obecný (*C. carassius*) a karas gengorobuna (*C. cuvieri*), u kterých jsou známy pouze diploidní jedinci (Sczczerbowski, 2002; Iguchi *et al*., 2003; Apalikova *et al*., 2011).

U zhruba 50 v současnosti známých taxonů - například několika živorodých ryb rodu *Poecilia* a *Poeciliopsis* (Vrijenhoek, 1994; Avise, 2008), nebo některých druhů rodu *Cobitis* (Janko *et al*., 2007a), které se množí gynogeneticky a sexuálně parazitují na sympatrických diploidních biotypech; a je známo, že gynogenetické polyploidní biotypy jsou výsledkem mezidruhového křížení diploidních, sexuálně se množících druhů. Z tohoto pohledu patří polyploidní karasi mezi výjimky, u kterých nebyl hybridní původ zatím potvrzen.

Nejnovější studie ale naznačují, že by to tak mohlo být. Ukázalo se, že hybridizace a polyploidie u karasů má úzkou souvislost. Mezhzherin *et al.* (2012) zjistili nejen vysoký počet hybridů v sledované populaci karasů, ale také to, že více než 70% hybridizací diploidních parentálních jedinců mělo za následek vznik polyploidních samic. Při hybridizaci tak musí docházet ke spojování chromozómových sádek a to v různých kombinacích. Genomový mechanismus vzniku těchto tzv. allopolyploidů není zcela jasný; důležitými faktory jsou biotypy (příslušnost k taxonu) a ploidní úrovně jedinců, kteří se na vzniku allopolyploida podílí (Knytl *et al.,* 2013).

Objasnění fylogeneze, genetického pozadí a biologických principů rozmnožování *C. gibelio* a *C. langsdorfii* komplex je žádoucí pro pochopení jejich ekologického potenciálu. Rychlé invazivní šíření a přizpůsobivost jejich polyploidních a gynogenetických biotypů má negativní vliv jak na produkční rybářství, tak na původní ichtyofaunu v nově osídlených oblastech. Wouters *et al.* (2012) a Mezhzherin *et al.* (2012) označují šíření jednotlivých taxonů pomocí hybridizace s taxony původními za tzv. tichou, nebo kryptickou invazi, která může vyústit v potlačení, nebo úplné nahrazení původního taxonu. Z toho hlediska se v současnosti jeví nejvíce ohroženým karas obecný.

Kryptická invaze je vedle již zmiňovaných dovozů ve formě příměsí u Koi (kaprů) (Kalous *et al*., 2007) jedním z dalších možných vysvětlení, jak se do evropských vod dostaly sublinie komplexu *C. langsdorfii*.

2.2. Zoogeografie rodu *Carassius*

Mnoho nejasností je také okolo geografického původu a současného rozšíření jednotlivých zástupců rodu. Za původní oblast kaprovitých ryb se považuje jihovýchodní Asie, která je centrem jejich evoluce. Odtud byly kolonizovány všechny oblasti, kde se nyní kaprovité ryby vyskytují – celý eurasijský kontinent, subsaharská Afrika a Severní Amerika. Tuto skutečnost dokládají i fosilní nálezy. Zatímco v Asii se kaprovití vyskytovali již v období Eocénu, na území dnešní Evropy a severní Ameriky se objevili až v Oligocénu. V Austrálii a Jižní Americe se přirozeně nevyskytují; byli sem zavlečeni až člověkem (Banarescu, 1991; Banarescu *et* Coad, 1991; Nelson, 2006).

Jako primárně sladkovodní druhy se mohou téměř všechny kaprovité ryby vyskytovat a pohybovat pouze v říčních a jezerních systémech. Možnost jejich šíření mezi jednotlivými povodími je tak velmi omezená. Z tohoto důvodu jejich rozšíření dobře odráží biogeografickou historii jednotlivých zástupců (Zardoya *et* Doadrio, 1999; Nelson, 2006).

Přes výše zmíněné informace je velmi obtížné stanovit geografický původ jednotlivých zástupců rodu karas; především kvůli mnohačetným introdukcím. Oblast výskytu rodu zahrnuje celou palearktickou oblast včetně Evropy, arktická povodí Sibiře a mírné a teplé oblasti střední a východníAsie (Szczerbowski, 2002). U polyploidních biotypů taxonu *C. gibelio* je ale známa i tolerance vůči vyšší salinitě vody. Jeho výskyt je zaznamenáván v příbřežních vodách Pobaltí a Skandinávie (Pihu *et al*., 2003; Vatemaa *et al*., 2005; Wouters

et al., 2012). Tato skutečnost potenciálně značně rozšiřuje možnosti jeho šíření mezi povodími.

Na území Evropy se vyskytuje pět popsaných taxonů – *C. carassius*, *C. gibelio*, *C. auratus*, *C. langsdorfii* a *C. sp. „M".* Původním je zřejmě pouze *C. carassius* (např. Kottelat *et* Freyhof, 2007) a diploidní biotyp *C. gibelio* (Kalous *et al*., 2012; Rylková *et al*., 2013).

C. carassius je původním evropským druhem. Dříve byl poměrně hojný, ale v současné době rychle mizí (Lusk *et al.,* 2010). Důvodů je několik. Jednak ubývání vhodných biotopů (stará říční ramena, tůně, záplavová území) (Kottelat *et* Freyhof, 2007; Baruš *et* Oliva, 1995) a jednak konkurenční boj s invazními druhy – triploidním biotypem *C. gibelio*, (Lusková *et al*., 2010; Rylková *et* Kalous, 2013b) pravděpodobně i *C. langsdorfii* komplex. Významné jsou i genetické ztráty způsobené křížením se všemi ostatními karasími taxony (Hanfling, 2005; Papoušek, 2008). Názorný příklad nahrazení populace *C. carassius* invazním biotypem *C. gibelio* je popsán v Rylková *et* Kalous (2013b). V České republice je karas obecný v současné době zařazen na Červený seznam druhů mihulí a ryb, a to v kategorii III – 1. Kriticky ohrožený (critically endangered, CE) (Lusk *et al.,* 2011).

Původní oblastí výskytu *C. auratus* je pravděpodobně východní Asie. Dříve bránily potvrzení a přesnému určení mnohačetné introdukce, díky kterým se karas zlatý rozšířil po celém světě (Szczerbowski, 2002; Baruš *et* Oliva, 1995). Na mnohých místech, kde byl chován jako okrasná ryba, unikl nebo byl záměrně vypuštěn do volné přírody, kde se usídlil a stal se trvalou součástí místní ichtyofauny. Tímto způsobem se v polovině devatenáctého století dostal i do Evropy (Balon, 2004). V teplejších oblastech, především na Pyrenejském a Balkánském poloostrově, ale např. i v Británii, se úspěšně aklimatizoval (Elvira, 2001). Stávající evropské populace *C. auratus* jsou tedy ferální, což potvrzují i fylogenetické práce (např. Rylková *et al*., 2010). Zajímavým příkladem jeho šíření je australský kontinent, kam byl zavlečen v šedesátých letech devatenáctého století a nyní se běžně vyskytuje v mnohých oblastech Nového Jižního Walesu, Viktorie, Jižní i Západní Austrálie; čímž se stal pravděpodobně vůbec nejrozšířenější australskou sladkovodní rybou (Brumley, 1996).

V případě polyploidních biotypů *C. gibelio* nelze původní oblast výskytu, jako u karase zlatého, určit zcela přesně. Pravděpodobně se šířil z povodí řeky Amur ve východní Asii (Baruš *et* Oliva, 1995; Kottelat *et* Freyhof, 2007). Diploidní biotyp *C. gibelio* je

pravděpodobně původní v severovýchodní Evropě (Kalous *et al*., 2012). Hraniční oblastí na západě je povodí řeky Odry. Přesné vymezení nelze v současnosti určit a to především z důvodu smísení původních diploidních a invazních polyploidních biotypů a následným introdukcím. Polyploidní biotyp karase stříbřitého byl do Evropy zavlečen v polovině minulého století. Expanzi povodím Dunaje (a následné osidlování vod na území tehdejšího Československa) podrobně popisují Holčík *et* Žitňan (1978) a Holčík (1980). Díky svým biologickým vlastnostem se stal invazní rybou, která se dnes vyskytuje na téměř celém kontinentu (Szczerbowski, 2002; Kottelat *et* Freyhof, 2007). Kromě sladkých vod je často pozorován i v příbřežních oblastech Baltského moře (Pihu *et al*., 2003; Vatemaa *et al*., 2005). Migrace mořskou vodou o nižší salinitě je jedním z potenciálních způsobů, jakým mohly polyploidní biotypy *C. gibelio* invandovat skandinávská povodí, kde se dříve *C. gibelio* vůbec nevyskytoval (Wouters *et al.,* 2012).

C. langsdorfii komplex je pro Evropu novým faunistickým prvkem. Poprvé byl zaznamenán v povodí řeky Labe (Kalous *et al.,* 2007). Následovaly další záznamy o jeho výskytu (Papoušek *et al*., 2008; Tsipas *et al*., 2009; Rylková *et al.,* 2010; Kalous *et al*., 2012; Rylková *et al*., 2013), které poukazují na to, že se nejedná o výskyt náhodný, ale s největší pravděpodobností se tento taxon v Evropě úspěšně aklimatizoval; ačkoliv původní oblastí výskytu ginbuny jsou pouze Japonské ostrovy. Z dostupných informací je zřejmé, že populace jsou životaschopné a rozmnožují se. Zjištěna byla i konkurenceschopnost a vyšší odolnost, než u původních druhů ryb (Rylková *et* Kalous, 2013). Zmíněné poznatky vedou k úvaze, že ginbuna může do budoucna představovat ekologickou hrozbu v případě jeho úspěšného šíření. Z genetické struktury komplexu *C. langsdorfii* je zřejmé, že se do Evropy dostaly minimálně dvě odlišné sublinie. Jejich přesná identita není vzhledem k problematice *C. langsdorfii* komplexu na samotných Japonských ostrovech známá; stejně tak i způsob jakým se do Evropských vod dostaly.

Posledním taxonem je mitochondriální linie *Carassius sp. „M".* Její výskyt v Evropě byl zatím zaznamenán ojediněle (Papoušek *et al*., 2008; Rylková *et al*., 2013).

Všechny výše zmíněné karasí taxony se přirozeně vyskytují také na různě rozsáhlých areálech Asie. Další, označované jako „buna" - *C. grandoculis*, *C. cuvieri*, *C. buergeri*, *C.a. subsp.*1 a C.*a. subsp.*2 jsou dle současných poznatků endemické pro Japonské souostroví (Temminck *et* Schlegel, 1842; Hosoya, 2000). Vzhledem k diverzitě komplexu *C. langsdorfii*

na území Evropy je otázkou, zda sem nebyl některý z těchto karasů zavlečen také (Rylková *et* Kalous, 2013).

Recentně popsaný taxon *C. a. argenteapthlamus* byl zaznamenán na území Vietnamu (Nguyen *et* Ngo, 2001). Geneticky zatím nebyl definován.

Fylogenetické analýzy (Kalous *et* Šlechtová, 2004; Rylková *et al*., 2008, Luo *et al*., 2014) odhalily několik dalších mitochondriálních linií, které nenáleží k žádnému doposud popsanému druhu. Všechny linie byly identifikovány na území střední a východní Asie. Tato skutečnost podporuje určení východní Asie jako centra evoluce rodu karas. K upřesnění areálu výskytu a fylogenetické pozice v rámci rodu je však zapotřebí obsáhlejší vzorkový materiál.

V současné době existují práce, které se zabývají problematikou diverzity rodu karas na základě molekulárních dat (z nejnovějších např. Takada *et al.,* 2010; Yamamoto *et al*., 2010; Gao *et al.,* 2012; Rylková *et al*., 2013; Wang *et al.,* 2013). Většina prací se ale zaměřuje pouze na mitochondriální analýzu. To dává jen omezený a do jisté míry i zkreslený pohled, jelikož přináší informace pouze o polovině genetické podstaty zkoumaných jedinců. Tyto analýzy tak nejsou schopné odhalit hybridní jedince, kterých je v karasích populacích mnoho (Hänfling *et al*., 2005; Mezhzherin *et al*., 2012; Wouters *et al*., 2012).

Konfrontace mitochondriální a jaderné analýzy umožní nahlédnout do skladby celého genomu. Tím bude možné identifikovat jak „čisté“ linie, tak odhalit případné hybridy a zjistit jejich četnost. Zároveň bude možné zjistit, které taxony se na hybridizaci podílejí a v jaké míře. Tento pohled může přinést zajímavé poznatky především o taxonech, v jejichž populacích se vyskytují polyploidní gynogenetické biotypy - *C. gibelio*, *C. langsdorfii*; případně také *C. auratus* a *C. sp. „M“*., u kterých byl potvrzen výskyt polyploidních jedinců (Rylková *et al.*, 2013) ale gynogeneze nebyla (ještě) zaznamenána. Původ polyploidních biotypů není jasný, ale jak naznačují výsledky prací Murakamiho *et al*. (2001) a Yamamoty *et al.* (2010), Mezhzerina *et al.* (2012), Wouterse *et al*. (2012) a Knytla *et al*. (2013) s největší pravděpodobností vznikají hybridizací několika diploidních taxonů.

U geneticky „čistých“ jedinců pak může být provedeno morfologické šetření, pro definování morfotypů a jejich variability v rámci druhu. Dosavadní morfologické studie

nemohou být považovány za věrohodné, jelikož jsou s velkou pravděpodobností znehodnoceny zahrnutím hybridních jedinců.

Kombinací výsledků mitochondriálně-jaderné analýzy a znalostí geografického původu vzorkového materiálu bude pravděpodobně možné zjistit také více o zoogeografii rodu. Nejednalo by se pouze o možné upřesnění geografického původu jednotlivých taxonů, ale i o případnou identifikaci míst, kde došlo ke vzniku polyploidních biotypů.

3. Vědecké hypotézy a cíle práce

Hypotéza

Polyploidní biotypy rodu *Carassius* jsou hybridního původu a jejich parentální taxony je možné identifikovat na základě molekulárně-genetických dat; zároveň je možné vysledovat původní areály rozšíření a místa, kde došlo k hybridizaci.

Cíle práce

Hlavním cílem této disertační práce je rozšíření molekulární studie rodu karas o analýzu jaderných genů a konfrontaci výsledků s mitochondriálními daty.

Dílčí cíle:

- doplnění vzorkového materiálu u vybraných taxonů
- genetická studie na molekulární úrovni:
    - sekvenace mitochondriálních a jaderných genů;
    - rekonstrukce fylogenetickýchvztahů;
    - zjištění struktury maternálních a paternálních linií a následná konfrontace výsledků
    - obou analýz (včetně případné identifikace hybridních komplexů)
- zjištění druhové diverzity
- rekonstrukce evoluce polyploidních taxonů s ohledem na zoogeografii rodu

4. Materiál a metody

4.1. Materiál

Celkově bylo v průběhu práce analyzováno na 420 tkáňových vzorků ryb, které pocházeli z přírodních lokalit Evropy, Asie a Severní Ameriky. Malé množství ryb představovali jedinci získaní z okrasných nebo produkčních chovů (převážně prošlechtěné formy *C. auratus*). U všech jedinců byla izolována celková DNA a osekvenován mitochondriální gen pro cytochrom *b* (cyt *b*). U vybraných jedinců byl následně osekvenován i jaderný gen pro ribozomální protein S7. U části jedinců byla určena ploidie. Většina použitého materiálu je uchována v podobě vzorků tkáně nebo izolátů DNA.

V případě, že byl k dispozici vzorek v podobě celého jedince, byla ryba také vyfotografována a byla provedena základní morfologická měření. Fotografie a všechny získané údaje jsou ve formě elektronické databáze uloženy stejně jako tkáňové vzorky a izoláty na katedře zoologie a rybářství ČZU v Praze.

Za účelem porovnání výsledků mitochondriální a jaderné analýzy bylo vybráno 93 jedinců tak, aby byly zastoupeny všechny mitochondriální linie. Byly vybírání jedinci jak diploidní, tak polyploidní. Dále bylo přihlédnuto i k zastoupení co nejrůznorodějších lokalit původu. Detailní informace o tomto datasetu jsou shrnuty v Tabulce 1.

Tabulka 1. Vzorkový materiál použitý pro rekonstrukci fylogenetických vztahů.

Jedinec	GenBank cyt *b*	Alely S7	Ploidie	Lokalita		Reference
aur1		1	2n	akvaristika, Praha	Česká republika	tato práce
aur2		2	2n	akvaristika, Praha	Česká republika	tato práce
aur3	EU663576	1	nd	akvaristika, Praha	Česká republika	Rylková et al. (2010)
aur4	EU663575	1	nd	akvaristika, Praha	Česká republika	Rylková et al. (2010)
aur5	DQ868923	1	nd	Rio Alviela	Portugalsko	Rylková et al. (2013)
aur6	DQ868927	1	nd	akvaristika, Mělník	Česká republika	Kalous et al. (2007)
aur7	DQ399923	1	nd	akvaristika, Mělník	Česká republika	Rylková et al. (2010)
aur8	DQ868908	1	nd	Guadiana, Brinches	Portugalsko	Rylková et al. (2013)
aur9	EU663584	1	nd	akvaristika, Praha	Česká republika	Rylková et al. (2010)
aur10	GU991386	1	3n	jezero Orchid	Albánie	Rylková et al. (2013)
aur11	GU991394	2	3n	jezero Prespa	Řecko	Rylková et al. (2013)
aur12	GU991389	2	3n	jezero Skadar	Černá Hora	Rylková et al. (2013)
aur13	GU991388	2	3n	jezero Skadar	Černá Hora	Rylková et al. (2013)
aur14	GU991390	1	2n	Ishern	Albánie	Rylková et al. (2013)
aur15	JN412519	2	3n	trh, Tsang Yuan	Čína	Rylková et al. (2013)
aur16	EU663598	1	2n	Yangtze, Nanking	Čína	Rylková et al. (2010)
aur17		1	nd	trh, Yangtian	Čína	tato práce
aur18		1	nd	trh, Yangtian	Čína	tato práce
aur19	EU663599	2	3n	Yangtze, Wuhan	Čína	Rylková et al. (2010)
aur20		1	nd	trh, Yangtian	Čína	tato práce
aur21		1	nd	trh, Yangtian	Čína	tato práce
aur22	GU991391	1	2n	Kylšutky, Ščušinsk	Kazachstán	Rylková et al. (2013)
gib1		1	2n	tůň Řehačka	Česká republika	tato práce
gib2		1	2n	rybník, Plön	Německo	tato práce
gib3	HM000007	2	nd	Jiu	Rumunsko	Rylková et al. (2013)
gib4		2	nd	tůn Lysá	Česká republika	tato práce
gib5		2	nd	Drivuša, Bosna	Bosna a Hercegovina	tato práce
gib6	HM008686	1	2n	Melleta	Itálie	Rylková et al. (2013)
gib7		2	3n	tůn Lysá	Česká republika	tato práce
gib8		2	nd	kanál, Porto Viro	Itálie	tato práce
gib9	HM000006	2	3n	rybí trh, Atény	Řecko	Rylková et al. (2013)
gib10	HM000022	2	3n	rybník, Talin	Estonsko	Rylková et al. (2013)
gib11	JN546046	2	nd	Berounka, Rokycany	Česká republika	Rylková et al. (2013)
gib12		1	3n	rybník, Kroměříž	Česká republika	tato práce
gib13		2	nd	Jezero Batlawa	Kosovo	tato práce
gib14		2	nd	Jijia	Rumunsko	tato práce
gib15		2	nd	Mures	Rumunsko	tato práce
gib16	JN546066	1	nd	Alma, Topoli	Ukrajina	Rylková et al. (2013)
gib17		2	nd	Doňana	Španělsko	tato práce
gib18		2	nd	Liběchov	Česká republika	tato práce
gib19		2	nd	Slezsko	Česká republika	tato práce
gib20	EU663593	2	3n	Ševarova Jagura, Cetina	Bosna a Hercegovina	Rylková et al. (2013)
gib21	HM008687	2	nd	jezero Krenica	Bosna a Hercegovina	Rylková et al. (2013)
gib22		2	nd	jezero Batlawa	Kosovo	tato práce
gib23		2	nd	tůň, Tomislavgrad	Bosna a Hercegovina	tato práce
gib24	HM008688	k82	3n	Cheon Jin Cheon, Pongho-ri	Korea	Rylková et al. (2013)
viet1		1	nd	rybí trh, Hué	Vietnam	tato práce
viet2		2	nd	rybí trh, Hué	Vietnam	tato práce
viet3		2	nd	rybí trh, Hué	Vietnam	tato práce
viet4		1	nd	tůň, Hué	Vietnam	tato práce

viet5		1	nd	tůň, Hué	Vietnam	tato práce
viet6		2	nd	Vihn Dien, Vihn Dien	Vietnam	tato práce
viet7		1	nd	rybí trh, Hoian	Vietnam	tato práce
viet8		2	nd	Ky Cung	Vietnam	tato práce
viet9		1	nd	Ky Cung, Lang Son	Vietnam	tato práce
viet10		1	nd	trh, Dong Miao Xiang	Čína	tato práce
viet11		1	nd	Bang Giang, Lang Son	Vietnam	tato práce
viet12		1	nd	Bang, Cao Bang	Vietnam	tato práce
viet13		2	nd	Bang, Cao Bang	Vietnam	tato práce
viet14		1	nd	trh, Dong Miao Xiang	Čína	tato práce
viet15		1	nd	trh, Dong Miao Xiang	Čína	tato práce
kor1		2	nd	Jin Geon Cheon, Jinju	Korea	tato práce
kor2		1	nd	Jin Geon Cheon, Jinju	Korea	tato práce
kor3		1	nd	Cheon Jin Cheon, Pongho-ri	Korea	tato práce
kor4		1	nd	Cheon Jin Cheon, Pongho-ri	Korea	tato práce
mon1	DQ868925	2	3n	jezero Uvs	Mongolsko	Rylková et al. (2013)
mon2	DQ868926	2	3n	jezero Uvs	Mongolsko	Rylková et al. (2013)
mon3		1	nd	Dyje	Česká republika	tato práce
mon4	HM008690	2	3n	Selenga	Mongolsko	Rylková et al. (2013)
lang1	GU942708	2	3n	rybník, Preetz	Německo	Rylková et al. (2013)
lang2	GU942707	3	3n	rybník, Preetz	Německo	Rylková et al. (2013)
lang3	DQ399932	2	3n	Chrudimka, Bojanov	Česká republika	Kalous et al. (2007)
lang4	JN412528	2	dn	rybník, Rodnoje	Ukrajina	Rylková et al. (2013)
lang5	JN412527	1	nd	Kako river, Honsyu	Japonsko	Rylková et al. (2013)
lang6	DQ399921	2	nd	jezero Abashiri, Hokkaido	Japonsko	Kalous et al. (2007)
lang7	JN412531	1	nd	Alma, vesnice Topoli	Ukrajina	Rylková et al. (2013)
lang8	GU942709	1	nd	kanál Sunca, řeka Neretva	Bosna a Hercegovina	Rylková et al. (2013)
lang9	GU942711	1	2n	Thessaloniki, rybí trh	Řecko	Rylková et al. (2013)
lang10	GU942710	1	nd	jezero Rama	Bosna a Hercegovina	Rylková et al. (2013)
lang11	JN412529	1	nd	tůň, Litvínovice	Česká republika	Rylková et al. (2013)
lang12	HM000036	1	nd	říčka Meletta	Itálie	Rylková et al. (2013)
cuv1	JN402304	2	nd	Lake Mikatako, Honsyu	Japonsko	Kalous et al. (2012)
car1	DQ399917	1	2n	rybník, Preetz	Německo	Kalous et al. (2007)
car2	GU991400	1	nd	rybí farma, Calverton	Anglie	Rylková et al. (2013)
car3	DQ399938	1	nd	Milevsko	Česká republika	Kalous et al. (2007)
car4	JN412540	1	nd	rybník, Hatterwusting	Německo	Rylková et al. (2013)
car5		1	nd	Labe	Česká republika	tato práce
car6		1	nd	Oldenburg	Německo	tato práce
car7	JN412547	1	nd	Skabersjø	Švédsko	Rylková et al. (2013)
car8		1	nd	Helsinky	Finsko	tato práce
car9	JN412548	1	nd	Angermanalven, Sandviken	Švédsko	Rylková et al. (2013)
car10	JN412549	1	2n	Višňová	Česká republika	Rylková et al. (2013)
car11	JN412550	2	nd	Višňová	Česká republika	Rylková et al. (2013)
car12		1	nd	Morava	Česká republika	tato práce
car13		1	nd	Lužnice	Česká republika	tato práce
car14		1	nd	Lužnice	Česká republika	tato práce
kapr		2		Mekong	Thajsko	Rylková et al. (2013)

4.2. Metodika

4.2.1. Izolace DNA, PCR a sekvenace

Celková DNA byla izolována ze vzorku čerstvé, mražené, nebo v ethanolu uchované tkáně. DNA byla izolována pomocí setu DNeasyBlood and TissueKit (Qiagen) přesně podle návodu výrobce.

Mitochondriální gen cyt *b* byl amplifikován polymerázovou řetězcovou reakcí (PCR) pomocí páru primerů: přímého Glu L. Ca14337-14359: GAA GAA CCA CCG TTG TTA TTC a zpětného Thr H. Ca15568-15548: ACC TCC RAY CTY CGG ATT ACA (Šlechtová *et al*., 2006). Reakční směs PCR obsahovala 3µl templátové DNA; 3 µl každého primeru; 15.5 µl Combi PPP Master Mix (Top-bio) a ddH2O byla doplněna do celkového obsahu 50 µl.

Profil PCR byl započat denaturací při 95°C po dobu 2 minut. Následovalo 35 cyklů, které se sestávali z denaturace při 95°C po 1 min, annealingu při 52°C po 30 vteřin a elongace při 72°C po 30 vteřin. PCR byla ukončena závěrečnou elongací při 72°C po dobu 10 minut.

K amplifikaci jaderného genu S7 byl použit pár primerů: přímý S7RPEX1F: TGG CCT CTT CCT TGG CCG TC a zpětný S7RPEX2R: AAC TCG TCT GGC TTT TGC CC Chow *et* Hazama (1998). Reakční směs obsahovala 3µl templátové DNA; 3 µl každého primeru; 25.5 µl Combi PPP Master Mix (Top-bio) a ddH_2O byla doplněna do celkového obsahu 50 µl.

Profil PCR byl započat denaturací při 95°C po dobu 5 minut. Následovali 2 cykly: 94°C po 1 min, 60°C po 1min 30 vteřin a 72°C po 2min; 2 cykly: 95°C po 1 min, 58°Cpo 1 min 30 vteřin a 72°C po 2 min; 2 cykly: 94°C po 1 min, 56°C po 1 min 30 vteřin a 72°C po 2 min; 30 cyklů: 94°C po 1 min, 54°C po 1 min 30 vteřin a 72°C po 2 min. PCR byla ukončena závěrečnou elongací při 72°C po dobu 7 minut. Pro PCR byl v obou případech použit přístroj MJ Mini^{TM} thermocycler (Bio-Rad).

Produkty PCR byly přečištěny a osekvenovány z obou konců (3´a 5´) analyzovaného fragmentu, aby byla získána celá sekvence genu (u cytochromu *b* cca 1140 bp, u S7 600-900 bp). Přečištění i sekvenace zajistila formou služby firma Macrogen Inc. (Korea, Amsterdam).

4.2.2. Rekonstrukce fylogenetických vztahů

Získané chromatogramy byly manuálně složeny a zkontrolovány proti výskytu případných chyb v softwarovém programu BioEdit 5.0.9. (Hall, 1999). Stejný program byl použit k sesazení hotových sekvencí pomocí algoritmu ClustalW. Závěrečné úpravy datasetu

byli provedeny pomocí on-line editoru FaBox (Villesen, 2007). V případě jaderné analýzy byly různé alely genu u jednoho jedince rozlišeny písmeny A a B.

Fylogenetické vztahy byly vymodelovány metodou maximální parsimonie (MP) v programu PAUP* ver. 4.0b10 (Swofford, 2000) a Bayesiánskou analýzou (BAY) s pomocí programu MrBayes ver. 3.0 (Huelsenbeck *et* Ronquist, 2001). Detailní postup fylogenetických rekonstrukcí je popsán v Rylková *et al*. 2010.

Jako outgroup pro zakořenění fylogenetických stromů byly použity sekvence kapra obecného (*Cyprinus carpio*).

Pro detailnější a jemnější vykreslení fylogenetických vztahů uvnitř komplexů *C. auratus* a *C. langsdorfii* byla s využitím statistické parsimonie (Templeton *et al.,* 1992) provedena haplotypová analýza v programu TCS 1.21 (Clement *et al.,* 2000). Limit propojení byl nastaven na 20 mutačních kroků. Detailní metodický postup je popsán v Rylková *et al.* (2010) a Rylková *et* Kalous (2013).

4.2.3. Měření ploidie

Ploidní úroveň vzorků, u kterých byla dostupná pouze tkáň, byla měřena pomocí průtokové cytometrie. Metoda podrobně popsaná v Lamatsch *et al.* (2000) je určena k použití ploutevní tkáně (finclip). Jako standard byly použity heparinizované červené krvinky kuřete (*Gallus gallus*) s genomovou velikostí 2.5pg/jádro (Vinogradov, 1998). U každého vzorku bylo měřeno nejméně 10 000 buněk. Ploidní úrovně byly rozděleny podle obsahu DNA pomocí „K-Means“ shlukové analýzy. Spočítána byla základní popisná statistika pro hodnoty každé ploidní úrovně a pro každou linii. Průkaznost výsledků byla otestována pomocí ANOVA. Pro všechna statistická vyhodnocení byl použit program STATISTICA ver. 9.1 (StatSoft, Inc., 2010). Podrobný metodický postup určení ploidie u analyzovaných karasů je popsán v Rylková *et al.* (2013).

U části vzorkového materiálu, u které byla možnost odebrat vzorek krve, byla použita metoda měření plochy jader erytrocytů. Při přípravě krevních nátěrů bylo postupováno podle metodiky popsané Flajšhansem (1997) upravené pro karase podle Kalous *et* Petrtýl (2004).

Sestava na snímání obrazu obarvených jader erytrocytů se skládala z mikroskopu Nikon Eclipse 600, analogové videokamery Hitachi HVC 20, počítačového softwaru NIS-Elements 3.2 (Laboratory Imaging s.r.o, Praha). Pro samotné snímání byl použit 100x zvětšující imerzní

objektiv. Celkové zvětšení tak bylo 1000x. Snímací systém je ve vlastnictví katedry veterinárních disciplin, ČZU v Praze, která v rámci projektové spolupráce umožnila její použití.

Měření plochy jader erytrocytů bylo provedeno pomocí programu QuickPHOTO Micro 3.0 (PROMICRA s.r.o., Praha). Program byl nejprve kalibrován podle snímku objektivového měřítka o rozlišení 1/100 mm, který byl pořízen v programu NIS-Elements.

U každé ryby byla následně změřena plocha nejméně 100 jader erytrocytů. Ze souboru naměřených hodnot byly odstraněny extrémní hodnoty (minimální a maximální). Pro ověření, zda soubor podléhá normálnímu rozdělení, byl použit Shapiro - Wilkův test. Pro následující testování byla data uspořádána dvojím způsobem. Zjišťovala se statisticky prokazatelná odlišnost mezi skupinou diploidních a triploidních jedinců a rovněž odlišnost mezi jednotlivými jedinci z obou skupin. Získané hodnoty byly zpracovány t-testem pro nezávislé výběry v programu STATISTICA ver. 9.1 (StatSoft, Inc., 2010).

5. Výsledky a diskuse

5.1. Výsledky práce publikované ve vědeckých časopisech

1) Rylková, K., Kalous, L., Bohlen, J., Šlechtová, V. 2010. Many branches one root: first evidence for monophyly of morphologicaly highly diverse goldfish (*Carassius auratus*). *Aquaculture* 302, pp. 36 – 41

Mitochondriální analýza cytochromu *b* různě prošlechtěných variant okrasných zlatých rybek, populací žijících divoce na území Evropy a populací z předpokládané oblasti původu karase zlatého potvrdila monofyletický původ domestikovaných forem, stejně tak i nepůvodnost Evropských populací. Jedná se o domestikované formy, které unikly, popř. byly vypuštěny a v oblastech s příhodným podnebím zdivočely. Zároveň oblast původu okrasných forem, na kterou ukazuje výsledek analýzy, je ve shodě s historickými záznamy: viz Příloha I.

2) Kalous, L., Rylková, K., Bohlen, J., Šanda, R., Petrtýl, M. 2012. New mtDNA data reveal a wide distribution of the Japanese ginbuna *Carassius langsdorfii* in Europe. *Journal of Fish Biology* 82 (2), pp. 703-707

3) Rylková, K., Kalous, L. 2013. New Finding of non indigenous Japanese cyprinid fish in the Czech Republic. *Scientia Agriculturae Bohemica* 44 (2), pp. 79-84

Na základě molekulárních znaků byl zaznamenán výskyt *C. langsdorfii* na několika vzdálených lokalitách v Evropě. Potvrdila se tak domněnka, že první nález nebyl ojedinělý a tento původně endemit Japonského souostroví se stává součástí evropské ichtyofauny. Genetická diverzita mezi analyzovanými jedinci ukazuje na více zdrojů zavlečení a tím i více nezávislých introdukčních událostí. Na území České republiky byli zaznamenané dvě ze tří mitochondriálních sublinií *C. langsdorfii*. Genetická vzdálenost mezi jednotlivými subliniemi je značná a proto se lze domnívat, že pod jméno *C. langsdorfii* může být v současnosti zahrnuto několik taxonů. O které se jedná, není známo. Pravděpodobně jde o skupinu taxonů podobnou *C. auratus* komplex a lze ji tak analogicky označit jako *C. langsdorfii* komplex: viz Příloha II. a Příloha III.

4) Kalous, L., Bohlen, J., Rylková, K., Petrtýl, M. 2012. Hidden diversity within the Prussian carp and designation of the neotype for *Carassius gibelio* (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwaters* 23 (1), pp. 11-18

Nejasnosti ohledně popisů jednotlivých druhů a objevení nepopsaných mitochondriálních linií vedlo k nutnosti znovu a jasně definovat karase stříbřitého *C. gibelio* jak morfologicky, tak geneticky. K tomuto účelu byl jako neotypní jedinec vybrán diploidní samec z oblasti Slezska, které spadá do území Blochem popsaného jako *terra typica*. Vybraný jedinec byl definován geneticky (mtDNA) i morfologicky. Zároveň byla věnována pozornost spornému původu *C. gibelio* v Evropě: viz Příloha IV.

5) Daněk, T., Kalous, L., Veselý, T., Krásová, E., Reschová, S., Rylková, K., Kulich, P., Pokorná, D., Knytl, M. 2012. Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2). *Diseases of Aquatic Organisms* 102 (2), pp. 87-95

Detailní analýza malé uzavřené populace, která byla plánovanou součástí disertační práce, nebyla provedena kvůli náhlému úhynu karasů na vytipované lokalitě krátce po prvním odběru vzorků. Důvodem úhynu byla vlna vysoce patogenního viru CyHV-2, který působil selektivně pouze na polyploidní biotyp karase stříbřitého (*C. gibelio*) a způsobil jeho 100% úmrtnost nejen na dané lokalitě, ale i v okolních vodách: viz Příloha V.

6) Rylková, K., Kalous, L., Bohlen, J., Lamatsch, D.K., Petrtýl, M. 2013. Phylogeny and biogeographic history of the cyprinid fish genus *Carassius* (Teleostei: Cyprinidae) with focus on natural and anthropogenic arrivals in Europe. *Aquaculture* 380, pp. 13-20.

Rozsáhlý screening evropských populací karasů zaměřený na detekování mitochondriálních linií a analýzu ploidní úrovně v rámci jednotlivých linií přinesl několik poznatků: odhalil jednotlivé mitochondriální linie v lokalitách mimo doposud známé oblasti rozšíření (např. *C. gibelio* v severní Itálii, ve Finsku). Dále rozšířil poznatky o populaci nepůvodního karase ginbuny *C. langsdorfii*. Zjištěna byla existence triploidního karase

zlatého *C. auratus*, který byl doposud považován za striktně diploidního. Všichni triploidní jedinci pocházeli z ferálních a divokých populací; nejednalo se tedy o prošlechtěné okrasné formy. Dále byla potvrzena existence nepopsané linie „*M*“, a to i na území Evropy, čímž se počet druhů žijících v Evropských vodách zvyšuje na pět. Výskyt původního karase obecného byl vyhodnocen jako zřídkavý, což koresponduje se současným trendem – mizení. Zároveň byla jeho populace geneticky velmi variabilní: viz Příloha VI.

7) Knytl., M., Kalous, L., Symonová, R., Rylková, K., Ráb, P. 2013. Chromosome studies of European Cyprinid Fishes: Cross-species painting revers natural allotetraploid origin of a *Carassius* female with 206 chromosomes. *Cytogenetic and Genome Research* 139 (4), pp. 276-283

Kombinací analýzy molekulárních znaků a chromozomové skladby genomu byl odhalen hybridní původ tetraploidního jedince. Jako parentální generace byla identifikována triploidní, gynogeneticky se rozmnožující samice karase stříbřitého (*C. gibelio*) a diploidní, sexuálně se rozmnožující samec karase obecného (*C. carassius*). Výsledkem hybridizace byla samice mající 156 chromosomů od své matky a 50 chromosomů pocházejících od otce. Hybridní samice s celkem 206 chromosomy je tak allotetraploidem: viz Příloha VII.

8) Manuskript : Ribeiro, F., Rylková, K., Moreno, R., Carrapato, C., Kalous, L.: *Carassius gibelio*: a cryptic invader arriving to Iberian Peninsula

Na iberijském poloostrově byla tradičně díky příznivému klimatu zaznamenávána početná ferální populace karase zlatého *C. auratus*. V posledních letech, a to především v souvislosti s rozšířením mobility rekreačních a sportovních rybářů, se objevily pochybnosti, zda je populace místních karasů skutečně tvořena pouze karasem zlatým. Mitochondriální analýza 15-ti jedinců pocházejících z 5ti lokalit Španělska a Portugalska určila, že se jedná o invazního karase stříbřitého *C. gibelio*. Po rozšíření tohoto taxonu v oblasti iberijského poloostrova lze konstatovat, že invaze polyploidního biotypu *C. gibelio* na evropském kontinentu je kompletní: viz Příloha VII.

5.2. Konfrontace mitochondriální a jaderné analýzy

5.2.1. Mitochondriální analýza

Celková délka konečného alingmentu byla 1083 bází, z toho 786 nukleotidových pozic bylo konstantních. Variabilita byla prokázána u 297 pozic, přičemž 204 pozic bylo parsimonně informativních a 93 parsimonně neinformativních.

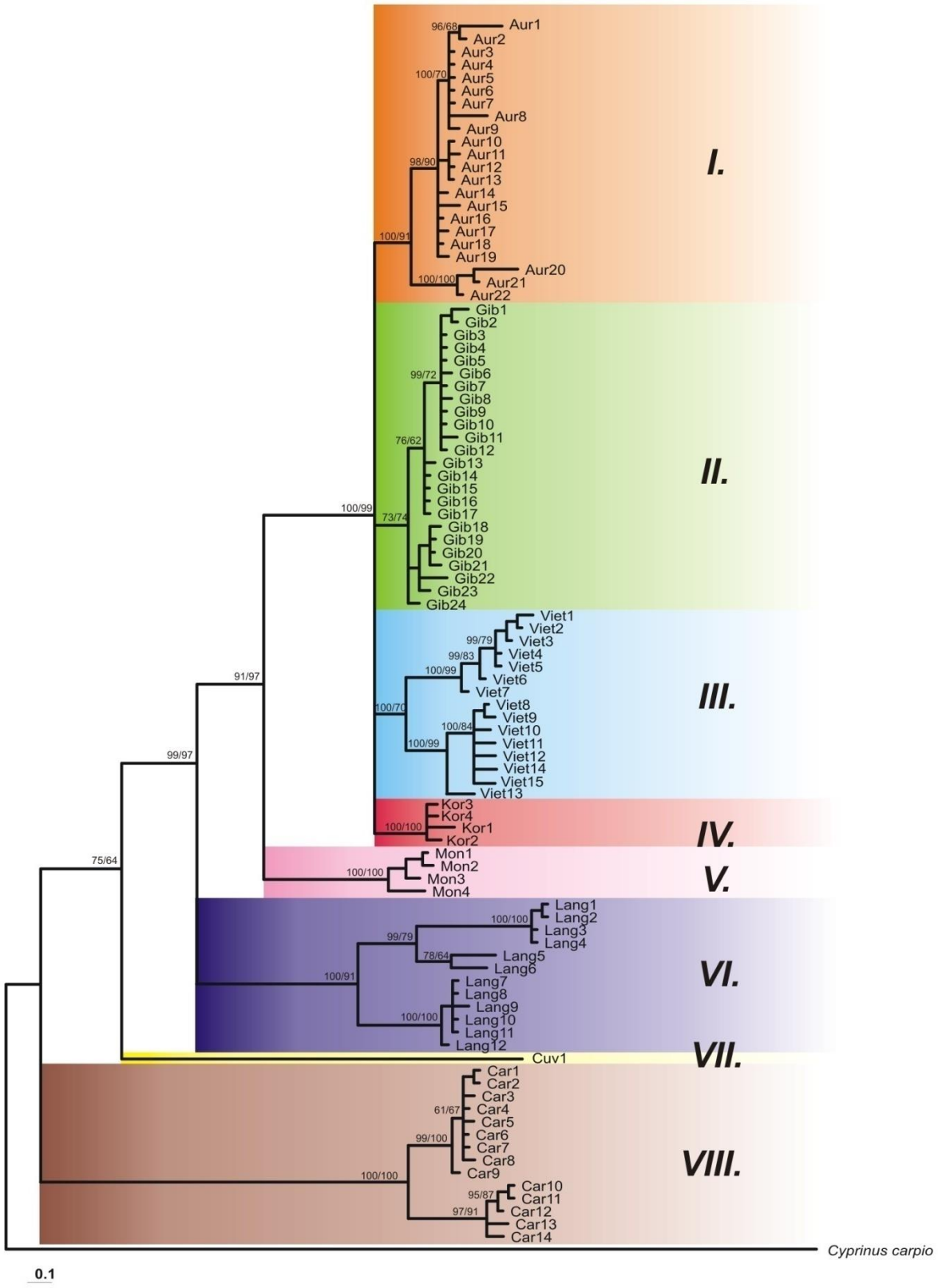
Analýza mitochondriálního genu pro cytochrom *b* rozdělila jedince do celkem osmi linií: *I*. *C. auratus, II*. *C. gibelio*, *III.* Linie „*V*“, *IV*. linie „*K*“, *V*. linie „*M*“, VI. *C. langsdorfii*, VII. *C. cuvieri* a VIII. *C. carassius*. Mitochondriální linie *I*., *II*. a *V*. –*VIII*. jsou definovány na základě taxonomické příslušnosti, linie *III*. a *IV*. sdružuje geografický původ a jsou odlišeny na základě genetické vzdálenosti, která je od ostatních linií odděluje na stejné úrovni, na které jsou od sebe separováni linie *C. auratus* a *C. gibelio*.

5.2.2. Jaderná analýza

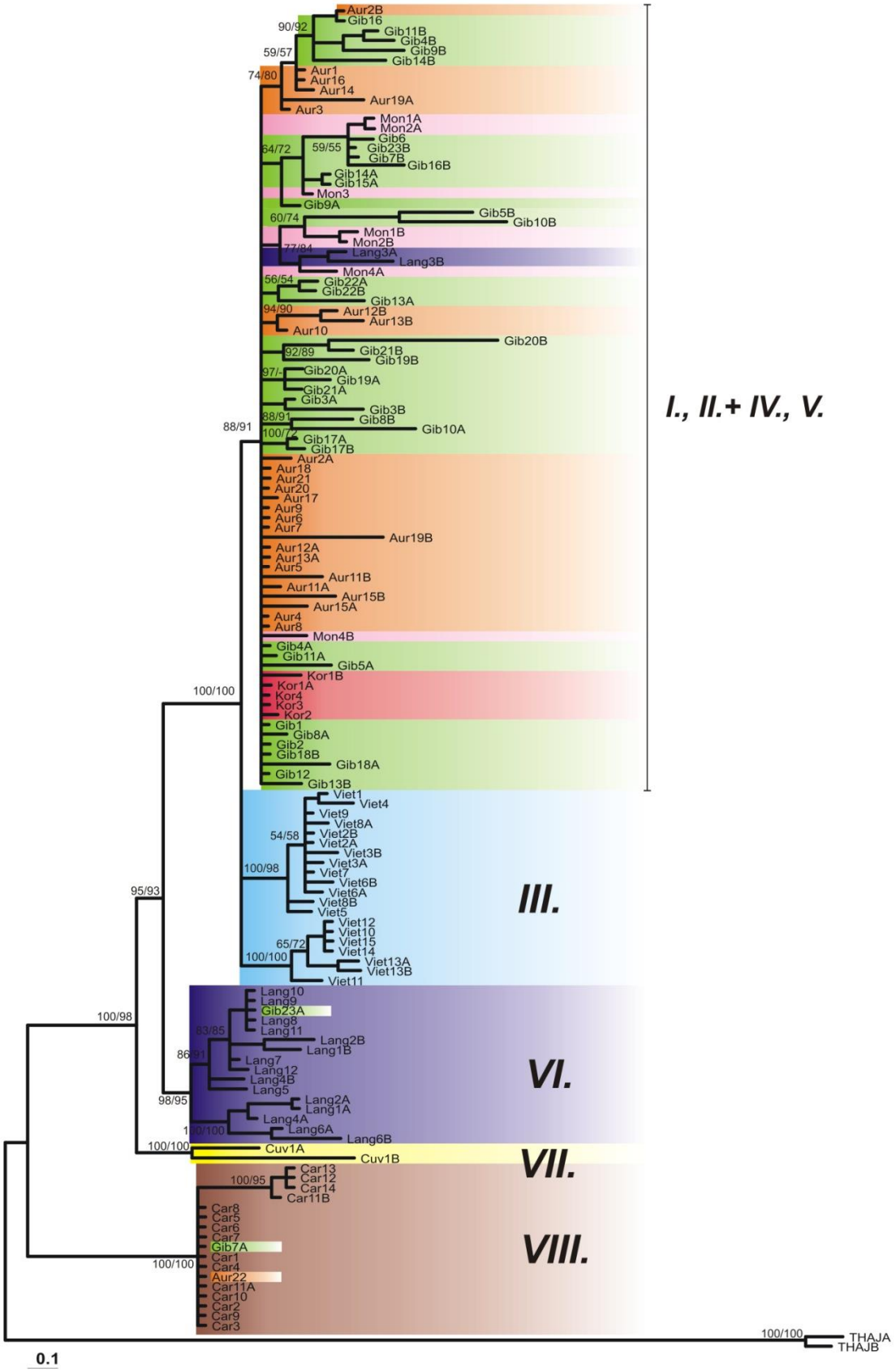
Celková délka konečného alingmentu byla 734 bází, z toho 427 nukleotidových pozic bylo konstantních. Variabilita byla prokázána u 307 pozic, přičemž 210 pozic bylo parsimonně informativních a 97 parsimonně neinformativních.

Výsledkem analýzy jaderného genu S7 je fylogenetický strom, který rozlišuje celkem pět klastrů. Tři z nich odpovídají mitochondriálním liniím *C. carassius*, *C. cuvieri* a *C. langsdorfii*. Další klastr tvoří linie *III „V“*, ale není monofyletický. Poslední klastr shlukuje mitochondriální linie *I.* - *C. auratus*, *II.* - *C. gibelio*, *IV.* - linie „*K*“ a *V.*- linie „*M*“.

Fylogenetický strom mitochondriálního genu pro cytochrom *b* je na obrázku 1. Fylogenetický strom jaderného genu S7 je na obrázku 2. Barevné značení jednotlivých vzorků odpovídá výsledkům mitochondriální analýzy. Hodnoty u jednotlivých větvení představují podporu bootsrappingu pro metody BAY/MP. Zobrazeny jsou hodnoty větší než 50%. Chybějící hodnoty jsou nahrazeny pomlčkou. Jako outgroup pro zakořenění stromů byly použity sekvence kapra obecného (*Cyprinus carpio*).



Obr. 1: Fylogenetický strom mitochondriální analýzy

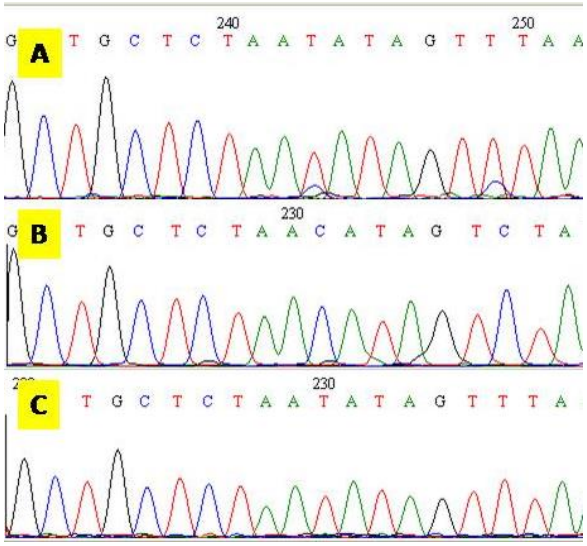


Obr. 2: Fylogenetický strom jaderného genu S7

Porovnání jaderného a mitochondriálního stromu ukazuje genetickou integritu *C. carassius*, *C. cuvieri, C. langsdorfii* a částečně i linie „V“.

V případě *C. carassius* je ve vzájemné shodě rozdělení větve na dvě části. Zajímavostí je i homogenita získaných jaderných sekvencí, které v případě karase obecného postrádali dvojtvárnost, a z každého vzorku s výjimkou Car11 byla získaná pouze jedna kopie. Z tohoto pohledu se *C. carassius* jeví jako homozygotní.

Ve dvou případech byla v jaderné linii *C. carassius* zaznamenána mitochondriální introgrese. Jedná se o jedince *C. auratus* - Aur22 a *C. gibelio* Gib7. Detailní analýza získaných sekvencí jaderných úseků ukázala, že v případě Aur22 se jednalo o homozygotního jedince. Zajímavější je případ vzorku Gib7. Jedná se o triploidního jedince, u něhož byly zaznamenány 2 kopie S7 *C. carassius* a jedna kopie *C. gibelio*. Tento fakt byl odhalen na základě poměru výšky genetického signálu, kdy „píky“ alel *C. carassius* (C) byly zhruba dvojnásobně vyšší, než „píky“ odpovídající *C. gibelio* (G) (obr. 3). Tento jev popisuje např. Janko *et al.* (2007b). Genetická skladba tohoto jedince by tak byla $CCG^{mtDNA\ G}$, což by znamenalo, že v parentální generaci muselo dojít ke spojení diploidní jikry (CG) a spermie s genomem (C). Tento způsob reprodukce byl popsán u některých jelců rodu *Leuciscus* (Alves *et al*., 2001). U karasů byla schopnost produkce neredukovaných diploidních gamet zjištěna při experimentálních pokusech, kdy byli záměrně kříženi karasi s kaprem (*Cyprinus carpio*) (Zhang *et al*., 2011). Diploidní gamety tvořili mezidruhoví hybridi v F2 a F3 generaci křížení.



Obr. 3: Porovnání sekvenačního signálu u jedince Gib7 (A) a odpovídající úseky vzorků Gib6 (B) a Car10 (C). Patrné jsou heterozygotní pozice 243 a 249.

Karas gengorobuna *C. cuvieri* se i přes malý vzorek vhodný k analýze jeví jako „dobrý“ druh. Stejně jako *C. carassius* si zachovává integritu i shodnou pozici v jaderném i mitochondriálním stromu. Ke stejnému výsledku došla rozsáhlá genetická studie Luo *et al.* (2014).

Stejné je to v případě linie *C. langsdorfii*. Obě analýzy se shodují v topologii větve v rámci celého stromu i v jejím vnitřním členění. Mitochondriální analýza jasně rozlišuje tři sublinie, které naznačují, že pod název *C. langsdorfii* se v současnosti zřejmě zahrnuje více druhů (Takada *et al*. 2010; Rylková *et al*., 2013). Jaderná analýza sice nezachovává přesné členění těchto tří sublinií, ale výraznější vnitří diverzitu zachovává. Stejně jako v případě *C. carassius* byla v jaderné linii *C. langsdorfii* (L) zaznamenána mitochondriální introgrese linie *C. gibelio*. Jedná se o jedince označeného jako Gib23. Ploidie tohoto jedince není známá a nebylo možné ji ani odhadnout na základě výšky „píků“. Jednalo se ale o heterozygota s kopiemi jaderného genu od obou rodičů ($LG^{mtDNA\ G}$).

Linie „*V*“ sdružující jedince z území Vietnamu a tří jedinců z hydrograficky přilehlých lokalit jižní Číny si také zachovává jak mitochondriální, tak jadernou integritu. Z pohledu jaderného lokusu ale není klastr monofyletický. Při podrobnějším pohledu na vnitřní strukturu linie „*V*“ je patrné, že obě analýzy jasně oddělili jedince podle geografického původu na dvě skupiny – „jižní“ a „severní“. V mitochondriálním stromu je rozdělení patrné uvnitř linie, kdežto jaderný strom tyto větve oddělil na vyšší úrovni. Identita této linie není známá. V roce 2001 popsali Nguyen et Ngo na území Vietnamu nový taxon, který nazvali *Carassius (auratus) argentheapthalmus.* V popisu tohoto taxonu uvádí, že jedním z hlavních rozlišovacích znaků *C. auratus* a *C. (a.) argentheapthalmus* je barva oční duhovky, kdy *C. (a). argentheapthalmus* má duhovku stříbrnou, zatímco *C. (a.) auratus* červenou. Z tohoto pohledu nelze linii „*V*“ s taxonem *C. (a.) argentheapthalmus* ztotožnit. Jedinci označení jako Viet10, Viet14 a Viet15 měli duhovku jasně červenou, zatímco ostatní jedinci stříbrnou; aniž by mezi nimi byl patrný genetický rozdíl. V práci Luo *et al.* (2014) se objevuje mitochondriální linie označená jako „C1“ sdružující jedince z Vietnamu a čínské provincie Fujian. Tato linie je s linií „*V*“ geneticky totožná a díky zahrnutí vzorkového materiálu z rozsáhlejší geografické oblasti ji dále vnitřně diverzifikuje.

Při porovnání topologií obou stromů je patrné, že mitochondriální linie *C. auratus*, *C. gibelio*, linie „*K*“ a linie „*M*“ postrádají v jaderném lokusu svou integritu. Nabízí se dvě základní možnosti, jak k tomuto stavu mohlo dojít.

A) Jedná o druhový komplex, slučující různé formy karasů z Evropy i Asie, které jsou v důsledku dlouhého samostatného evolučního vývoje poměrně hluboce mitochondriálně divergované, ale nesou blízce příbuznou jadernou DNA. Vysvětlením může být retence ancestrálního polymorfismu, kdy došlo v daném jaderném lokusu u těchto taxonů k neúplnému vysortování linií (incomplete lineage sorting, ILS).

B) Mohlo by se ale jednat i o blízce příbuzný druhový komplex, který vznikl na základě sekundárního genetického toku - mezidruhových hybridizací již oddělených taxonů. Četnost hybridizací setřela druhovou integritu, která je pozorovatelná v podobě mitochondriálních linií. V tomto případě bychom mohli mluvit o tzv. hybridním (genetickém) roji. Tento jev je známý například u vranek (*Cottus sp*.) (Stemshorn *et al*., 2011) nebo afrických cichlid (Seehausen, 2004). Teorii hybridního roje v případě karasů by podporovaly výsledky práce Luo *et al.* (2014).

V rámci tohoto klastru je dále zajímavá pozice linie „*M*“. Ta je z pohledu mitochondriálního stromu nejen jednoznačně oddělena, ale i vyčleněna ze skupiny *C. auratus* komplex, kdežto stopa jaderné DNA se v komplexu *C. auratus* ztrácí. Zatímco linie „*V*“, která je mitochondriálně na nižší taxonomické úrovni, si svoji jadernou integritu zachovala, linie „*M*“ se ztrácí ve směsném klastru. Tato skutečnost může být výsledkem jednak malého počtu analyzovaných jedinců a také pravděpodobně souvisí s neznámou oblastí původu linie „*M*“. Je možné, že dostupný materiál pochází z marginálních oblastí výskytu, kde se linie „*M*“ setkává s populacemi *C. gibelio* a *C. auratus* a z pozice invazního druhu se zde s těmito geneticky mísí. Tuto domněnku by potvrzoval případ jedinců linie „*M*“ pocházejících z České republiky (v této analýze zastoupeni vzorkem Mon3). Zde byli tito v malém počtu zaznamenáni v přírodní populaci diploidních *C. gibelio*. Podobným způsobem došlo pravděpodobně k introgresi jedince mitochondriálně určeného jako *C. langsdorfii* (Lang3) do směsného klastru.

Vzhledem k faktu, že podobná jaderná analýza nebyla dosud publikována, neexistují data, se kterými by bylo možné výsledky srovnat. Autoři Yamamoto *et al*. (2010) dospěli na základě analýzy polymorfismu délek amplifikovaných fragmentů (AFLP) k závěru, že výsledky

jaderných a mitochondriálních analýz v rámci rodu karas dávají principielně podobné výsledky. Stejné výsledky prezentují i Luo *et al*. (2014), kteří rekonstruovali fylogenezi karasů pomocí tří jaderných genů (MIF, StAR a GH).

6. Závěry a doporučení pro využití poznatků v praxi nebo pro další rozvoj oboru

Na základě těchto výsledků lze říct, že diverzita rodu je vyšší, centrem diverzity je jihovýchodní Asie. Za „dobré“ druhy lze zatím označit *C. carassius*, *C. cuvieri* a *C. langsdorfii*. Pro upřesnění statutu ostatních taxonů, zejména těch spadajících do skupiny *C. auratus* komplex, je zapotřebí detailnější a širší molekulární a cytologická analýza, přičemž větší vypovídací hodnotu bude mít spíše podrobnější studium vybraných jedinců, než letmý screening obsáhlého vzorkového materiálu. Cestou k odpovědím pravděpodobně bude sekvenování více jaderných genů s různou rychlostí mutace a zaklonování DNA do bakterií, které jednoznačně oddělí jednotlivé kopie genu u heterozygotních jedinců.

Výsledky pomohou vyřešit nejen otázky taxonomické. V současnosti jsou karasi jedním z nejpoužívanějších laboratorních modelů, který slouží při různorodých studiích nejen základního výzkumu. V těchto studiích ale není věnována jakákoliv pozornost genetické podstatě samotných použitých ryb; proto jsou porovnávány výsledky pokusů, které byly provedeny na geneticky rozdílných populacích. Práce jako tato může napomoci sjednocení a například i selekci konkrétních laboratorních kmenů.

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Many branches, one root: First evidence for a monophyly of the morphologically highly diverse goldfish (*Carassius auratus*)

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ABSTRACT

Goldfish is one of the most important pet and laboratory fishes of the world that is nowadays pan-globally distributed and well known to everybody. Despite the wide phenotypic variability of the ornamental forms, all goldfish are traditionally considered to be *Carassius auratus*, a species that developed via domestication from the Silver Prussian carp, *C. gibelio*. However, the postulated monophyly of goldfish has never been proven, and multiple domestication events may have occurred. Here we present the results of a reconstructed genealogy of 49 individuals of the genus *Carassius* based on unique sequences of mitochondrial gene cytochrome *b*. The samples originated from different parts of Eurasia and include different varieties of domesticated goldfish as well as feral populations and specimens of other representatives of the genus *Carassius*. The results indicate that goldfish indeed forms a monophyletic lineage and point on a single domestication event as source of all goldfish varieties. However, the monophyletic goldfish lineage was not nested within the samples of *C. gibelio*, but formed a sister lineage.

1. Introduction

The goldfish can be considered as the most well known fish in history (Balon, 2004). Today there is no other ornamental fish so popular and easy to obtain as goldfish. Due to its easy availability and hardiness, goldfish became one of the most commonly used laboratory animals. Plenty of scientific studies, especially in the field of physiology, used goldfish as animal model. For the comparability of the results of such studies carried out in different laboratories across the whole world it is crucial to ensure that they have used the same animal model. However, often enough the experimental animals are bought just from the next local ornamental fish dealer and without any knowledge of its origin or if it forms a monophyletic lineage with other strains of the same animal.

The goldfish was described by Linnaeus (1758) on the basis of an orange coloured specimen with trilobed caudal fin. It is generally considered to represent a domestic form of the silver Prussian carp, *C. gibelio* (Gentry et al., 2004) although unsaid; this assumption implies that all goldfish represent a monophyletic lineage despite the impressive phenotypic variability of the ornamental forms. However, this monophyly has never been proven, and since *C. gibelio* has a vast area of distribution across most of Eurasia (Kottelat and Freyhof, 2007), multiple domestication events may have occurred. In a similar way, multiple origins have recently been detected for other domesticated animals, namely sheep, goat and dog. (Luikard et al., 2001; Pedrosa et al., 2005; Hiendleder et al., 2002; Vilà et al., 1999). In these cases, a phylogenetic analysis of specimens with different geographic origins has revealed several lineages inside the domesticated species that can only be explained by multiple domestication events. The multiple origins were not detected by analysis of morphologic data, but of genetic characters, namely by analysis of mitochondrial sequence data. Especially in morphologically variable animals like fancy goldfish, genetic makers can be expected to be much more reliable than morphologic characters.

Today there are more than 300 morphologic forms of fancy goldfish known (Brokenshire, 2008) and it would be impossible to include them all into a single study. However, to cover the broad morphologic diversity of goldfish in a genetic study, the sampling scheme has to focus on three groups of samples: First, a number of most advanced forms (those with aberrations of vertebral column, absence of fins and duplication and enlargement of other fins), because these have passed through careful human care during the last centuries and can be considered pure-blooded. Second, the simplest morphs of goldfish have to be included; this means specimens that differ only in colour from wild goldfish. These morphs represent the most ancestral morphologic type and were for long time the only known form of domestic goldfish. These simply red morphs could have developed several times independently from a brown ancestral species and therefore are necessary in a study testing the possibility of a multiple origin of goldfish. Third, some samples of feral populations of goldfish have to be included to find its phylogenetic relations to

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Table 1
Origin of the samples.

Scientific name	Breed	Location	GenBank accession no. (reference)	Haplotype
Carassiu auratus	Shubunkin	Ornamental fish trade	EU663576[a]	H1
	Lionhead	Ornamental fish trade	EU663578[a]	H1
	Telescope eye	Ornamental fish trade	EU663579[a]	H1
	Telescope eye	Ornamental fish trade	EU663580[a]	H1
	Ryukin	Ornamental fish trade	EU663581[a]	H1
	Ryukin	Ornamental fish trade	EU663582[a]	H1
	Lionhead	Ornamental fish trade	EU663583[a]	H1
	Oranda	Ornamental fish trade	EU663585[a]	H1
	Shubunkin	Ornamental fish trade	EU663589[a]	H1
	Ranchu	Ornamental fish trade	EU663590[a]	H1
		Ornamental fish trade	DQ399923 (Kalous et al., 2007)	H1
		Ornamental fish trade	DQ868927[a]	H1
		Ornamental fish trade	DQ868928[a]	H1
	Oranda	Ornamental fish trade	EU663584[a]	H2
	Panda	Ornamental fish trade	EU663586[a]	H2
	Panda	Ornamental fish trade	EU663587[a]	H2
	Panda	Ornamental fish trade	EU663588[a]	H2
	Shubunkin	Ornamental fish trade	EU663574[a]	H3
	Telescope eye	Ornamental fish trade	EU663575[a]	H4
	Lionhead	Ornamental fish trade	EU663577[a]	H5
		Wuhan (Yangtze basin, China)	EU663599[a]	H6
		Nanking (Yangtze basin, China)	EU663597[a]	H8
		Nanking (Yangtze basin, China)	EU663598[a]	H8
		Vodňany (Elbe basin, Czech Republic)	DQ868897[a]	H1
		Guadiana (Guadiana basin, Portugal)	DQ868906[a]	H1
		Guadiana (Guadiana basin, Portugal)	DQ868907[a]	H1
	Telescope eye	Shizuoka (Japan)	AB379915 (Komiyama et al., 2009)	H1
	Shubunkin	Shizuoka (Japan)	AB379916 (Komiyama et al., 2009)	H1
	Ranchu	Shizuoka (Japan)	AB379917 (Komiyama et al., 2009)	H1
	Chotengan	Shizuoka (Japan)	AB379918 (Komiyama et al., 2009)	H1
	Oranda	Shizuoka (Japan)	AB379919 (Komiyama et al., 2009)	H1
	Ranchu	Shizuoka (Japan)	AB379920 (Komiyama et al., 2009)	H1
	Chinese Ranchu	Guangzhou (China)	AB379921 (Komiyama et al., 2009)	H1
		Kai-Ping (China)	AB379922 (Komiyama et al., 2009)	H7
	Hongmao	Ornamental fish trade	EU528842	–
	Honglongjing	Ornamental fish trade	EU528843	–
	Hutou	Ornamental fish trade	EU528844	–
	Liujin	Ornamental fish trade	EU528845	–
	Molong	Ornamental fish trade	EU528846	–
	Shuipao	Ornamental fish trade	EU528848	–
	Zhenzu	Ornamental fish trade	EU528849	–
Carassius gibelio		Sevarova jagura (Cetina basin, Bosnia and Hercegovina)	EU663591[a]	H9
		Dyje River (Danube basin, Czech Republic)	DQ399939 (Kalous et al., 2007)	H10
		Canal de Fougéres (Loire basin, France)	EU663594[a]	H10
		Shanghai (Yangtze basin, China)	DQ868918[a]	H11
		Pengze Lake (China)	EU528847	–
Carassius langsdorfii		Chrudimka River (Elbe basin, Czech Republic)	DQ399930 (Kalous et al., 2007)	–
Carassius cuvieri		Unknown	AB045144	–
Carassius carassius		Milevsko (Elbe basin, Czech Republic)	DQ399938 (Kalous et al., 2007)	–
Cyprinus carpio		Yangtze River, China	AY347291	–
		Germany	AY347293	–
	Koi	Japan	AY347285	–
		Lysimacheia Lake, Greece	DQ868872 (Tsipas et al., 2009)	–

[a] This study.

other species of *Carassius*. These relations may be more difficult to extract from domestic forms due to strong selection.

In the present study, we reconstructed a phylogeny of representatives of the genus *Carassius* in order to test the phylogenetic origin of the goldfish. We included in the study a number of different breeds of the goldfish, the more simple ones, highly bred forms and some wild populations. We tested the proposed monophyly of the domestic goldfish as well as its phylogenetic relation to C. gibelio.

2. Materials and methods

2.1. Samples

Altogether, we have analysed a set of 53 samples: 49 individuals of the genus *Carassius* including 36 specimens of the most common breeds of Goldfish (Oranda, Telescope, Lionhead, Ranchu, Ryukin, Panda and Shubunkin) and 13 specimens coming from wild or feral population of Eurasia. As outgroup we have used four individuals of common carp, *Cyprinus carpio*. In addition to our original samples the dataset contains 18 sequences obtained from GenBank. Detailed information about material used in this study is listed in Table 1.

2.2. DNA isolation, PCR amplification, and sequencing

Genomic DNA was isolated from ethanol preserved tissue using DNeasy Tissue Kit (Qiagen) according to manufacturer's instructions.

With regard to expected genetic distances within the genus *Carassius* we have chosen analysis based on comparison of mitochondrial gene cytochrome *b* which is a very common marker for phylogenetic and biogeographic studies on closely related species and intraspecific studies (Avise, 1986; Boore and Brown, 1998).The mitochondrial gene cytochrome *b* was amplified using the primers Glu L. Ca14337-14359: GAA GAA CCA CCG TTG TTA TTC AA and Thr H. Ca15568-15548: ACC TCC RAT CTY CGG ATT ACA (Šlechtová et al., 2006). PCR amplification was performed in 50 µl reaction volumes containing 10 mM Tris–HCl, 50 mM $(HN_4)_2SO_4$, 0.1% Triton X-100, 1.2–1.8 mM $MgCl_2$, 2 mM TMA oxalate (PCR enhancer), 10 nmol of each nucleotide, 2.5 U Taq polymerase (all chemicals by Top-Bio), and 25 pmol of each primer. The PCR profile (carried out on MJ Research thermocycler) started with 10 min period of initial denaturation at 95 °C, followed by 34 cycles each consisting of denaturation step at 94 °C for 30 s, a primer annealing step at 54 °C for 30 s and an elongation step at 72 °C for 1 min. PCR was terminated by final elongation period of 72 °C for 10 min. PCR products were purified

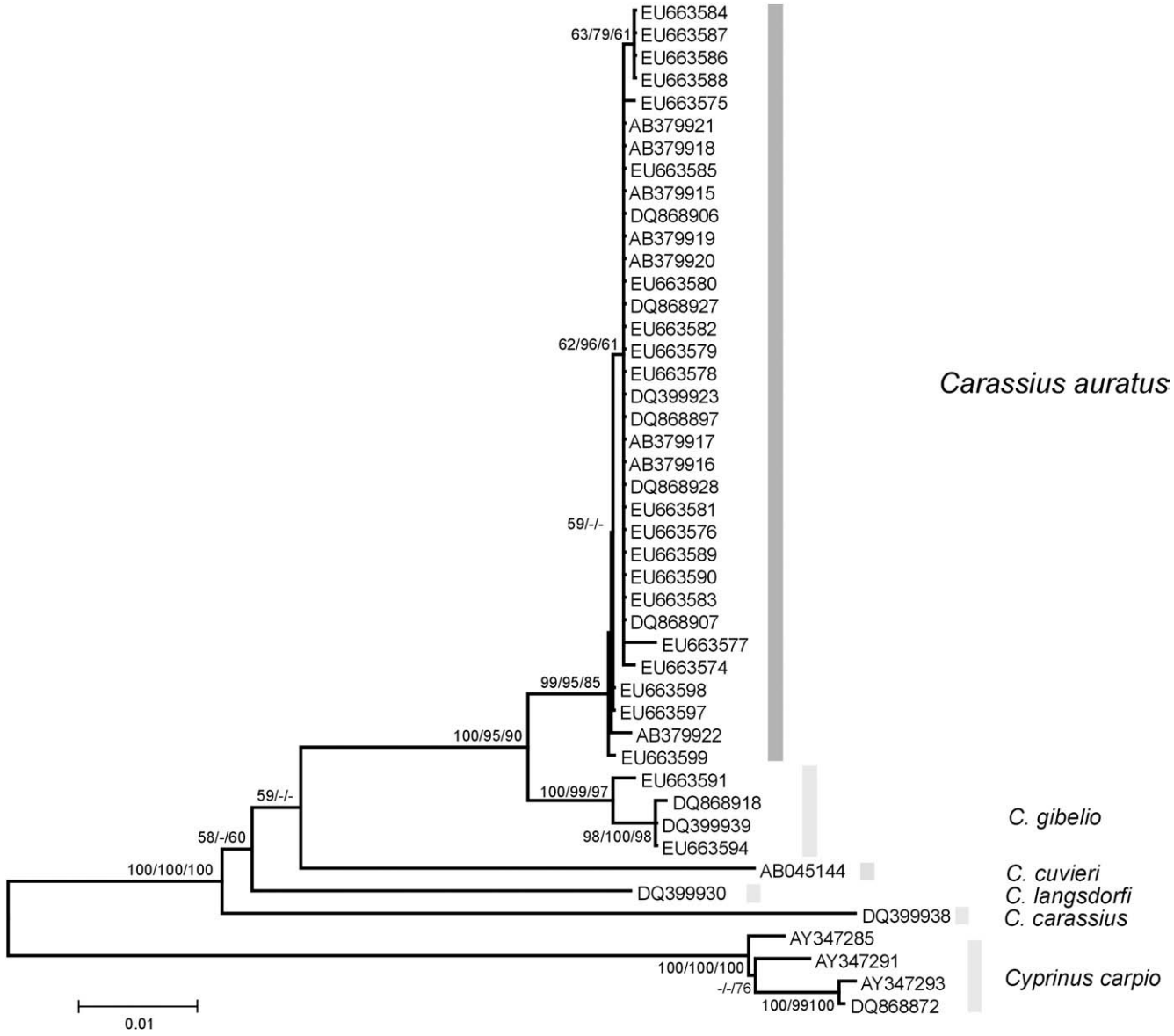

Fig. 1. Reconstructed phylogeny of the cyt *b* sequences of *Carassius* included into the present study resulting from the analyses of "the long" dataset. The numbers at the nodes represent statistical supports for NJ, BAY and ML, respectively. The bootstrap supports below 50 and Bayesian posterior probabilities below 0.75 are not shown or are represented by a dash.

with QIAquick PCR Purification Kit (Qiagen). Sequencing reaction of purified PCR products was performed with BigDye™ Terminator Cycle Sequencing Kit 1.1 (BE Applied Biosystems) according to manufacturer´s instructions. Sequencing products purified with DyeEx Spin Kit (Qiagen) were resolved on 3130 Genetic Analyser (3130 GA, Hitachi, Applied Biosystem). Each sample was sequenced from both (3′ and 5′) ends of the both fragments with the same primers as used for double strand PCR amplification.

2.3. Molecular data analyses

The raw chromatograms were manually assembled and checked by eye for potential mistakes using the computer software BioEdit 5.0.9 (Hall, 1999); the same program that was used to align the sequences using the ClustalW algorithm. In order to determine the best fitting model of nucleotide substitution for the model-based methods of phylogenetic analysis, the aligned sequences were tested by the program Modeltest 3.06 (Posada and Crandall, 1998). Under Akaike Information Criterion, the general time reversible model with gamma distribution of rate heterogeneity (GTR + Γ) was selected.

We have created two datasets; one using only those 41 sequences of *Carassius* that are longer than 1111 basepairs (plus four outgroup sequences of *C. carpio*). The second dataset included additional shorter sequences available from GenBank (altogether 49 ingroup and four outgroup taxa) but had to be shortened to 597 basepairs (the shared gene stretch).

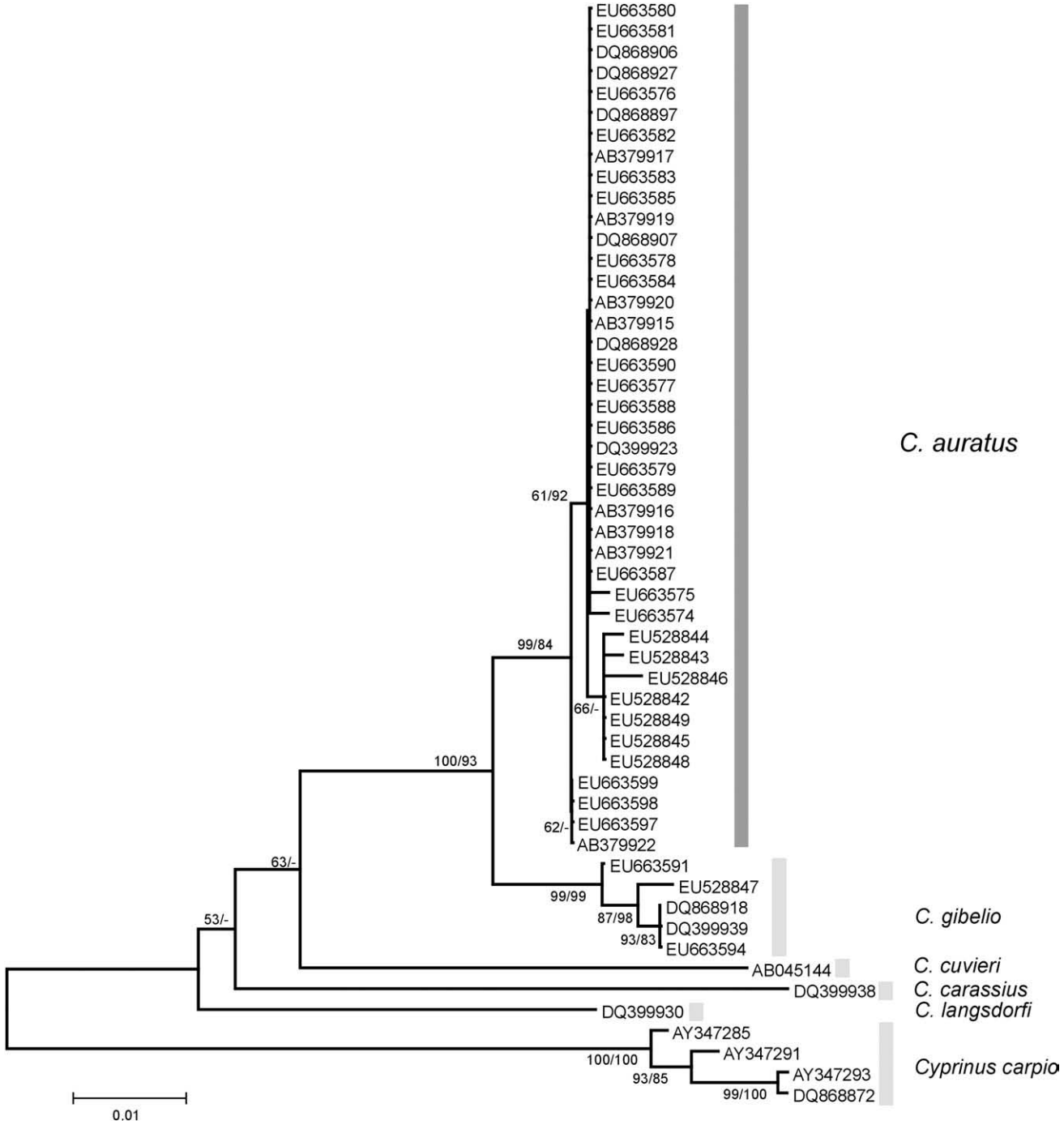

Fig. 2. Reconstructed phylogeny of the cyt *b* sequences of *Carassius* resulting from the analyses of "the short" dataset. The numbers at the nodes represent statistical supports for NJ, BAY and ML, respectively. The bootstrap supports below 50 and Bayesian posterior probabilities below 0.75 are not shown or are represented by a dash.

Statistical information on the dataset and estimates of haplotype (h) and nucleotide (π) diversities (Nei, 1987) across the dataset as well as within the *C. auratus* lineage were obtained with DNsp 4.10.3 (Rozas et al., 2005).

The phylogenetic relationships were estimated using the methods of neighbour joining (NJ) in PAUP* version 4.0b10 (Swofford, 2000), Bayesian analyses (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck and Ronquist, 2001) and maximum likelihood analyses (ML) were performed in GARLI v. 0.95 (Zwickl, 2006; www.bio.utexas.edu/faculty/antisense/garli/Garli.html). For the model-based methods, the datasets were analysed for the best fitting evolutionary model of nucleotide substitution using Modeltest 3.06 (Posada and Crandall, 1998). The NJ analyses were performed under best-fit model and the node support of tree generated by NJ analyses was assessed by 1000 non-parametric bootstrap replications. The Bayesian analyses were based on estimated models (GTR + Γ) taking into consideration six rate categories and the gamma distribution of mutation rates. Starting from a random tree, two parallel runs, each consisting of six Monte Carlo Markov Chains (MCMC) were running simultaneously for 1,000,000 generations with sampling frequency of trees as well as likelihood scores of 100. The number of trees to discard was estimated from log-likelihood plots. From the resulting trees, 500 were discarded as "burnin" and the remaining trees were used to build a 50% majority rule consensus tree. The Bayesian posterior probabilities were used to indicate branch supports. Posterior probabilities of 90% and larger were considered as significant. ML analyses were performed under best-fit model with parameters estimated by GARLI no starting topology was specified. To assess the statistical support of the nodes, we used 1000 non-parametric bootstrap resamplings for each of the datasets, and the resulting trees were used to build 50% majority rule consensus trees in PAUP*.To estimate the "fine scaled" relationships among *C. auratus* and *C. gibelio* haplotypes, we constructed a haplotype network (Fig. 3.) employing the statistical parsimony (Templeton et al., 1992) implemented in the TCS 1.21 program (Clement et al., 2000). The connection limit was set to 20 mutation steps.

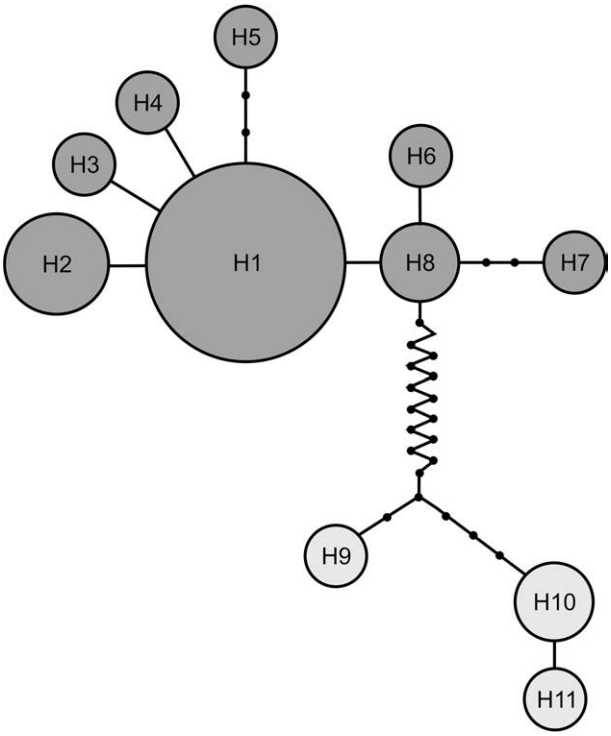

Fig. 3. Unrooted haplotype network based on cyt *b* sequences of analysed *C. auratus* and *C.gibelio*. The haplotype numbers refer to H-numbers in Table 1. The circle area is proportional to the haplotype frequencies.

3. Results

In the first dataset consisting of 1111 bp there were 172 variable (15.5%) and out of that 65 parsimony informative sites (5.9%). The analysed sequences showed a very low nucleotide diversity ($\pi = 0.01$); $\pi < 0.001$ within the *C. auratus* clade. The shorter dataset (597 bp) contained 134 variable and 92 parsimony informative sites, which correspond to 16.4 and 6.7%, respectively.

All three phylogenetic analyses methods — NJ, BAY and ML, recovered trees with congruent topologies that agreed on five well supported lineages within the analysed samples of *Carassius*: *C. carassius*, *C. langsdorfi*, *C. cuvieri*, *C. gibelio* and *C. auratus*. *C. auratus* and *C. gibelio* represent sister lineages that are reciprocally monophyletic with mean uncorrected genetic distance $p = 0.02$ (Figs. 1, 2).

Within the lineage consisting of sister clades *C. auratus* and *C. gibelio* we identified altogether 11 haplotypes among 38 sequences of these two taxa: eight haplotypes of *C. auratus* and three haplotypes of *C. gibelio*. The minimal separation between any two haplotypes from these two taxa was 18 mutation steps (Fig. 3).

4. Discussion

Despite substantial morphological variability among the sampled individuals, the analyses of sequence of mitochondrial gene cytochrome *b* have shown that all analysed samples of goldfish form a monophyletic lineage, independently if they expressed simple morphological changes like only a orange colour of body and fins (e.g. Oranda form) or massive morphologic rearrangements including shape of vertebral column, absence or doubling of fins or shape of eye (e.g. Lion head, Ranchu). Also all specimens from European feral populations fell into this monophyletic lineage, strengthening the assumption that these populations originated from released fancy goldfish. Consequently, the present data do not give indication that the domestic animal generally known as goldfish includes more than one lineage. Since we used a mitochondrial marker it at least demonstrates that all analysed samples have the same maternal origin. Most likely, all ornamental goldfish are the result of a single domesticating event. Including the GenBank sequences has shown that one specimen (AB379922) treated in GenBank under the taxonomic identity as *C. auratus gibelio* belongs in our results to the lineage of *C. auratus* and one specimen (EU528847) treated under the taxonomic name *C. auratus* belongs to *C. gibelio* lineage. Both samples are from China where wild *C. auratus* and *C. gibelio* co-occur. Moreover taxonomical disagreements are known from variable literature (Kottelat, 1997) as well as morphological similarities between feral *C. auratus* and *C. gibelio* (Hensel, 1971; Kottelat and Freyhof, 2007). We suppose that this is a case of misidentification or taxonomical confusion.

The monophyletic lineage of goldfish turned out to represent a sister clade to another monophyletic lineage that includes all specimens of silver Prussian carp. The separation of the two types of *Carassius* into two separate lineages does not support the hypothesis that the domestic goldfish is a morph of silver Prussian carp. In this case, the phylogenetic position of goldfish would be nested inside silver Prussian carp. The observed separation of goldfish and silver Prussian carp rather indicate that they represent two distinct species. This conclusion is further supported by the basal position of the wild goldfish population within the goldfish lineage. The wild goldfish from the Yangtze River basin (EU663597–EU663599) are basal to all domestic forms, supporting the hypothesis that a wild ancestor of goldfish that is different from the silver Prussian carp exists in central China. Interestingly, the domestication of goldfish is believed to have started in the Yangtze River basin, in the cities Kiahsing, Nanking and Hangchow (Balon, 2004). Here, xanthic aberrations of wild fishes have been kept in so-called "ponds of mercy" during early Sung

dynasty (960 to 1279 CE). Since the beginning of the 12th century only xanthic fishes were selected to be bred in ponds, marking the beginning of selected domestication of goldfish (Hofmann and Novák, 1996). Around the year 1500, the goldfish was imported to Japan and became as popular as in China (Pereira, 1937). Our data show that Chinese, Japanese and European goldfish of simple as well as of advanced breeds still form a monophyletic lineage and conclude that a single domestication event has occurred in this important domestic animal.

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BRIEF COMMUNICATION

New mtDNA data reveal a wide distribution of the Japanese ginbuna *Carassius langsdorfii* in Europe

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In this study, records on the occurrence of the Japanese ginbuna *Carassius langsdorfii* from northern Germany, north-western Italy and southern Bosnia and Herzegovina are presented. The new findings, in addition to former studies reported in the Czech Republic and Greece, show that *C. langsdorfii* is much more widespread in Europe than was previously believed.

Key words: alien species; introduction; molecular genetics; ornamental fish.

The genus *Carassius* includes four species that are distributed across most of Europe (Kottelat & Freyhof, 2007). Two species, the Crucian carp *Carassius carassius* (L. 1758) and the Prussian carp *Carassius gibelio* (Bloch 1782) have been recorded in European waters over a long period of time, while the goldfish *Carassius auratus* (L. 1758) was domesticated in China and introduced to Europe in the 17th century (Kottelat & Freyhof, 2007). Recent analyses revealed the existence of many feral populations of *C. auratus* in southern Europe and in the British Isles (Economidis *et al*., 2000; Doadrio, 2001; Hänfling *et al*., 2005; Ribeiro *et al*., 2009; Rylková *et al*., 2010). In 2007, two specimens of the Japanese ginbuna *Carassius langsdorfii* Temminck & Schlegel 1846 were recorded from the Elbe River system in the Czech Republic (Kalous *et al*., 2007). Evidence for the existence of *C. langsdorfii* in western Greece came from mitochondrial (mt) DNA sequences analysed by Tsipas *et al*. (2009) and Takada *et al*. (2010). *Carassius gibelio*, *C. auratus* and *C. langsdorfii* are morphologically very similar and often a reliable identification on the basis of morphological characters is not possible. Therefore, genetic characters, namely mtDNA sequences, are used for the first identification that can be cross-checked with morphological characters when necessary.

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The process by which *C. langsdorfii* entered Europe remains unclear; it was most probably introduced by unintended imports together with koi carps *Cyprinus carpio* L. 1758 (Kalous *et al*., 2007). This article gives additional findings of *C. langsdorfii* in European open water systems which suggest that the species is more widespread and common than was formerly believed.

Altogether, 37 specimens of *Carassius* spp. from 13 European and two Asian countries were included in this analysis (Table I). The mt *cytochrome b* gene was amplified using the methods described in the study of Rylková *et al*. (2010), using primers Glu L. Ca14337-14359: GAA GAA CCA CCG TTG TTA TTC AA and Thr H. Ca15568-15548: ACC TCC RAT CTY CGG ATT ACA (Šlechtová *et al*., 2006). The phylogenetic relationships were estimated from aligned sequences using the method of maximum parsimony (MP) performed in PAUP* version 4.0b10 (Swofford, 2000) and Bayesian analyses (BAY) using the programme MrBayes ver. 3.0 (Huelsenbeck & Ronquist, 2001) as described by Šlechtová *et al*. (2004). Both reconstructions reveal trees of very similar topologies with high statistical support. A sequence of common carp *C. carpio* was used as the outgroup.

The final matrix of the *cytochrome b* sequences consists of 1080 characters and contains 245 (22·7%) variable characters and 155 (14·4%) parsimony informative sites. All the methods used for phylogenetic analysis sort the sequences into five well-supported lineages corresponding to *C. carassius*, *C. langsdorfii*, *Carassius cuvieri* Temminck & Schlegel 1846, *C. auratus* and *C. gibelio* (Fig. 1). The lineage of *C. langsdorfii* is divided into three distinct sublineages. One of the sublineages (sublineage 2; Fig. 1) contains only specimens from Hokkaido and Honshu Islands (Japan), sublineages 1 and 3 (Fig. 1) contain only European samples or a mix of European and Japanese samples, respectively.

The results of this study indicate that *C. langsdorfii* has a much wider distribution in Europe than originally known. Besides previous records from the Czech Republic and Greece, the new data reveal occurrence of *C. langsdorfii* also in northern Germany, north-western Italy and the Neretva basin in Bosnia and Herzegovina. As there are currently no known characters that allow easy and unambiguous identification of *C. langsdorfii* in the field, these records were gathered randomly and the species might occur in many more places in Europe than are currently identified.

The mt haplotypes of the identified specimens of *C. langsdorfii* in this study belong to two groups that differ from each other in *c*. 13 mutation steps. One group (sublineage 1) includes all specimens from Germany and the Czech Republic as well as two specimens from Greece. The sister sublineage to this clade is formed by specimens from two localities in Japan. The other haplotype group comprises specimens from Europe, including all specimens from Italy and Bosnia and Herzegovina, together with four specimens from Greece and one specimen from Japan. The close relation of all European populations of *C. langsdorfii* with populations from distant sites in Japan confirms the assumption that the species is not native to Europe, but was introduced from Japan. In addition, the presence of two distant genetic lineages in Europe, in combination with the wide distribution in Europe, indicates that the species was introduced from more than one place of origin in Japan, and to more than one place of destination in Europe. As the species of *Carassius* are not traded as food fishes in Europe, and as there is very limited intentional stocking, it is more likely that the species was accidentally introduced together with other fishes from Japan. The most important fishes for export from Japan to Europe are *C. auratus*

TABLE I. Specimens of *Carassius* used for mtDNA analyses. The sequence of *Cyprinus carpio* was used as an outgroup. Code numbers marked (*) represent new data

Scientific name	Location	GenBank number	Reference
Carassius langsdorfii	Neretva River, Sunca canal, BIH	GU942709*	
	Rama Lake, BIH	GU942710*	
	Fish market, Thessaloniki, GR	GU942711*	
	Lysimacheia Lake, GR	DQ868876	Tsipas *et al*. (2009)
	Trichonida Lake, GR	DQ868877	Tsipas *et al*. (2009)
	Ozeros Lake, GR	DQ868878	Tsipas *et al*. (2009)
	Amvrakia Lake, GR	DQ868879	Tsipas *et al*. (2009)
	Abashiri Lake, Hokkaido, J	DQ399920	Kalous *et al*. (2007)
		DQ399921	
		DQ399922	
	Chrudimka River, Bojanov, CZ	DQ399930	Kalous *et al*. (2007)
		DQ399932	
	Lysimacheia Lake, GR	EU186830	Tsipas *et al*. (2009)
	Urano River, Honshu, J	AB368688	Takada *et al*. (2010)
	Meletta River, Carmagnola, I	HM000036*	
		HM008691*	
	Kühren Lake, Preetz, D	GU942707*	
		GU942708*	
	Floodplain, Chomoutov, CZ	FJ169953	Papoušek *et al*. (2008)
	Okinawa, J	AB368679	Takada *et al*. (2010)
Carassius carassius	Kühren Lake, Preetz, D	DQ399917	Kalous *et al*. (2007)
	Fish farm, Milevsko, CZ	DQ399938	Kalous *et al*. (2007)
	Fish farm, Calverton, GB	GU991400*	
Carassius cuvieri	Unknown	AB045144	
Carassius gibelio	Nitra River, SK	DQ868911*	
	Dyje River, CZ	HM000031*	
	Danube River, Olteniţa, RO	HM000008*	
	Shanghai, CN	DQ868918	Rylková *et al*. (2010)
	Chrudimka River, Bojanov, CZ	DQ399929	Kalous *et al*. (2007)
	Sluch River, Krasna Siolka, UA	DQ868903*	
	Loire River, Canal de Fougéres, F	EU663594	Rylková *et al*. (2010)
Carassius auratus	Guadiana River, P	DQ868906	Rylková *et al*. (2010)
	Yangtze River, Wuhan CN	EU663599	Rylková *et al*. (2010)
	Yangtze River, Nanking CN	EU663597	Rylková *et al*. (2010)
	Okinawa, J	AB368696	Takada *et al*. (2010)
	Ohrid Lake, AL	GU991386*	
	Skadar Lake, MNE	GU991388*	
Cyprinus carpio	Mekong River, T	HM008692*	

AL, Albania; BIH, Bosnia and Herzegovina; CN, China; CZ, Czech Republic; D, Germany; F, France; GB, Great Britain; GR, Greece; I, Italy; J, Japan; MNE, Monte Negro; P, Portugal; RO, Romania; SK, Slovakia; T, Thailand; UA, Ukraine.

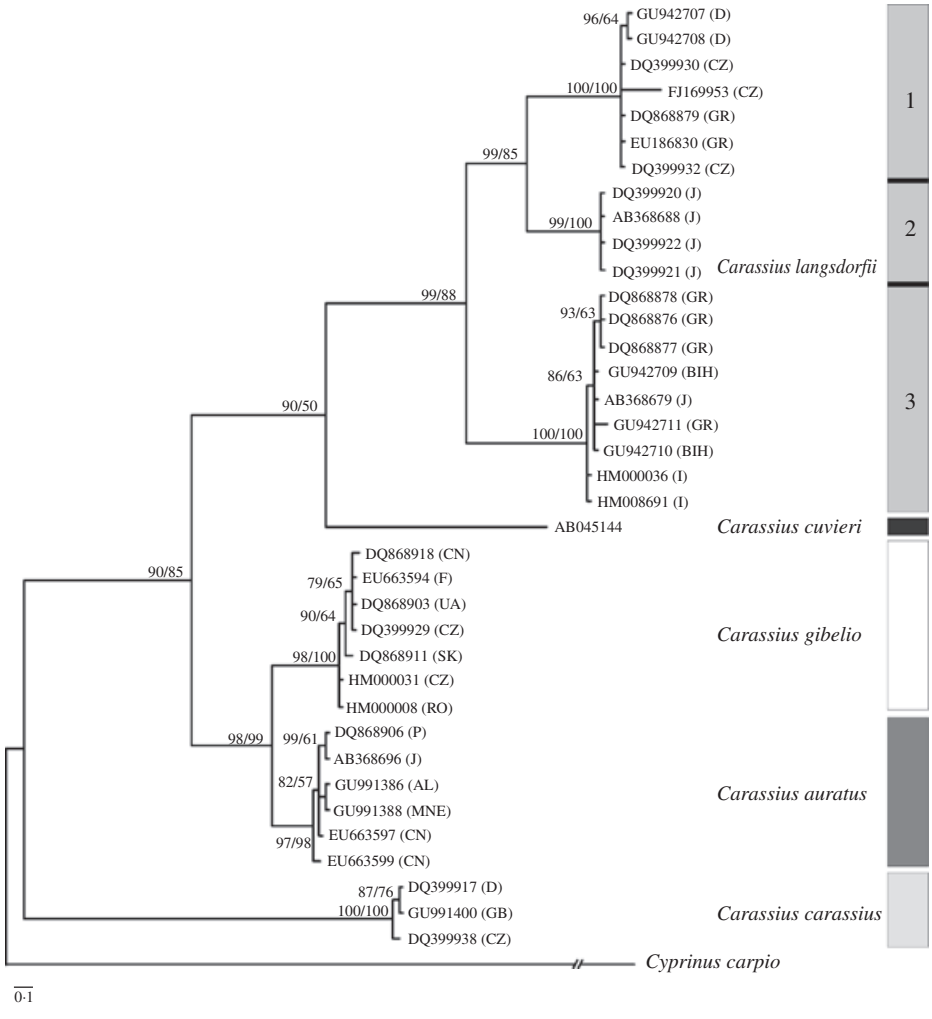

FIG. 1. Reconstructed phylogeny of the *cytochrome b* sequences of species of *Carassius*. The numbers at the nodes represent statistical supports for Bayesian and maximum parsimony analyses, respectively. The bootstrap supports <50 and Bayesian posterior probabilities <0·75 are not shown.

and koi carp *C. carpio*. Unintended introductions might have come with imports of juveniles of these species.

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NEW FINDING OF NON-INDIGENOUS JAPANESE CYPRINID FISH IN THE CZECH REPUBLIC*

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Ginbuna *Carassius langsdorfii* endemic species of Japanese archipelago have been found in European waters. The origin of European records and the environmental impact remain unclear. The present paper reports on the population of this species discovered in a small natural pool in South Bohemia. Genetic markers were employed since morphological characters failed in species identification. Although the population was clustered in the mitochondrial lineage of *C. langsdorfii*, genetic distance and morphological difference were found largely significant when comparing to other ginbuna individuals found in Europe. The phylogenetic position is further discussed.

Carassius; introduction; South Bohemia; cytochrome *b*; phylogeny

INTRODUCTION

Although the Czech waters are not much rich for freshwater ichthyofauna in worldwide context, they encompass a high percentage of non-native fishes that are represented by 41 species (Lusk et al., 2010). The reasons for introductions in the last century were mainly aquacultural as well as experimental to fill empty niches in semi-natural environment of Czech rivers and streams. Some introductions were also promoted to satisfy the enlarging community of recreational fishermen as it was for example in the case of *Oncorhynchus mykiss* (Lusk et al., 2010).

One of the globally successful genera that undergone naturalization in many places of the world is the genus *Carassius* (Brumley, 1996; Dyer, 2000; Elvira, 2001; Copp et al., 2005; Musil et al., 2010).

Four species of the genus *Carassius* (*sensu* Rylková et al., 2010) are recognized in the Czech water bodies: Crucian carp *C. carassius* (L.), invasive gynogenetic biotype of Prussian carp *C. gibelio* (Bloch, 1782), domesticated or feral forms of introduced Goldfish *C. auratus* (L.), and recently recorded ginbuna *C. langsdorfii* (Temminck, Schlegel, 1842). The last three mentioned species, namely *C. gibelio*, *C. auratus,* and *C. langsdorfii,* are included in so called *Carassius auratus* complex, mainly because of their morphological similarity, hybridization, and not completely solved taxonomical status (Takada et al., 2010).

C. gibelio and *C. langsdorfii* are characteristic for their capability of clonal reproduction via gynogenesis, occurrence of all female populations consisting of polyploid individuals which sexually parasite on other cyprinid fishes (e.g. Gui, Zhou, 2010). These features, like all female population and clonality, allow rapid invasive spreading into new areas and led to the consideration of fishes from *C. auratus* complex as of animals with high environmental impact (Savini et al., 2010).

The ginbuna originated from Japanese archipelago where it is considered a common species (Hosoya, 2000) but its appearance in Europe was evaluated as accidental and rare (Kalous et al., 2007). The same authors tentatively attributed its introduction to Europe as results of the Koi carps imports. However, after Kalous et al. (2007), another finding from the Elbe River basin was recorded in Greek lakes (Tsipas et al., 2009; Takada et al., 2010). Latest screening of Kalous et al. (2013) revealed the presence of *C. langsdorfii* at other five European localities. Beside the south European countries (Italy, Bosnia and Herzegovina, and Greece) it was found also in northern Germany. Another finding of *C. langsdorfii* from South Bohemia with distinct morphology and phylogenetic position is presented herein.

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MATHERIAL AND METHODS

Samples

In 2007 several fish of atypical appearance (Fig. 1) were caught in a small natural pool near Litvínovice (South Bohemia; 48°57′34.96″N, 14°27′13.70″E). These individuals were brown-green at dorsal side and dark yellow up to orange at ventral side. All fins were reddish-brown in colour. Upper edge of the dorsal fin was slightly concave up to almost straight. Number of scales in lateral line 30–33; number of scales both above and below lateral line 6–7; number of dorsal fin rays III 17 ($n = 6$). With respect to this, morphological characters were not typical of any *Carassius* species occurring in European waters. Since morphological characters are known to be not much reliable in determination of species within the genus *Carassius* (Hensel, 1971; Vasileva, 1990), molecular markers were employed to identify the species affiliation.

Altogether 41 specimens of *Carassius* were included into the analysis. As outgroup, the sequence of common carp *Cyprinus carpio* was used. Detailed information on the samples origin and GenBank Accession Nos. are listed in Table 1.

DNA isolation, PCR amplification and sequencing

Genomic DNA was isolated from ethanol preserved tissue using DNeasy Blood and Tissue Kit (Qiagen, Valencia, USA) according to manufacturer's instructions. Mitochondrial gene cytochrome *b* was amplified using the forward primer Kai_F (5′ GAA GAA CCA CCG TTG TTA TTC 3′) and reverse primer Kai_R (5′ TTA GTT TCT TTT CCT CCG CT 3′) (Šlechtová et al., 2006). PCR was performed in 50 µl reaction volumes as described in Rylková et al. (2010). The PCR profile (carried out on MJ Mini thermocycler, Bio-Rad, Hercules, USA) started with 10 min period of initial denaturation step at 94°C, followed by 34 cycles, each consisting of denaturation step at 94°C for 30 s, a primer annealing step at 54°C for 30 s, and an elongation step at 72°C for 1 min. PCR was terminated by final elongation period at 72°C for 10 min. PCR products were purified and sequenced from both (3′and 5′) ends of fragments using the same pair of primers as used for double strand PCR amplification. Purification and sequencing were performed by Macrogen Inc., Seoul, Korea.

Fig. 1. Specimen from the Litvínovice pool (photo by J. Okrouhlík)

Molecular data analyses

The raw chromatograms were manually assembled and checked by eye for potential mistakes using the computer software BioEdit 5.0.9. (Hall, 1999); the same program was used to align the sequences using the ClustalW algorithm.

The phylogenetic relationships were estimated using the methods of maximum parsimony (MP) in PAUP*, version 4.0b10 (Swofford, 2000) and Bayesian analysis (BAY) using the program MrBayes, version 3.0 (Huelsenbeck, Ronquist, 2001) as described in Rylková et al. (2010).

To estimate the "fine scaled" relationships among *C. langsdorfii* haplotypes, we constructed a haplotype network employing the statistical parsimony (Templeton et al., 1992) implemented in the TCS 1.21 program (Clement et al., 2000). The connection limit was set to 20 mutation steps.

RESULTS AND DISCUSION

The final matrix of the cytochrome *b* sequences consisted of 1082 basepairs containing 255 variable characters with 159 parsimony informative sites. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted the sequences into 5 well-supported lineages corresponding to *C. langsdorfii*, *C. auratus*, *C. gibelio*, *C. cuvieri*, and *C. carassius,* respectively (Fig. 2).

There are 21 haplotypes within the clade of *C. langsdorfii* showing high genetic diversity within this taxon. The whole lineage is clearly divided into 3 clusters: cluster *I* (haplotypes 1–12), cluster *II* (haplotypes 13–15), and cluster *III* (haplotypes 16–21). Specimens coming from South Bohemia belong to haplotype *Clan16* nested in cluster *III*.

Haplotype network analysis (Fig. 3) divided the lineage of *C. langsdorfii* into three separate groups corresponding to cluster *I*, *II,* and *III* of the phylogenetic tree.

The presented analysis showed a high phylogenetic diversity within the lineage of *C. langsdorfii*. Specimens coming from South Bohemia are quite distant from those recorded in the upper part of the Elbe Basin (Kalous et al., 2007) what is further accompanied by different values on morphological characters. Origin of the fish from both Czech findings remains unclear, but most probably each population belongs to different introduction events. The South Bohemian population

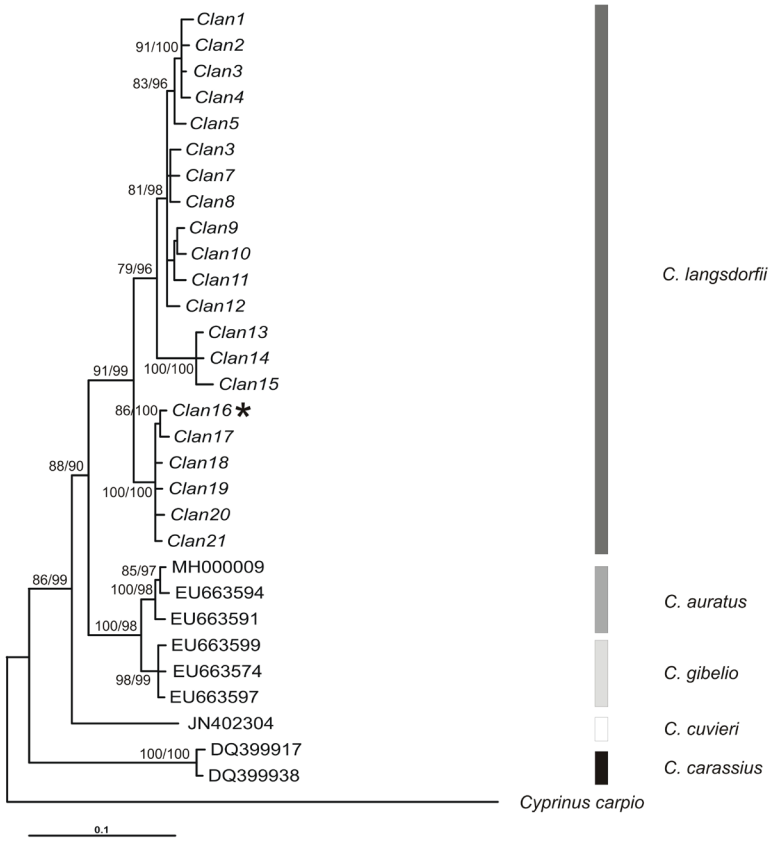

Fig. 2. Reconstructed phylogeny of the cyt b sequences of Carassius included into the present study. Numbers at the nodes represent statistical supports for maximum parsimony (MP) and Bayesian analysis (BAY), respectively. *haplotype including the fish from Litvínovice

(*Clan16*) is clustered with samples deriving from Ryukyu Island (*Clan16*, *Clan18*-*Clan21*) but that from the Chrudimka River (*Clan13*) is linked to samples from Honshu Island (*Clan15*). The population of *C. langsdorfii* from several Greek lakes is also interesting (T s i p a s et al., 2009). Part of it shares the same haplotype with the specimens from the Chrudimka River, while the other part (*Clan17*) is very closely related to South Bohemian population. This indicates that both clusters of *C. langsdorfii* are more spread in European waters.

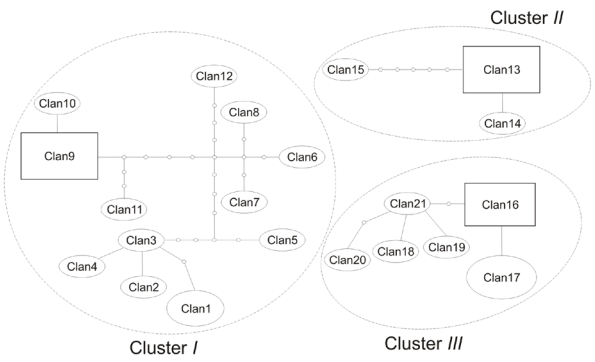

Fig. 3. Unrooted haplotype network based on cyt b sequences of C. langsdorfii analyzed. The haplotype numbers refer to numbers in Table 1. and Fig. 2. The oval area is proportional to the haplotype frequencies

The haplotype analysis sorted the samples of the *C. langsdorfii* lineage into three separate groups. This fact further supports the presumption that *C. langsdorfii* may consist of more taxa. It has already been mentioned by M u r a k a m i et al. (2001) and T a k a d a et al. (2010) that several species are probably taxonomically treated under the name *C. langsdorfii*. This fact must be firstly proven and resolved at the place of natural occurrence of these fishes. Having in mind all the above-mentioned information, we recommend to treat the population found in South Bohemia as *Carassius* cf. *langsdorfii* since its taxonomical status seems to be problematic.

The population of ginbuna has shown itself ecologically very strong in a small pool in the inundation area; in fact it was dominant throughout many years of observations (1999–2006). It represented the majority (> 50%) of the fish community; the supplemental species were topmouth gudgeon (*Pseudorasbora parva*) and common tench (*Tinca tinca*). Ginbuna was apparently reproducing itself in the pool as the young-of-the-year fish always dominated. The dominant position of ginbuna was most likely supported by the harsh oxygen conditions during the winter to which the *Carassius* species are known to be more tolerant than the other fish (B l a ž k a , 1958; B l a ž k a et al., 2006). The pool at Litvínovice was flooded by the Vltava River water

Table 1. Material used for the genetical analyses

Species	Haplotype	Frequency	GenBank Acc. No.	Origin	Reference
C. langsdorfii	*Clan1*	2	AB368693	Kako River, Honshu, Japan	Takada et al. (2010)
			JN412527	Kako River, Honshu, Japan	present study
	Clan2	1	AB368690	Biwa Lake, Honshu, Japan	Takada et al. (2010)
	Clan3	1	AB368692	Urano River, Honshu, Japan	Takada et al. (2010)
	Clan4	1	AB368694	Shimanto, Shikoku, Japan	Takada et al. (2010)
	Clan5	1	AB368695	Tanegashima Island, Japan	Takada et al. (2010)
	Clan6	1	AB368683	Okinawa Island, Japan	Takada et al. (2010)
	Clan7	1	AB368686	Shigenobu, Shikoku, Japan	Takada et al. (2010)
	Clan8	1	AB368684	Urano River, Honshu, Japan	Takada et al. (2010)
	Clan9	4	DQ399920	Abashiri Lake, Hokkaido, Japan	Kalous et al. (2007)
			DQ399921	Abashiri Lake, Hokkaido, Japan	Kalous et al. (2007)
			DQ399922	Abashiri Lake, Hokkaido, Japan	Kalous et al. (2007)
			AB368688	Urano River, Honshu, Japan	Takada et al. (2010)
	Clan10	1	AB368687	Nagara, Honshu, Japan	Takada et al. (2010)
	Clan11	1	AB368689	Urano River, Honshu, Japan	Takada et al. (2010)
	Clan12	1	AB368685	Tanegashima Island, Japan	Takada et al. (2010)
	Clan13	4	DQ399930	Chrudimka River, Czech Republic	Kalous et al. (2007)
			DQ399932	Chrudimka River, Czech Republic	Kalous et al. (2007)
			EU186830	Lysimacheia Lake, Greece	Tsipas et al. (2009)
			DQ868879	Amvrakia Lake, Greece	Tsipas et al. (2009)
	Clan14	1	AB368677	Taktsu, Honshu, Japan	Takada et al. (2010)
	Clan15	1	FJ169953	floodplain, Chomutov, Czech Republic	Papoušek et al. (2008)
	Clan16	3	JN412529	pool at Litvínovice, Czech Republic	present study
			JN412530	pool at Litvínovice, Czech Republic	present study
			AB368679	Okinawa Island, Japan	Takada et al. (2010)
	Clan17	3	DQ868878	Ozeros Lake, Greece	Tsipas et al. (2009)
			DQ868877	Trichonida Lake, Greece	Tsipas et al. (2009)
			DQ868876	Lysimacheia Lake, Greece	Tsipas et al. (2009)
	Clan18	1	AB368681	Amami-oshima Island, Japan	Takada et al. (2010)
	Clan19	1	AB368682	Tokunoshima Island, Japan	Takada et al. (2010)
	Clan20	1	AB368680	Okinawa Island, Japan	Takada et al. (2010)
	Clan21	1	AB368678	Iki Island, Japan	Takada et al. (2010)
C. auratus			EU663574	pet shop, Czech Republic	Rylková et al. (2010)
			EU663599	Wuhan, Yangtze, China	Rylková et al. (2010)
			EU663597	Nanking, Yangtze, China	Rylková et al. (2010)
C. gibelio			EU663591	Cetina River, Bosnia and Herzegovina	Rylková et al. (2010)
			EU663594	Canal de Fougeres, Loire River, France	Rylková et al. (2010)
			HM000009	Czerskie Rumunki, Poland	Kalous et al. (2012)
C. cuvieri			JN402304	Lake Mikatako, Honshu	Kalous et al. (2012)
C. carassius			DQ399938	Milevsko, Elbe drainage, Czech Republic	Kalous et al. (2007)
			DQ399917	pond, Plon, Germany	Kalous et al. (2007)
Cyprinus carpio			HM008692	Mekong River, Thailand	Kalous et al. (2012)

during the 1000-year flood in 2002 (it is located at the inundation area). During this event ginbuna offspring could colonize many other locations in the Vltava catchment. Other fish could colonize the pools during the flooding but the apparently vanished and were not found in subsequent sampling during 2003 and 2006 (Kubečka, Okrouhlík, personal communication). These facts indicate that ecological impact of ginbuna on original ichthyofauna is probably significant and worth of further following up.

CONCLUSION

C. langsdorfii is most probably more widespread than has recently been known but its existence seems to remain hidden usually due to mistaken identity based on morphological similarity with the other species of the genus *Carassius*.

It seems to be important to gain more data for estimating its possible ecological impacts in newly inhabited areas, e.g. food competition, sexual parasitism or genetic contamination of native European populations of other *Carassius* species via hybridization (Hänfling et al., 2005). Unfortunately, until now the information on *C. langsdorfii* in European waters has been very limited.

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Hidden diversity within the Prussian carp and designation of a neotype for *Carassius gibelio* (Teleostei: Cyprinidae)

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A phylogeny of the genus *Carassius* using the mitochondrial cytochrome *b* gene supported the monophyly and distinctness of the species *C. carassius*, *C. auratus*, *C. langsdorfii* and *C. cuvieri*. In contrast, the samples of *C. gibelio* did not form a monophyletic lineage, but separated into two clades, suggesting the inclusion of two species under the name *C. gibelio*. In order to clarify the identity of *C. gibelio*, a neotype is designated and briefly described.

Introduction

The cyprinid genus *Carassius* is widespread across Europe and North and East Asia. At least five species are considered as valid: *C. carassius* (Linnaeus, 1758) in most of Europe and western Siberia (Kottelat & Freyhof, 2007), *C. langsdorfii* (Temminck & Schlegel, 1846) and *C. cuvieri* (Temminck & Schlegel, 1846) in Japan (Hosoya, 2002; Yamamoto et al., 2010), *C. auratus* (Linnaeus, 1758) in mainland East Asia (Rylková et al., 2010) and *C. gibelio* (Bloch, 1782) in Europe, Siberia and Northeast Asia (Berg, 1949; Kottelat & Freyhof, 2007; Szczerbowski, in Bănărescu & Paepke, 2002). Some authors recognise additionally the species *C. grandoculis* and *C. buergeri* from Japan (Kawanabe & Mizuno, 1989; Suzuki et al., 2005). *Carassius argenteaphthalmus* Nguyen & Ngo, 2001 from Northern Vietnam is too poorly described to comments on its identity or validity.

Due to the high morphological similarity between species of *Carassius* and the intraspecific variability of morphological characters (Hensel, 1971; Lusk & Baruš, 1978; Vasileva, 1990; Vasileva & Vasilev, 2000) the definition of species is not always sure, especially in the case of the most widespread species *C. gibelio* and *C. auratus*. In the case of *C. gibelio*, the situation is more complicated by the simultaneous occurrence of diploid ($2n = 100$) and triploid ($2n \approx 150$) individuals in many populations (Halačka et al., 2003; Lusková et al., 2004; Abramenko et al., 2004; Mezherin & Lisetskii, 2004; Apalikova et al., 2008). The triploid individuals are usually females that reproduce asexually by gynogenesis and represent clonal lineages (Golovinskaya et al., 1965; Peňáz et al., 1979; Gui & Zhou, 2010), but triploid males have been reported also (Halačka et al., 2003; Abramenko et al., 2004). Kottelat & Freyhof (2007) pointed out that the conspecificity of

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populations with different mode of reproduction or different ploidy level remains to be demonstrated. During the last years, genetic markers have been established that allows differentiation between species. Such genetic analyses have recently shown that *C. auratus* represents a monophyletic lineage that is distinct from *C. gibelio* (Rylková et al., 2010). A number of local lineages within *C. langsdorfii* in Japan, uncovered first by morphological analyses (Hosoya, 2002), later accompanied by genetic data (Takada et al., 2010; Yamamoto, 2010); and genetic data have revealed the existence of at least two lineages among the *C. langsdorfii* that have been introduced to Europe (Kalous et al., 2007; Takada et al., 2010; Tsipas et al., 2009).

While the diversity of *Carassius* in Japan has been objected in several studies (Murakami et al., 2001; Iguchi et al., 2003; Yamamoto et al., 2010; Takada et al., 2010), most *Carassius* in mainland Eurasia are still referred to as *C. gibelio*, despite the fact that the monophyly of these populations has never been confirmed. Kottelat (1997, 2006) pointed out that the basal problem is the poor definition of *C. gibelio*, this means the identity of the species that was described by Bloch (1782) from a European population (in 'Schlesien') under the name *Cyprinus gibelio*.

In the present note we report the presence of two independent lineages within *C. gibelio* as revealed by an analysis of mitochondrial cytochrome *b* sequences. In order to clarify the taxonomic status of *C. gibelio*, a neotype is designated and briefly described.

Material and methods

Phylogenetic analyses. Thirty-four specimens of *Carassius* from European and Asian countries were included in the analysis. In addition to our original samples the dataset contains four sequences coming from previous studies and four sequences obtained from GenBank. As outgroup we have used sequence of common carp, *Cyprinus* sp. Detailed information about used material is listed in Table 1.

Genomic DNA was isolated from ethanol preserved or fresh tissue using DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The mitochondrial cytochrome b gene was amplified using primers Glu L. Ca14337-14359: GAA GAA CCA CCG TTG TTA TTC AA and Thr H. Ca15568-15548: ACC TCC RAT CTY CGG ATT ACA (Šlechtová et al., 2006). PCR amplification was performed in 50 µl reaction volumes containing 15.5 µl Combi ppp Master Mix (Top-Bio s.r.o., Praha, Czech Republic), 3 µl of each primer and template DNA. The PCR profile started with 10 min period of initial denaturation at 95 °C, followed by 34 cycles each consisting of denaturation step at 94 °C for 30 s, annealing step at 54 °C for 30 s and elongation step at 72 °C for 1 min. PCR was terminated by final elongation step at 72 °C for 10 min. PCR was carried out on MJ Mini thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea.

The raw chromatograms were manually assembled and checked by eye for potential mistakes using computer software BioEdit 5.0.9 (Hall, 1999); the same program was used to align sequences using the ClustalW algorithm.

The phylogenetic relationships were estimated from aligned sequences using the method of maximum parsimony (MP) performed in PAUP* version 4.0b10 (Swofford, 2000) and Bayesian analysis (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck & Ronquist, 2001) as described in Šlechtová et al. (2004).

Morphological data. Measurements and counts were done according to Kottelat & Freyhof (2007) using digital callipers. All measurements were recorded to the nearest 0.1 mm. Number of fin rays, vertebrae and ribs were taken from digital high-resolution radiographs, using a digital X-ray device Faxitron LX-60.

Ploidy determination. The ploidy level was determined using the measurements of erythrocyte nuclei area by computer-assisted image analyses as was proposed by Flajšhans (1997). Prior to any handling, the fish were anaesthetized with 0.6 $ml \cdot l^{-1}$ 2-phenoxyethanol (Merck KGaA). Blood was taken from the heart by a heparinised syringe; blood smears were prepared on clean microscope slides one for each specimen and fixed by few drops of 90 % ethanol. Slides were stained in a 20 % Giemsa-Romanowski solution for 10 minutes. Computer-assisted image analysis was carried out using a system that was composed from a microscope Nikon Eclipse 600 with immersion objective 100 ×, an analogue video camera Hitachi HVC 20 and the software L.U.C.I.A

ver. 4.71. The mean area of nuclei was calculated from 247 erythrocytes of the neotype and 220 and 200 erythrocytes of diploid and triploid reference specimens, respectively. The triploid reference specimen (156 chromosomes) originated from Řehačka backwater (alluvium of Elbe River), Central Bohemia, Czech Republic 50°10'39"N 14°48'27"E and it is deposited in the National Museum Prague (NMP P6V140484). The diploid reference specimen is a goldfish *(Carassius auratus)* var. Oranda (100 chromosomes) from petshop in Prague. Karyotype analyses of reference specimens were performed according to Ráb & Roth (1988). The calculated nuclei areas were compared by t-test in programme STATISTICA ver. 9.

Results

The final matrix of the cytochrome b sequences consisted of 931 characters containing 222 variable characters with 157 parsimony informative sites. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted sequences into six well-supported lineages (Fig. 1).

The neotype specimen had a mean erythrocyte nuclei area of 15.32 μm^2 (SD 2.06 μm^2), which is significantly smaller (t-test, $p < 0.01$) than erythrocyte nuclei of the triploid reference specimen (21.58 μm^2, SD 3.12 μm^2) and corresponds to the values of the diploid reference specimens

Table 1. Material used for the genetic analyses. Sources: a, Takada et al. (2010); b, Rylková et al. (2010); **c,** Kalous et al. (2007); n, GenBank database – unpublished; *, present study.

taxon	Acc. No.	source	origin
Carassius auratus	EU663599	b	Wuhan, Yangtze River, China
	GU991398	*	Gyeongju , Miho-cheon River, Korea
	EU663597	b	Nanking, Yangtze River, China
	GU991392	*	Nanking, Yangtze River, China
	GU991386	*	Ochrid Lake, Albania
	GU991390	*	Ishem River, Albania
	GU991395	*	Prespa Lake, Greece
	EU663574	b	pet shop, Czech Republic
	GU991391	*	Shuchinsk, Ishim River drainage Kazakhstan
C. gibelio I	HM000009	*	Czerskie Rumunki, Vistula River, Poland
	HM000020	*	Haaslava, Estonia
	GU170378	n	Volga River, Russia
	FJ822041	n	Hanka (Khanka) Lake, Primorye, Russia
	FJ478019	n	Lake near Dalnegorsk, Primorye, Russia
	AB368700	a	Amur River, Russia
	HM000008	*	Olteniţa, Danube River, Romania
	HM008678	*	Varna, Bulgaria
	JN402305	*	neotype; Český Těšín, Olza River, Czech Republic
	HM008684	*	Byur Lake, Amur drainage, Mongolia
	HM008685	*	Byur Lake, Amur drainage, Mongolia
C. gibelio II	DQ868924	*	Uvs Lake, Mongolia
	DQ868925	*	Uvs Lake, Mongolia
	DQ868926	*	Uvs Lake, Mongolia
	HM008690	*	Bulgan, Selenga River, Mongolia
C. langsdorfii	AB368690	a	Honshu, Biwa Lake, Japan
	DQ399920	c	Hokkaido, Abashiri Lake, Japan
	AB368677	a	Taktsu, Honshu, Japan
	AB368678	a	Iki Island, Japan
	AB368680	a	Okinawa Island, Japan
C. cuvieri	AB045144	n	unknown
	JN402304	*	Honshu, Lake Mikatako, Japan
C. carassius	DQ399917	c	Plön, Germany
	GU991400	*	Calverton, Great Britain
	DQ399938	*	Milevsko, Elbe drainage, Czech Republic
Cyprinus sp.	HM008692	*	Mekong River, Thailand

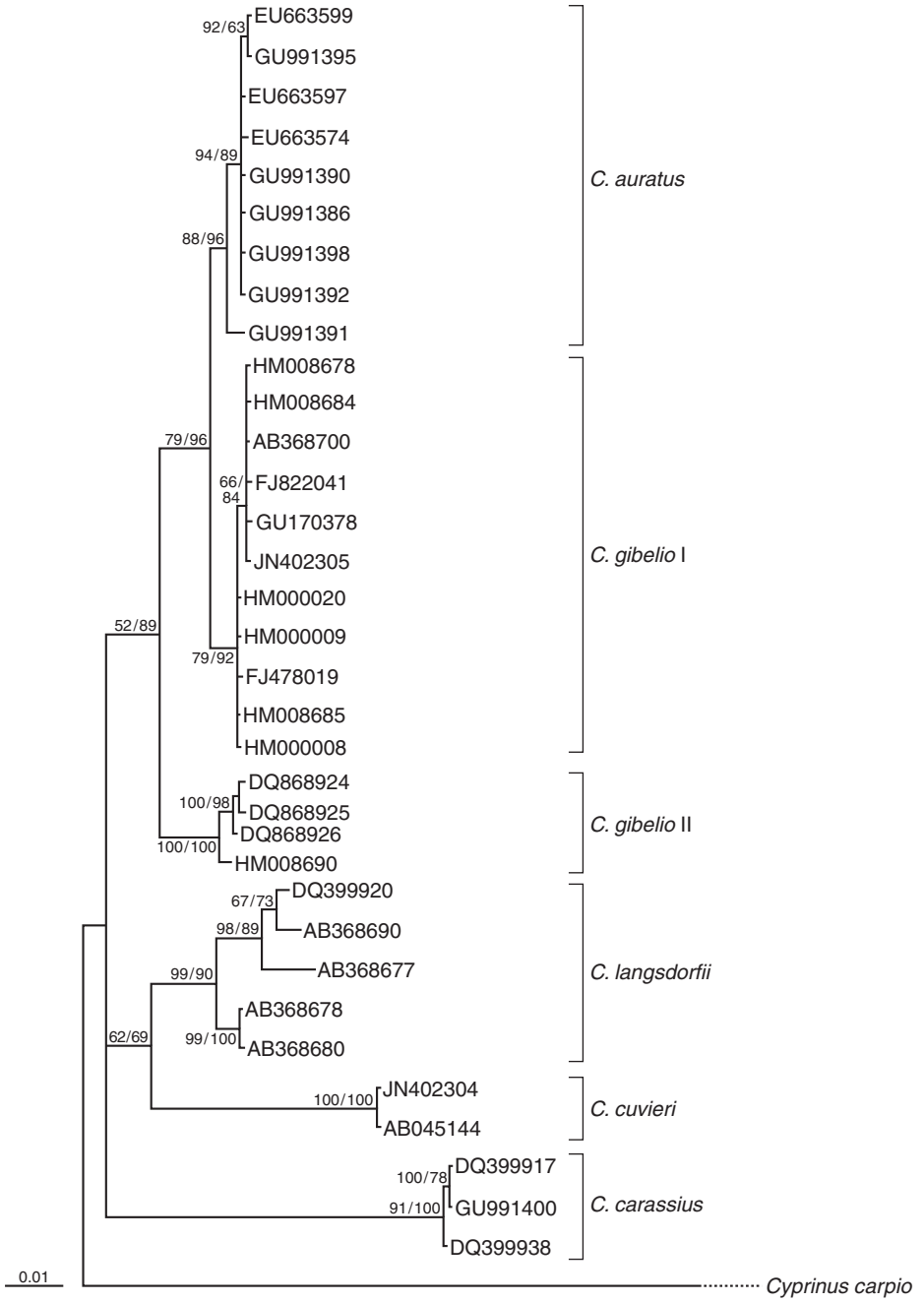

Fig 1. Reconstructed phylogeny of the cytochrome *b* sequences of *Carassius* samples included in present study. Numbers at nodes represent statistical supports for BAY and MP analyses respectively.

(14.82 μm², SD 1.8 μm²). The respective values are in agreement with published results confirmed by karyological analyses where triploids and diploids of *C. gibelio* are characterized by nuclei area of 14.03 ± 1.46 μm² and 20.71 ± 1.76 μm² respectively (Kalous & Petrtýl, 2004).

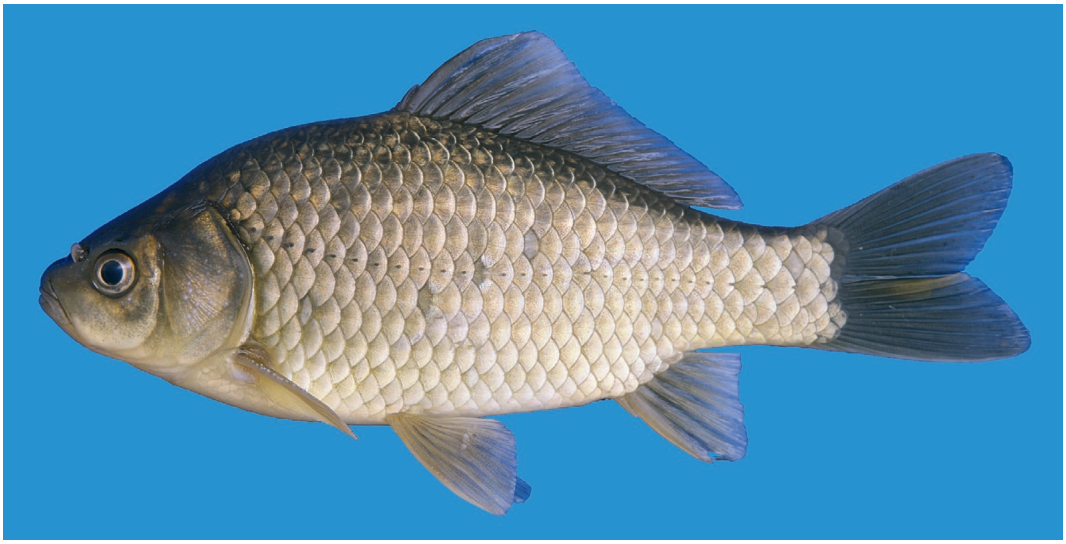

Fig. 2. *Carassius gibelio*, ZMB 33979, neotype, male, 127.4 mm SL; Czech Republic: Silesia: Odra River system.

Discussion

Our data show six separate genetic lineages within the genus *Carassius*. Four of these correspond to the species *C. carassius*, *C. auratus*, *C. langsdorfii* and *C. cuvieri*, respectively. In contrast, the samples of *C. gibelio* do not form a monophyletic lineage, but separate into two clades. One of these clades contains all samples of *C. gibelio* from western Mongolia, while the other clade collects samples of *C. gibelio* from Europe, Russian Federation, eastern Mongolia and China. The two clades do not have sister-relation to each other; instead, the Europe-China clade is more closely related to *C. auratus* than to the second clade of *C. gibelio*.

The present results suggest that the genus *Carassius* contains a higher diversity than formerly known, and that at least two species are included within what is presently considered as *C. gibelio*. Prussian carps from western Mongolia have been morphologically investigated by Peňáz & Dulmaa (1987) and identified as *C. gibelio*. Kottelat (2006) pointed on the problems of identification and nomenclature of Mongolian *Carassius* that come from the missing definition of *C. gibelio*. For further studies on the taxonomy of Prussian carps it is important to define which of the two species represents *C. gibelio*.

Cyprinus gibelio was described by Bloch (1782) and was stated to occur in 'Churmark, Pommern, Schlesien und Preussen', corresponding nowadays to most of eastern Germany, Poland and a part of north-eastern Czech Republic. Bloch did not explicitly designate a holotype; consequently all specimens included by Bloch are syntypes (ICZN, 1999, arts. 72.2, 73.4). In the part of Bloch's collection still present in Museum für Naturkunde (ZMB) in Berlin, a single lot is catalogued as *C. gibelio*, but nowadays this lot contains a specimen of *C. carassius* (see Paepke, 1999). Paepke (1999) demonstrated that the original syntype of *C. gibelio* has been replaced by a specimen of *C. carassius* during former investigations. No other potential types have ever been reported. During a research visit in ZMB in 2001, LK together with the staff of the museum searched the collection again for potential syntypes of *C. gibelio* but failed to find any. We therefore conclude that all type specimens of *C. gibelio* are lost. Since the present study indicates that more than one species is hidden under the name *C. gibelio*, a neotype designation is needed to fix the name *C. gibelio* to one of the identified species. We here designate specimen ZMB 33979 as neotype of *C. gibelio* (Fig. 2). The specimen originated from an alluvium area close to Český Těšín in the historical area of Silesia [Schlesien], one of the areas mentioned in the original description of *C. gibelio*. In accordance with ICZN (1999) art. 73.3.6, the neotype therefore comes from a locality that is part of the type locality. The neotype

corresponds in all investigated morphologic characters to the description of *C. gibelio* as given by Bloch (1782). In order to avoid taxonomical problems that might arise from the high percentage of polyploid specimens of gynogenetically reproducing lineages, we selected an adult male as neotype as indicated by the presence of spawning tubercles. According to the size of its erythrocyte nuclei, the neotype specimen is diploid (2n = 100). A description of the neotype is given below.

Table 2. Morphometric and meristic data of neotype of *Carassius gibelio*, ZMB 33979.

	in mm	in % SL
Total length	163.0	127.9
Standard length	127.4	100.0
Lateral head length	37.5	29.4
Predorsal length	63.6	49.9
Prepectoral length	36.1	28.3
Prepelvic length	61.8	48.5
Preanus length	91.7	72.0
Preanal length	94.6	74.3
Snout length	11.8	9.3
Horizontal eye diameter	7.3	5.7
Interorbital width	15.6	12.2
Head depth at eye	23.2	18.2
Head depth at nape	33.2	26.1
Body depth at dorsal-fin origin	51.1	40.1
Body depth at anal-fin origin	36.3	28.5
Depth of caudal peduncle	19.5	15.3
Length of caudal peduncle	21.0	16.5
Head width at eye	19.2	15.1
Maximum head width	25.4	19.9
Body width at dorsal-fin origin	26.0	20.4
Body width at anal-fin origin	17.2	13.5
Height of dorsal fin	24.3	19.1
Length of upper caudal-fin lobe	34.7	27.2
Length of middle caudal-fin ray	20.6	16.2
Length of lower caudal-fin lobe	36.8	28.9
Height of anal fin	20.9	16.4
Length of pelvic fin	26.0	20.4
Length of pectoral fin	25.9	20.3
Number of pores in lateral line	28	
Number of scales along lateral line	26+2	
Number of transverse scales between lateral line and origin of dorsal fin	½6	
Number of transverse scales below lateral line in front of pelvic fin	8	
Number of rows of scales around caudal peduncle	16	
Number of branched dorsal-fin rays	18½	
Number of branched caudal-fin rays	9+8	
Number of branched anal-fin rays	5½	
Number of pelvic-fin rays	9	
Number of pectoral-fin rays	18	

The neotype was analysed genetically for the present study and is part of the Europe-China clade of *C. gibelio*. Consequently, the name *C. gibelio* can be used for this lineage, while a different name has to be given for the Mongolian clade. We do not have sufficient material for a detailed morphological analysis of the Mongolian clade; but preliminary data (LK, unpubl.) suggest that this lineage does not correspond to any of the already available species names.

Our genetic analyses grouped most specimens from Albania and Greece (river Ischem and Lakes Prespa and Ochrid) to *C. auratus*, although only *C. gibelio* was formerly reported from the Balkan region (e. g. Perdikaris et al. 2012). It is possible that feral populations of *C. auratus* have been wrongly identified as *C. gibelio*. Further investigation is needed to identify the species occurring in the Balkan region.

Carassius gibelio
(Fig. 2)

Neotype. ZMB 33979, male, 127.4 mm SL, Czech Republic: pond in alluvium area of Olza River (tributary of Odra River) at Český Těšín; 49°47' 11" N 18°35'24" E; Lukáš Choleva, 5 May 2011.

Description. Morphometric and meristic data of neotype are shown in Table 2. Head and body laterally compressed; body high, greatest depth before dorsal-fin origin. Dorsal fin with 5 unbranched and 18½ branched rays; last unbranched ray with 10 spines along posterior edge on distal 60 % of length. Caudal fin with 9+8 branched rays, lower lobe slightly longer than upper. Pelvic fin with 9 rays, not reaching anus, which is located directly before anal-fin origin; origin under last unbranched dorsal-fin ray. Pectoral fin with 18 rays, reaching backwards to base of pelvic fin. Anal fin with 3 unbranched and 5½ branched rays, origin under branched dorsal-fin ray 13; last unbranched ray with 10 spines along posterior edge on distal 60 % of length; base reaching posteriorly beyond base of dorsal fin. Anal fin not reaching caudal fin. Breeding tubercles under eye and on opercle, along dorsal surface of first pectoral-fin ray and on median branched pelvic-fin rays at about 60 % of their length. Number, size and arrangement of tubercles of left and right sides different. First gill arch with 47 gill rakers. Total number of vertebrae 28, 14 ribs on each side.

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DISEASES OF AQUATIC ORGANISMS
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Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2)

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ABSTRACT: From 22 May to 10 June 2011 massive mortality of Prussian carp *Carassius gibelio* was observed in alluvial Lake Řehačka close to the Elbe River in the Czech Republic. More than 1400 kg of dead fish were collected and no other fish species were affected. Further molecular and cytogenetic investigation of fish (n = 232) revealed that the Řehačka population of Prussian carp consisted exclusively of gynogenetic triploid females. The causative agent was identified by means of molecular and electron microscopy as a herpesviral hematopoietic necrosis virus (*Cyprinid herpesvirus* 2, CyHV-2). This is the first report of CyHV-2 from the Czech Republic and the second finding worldwide of CyHV-2 causing mass mortality of *C. gibelio*. Some other localities in the upper Elbe River basin where *C. gibelio* was affected are also noted. We assume that the massive wave of deaths of all female gynogenetic Prussian carp can be attributed to limited genetic variation and the favourable conditions for development of viral disease.

KEY WORDS: Cypriniformes · Herpesvirus · Triploid · Gynogenetic · Mortality

INTRODUCTION

The triploid biotype of Prussian carp *Carassius gibelio* Bloch 1782 is considered the most successful non-native fish in Europe. It easily becomes one of the dominant species in newly inhabited areas, and it has a severe impact on the environment and aquaculture (Economidis et al. 2000, Varadi et al. 2000, Özcan 2007, Lusková et al. 2010, Savini et al. 2010). The type of reproduction significantly facilitates spreading of all-female populations due to rapid multiplication realized through sperm-dependent parthenogenesis.

When the eggs of *Carassius gibelio* are inseminated by males of other species, the heterologous sperm triggers development but does not contribute significantly to the formation of the zygote (Gui & Zhou 2010). This is known as gynogenesis and it leads to all-female offspring, each of which is considered a clone of the mother (Lamatsch & Stöck 2009).

The first record of the invasive triploid form of *Carassius gibelio* in the Czech Republic was in the lower stretches of the Dyje River (Lusk et al. 1977). The population was derived from a Danubian invasion (Holčík & Žitňan 1978) and consisted exclusively of triploid gynogenetic females (Peňáz et al. 1979). Triploid Prussian carp subsequently invaded all 3 main hydrological systems of the Czech Republic (Lusk et al. 1980, 1998, Lusk 1986), and aquaculture activities were considered the key factor accounting for its spread (Slavík & Bartoš 2004). In the Elbe River basin, an all-female population of *C. gibelio* was recorded for the first time in 1989 (Kubečka 1989) and later became a natural component of all suitable habitats (Halačka et al. 2003). In the early 1990s, the

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first males and diploids within the population of *C. gibelio* in the Dyje River alluvium (Danube basin) were found (Halačka et al. 2003, Lusková et al. 2004). In a relatively short time the former all-female triploid population changed to a diploid–polyploid complex with up to 43% males (Vetešník 2005). Similar situations were described in other European localities (Černý & Sommer 1992, Abramenko et al. 1998, 2004, Tóth et al. 2005).

Certain localities are now represented either by all-female gynogenetic populations or by the concurrent occurrence of fish with different ploidy and various proportions of males. However, low genetic variability in introduced all-female populations of Prussian carp is expected due to the low number of initial founders combined with the gynogenetic type of reproduction (Hänfling 2007). The all-female clonal populations could also be classified as vulnerable, since antigen recognition and killing by T-cells is genetically restricted by the major histocompatibility complex (MHC) (Somamoto et al. 2009). Lower tolerance to parasites has been reported in gynogens (Lively et al. 1990, Moritz et al. 1991, Poulin et al. 2000, Hakoyama et al. 2001).

In the present paper we report the rapid, massive and selective mortality of thousands of morphologically and genetically identified Prussian carp from Lake Řehačka with further investigation of ploidy level and sex ratio of the Prussian carp and identification of the causative agent. The concurrent occurrence of a massive kill of Prussian carp at several other localities in the upper Elbe River basin is also noted.

MATERIALS AND METHODS

Locality

Řehačka alluvial lake (50° 10′ 39″ N, 14° 48′ 27″ E) is situated close to the Elbe River. The lake covers 12.4 ha and represents an old oxbow of the River Elbe connected with an old flooded sand pit that is linked with the river by a pipeline connection. The water body is administered by the Czech Anglers Union, Local Organization Čelákovice, as a part of fishing district 'Labe 19 A' and includes common fish stock. According to local fishing statistics and an ichthyological survey (T. Daněk unpubl. data), the fish stock consists of: *Cyprinus carpio*, *Esox lucius*, *Sander lucioperca*, *Blicca bjoerkna*, *Tinca tinca*, *Anguilla anguilla*, *Silurus glanis*, *Abramis brama*, *Ctenopharyngodon idella*, *Aspius aspius*, *Perca fluviatilis*, *Gymnocephalus cernuus*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Alburnus alburnus*, *Scardinius erythrophthalmus*, *Rutilus rutilus*, *Leuciscus idus*, *Rhodeus amarus*, *Ameiurus nebulosus* and *Carassius gibelio*.

Mortality evaluation

Dead fish were collected from the lake by members of the local organization of the Czech Anglers Union, and quantities were recorded with information on water temperature. Additionally, a survey was conducted of representatives of other local organizations by the Czech Anglers Union along the Elbe River, and the authors of this study personally carried out inspections of localities reporting mass mortalities of *Carassius gibelio*.

Fish identification and sex determination

Moribund and dead fish were identified morphologically according to Kottelat & Freyhof (2007) and additionally by sequencing of mitochondrial DNA of 8 specimens. The cytochrome *b* gene was amplified by the methods described in Rylková et al. (2010), using forward primer Kai_F (GAA GAA CCA CCG TTG TTA TTC) and reverse primer Kai_R (ACC TCC RAY CTY CGG ATT ACA) (Šlechtová et al. 2006). PCR products were subsequently sequenced in both directions (Macrogen) and aligned. Sequences were compared with the Genetic sequence database (GenBank) at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLASTn) program.

The sex of fish was determined by inspection of gonads at autopsy of 200 dead fish and in specimens used in ploidy level determination.

Determination of ploidy level

Thirty-two moribund Prussian carp were used for ploidy level determination. The ploidy level was determined by computer-assisted image analyses using the measurements of mean erythrocyte nuclei area (MENA) as was proposed by Flajšhans (1997). The blood was obtained with a heparinised syringe from the fish heart, and blood smears were prepared as for conventional haematological examination, then air-dried, fixed in 90% ethanol, and stained with 4% Giemsa-Romanowski. Blood smears were then processed on a system consisting of microscope

(Nikon Eclipse 600, immersion objective 100×), analogue video camera (Hitachi HVC 20) and software (L.U.C.I.A version 4.71, Laboratory Imaging spol. sr. o.). The mean area of the nuclei was calculated from 200 erythrocytes for each specimen.

Moreover, the chromosome preparation according to Ráb & Roth (1988) was performed on 3 of 32 diseased fish. Nuclear suspensions were dropped on slides, stained with 4% Giemsa-Romanovski, and examined microscopically. The chromosome counts were realized on the 10 best metaphase plates per specimen. The ploidy levels of 3 specimens obtained by chromosomes counts were used as a reference for the ploidy level determination by MENA and possible differences were tested by *t*-tests using the program Statistica version 9.1. (StatSoft).

Identification of causative agent

Five moribund fish originating from 3 separate samplings (sample no. 1736/1 contained 1 fish collected on 27 May 2011; sample no. 1736/2 contained 3 fish collected on 30 May 2011; sample no. 1736/3 contained 1 fish collected on 5 June 2011) were frozen (–20°C) and transported to the laboratory. Standard pathological and parasitological examinations (Ergens & Lom 1970) were carried out, and an ELISA test for spring viremia of carp virus (SVCV) formerly known as *Rhabdovirus carpio* (RVC) was undertaken (Test-Line). Virological examination consisted of isolation on tissue culture and identification of the pathogen by electron microscopy and PCR.

Preparation of tissue homogenates

Pooled fish tissues were homogenized in a mortar with sterile sea sand, supplemented with Eagle's medium Tris MEM (minimal essential medium, Sigma), pH 7.6, enriched with 10% FBS (foetal bovine serum, GIBCO) and centrifuged (4°C, 1500 × *g*, 15 min). The supernatant was incubated overnight at 4°C with the addition of antibiotics (100 IU ml^{-1} of penicillin and 100 µg ml^{-1} of streptomycin) and afterwards used for cell line virological testing and for DNA extraction.

Isolation of viruses on tissue cultures

Monolayers (24 h) of bluegill fibroblast (BF-2), epithelioma papulosum cyprini (EPC), rainbow trout gonad (RTG-2) and fathead minnow (FHM) cell lines in 24-well plates (NUNC) were used for viral isolation. Cultures were inoculated with 3 serial tenfold dilutions of the examined samples and incubated at 15 and 23°C for 7 d. The cell lines were monitored by microscope every day for the development of cytopathic effect (CPE). If no CPE was observed, the cultures were frozen, thawed and subcultured for an additional 7 d. If CPE was not observed after the subculture, the samples were considered negative. Cell cultures exhibiting CPE were used for identification of viruses.

Electron microscopy

Supernatant from cell cultures displaying CPE were studied by electron microscopy. Subcultured samples were negatively stained with ammonium molybdate and examined using a Philips 208 transmission electron microscope (TEM) at 18000× magnification and an accelerating voltage of 90 kV.

DNA extraction

Tissue cultures with CPE were used for DNA isolation. The nucleic acid extraction was performed with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions.

PCR and sequencing

Four primer pairs were used in this study; 2 pairs were used for a nested PCR on the thymidine kinase of CyHV-3 and 2 other pairs for a nested PCR on the DNA polymerase of cyprinid herpesviruses (Table 1). PCR products were subsequently sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were aligned using BioEdit version 5.0.9 and compared with GenBank using the BLASTn program.

RESULTS

Mortality evaluation

More than 1400 kg of dead fish were removed from Lake Řehačka within the period 22 May to 10 June 2011. On 22 May only 30 kg were collected,

Table 1. Primer pairs used for identification of the virus. Nested PCRs targeted the thymidine kinase gene of herpesviral hematopoietic necrosis virus (CyHV-3) and the DNA polymerase gene of CyHV. One of 2 possible cycling conditions was used: (A) 40 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and the reaction was preceded by 94°C for 5 min and finished at 72°C for 10 min, or (B) 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and the reaction was preceded by 94°C for 5 min and finished at 72°C for 10 min. Centre for Environment, Fisheries & Aquaculture Science (CEFAS) protocol can be obtained at www.cefas.defra.gov.uk

Primer sequences (5′–3′)	Cycle conditions	Size (bp)	Source
CyHV-3 thimidine kinase			
Outer forward: GGG TTA CCT GTA CGA G	A	409	Bercovier et al. (2005)
Outer reverse: CAC CCA GTA GAT TAT GC			
Inner forward: CGT CTG GAG GAA TAC GAC G	B	348	CEFAS protocol
Inner reverse: ACC GTA CAG CTC GTA CTG G			
DNA polymerase gene of cyprinid herpesviruses			
Outer forward: CCC AGC AAC ATG TGC GAC GG	A	362	Jeffery et al. (2007)
Outer reverse: CCG TAR TGA GAG TTG GCG CA			
Inner forward: CGA CGG AGG CAT CAG CCC	B	339	Jeffery et al. (2007)
Inner reverse: GAG TTG GCG CAY ACY TTC ATC			

with the remaining fish collected on 27 May (700 kg), 30 May (300 kg), 1 June (300 kg), 5 June (100 kg), and the last dead specimens occurring on 10 June. Water temperature at the locality during this period was between 16.1 and 20.5°C. To our knowledge, no other fish species were affected besides *Carassius gibelio* and no newly dead fish were observed after 10 June.

The survey among representatives of the local organization of the Czech Anglers Union also revealed the occurrence of a selective kill of Prussian carp in 4 other localities within the upper Elbe Basin (Fig. 1, numbers 2 to 5; Table 2).

Identification and sex determination of fish from the Řehačka mortality event

All 232 investigated fish (200 examined grossly and a further 32 for ploidy determination) were females and were morphologically identified as *Carassius gibelio* sensu Kottelat & Freyhof (2007). Eight fish that were also investigated genetically shared 1 haplotype of Cyt *b* mt DNA (final length of sequences consisted of 1027 characters). Sequence of the haplotype was compared in the program BLASTn that evaluates the percentage of sequence similarity (%S) and the percentage of sequence overlap (%O) with the reference sequence of *C. gibelio* from GenBank (Table 3).

Determination of ploidy level

Chromosome preparation of 3 of the 8 specimens (Table 3) revealed they were triploids with modal chromosome numbers of 156 (60% of investigated metaphases), 156 (50% of investigated metaphases), and 150 (60% of investigated metaphases). Ploidy level determination by MENA showed that all 32 investigated specimens were triploids with values of the nuclei area mean (± SD) ranging from 20.7 ± 2.2

Table 2. *Carassius gibelio*. Selective mortalities of Prussian carp in the upper Elbe Basin. Biomass: biomass of dead fish, which were removed from the locality by local organisations of the Czech Anglers Union during 2011. See Fig. 1 for locations of localities. nd: no data

Locality number	Locality name	Period	GPS position	Biomass (kg)	% mortality
1	Řehačka	22 May–10 June	50° 10′ 37.956″ N, 14° 48′ 22.419″ E	>1400	>95
2	Pňov	30 June–5 July	50° 5′ 36.024″ N, 15° 9′ 11.413″ E	>700	nd
3	Nová Ves	30 June–25 July	50° 3′ 24.666″ N, 15° 9′ 35.738″ E	>5600	>95
4	Trnávka	1 June–11 June	50° 1′ 56.253″ N, 15° 27′ 48.229″ E	150	nd
5	Hrobice	5 June–19 June	50° 6′ 28.754″ N, 15° 47′ 23.408″ E	100	nd

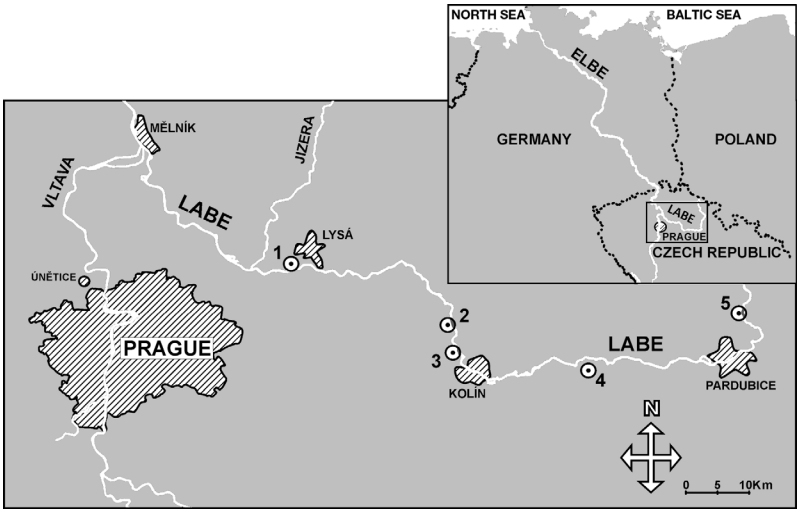

Fig. 1. Localities with selective Prussian carp *Carassius gibelio* mortalities. Cases (numbers 1 to 5 in main map) are described in Table 2

Table 3. *Carassius gibelio.* Genetic and cytogenetic identification of 8 specimens of Prussian carp from Lake Řehačka (CgTL006, CgTL008, CgTL013, CgTL046, CgTL078, CgTL114, CgTL144 and CgTL113). Basic local alignment seach tool (BLASTn) comparisons used reference sequence from *Carassius gibelio* DQ399929 (Kalous et al. 2007). %S: percentage of overlap sequence similarity; %O: percentage of sequence overlap; MENA: mean erythrocyte nuclei area; Chro: modal chromosome number

Specimen	Morphological identification	Ploidy level MENA	Chro	GenBank Accession No.	%S	%O
CgTL006	*Carassius gibelio*	3n	156	JN546055	100	98
CgTL008	*Carassius gibelio*	3n	156	JN546056	100	98
CgTL013	*Carassius gibelio*	3n	150	JN546057	100	98
CgTL046	*Carassius gibelio*	3n	–	JN546058	100	98
CgTL078	*Carassius gibelio*	3n	–	JN546043	100	98
CgTL114	*Carassius gibelio*	3n	–	JN546041	100	98
CgTL144	*Carassius gibelio*	3n	–	JN546040	100	98
CgTL113	*Carassius gibelio*	3n	–	JN546034	100	98

to 23.2 ± 2.5 µm^2. Comparison of values using a *t*-test confirmed no statistically significant difference ($p > 0.05$) between the 3 reference specimens and the remaining specimens (Fig. 2). All values are in agreement with those for triploid *Carassius gibelio* (Kalous & Petrtýl 2004).

Identification of causative agent of mortality

Diseased Prussian carp had pinpoint red foci at the base of their fins, red foci in the eyes, haemorrhaging of the gills, and some specimens showed pink-colored skin in the abdominal region and fins (Fig. 3). Internal organs were soft and reddened. Standard pathological and parasitological examination failed to detect parasites or evidence of spring viremia of carp. Given that the massive mortality was selective for *Carassius gibelio*, toxicosis was also excluded.

Isolation of viruses on tissue cultures and electron microscopy

Three separate fish samples (1736/1, 1736/2 and 1736/3) produced CPE on first passage in all 4 cell lines incubated at 23°C and in BF-2, FHM and RTG 2 cell lines incubated at 15°C. Cell cultures with clear CPE were examined by TEM, and viral particles morphologically similar to a herpesvirus were observed (Fig. 4). These samples were tested by PCR.

PCR and sequencing

Samples with CPE and herpesviral particles observed in TEM were investigated by nested PCR using primers specific for koi herpesvirus (CyHV-3) (Table 1). Negative results were obtained. Subsequently generic primers for the DNA polymerase gene of cyprinid herpesviruses were used (Table 1) and a primary product of 362 bp was obtained in 2 of 3 separate samples. The nested PCR resulted in a specific product of 339 bp in all 3 samples (Fig. 5). Products were sequenced and the sequences were deposited in GenBank (accession nos. JQ740764, JQ740765 and JQ740766). Nucleotide sequences were compared with GenBank using the BLASTn program and exhibited 100% identity with cyprinid herpesvirus 2 DNA polymerase gene (GenBank accession no. DQ085628.1) (Goodwin et al. 2006a).

DISCUSSION

The causative agent of a massive kill of Prussian carp was identified as CyHV-2, family *Alloherpesviridae*, genus *Cyprinivirus* (Davison et al. 2009). This virus shares morphological similarities with carp pox herpesvirus (CyHV-1) and koi herpesvirus

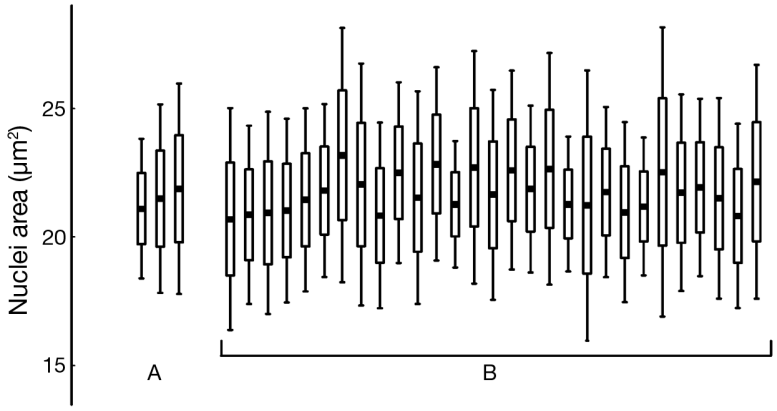

Fig. 2. *Carassius gibelio*. Obtained erythrocyte nuclei area of triploid reference specimens (A) and the remaining 29 specimens (B) from Lake Řehačka. Thick black line: mean; box: SD; whiskers: 1.96 × SD

Fig. 3. *Carassius gibelio*. Freshly dead fish from Lake Řehačka affected by CyHV-2 showing pinpoint foci at the base of fins and in the eyes

(CyHV-3), but it differs in the clinical manifestation, host range, antigenic properties, and growth characteristics (Waltzek et al. 2005). (CyHV-2) is a pathogen of goldfish *Carassius auratus* (Goodwin et al. 2006b, Jeffery et al. 2007), but recently it was also identified in *C. gibelio* in Hungary (Doszpoly et al. 2011). In the case of Lake Řehačka, all affected fish were *C. gibelio*. CyHV-2 is associated with mortality in at least 2 species of the genus *Carassius* — *C. auratus* and *C. gibelio* — but it seems not to be pathogenic for *C. carassius* (Jeffery et al. 2006) or for common carp *Cyprinus carpio* (Jung & Miyazaki 1995).

CyHV-2 was originally described in Japan (Jung & Miyazaki 1995) but it probably has a global distribution (Waltzek et al. 2009), with mortality reported in the United Kingdom (Jeffery et al. 2007), USA, Taiwan (Goodwin et al. 2006a) and Australia (Stephens et al. 2004).

The high mortality within goldfish can be attributed to their low genetic diversity (Rylková et al. 2010) since this species has experienced intensive selection during its breeding history (Balon 2004). We presume that some of the differences in the manifestation of the disease at Lake Řehačka in comparison to previously described symptoms (Jung & Miyazaki 1995, Stephens et al. 2004, Jeffery et al. 2007) could be influenced by the heterogeneity of affected species within the genus *Carassius*.

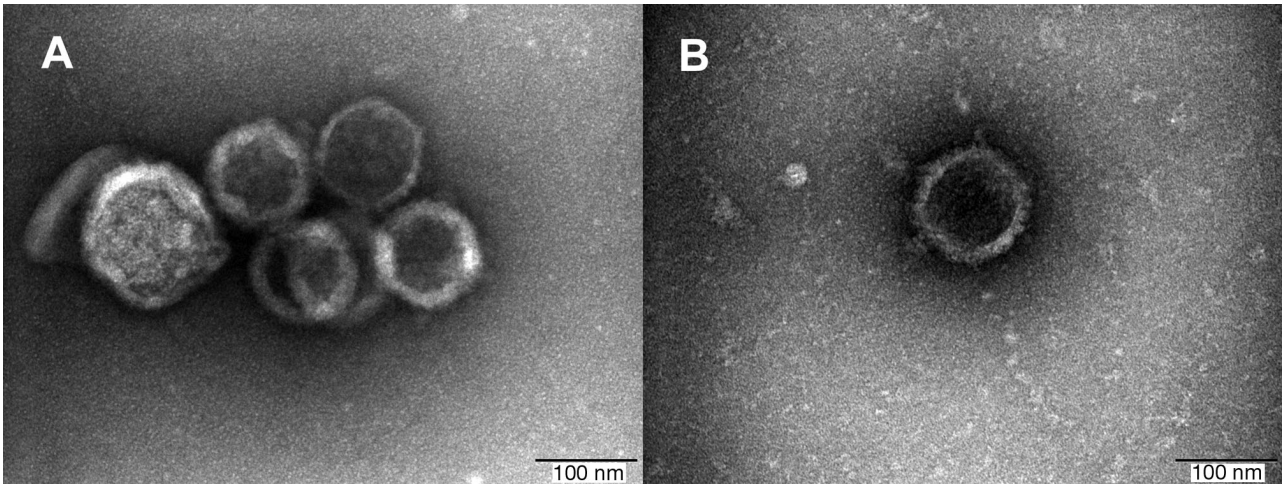

Fig. 4. Viral particles with a herpesviral morphology isolated in different cell lines and temperatures. (A) Rainbow trout gonad cell line, 15°C. (B) Epithelioma papulosum cyprini cell line, 23°C. Transmission electron microscopy; negative staining

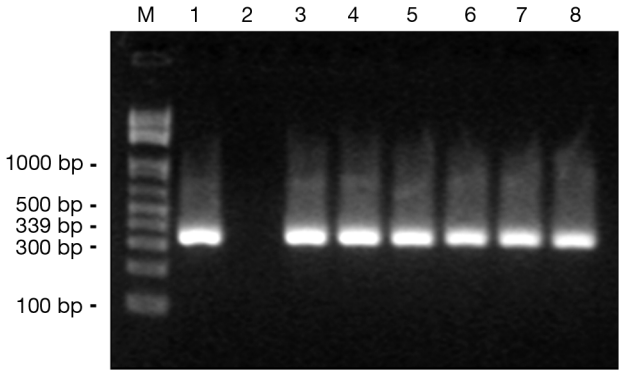

Fig. 5. Nested generic PCR of the DNA polymerase gene of CyHV. Lane M: mass ladder (TrackIt 1 Kb Plus DNA Ladder, Invitrogen); Lane 1: positive control; Lane 2: negative control; Lane 3: Sample 1736/1; Lane 4: Sample 1736/2; Lane 5: Sample 1736/3; Lanes 6 to 8: Same samples after virus multiplication in cell line RTG-2, 23°C

The natural populations of *Carassius gibelio* consist of clonal lineages with the sympatric occurrence of sexually reproducing individuals (Gui & Zhou 2010). In contrast, in newly inhabited areas the populations are characterized by the dominance of females that take advantage of rapid multiplication due to sperm-dependent parthenogenetic reproduction (Hänfling 2007). Our data suggest that the *C. gibelio* population from Lake Řehačka is gynogenetic since not a single male was found. Moreover, all investigated specimens were shown to be triploids, and only 1 haplotype of Cyt *b* mtDNA was shared among 8 sequenced fish.

Populations of asexually reproducing vertebrates are often considered to be less resistant to pathogens due to reduced genetic variability of the host (Neiman & Koskella 2009). In natural populations that reproduce sexually, usually only a fraction of individuals infected by viruses show symptoms of disease, and a significant part of the clinical variability observed within populations is explained by the host genetic background that plays an important role in the susceptibility to infections (Verrier et al. 2012). This phenomenon changes in a genetically uniform population caused by artificial selection. Within the common carp *Cyprinus carpio*, strains more or less susceptible to CyHV-3 have been identified (Shapira et al. 2005, Ødegård et al. 2010). High stock densities and low genetic variability can result in mass mortalities from virus, e.g. in koi carp (Hedrick at al. 2000), or even in introduced common carp in a natural environment (Garver et al. 2010). Animals that reproduce clonally face the same population breakdown possibility when infected by highly pathogenic viruses due to their obvious genetic similarity. Although we did not completely define the genotype diversity of Prussian carp in Lake Řehačka, only a few clonal lineages are likely to be present due to the bottle-neck effect associated with introduction and the gynogenetic type of reproduction. Our cytogenetic data showed 2 different modal chromosome numbers. The triploid biotype of *Carassius gibelio* is known to bear various numbers of chromosomes from 150 to 162 (Kalous & Knytl 2011). Particular clones are then usually characterized by a specific chromosome number (Zhou & Gui 2002). In the case of Lake Řehačka, there are at least 2 clones, although the cytogenetic data in the study are very restricted.

After the first dead fish appeared on 22 May, numbers rapidly increased within 5 d. One week after the peak of mortality, only a few newly dead specimens were found. Estimating mortality in natural waterbodies is very difficult. However, based on information from the local organization of the Czech Anglers Union and the complete lack of Prussian carp caught at the locality by anglers in the period 10 June 2011 to July 2012, we assume that all or nearly all of the *Carassius gibelio* in Lake Řehačka were eliminated by the pathogen during this short period.

The rapid progress of the pathogen was also reported in controlled conditions when fish began to die at 3 to 6 d post-inoculation and cumulative mortality ranged from 60 to 100%, depending on viral titre, within 13 d at 20°C (Jung & Miyazaki 1995). CyHV-2 is often present as an inapparent infection and could be widespread in nature (Goodwin et al. 2009). However, when infected fish are subjected to stress such as a temperature change, there is a greater probability of disease outbreaks.

We believe the stress/temperature hypothesis may explain the initiation of the disease outbreak at Lake Řehačka and other locations in the upper Elbe River because a sharp drop in water temperature occurred from 13 May to 16 May 2011. Additionally our data showed that the virus replicated well in the temperature range 15 to 23°C, which is in agreement with Jung & Miyazaki (1995) who noted the optimum for virus propagation ranging between 15 and 25°C.

We conclude that the massive wave of deaths of Prussian carp at Lake Řehačka and at other localities in the upper Elbe River basin can be attributed to limited genetic variation of Prussian carp and the favourable conditions for propagation of CyHV-2.

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Phylogeny and biogeographic history of the cyprinid fish genus *Carassius* (Teleostei: Cyprinidae) with focus on natural and anthropogenic arrivals in Europe

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ABSTRACT

Freshwater fishes of the genus *Carassius*, widespread throughout Europe and Asia, are important aquaculture fishes and include the world's most important pet fish, the goldfish. The high morphologic similarity between the species, however, has up to now prevented reliable conclusions on their taxonomy, biogeography and introduction history. A phylogeny of the fish genus *Carassius* based on the cytochrome *b* sequence of 404 specimens collected from aquaculture and open water localities across Eurasia identifies most of the presently recognised species as monophyletic lineages, but also that at least one lineage exists that does not correspond to any described species. Within Europe, feral populations of *Carassius auratus* occur mainly in the Mediterranean area and Great Britain, while *Carassius gibelio* is found in most of non-Mediterranean Europe and some localities in Italy. *Carassius langsdorfii* has very scattered points of occurrence in at least six European countries. *C. auratus* and *C. langsdorfii* are not native to Europe. The populations of *C. gibelio* in eastern Central Europe and parts of Eastern Europe are considered as resulting from a natural postglacial range expansion, while the rest of Europe was colonised due to anthropogenic impact. The presence of diploid (2n = 100) as well as triploid (3n = 150) specimens in the three most widespread species indicates that ploidy level is not a character to identify the species of *Carassius*. A remarkably low genetic divergence in *C. gibelio* can be the result of clone selection in the gynogenetic populations. In general, our data present the first comprehensive overview about the genus *Carassius* in Europe based on genetic data.

1. Introduction

The first and most important step in any study dealing with biological material is the proper identification of the investigated species, otherwise studies on biodiversity and distribution cannot be carried out, and these animals cannot be used as models in any type of investigation. The traditional and most practical way to identify fish species is based on morphological characters, but in cases of morphologically similar species genetic data can also help in the identification (Avise and Hamrick, 1996; Ogden, 2008).

Freshwater fishes of the genus *Carassius* are closely related to the common carp and include the goldfish, one of the best-known fishes at all. The genus occurs frequently across Eurasia from Portugal in the west to Japan in the east and from the Siberian Rivers in the north to southern China and Vietnam in the south (Szczerbowski, 2002a,b). They are farmed for aquaculture in East Asia (annual production in China about 2 million tonnes Gui and Zhou, 2010), Eastern Europe and Central Asia (FAO, 2011) and for this purpose they have been introduced from Asia to Europe several times (Savini et al., 2010). Especially the introduced forms from Asia are supposed to have a negative impact on European river ecosystems (Richardson et al., 1995). However, due to the morphological similarity of the species, the understanding of the taxonomy of the genus *Carassius* and the detailed distribution of species in Europe are poor. At present, five species are considered valid: *Carassius carassius* in most of Europe and western Asia, *Carassius langsdorfii* and *Carassius cuvieri* in Japan, *Carassius auratus* in China and *Carassius gibelio* in Europe (introduced lineages from Asia as well as native populations), Siberia and East Asia (Bănărescu, 1991; Hosoya, 2000; Szczerbowski, 2002a,b). Morphologically, *C. carassius* is the only species that can be identified easily (Kottelat and Freyhof, 2007), while the remaining species differ only slightly in morphological characters and will further on be referred to as the *C. auratus* complex.

The identification of species is even more complicated in many populations by the occurrence of specimens with different ploidy level (Abramenko et al., 1997; Jakovlić and Gui, 2011; Lusková et al., 2004). For a long time it has been believed that *C. gibelio* and *C. langsdorfii* are triploid (3n = around 150), while the other species are diploid (2n = 100); therefore the ploidy level has been taken as important character to identify species (Vasil'eva and Vasil'ev, 2000). In recent times it has

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been shown that diploid specimens also occur within *C. gibelio* and *C. langsdorfii* and triploid specimens have been found in *C. auratus* (Abramenko et al., 1997; Takada et al., 2010; Xiao et al., 2011). Triploid specimens are nearly all females and are considered to reproduce by gynogenesis after mating with any other cyprinid species (Cherfas, 1966; Flajšhans et al., 2008), but may incorporate sperm nuclei when coexisting with diploid bisexual populations of *Carassius* (Fan and Shen, 1990; Lamatsch and Stöck, 2009; Tóth et al., 2005; Zhou et al., 2000). Moreover, hybrids between *C. carassius* and *C. auratus* exist at least in Great Britain and can be difficult to identify (Hänfling et al., 2005).

The combination of morphologically similar species, of hybrid specimens and of gynogenetic lineages within species, hampers studies aiming to clarify the biogeography and taxonomy of the genus *Carassius*. Moreover, these fishes have a long history of introductions and translocations by man (Burmakin, 1963; Copp et al., 2005a). Their outstanding hardiness when transported in wet grass and a great possibility to survive and grow even in small ponds with eutrophic conditions made the species of *Carassius* one of the first candidates for stocking throughout and outside their natural distribution areas. In Europe and China, anthropogenic translocations of *Carassius* have occurred for hundreds of years (Balon, 2004). During Medieval times, monks developed an early carp aquaculture across Europe, which later developed into a flowering carp industry with global exchange of stocking material in the 19th and 20th century, and the unintended propagation and translocation of *Carassius* together with juvenile carps are until today a common side product of carp aquaculture worldwide (Copp et al., 2005a; Tóth et al., 2005). Moreover, the goldfish became the most popular pet fish of all times and was globally distributed, leading to uncounted events of release to open waters (Kottelat, 1997). Today, records of goldfish from open waters come from nearly all climatically suited parts of the world (Elvira, 2001; Kumar, 2000; Olden et al., 2008; Seegers et al., 2003). The fact that feral goldfish lose their orange colour and fancy fin shapes within a few generations and return to the wild phenotype has added to the problem of identification of species of the genus *Carassius*.

The distribution of species of *Carassius* in Europe and their translocations can be summarised as following: *C. carassius* occurs from the Rhine basin eastwards through most of Europe except of the Mediterranean basin. It has been introduced to the large part of Great Britain, Italy and France (Kottelat and Freyhof, 2007). *C. gibelio* was originally described from north-eastern Central Europe by Bloch (1782) but was at that time not mentioned or its status as species was doubted by ichthyologists in more western parts of Europe and the Danubian basin (Balon, 1962; Changeux and Pont, 1995; Holčík and Žitňan, 1978; Verreycken et al., 2007). Since 1940 it was recorded in the Danube River basin, first from Bulgaria and Romania, most likely as a result of introductions from the Russian part of the distribution area and since then has spread across most of Europe (Bănărescu, 1964; Drensky, 1948; Szczerbowski, 2002a,b). The goldfish *C. auratus* arrived as valuable pets in the 17th century to Portugal, France and Great Britain (Balon, 2004; Hervey and Hems, 1968; Kottelat, 1997). They were soon reproduced in captivity and spread across Europe. Distribution as ornamental species and stocking by private persons can be considered as a general and major way for the goldfish into open water systems (Copp et al., 2005b; Kottelat, 1997). Recently the Japanese species *C. langsdorfii* was discovered in the Elbe river system and Greece (Kalous et al., 2007; Takada et al., 2010; Tsipas et al., 2009).

In the present study, we identify more than 400 individuals of *Carassius* based on mitochondrial DNA and reconstruct their phylogeny. The ploidy level of a subsample of the analysed specimens of *Carassius* was estimated in order to test if the ploidy level is responsible for splits between species or lineages within species. Focusing on the relationships of the European populations we evaluate the number of introductions and the distribution of introduced species. On the base of the voluminous records of *Carassius* introductions and translocations, we reconstruct the biogeographic history of the introduced species of the *C. auratus* complex in Europe and report on the existence of at least one undescribed species in northeast Asia.

2. Material and methods

Altogether, 404 samples of *Carassius* from European, Asian and North American water bodies were included into the analysis. The dataset includes 183 new sequences, 121 sequences from our previous studies and 100 sequences obtained from GenBank. Our original samples were obtained via random sampling. As an outgroup, we used the common carp, *Cyprinus carpio*. Detailed information of sample origin and GenBank accession numbers are listed in Supplementary Table 1.

2.1. Reconstruction of phylogeny

Genomic DNA was isolated from ethanol preserved tissue using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocol. The mitochondrial gene cytochrome *b* was amplified using forward primer Kai_F (GAA GAA CCA CCG TTG TTA TTC) and reverse primer Kai_R (ACC TCC RAY CTY CGG ATT ACA) (Šlechtová et al., 2006). Polymerase chain reaction (PCR) consisted of 3 μl template DNA, 3 μl of each primer, 15.5 μl of Combi PPP Master Mix (Top-bio) and ddH_2O up to 50 μl of reaction mix. The profile of the PCR, carried out on an MJ Mini™ thermocycler (Bio-Rad), started with initial denaturation at 95 °C for 2 min followed by 35 cycles consisting of denaturation step at 95 °C for 1 min, annealing step at 52 °C for 30 s and elongation at 72 °C for 30 s; the last step was final elongation at 72 °C for 10 min.

The PCR products were purified and sequenced from both ends to gain complete sequence of the gene. Purification and sequencing were performed by Macrogen Inc., Korea.

The raw chromatograms were manually assembled and checked by eye for potential mistakes using the computer software BioEdit 5.0.9. (Hall, 1999); the same program was used to align the sequences using the ClustalW algorithm.

The phylogenetic relationships were estimated using the methods of maximum parsimony (MP) in PAUP* version 4.0b10 (Swofford, 2000) and Bayesian analyses (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck and Ronquist, 2001).

2.2. Ploidy level determination

Measurements of ploidy level followed the method described by Lamatsch et al. (2000) for fixed fin clips. Heparinised red blood cells from female chicken (*Gallus gallus*) cells were used as the internal standard (genome size 2.5 pg/nucleus; Vinogradov, 1998).

Ethanol fixed fin clips were minced in 2.1% citric acid/0.5% Tween 20 and incubated for 15 min with gentle shaking at room temperature (RT). Fish cells and 100 μl of the chicken erythrocyte solution in phosphate buffered saline (PBS) were centrifuged for 5 min at 300 ×g, the supernatant was discarded and the cell pellets were resuspended in 400 μl 0.5% pepsin in 0.1 M HCl. After incubating at RT for 10 min with gentle shaking, the cells were stained overnight at 4 °C by adding 1100 μl DAPI solution (5.9% citric acid trisodium salt $2H_2O$, 0.0002% DAPI). Immediately before analysis the fish samples were filtered through a 50 mm nylon mesh (Celltrics, Partec©), to prevent obstruction of the flow chamber by fin rays. To certify that nuclei were stained completely, 1:10 dilutions with DAPI were used for measurements. For each measurement fish fin clip cells and chicken red blood cells were mixed in such a way that similar final concentrations were obtained. All measurements were conducted on a Partec Ploidy Analyser PA-II applying a mercury lamp (Partec, Münster, Germany). At least 10,000 cells were measured per sample.

Ploidy level was determined for 128 specimens (67 samples original to this study). Cluster analysis based on K-means clustering method

was used for the separation of two ploidy levels based on the values of DNA content. Basic descriptive statistics were then calculated for all values of each ploidy level and for each lineage. Significance of the results of cluster analysis was tested by ANOVA. All computations were done using STATISTICA ver. 9.1 (StatSoft, Inc., 2010).

3. Results

The 404 analysed specimens of *Carassius* revealed the existence of six monophyletic lineages. Five of these lineages correspond to the species *C. carassius*, *C. cuvieri*, *C. langsdorfii*, *C. gibelio* and *C. auratus*, but one lineage containing samples of *C.* cf. *gibelio* from Mongolia could not be assigned to any described species. The samples of *C. langsdorfii* and *C. auratus* split into three and two sublineages, respectively, that were identified in both analyses with high statistical support (Fig. 1).

3.1. Species of Carassius and their distribution in Europe

Our dataset identifies four species of *Carassius* from localities in Europe, namely 1) *C. carassius* from Austria, Czech Republic, Germany, Great Britain and Sweden; 2) *C. auratus* from Albania, Germany, Greece, Montenegro, Portugal and Spain; 3) *C. langsdorfii* from Bosnia-Herzegovina, Czech Republic, Germany, Greece, Italy and Ukraine and 4) *C. gibelio* from Austria, Bosnia-Herzegovina, Bulgaria, Croatia, Czech Republic, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Poland, Romania, Slovakia, Turkey and Ukraine (Fig. 2a).

3.2. Species of Carassius and their distribution in Asia

Four species of *Carassius* were identified in Asia: 1) *C. auratus* in China, Japan, Kazakhstan, Korea and Taiwan; 2) *C. gibelio* in China, Korea, Mongolia and Uzbekistan; 3) *C. langsdorfii* in Japan; and 4) *C. cuvieri* in Japan (Fig. 2b).

3.3. Species of Carassius and their distribution in USA

Five individuals of *C. auratus* were identified in one locality of the USA. All of them shared the same haplotype *Ca1* which is the most common within this taxon and gather mainly ornamental and feral specimens (Fig. 2c).

3.4. Reconstruction of phylogeny

The final matrix of the cytochrome *b* sequences consisted of 1023 basepairs containing 296 variable characters with 214 parsimony informative sites. Altogether, the analysed specimens of *Carassius* revealed 104 haplotypes. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted sequences into six well-supported lineages corresponding to taxonomical division of the genus *Carassius*; further divided into nine sublineages. Sublineages occur within the lineage of *C. auratus* and *C. langsdorfii* (Fig. 1). The geographical distribution of particular lineages is shown in Fig. 2.

3.5. Ploidy determination

Based on the results of cluster analyses two groups of ploidy level are separated with a mean value of DNA content ($\pm$SD) of 4.38 $\pm$ 0.49 pg for diploid specimens and 6.74 $\pm$ 0.49 pg for triploid specimens. The total range of values in the group of diploids and triploids was between 3.6–5.42 pg and 5.75–7.33 pg respectively. ANOVA confirmed the results of cluster analysis as statistically significant ($P<0.01$). As expected, all investigated *C. carassius* are diploid ($n=6$). *C. gibelio* ($n=61$) show diploid ($n=12$) and triploid ($n=49$) individuals. *C. auratus* ($n=39$) show ($n=19$) diploid and ($n=20$) triploid individuals, and in *C. langsdorfii* ($n=19$) there were ($n=5$) diploid and ($n=14$) triploid individuals. The three investigated individuals from *Carassius* sp. "*M*" all showed DNA content comparable to three chromosome sets. Values of DNA content of the blood cells separately for each lineage as well as basic descriptive statistics for diploids and triploids are summarized in Table 1.

4. Discussion

4.1. Genetic diversity within species

Three of the species analysed in the present study, *C. auratus*, *C. gibelio* and *C. langsdorfii*, have been sampled with a comparably high number of specimens from a broad variety of localities in Europe as well as in East Asia. It appeared that the genetic diversity (individuals/haplotypes) within *C. auratus* (174/43) and *C. langsdorfii* (46/25) is considerably higher than within *C. gibelio* (150/21). The diversity within *C. auratus* and *C. langsdorfii* is 1.77 and 3.88 times higher, respectively, than within *C. gibelio*. This result is at first surprising, because *C. gibelio* has the largest distribution area of the three species, occurs in very high density in many localities and did not pass through such a strong bottleneck like *C. auratus* when humans selected the rare red specimens at the beginning of domestication (Komiyama et al., 2009). However, the clonal mode of reproduction of many populations of *C. gibelio* might have been responsible for their restricted genetic diversity. In sexually reproducing populations, the natural (or artificial) selection pressure increases or decreases the frequency of certain nuclear gene haplotypes that are coding phenotypic features, but does not select mitochondrial genes. In clonal lineages, selection pressure changes the frequency of clones within the population, leading to the strong dominance of one or few clones, each carrying only one mitochondrial copy (Vrijenhoek, 1998).

4.2. Ploidy level of the species

For a long time, the ploidy level has been considered an important character for the species determination within *Carassius*, especially to distinguish the diploid *C. auratus* from the triploid *C. gibelio* (Szczerbowski, 2002a,b; Vasil'eva and Vasil'ev, 2000). Recent investigations have reported the occurrence of triploid specimens of *C. auratus* (Takada et al., 2010; Xiao et al., 2011), and in the populations of *C. gibelio* more and more diploid specimens are observed (Abramenko et al., 1997; Apalikova et al., 2008; Brykov et al., 2005; Lusková et al., 2004). Our present data show that both species contain diploid as well as triploid specimens and that ploidy level is an inaccurate character to distinguish them. Similar to *C. gibelio*, *C. langsdorfii* has also been considered to be a uniquely triploid species for a long time, but recent studies have shown that diploid as well as triploid specimens occur (Takada et al., 2010).

4.3. Origin of European populations of C. auratus complex

Our results show that the European populations of *C. auratus*, *C. gibelio* and *C. langsdorfii* are very closely related to the Asian populations and that in all three species certain haplotypes are found in Europe as well as in Asia. This observation corresponds to a very recent origin of the European populations.

The fact that populations of *C. auratus* are only observed in southern Europe can be attributed to the suited climatic conditions; it looks like the goldfish requires higher winter temperatures than other species of *Carassius*. Moreover, most populations in Portugal, Spain, and Italy consist of diploid specimens and share the same haplotype, supporting their joint origin. In contrast, nearly all specimens of *C. auratus* in the Balkans (Albania, Montenegro and Greece) are triploids, indicating a different origin. It has been recorded that Albania introduced 'Chinese carps' during the 1960s directly from China and stocked them into Lake Skadar (Flloko, 2005; Shumka et al., 2008). Since these fishes

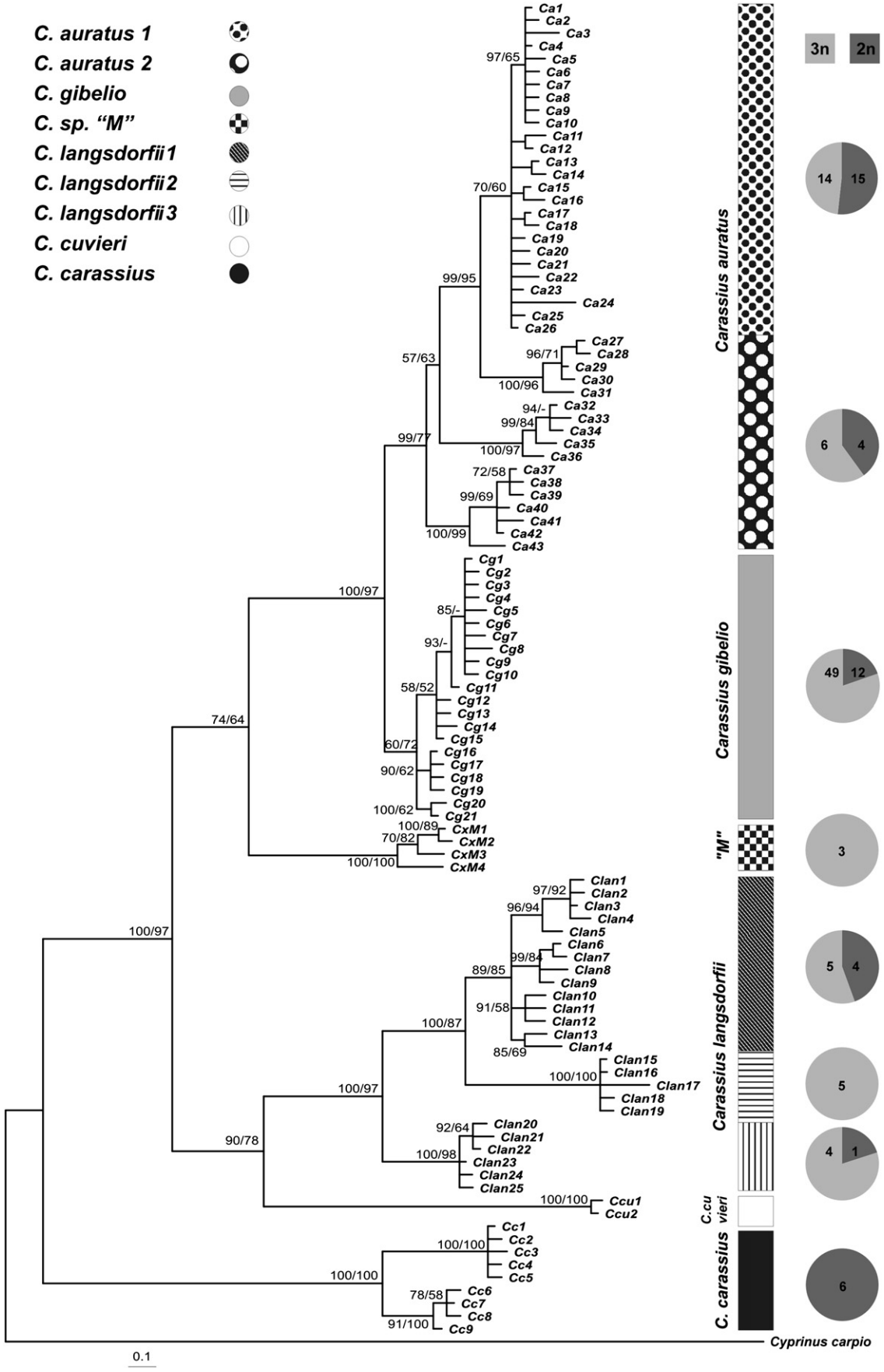

Fig. 1. Phylogenetic tree: reconstructed phylogeny of the Cyt. *b* sequences of *Carassius*. The numbers at the nodes represent statistical supports for BAY and MP analyses respectively. The bootstrap supports below 50 and Bayesian posterior probabilities below 0.75 are not shown. Analyses divided samples into a total of 105 haplotypes as follows: 43 *C. auratus*, 21 *C. gibelio*, 4 *Carassius* sp. "*M*", 25 *C. langsdorfii*, 2 *C. cuvieri* and 9 *C. carassius*.

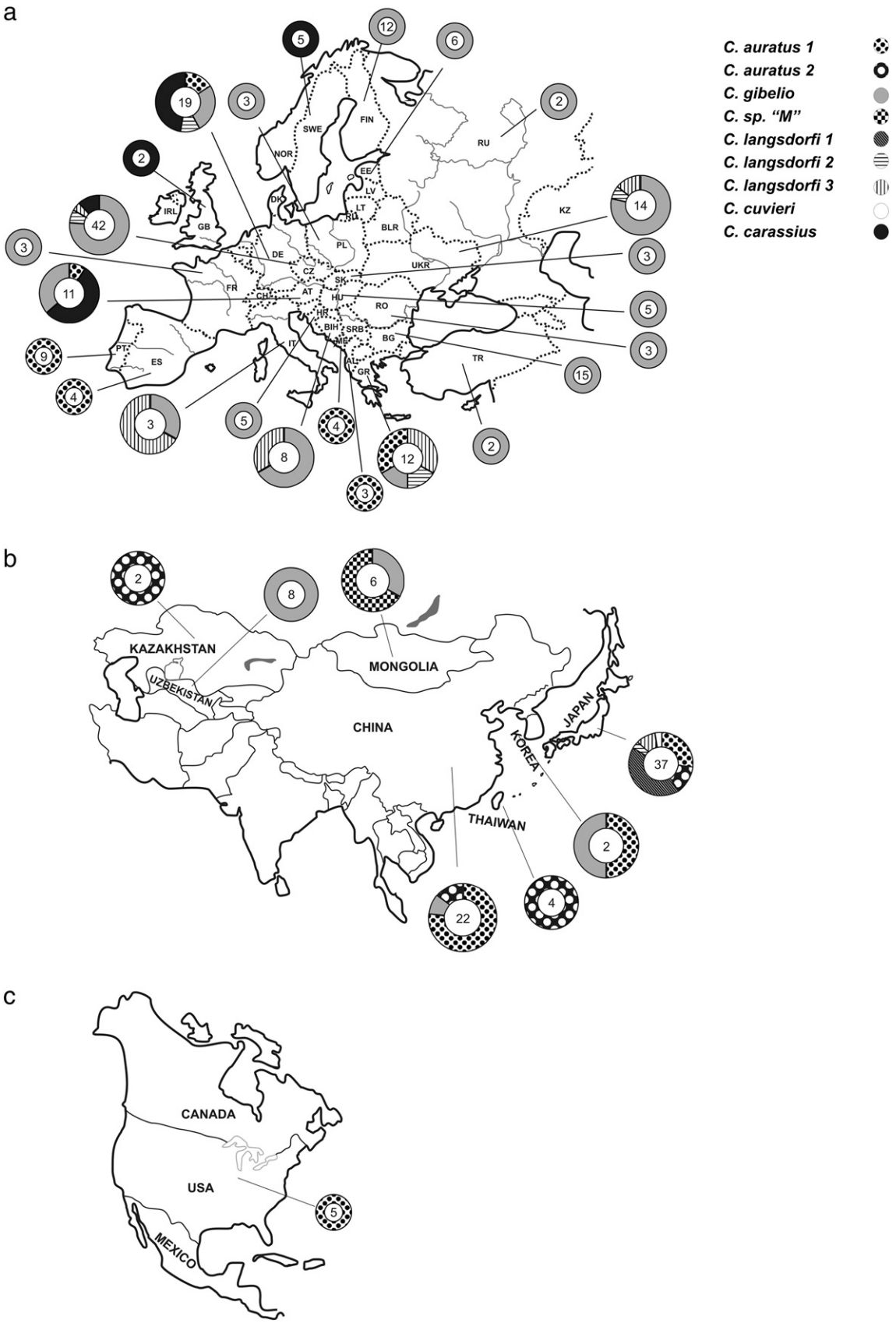

Fig. 2. Map showing the distribution of particular lineages of the genus *Carassius* within Europe (a), Asia (b) and North America (c). Patterns of the lineages in the circle correspond to presented phylotree (Fig. 1). Number in the circles represents the number of analysed specimens. Individuals of unknown origin and those clearly coming from ornamental trade were not included.

Table 1
Number of examined specimens within each lineage with mean values of DNA content (pg) including basic statistics separately for groups of diploids and triploids. Symbol n.a. refers to absent data.

Lineage	2n	DNA content pg ± S.D.	3n	DNA content pg ± S.D.	Sequences	Haplotypes
C. auratus I	15	4.25 ± 0.74	14	6.56 ± 0.13	147	26
C. auratus II	4	4.66 ± n.a.	6	6.76 ± 0.07	27	17
C. gibelio	12	4.40 ± 0.64	49	6.81 ± 0.42	150	21
Carassius sp. *"M"*	0	n.a.	3	6.78 ± 0.4	5	4
C. langsdorfii I	4	n.a.	5	n.a.	20	14
C. langsdorfii II	0	n.a.	5	6.65 ± 0.03	9	5
C. langsdorfii III	1	4.81 ± n.a.	4	n.a.	17	6
C. cuvieri	0	n.a.	0	n.a.	3	2
C. carassius	6	4.65 ± 0.3	0	n.a.	26	9
Mean ± S.D.		4.38 ± 0.49		6.74 ± 0.36		
Min.–max.		3.6–5.42		5.75–7.33		

were imported as fry that had been collected in natural rivers in China, other fish species than carps may also have been imported unintentionally, goldfish dispersed through the Ohrid-Drin-Skadar system into the territories of Albania, Montenegro and Greece and here were transported to neighbouring water systems like Prespa Lake (Liasko et al., 2010). Although these two groups can be distinguished, both of them belong to one mitochondrial lineage (sublineage *C. auratus I*). In contrast, populations from Asia display much larger genetic variability (beside of *C. auratus I* also more diversified sublineage *C. auratus II*). This is consistent with the findings of Gao et al. (2012).

The species *C. langsdorfii* was reported for the first time from Europe in 2000 (Kalous et al., 2007) and seems to have been only recently introduced. The ways of introduction to Europe are not known, but most likely it has been an unintended introduction with other cyprinid fishes. Up to now, only few single reports have been published, but our results suggest that the species is by far more widespread than originally believed. Due to its very recent finding, nothing is known about the establishment of stable populations in Europe, but the fact that the specimens from Bosnia-Herzegovina and from northern Germany were juveniles of 3–5 cm TL suggests that they are naturally reproducing there. Takada et al. (2010) and Yamamoto et al. (2010) have recently demonstrated that *C. langsdorfii* in Japan includes a number of very different lineages that have non-overlapping geographic distribution. Comparing our haplotypes with the data presented in these studies, we conclude that the geographic origin of *C. langsdorfii* type *II* is related to Honshú Island and the origin of *C. langsdorfii* type *III* is in the Ryúkyú Islands. The type *I* of *C. langsdorfii* was not detected in Europe. The presence of two different sublineages of *C. langsdorfii* in different localities across Europe indicates introductions from more than one source population.

The most abundant and widespread species of the *C. auratus* complex in Europe is *C. gibelio*. It has been mentioned by Bloch (1782) to occur in the area that nowadays is eastern Germany and Poland, by Gąsowska (1934) from an area nowadays in western Ukraine and by Slynko et al. (2011) as being historically present in Dnieper River. In contrast, it seems to have been unknown to historical authors in Western Europe and the Danube basin. The earliest records from the Danube basin (lower Danube in Bulgaria and Romania) are from the 1940s (Bănărescu, 1964), but catches were rather negligible until the 1960s, when the species became rapidly more abundant and started to spread (Holčík and Žitňan, 1978). Subsequently, the species was observed more and more westward in the Danube system and finally also in several western European river basins. These late records together with the observation that *C. gibelio* in the Danube basin carried parasites that were new to the region (Žitňan, 1974) suggest that the species is a recent invader in these areas. In fact it has been speculated that *C. gibelio* is a non-native species for all of Europe (Lusková et al., 2004), while other authors considered it native at least in the eastern parts of Central Europe (Kottelat, 2006; Kottelat and Freyhof, 2007). Here we extend the later opinion and suggest that *C. gibelio* is native from eastern Central Europe eastwards through Siberia for the following reason: throughout Siberia until the Amur basin, *C. gibelio* is a common species similar to other lowland species like *Cobitis melanoleuca* and *Rhynchocypris percnurus* (Bogutskaya and Naseka, 2002; Kottelat and Freyhof, 2007; Reshetnikov et al., 1997). These three species further have in common that their closest related species live in East Asia; indicating that they spread from northern East Asia through Siberia to Europe. The presence of the same species in Europe, East Asia and across Siberia together with the close genetic relationship between Asian and European populations (demonstrated for *C. melanoleuca* by Tang et al. 2008 and for *C. gibelio* by the present study) suggests this spreading to have occurred postglacially, most likely via the swamp areas created by the melting ice. While this postglacial range extension carried *C. melanoleuca* westwards until the River basins of Volga and Don, the small swamp-inhabiting *R. percnurus* was able to colonise Europe westwards as far as into the Odra river basin in present-day western Poland (Kottelat and Freyhof, 2007). Having in mind that *R. percnurus* and *C. gibelio* inhabit the same habitat, often co-occur in large parts of their distribution area and that *C. gibelio* is much hardier than *R. percnurus* to withstand habitat degradation, it seems highly likely that the native distribution area of *C. gibelio* stretches at least as far westwards as that of *R. percnurus*. Unfortunately, the low genetic diversity within *C. gibelio* does not bring further information about a single or double origin of the European populations of *C. gibelio*. However, since the genetic diversity among East Asian populations is comparably low and the proposed colonisation of Europe has happened maximally 8000 years ago, the absence of any statistical differences in the cytochrome *b* between populations from eastern Central Europe and the Danubian basin cannot be taken as a sign that they have the same colonisation history, but that this marker is not suited to answer this question.

5. Conclusions

Our data show that the present day knowledge about the taxonomy as well as the distribution of the genus *Carassius* still bears many weaknesses. Genetic data give indications of at least one undescribed species and reveal a much wider distribution of *C. langsdorfii* and *C. auratus* in Europe than formerly believed. It appears that *C. auratus* is nearly restricted to the Mediterranean basin and Great Britain, while *C. gibelio* inhabits most of Central and Eastern Europe and *C. langsdorfii* has a very scattered distribution across Central and South-eastern Europe. It is demonstrated that genetic data can be used to provide valuable insights about the taxonomy and distribution of fishes with difficult morphological species-recognition, to evaluate the value of ploidy changes in taxonomy and to reconstruct their biogeographic history. Therefore it can stimulate future studies on these commercially important fishes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2012.11.027.

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Chromosome Studies of European Cyprinid Fishes: Cross-Species Painting Reveals Natural Allotetraploid Origin of a *Carassius* Female with 206 Chromosomes

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Key Words
Fish cytogenetics · Genome addition · GISH · Leaky gynogenetic reproduction · Polyploid cyprinids

Abstract
A single female with 206 chromosomes and another 26 females with 156 chromosomes identified as Prussian carp, *Carassius gibelio*, and 5 individuals with 100 chromosomes identified as crucian carp, *C. carassius,* were sampled during field survey in one locality in the upper Elbe River. To identify the origin of females with high chromosome numbers, comparative karyotype analysis, GISH, with whole *C. carassius* DNA as probe and phylogenetic positions of sampled individuals revealed by cytochrome *b* mitochondrial marker were performed. GISH showed consistently bright labeling of 50 chromosomal elements out of 206, corresponding to the haploid chromosome number of *C. carassius.* The position of these females with high chromosome numbers in a reconstructed phylogenetic tree was within the clade of *C. gibelio*, documenting its affiliation to *C. gibelio* mitochondrial, i.e. maternal lineage. Our findings indicated that the mother of the female with high chromosome numbers was a gynogenetically reproducing 156-chromosome *C. gibelio* female and the father a bisexually reproducing *C. carassius* male. We, therefore, hypothesized that the *C. gibelio* × *C. carassius* allopolyploid female with 206 chromosomes arose by a mechanism of sperm genome addition to an unreduced egg of the mother.

Interspecific hybridization and production of viable hybrid offspring is well known among lower vertebrates [Dawley and Bogart, 1989; Vrijenhoek, 1998; Neaves and Bauman, 2011]. The relatively higher frequency of cross-species breeding among fishes is caused by overall predominance of external fertilization in aquatic environment. Moreover, many cyprinid species share similar spawning grounds in the same time that indeed increases the probability of hybridization events [Wheeler and Easton, 1978]. Fishes of the genus *Carassius* are represented in Europe by 4 taxa: (1) native and highly endangered crucian carp (*C. carassius* L.) [Kottelat and

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Freyhof, 2007], (2) pan-globally distributed feral goldfish (*C. auratus* L.) [Szczerbowski, 2002], (3) recently found Japanese Ginbuna (*C. langsdorfii*, Temminck and Schlegel 1846) [Kalous et al., 2007], and (4) native Prussian carp (*C. gibelio*, Bloch 1782) [Lusková et al., 2010; Kalous et al., 2012]. Moreover, diploid-polyploid complexes within the genus *Carassius* exist throughout the vast territory of its occurrence including biotypes comprising individuals with approximately 150 chromosomes ('triploids') [Kalous and Knytl, 2011] that are often represented almost exclusively by females [Halačka et al., 2003]. These females are sperm dependent parthenogens (gynogens) that require sperm of another related species for their reproduction [Peňáz et al., 1979]. Interestingly, when the heterologous sperm enters the unreduced egg of a gynogenetic female, the biological function of a sperm is reduced to the triggering of egg development, and the resulting offspring is a clone of the mother with the same ploidy level [Golovinskaya and Romashov, 1947; Yamashita et al., 1993; Vrijenhoek, 1998; Gui and Zhou, 2010]. An appearance of a small amount of male genetic material in the genome of a gynogenetic offspring was described by Yi et al. [2003], and this phenomenon is known as paternal leakage [Tóth et al., 2005; Lamatsch and Stöck, 2009]. It was also hypothesized that such leakage can be the reason for sudden male appearance within the whole female gynogenetic populations, due to a possible interspecific transfer of sex-determining genes [Arai et al., 1995; Janko et al., 2007; Loewe and Lamatsch, 2008; Neaves and Bauman, 2011]. Hybridization between different *Carassius* species and biotypes with various ploidy levels appears to be quite common [Mezhzheryn et al., 2012]. Hybridization between *C. carassius* and *C. gibelio* in alluvium of the Thaya River, Danube River basin, was recently demonstrated by microsatellite analyses [Papoušek et al., 2008]. Similarly, a recent ongoing hybridization process between native crucian carp *C. carassius* and introduced goldfish *C. auratus* was described from the British Isles by Hänfling et al. [2005] and from Sweden by Wouters et al. [2012]. Even intergeneric hybridization between the fishes of the genera *Carassius* and *Cyrpinus* were revealed by several studies [Hänfling et al., 2005; Zhu and Gui, 2006; Liu, 2010]. On the other hand, the morphological recognition and identification of such hybrids is very difficult, due to high external similarities. Here, we report a discovery and identification of a natural allotetraploid female resulting from hybridization of bisexually reproducing *C. carassius* and gynogenetically reproducing *C. gibelio*.

Materials and Methods

Fish Sampling

A single female with 206 chromosomes and another 26 females with 156 chromosomes identified morphologically as Prussian carp, *C. gibelio*, and 5 individuals with 100 chromosomes identified as crucian carp, *C. carassius*, were collected during a field survey of ichthyofauna in alluvial ponds and old oxbows of the Elbe River close to the city of Lysá nad Labem (recognized as Byšičky, GPS: 50°10′33,7″ N, 14°46′25,4″ E). The specimens examined were not deposited as vouchers.

Chromosome Analysis

Mitotic activity was stimulated by intraperitoneal injection of 0.1% $CoCl_2$ (1 ml $CoCl_2$/100 g body weight) 24 h before chromosome preparation. Standard direct procedures for chromosome preparation from cephalic kidney followed Ráb and Roth [1988]. To arrest cell division in metaphase, 0.1% colchicine (1 ml colchicine/100 g body weight) by intraperitoneal injection was used. Valid animal use protocols were in force at IAPG and CULS during this study.

Microscopy and Image Processing

Metaphase chromosomes stained in 4% Giemsa-Romanowski solution in phosphate buffer (pH = 7) were observed with a microscope BX41TF equipped with a digital camera Olympus SP-350, and chromosomes were counted by PC software QuickPhoto Micro. Karyotypes were constructed using PC software Ikaros (karyotyping system) version V 3.4.0 and Adobe Photoshop version CS5. Chromosome morphology was determined according to Levan et al. [1964]. Analyzed slides with recorded coordinates of selected metaphases were distained in fixative (methanol and acetic acid; 3:1, v/v) for 3 min and stored at +4°C until the GISH experiment.

Isolation of Genomic DNA

Total genomic DNA was isolated from ethanol preserved tissue using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol.

Genomic in situ Hybridization

Genomic DNA from *C. carassius* was indirectly labeled by a standard nick translation reaction using nick translation mix for in situ probes according to the manufacturer's instructions (Roche, Mannheim, Germany). Total 25 µl of hybridization mixture, containing nick translation mix, NT-dNTPs, labeled dUTPs, DNA template, and H_2O, was incubated for 90 min at 15°C. DNA was precipitated with salmon sperm (100 µg/ml), 3 M sodium acetate pH = 5.2 (25°C) and 96% ethanol. The biotin-dUTP labeled probes (Roche) were detected by either the Invitrogen (Karlsruhe, Germany) CyTM3-Streptavidin or by FITC-Streptavidin. The digoxigenin-dUTP labeled probes (Roche) were detected by either the Roche anti-digoxigenin-fluorescein or by anti-digoxigenin-rhodamin diluted according to manufacturer's instructions. Chromosome preparations were dehydrated through ethanol series (70, 80 and 96% for 3 min each) on ice and air-dried. Chromosome preparations were aged for 1 h at 37°C before and after pepsinization (3 min at 37°C 50 µl aliquot pepsin, 1 N HCl and distilled H_2O).

Hybridization and detection during GISH experiments were carried out as described by Cremer et al. [2008]. Slides were dehydrated through ethanol series, air dried and aged again for 45 min at 37°C. Chromosomal denaturation was carried out in 75% for-

Table 1. Material used in molecular analyses

Fish	n	Sex	Ploidy level	Chromosome number	Locality	GenBank number
C. carassius	5	–	2n	100	Byšičky, Elbe River, Czech Republic	JQ763597
C. gibelio	5	f	3n	156	Byšičky, Elbe River, Czech Republic	JQ763598
C. gibelio/ *C. carassius*	1	f	4n	206	Byšičky	JQ763599
C. gibelio (neotype)	1	f	2n	–	Český Těšín, Olza River, Czech Republic	JN402305*
C. auratus	1	–	–	–	Nanking, Yangtze River, China	EU663598**
Cyprinus sp. (outgroup)	1	–	–	–	Mekong River, Thailand	HM008692*

* Sequences from Kalous et al. [2012]; ** sequence from Rylková et al. [2010]; cytochrome *b* haplotypes are deposited under the corresponding number in the GenBank.

mamid in 2× SSC (pH = 7) for 3 min at 74°C and quickly dehydrated through –20°C ethanol series and air dried. Hybridization mixture with Salmon sperm was denaturated at 86°C for 6 min and then cohybridized to target slide with the denatured metaphases from the tetraploid *Carassius* female under a 24 × 50 mm coverslip. The slides were incubated for 48 h at 37°C in a dark room. After hybridization, slides were then washed for 2 × 10 min each in 50% formamid with 2× SSC, 3 × 7 min each in 1× SSC in water bath at 42°C and 1 × 20 s in 4× SSC at room temperature. After performing series of stringency washes, stop reaction was carried out with 2.5% BSA/4× SSC/Tween for 20 min at 37°C under a 24 × 50 mm coverslip. Chromosomes were counterstained with DAPI (4′,6-diamino-2-phenylindol) combined with a mounting media (Starfish, Cambio, Cambridge, UK).

Microscopy and Image Processing

GISH images were captured with a cooled CCD camera Olympus DP30BW (equipped with a B&W CCD-Chip Sony ICX285-AL) coupled to an epifluorescence microscope Olympus AX70 equipped with a set of 3 narrowband fluorescent filters. Micrographs were captured with the Olympus Acquisition Software, and B&W images were processed with the software MicroImage. The pseudocolor images were analyzed using the Adobe Photoshop Version CS5. Altogether, 30 metaphases for each specimen were analyzed.

Molecular Analysis

Detailed information about material used for molecular analysis is listed in table 1. The cytochrome *b* gene was amplified using the methods described in Rylková et al. [2010], with the forward primer Kai_F 5′-GAAGAACCACCGTTGTTATTC-3′ and reverse primer Kai_R 5′-ACCTCCRAYCTYCGGATTACA-3′ [Šlechtová et al., 2006]. PCR products were purified and sequenced by Macrogen Incooperation (Seoul, Korea).

The raw chromatograms were manually assembled and checked by eye for potential mistakes using computer software BioEdit 5.0.9. [Hall, 1999]; the same program was used to align the sequences using the ClustalW algorithm. Dataset was created for cytochrome *b* analysis, and the phylogenetic relationships were estimated using the methods of maximum parsimony in PAUP* version 4.0b10 [Swofford, 2000] and Bayesian analysis using the program MrBayes version 3.0 [Huelsenbeck and Ronquist, 2001].

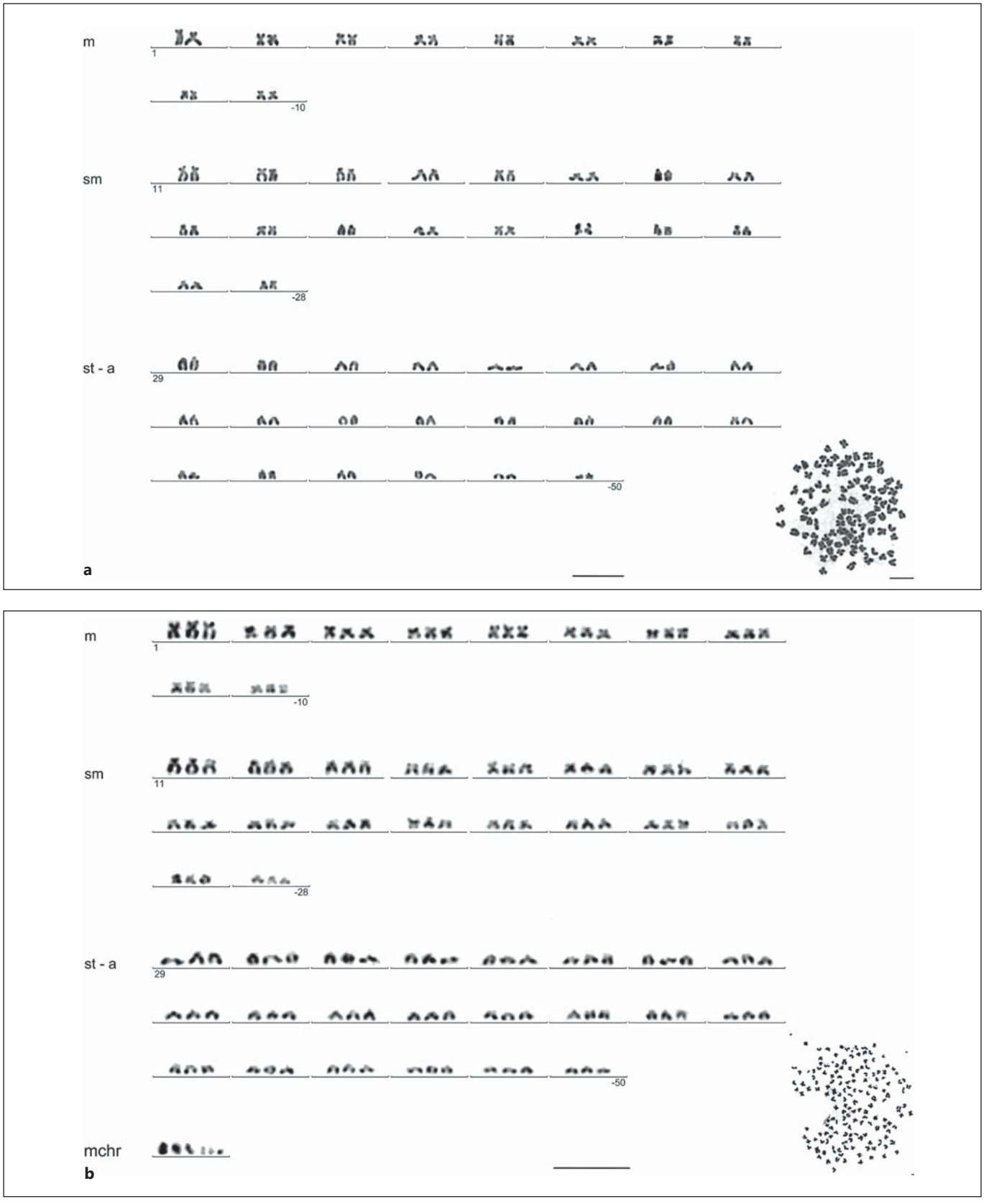
Results

Chromosome Analysis

The analyzed individuals included 3 different categories. Individuals with 2n = 100 chromosomes were unambiguously identified as *C. carassius*, and their karyotypes consisted of 10 pairs of metacentric (m), 18 pairs of submetacentric (sm) and 22 pairs of subtelo- (st) to acrocentric (a) chromosomes (fig. 1a). All other fishes were identified as females of *C. gibelio*, where 26 individuals possessed 3n = 156 chromosomes with a karyotype composed of 30 m, 54 sm, 66 st to a, and 6 microchromosomes (fig. 1b), while one female had 4n = 206 and a karyotype composed of 40 m, 72 sm, 88 st to a, and 6 microchromosomes (fig. 1c).

Genomic in situ Hybridization

Biotin-labeled *C. carassius* genomic DNA hybridized to the chromosomes of tetraploid female and provided consistently intensive fluorescent signals on 50 chromosomes (fig. 2b) with distinctly pink fluorescence well discriminated from other blue-stained chromosomes. The positively hybridized chromosomes were graphically sep-



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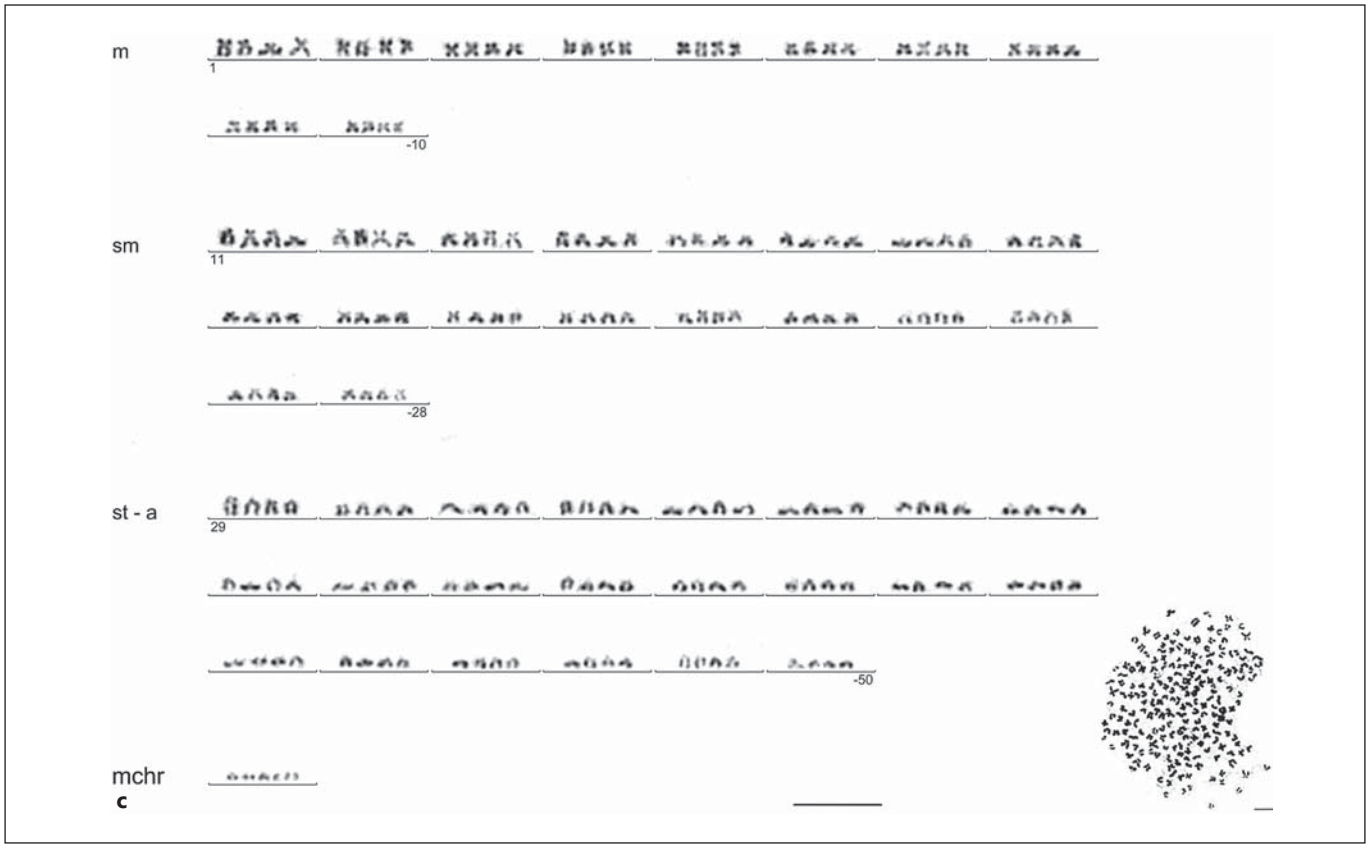

Fig. 1. Karyotype of male *C. carassius* (**a**), of female *C. gibelio* (**b**) and of female allotetraploid hybrid of *Carassius* (**c**), arranged from Giemsa-stained chromosomes (shown as inlay). m = Metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric chromosomes; mchr = microchromosomes. Scale bar = 10 μm.

arated in the karyotype of tetraploid individual (fig. 3) and very likely corresponded to haploid chromosomes of *C. carassius* (fig. 1a).

Molecular Analyses

The final matrix of the cytochrome *b* sequences consisted of 1,110 bp containing 152 variable characters with 45 parsimony informative sites. Both employed methods have recovered topologies with high statistical supports. All 5 sequences of *C. carassius* from Byšičky showed one haplotype (GenBank accession number JQ763597); in case of *C. gibelio* from Byšičky one haplotype was found (GenBank accession number JQ763598). The position of *C. gibelio* × *C. carassius* hybrid (GenBank accession number JQ763599) in reconstructed phylogenetic tree (fig. 4) is within the clade of *C. gibelio* mitochondrial lineage indicating that the mother of the allotetraploid specimen was *C. gibelio*. Moreover, the hybrid and 5 individuals of *C. gibelio* from Byšičky shared the same haplotype.

Discussion

Our finding and subsequent genetic analyses of a natural allotetraploid female of the genus *Carassius* revealed its interspecific origin and combination of 3 chromosome sets of *C. gibelio* and a haploid one of *C. carassius* within one individual genome.

It was clearly demonstrated that the paternal chromosome haploid set detected by GISH analysis originated from *C. carassius*, while the maternal triploid set could be assigned by mtDNA markers to *C. gibelio*, but final confirmation must explore nuclear markers. This conclusion confirmed the previous assumptions because the males of *C. gibelio* were not found at the locality, and the local Prussian carp population consisted of triploid gynogenetic females only [Daněk et al., 2012].

Such occasional sperm genome additions in otherwise gynogenetically reproducing *Carassius* fishes are likely more common. Zhu and Gui [2006] reported on an inter-

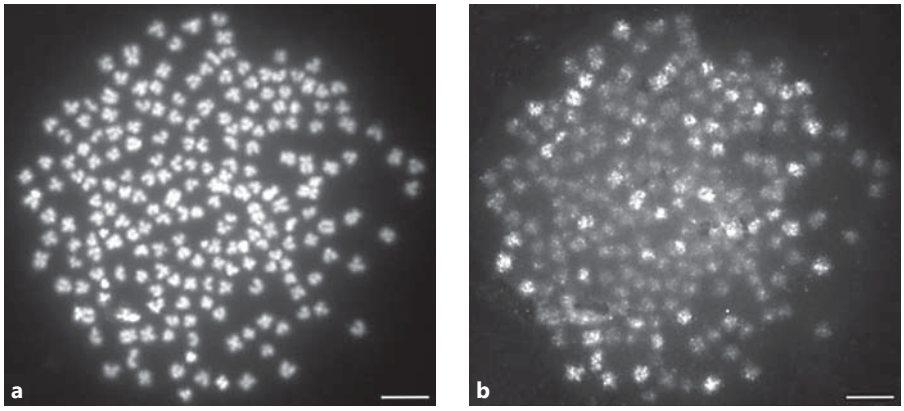

Fig. 2. Genomic in situ hybridization experiment using biotin-labeled *C. carassius* genomic DNA to the chromosomes of a tetraploid individual. Metaphase counterstained by DAPI shows all 206 chromosomes (**a**), metaphase image stained by biotin with Cy3 filter shows 50 chromosomes originating from *C. carassius* (**b**). Scale bar = 10 μm.

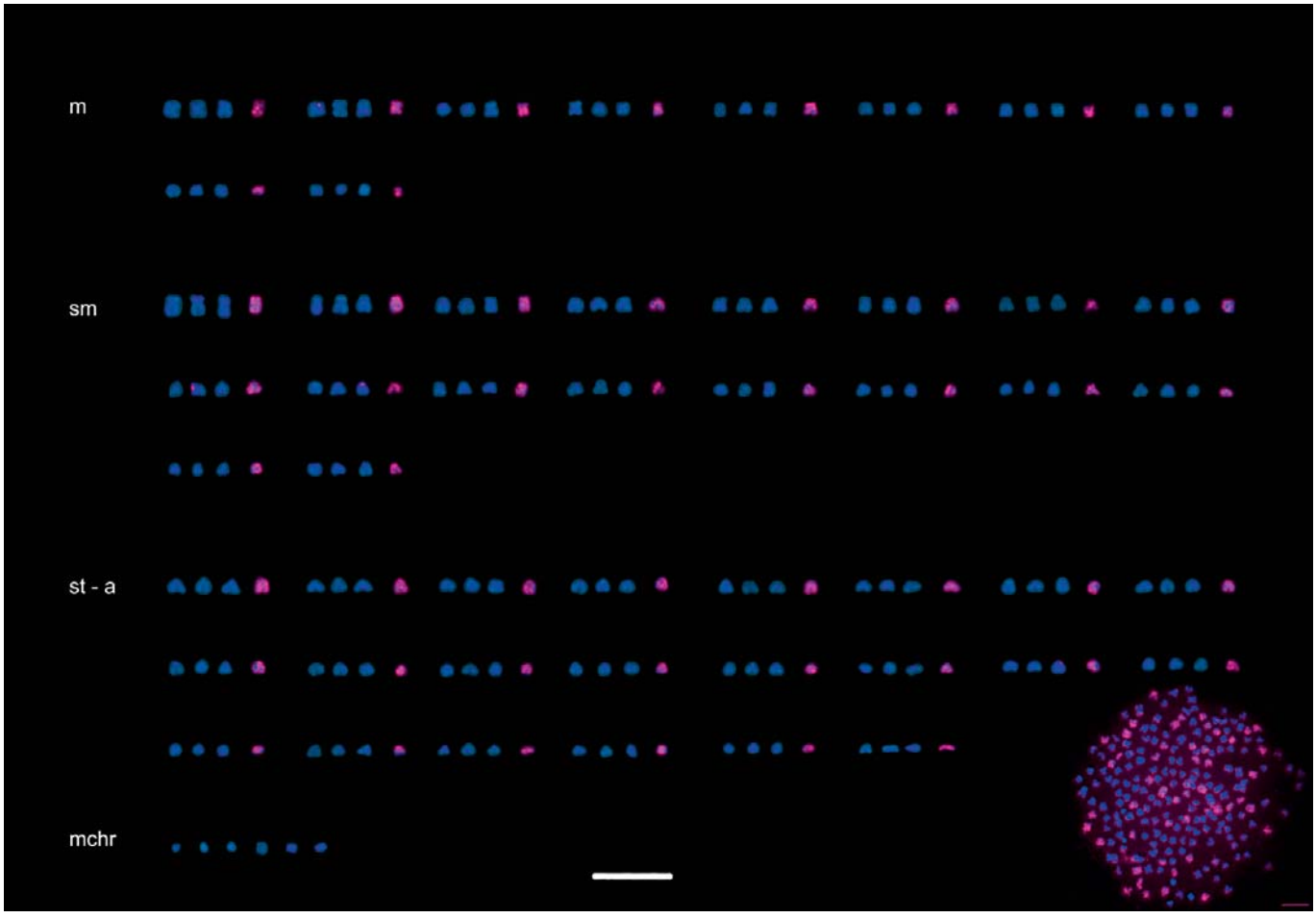

Fig. 3. Karyotype of an allotetraploid hybrid *Carassius* female with 206 pseudocolored chromosomes, DAPI (blue) and Cy3 filter (red). Scale bar = 10 μm.

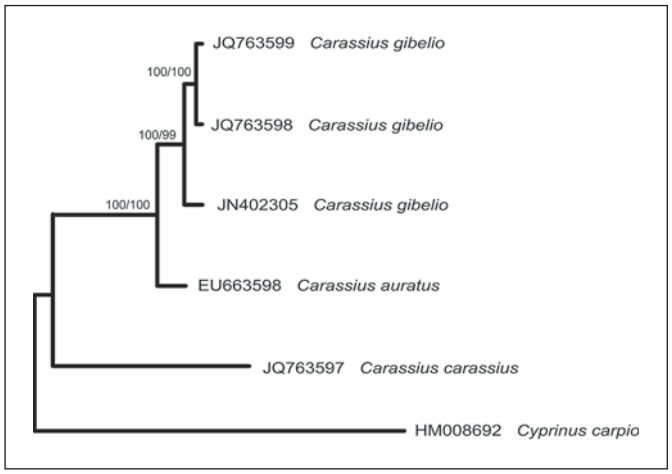

Fig. 4. Phylotree: reconstructed phylogeny of the cytochrome *b* haplotype sequences. The numbers at the nodes represent statistical supports for Bayesian and maximum parsimony analyses, respectively. As outgroup, the sequence of *Cyprinus carpio* was used.

generic hybrid from China and the cytogenetic analysis clearly showed the ratio 3 *Carassius*:1 *Cyprinus* genomes combination in the tetraploid fish. The ratio of representative genomes *(C. gibelio:C. carpio)* in genome of allotetraploid hybrids is the same as in our study *(C. gibelio:C. carassius)*, thus reproducing mechanism should be at least similar in both cases.

Besides the report of Zhu and Gui [2006], our finding proved that gynogenetic triploid females of *C. gibelio* have not only the capability to maintain all chromosomes, but also the ability to elevate ploidy level via sperm incorporation.

The molecular evidence for an occurrence of natural hybrids between *C. gibelio* and *C. carassius* was already described by Papoušek et al. [2008], but all of them were considered as diploids (i.e. with 100 chromosomes) with the assumption that only diploid biotypes of *C. gibelio* could have hybridized with *C. carassius* following the model of *C. auratus* and *C. carassius* hybrids in Great Britain [Hänfling et al., 2005].

The explanation of allopolyploidization in gynogenetic Prussian carp can be attributed to the dual reproduction modes, i.e. gynogenesis and sexual reproduction as it has been described in Chinese population of polyploid biotypes of the genus *Carassius*. These 2 modes are based on recognition of homologous and heterologous sperm by ovum. When heterologous sperm enters the egg, the gynogenesis takes place; the entered sperm does not decondense until the first cleavage and triggers embryogenesis. Contrary, when a homologous sperm enters the egg, the responding development mode is sexual reproduction; the entered sperm decondenses and forms a male pronucleus that fuses with the female pronucleus. The established zygote undergoes recombination and removes approximately half of the maternal chromosomes from the egg or dissolves them in cytoplasm [Zhou et al., 2000; Gui and Zhou, 2010].

It seems that the ability of sperm recognition and sperm genome elimination is not flawless, and possible mistakes can result in the presence of a certain number of tetraploid individuals within the triploid and diploid biotypes of the genus *Carassius*. Although the tetraploids are rare in the natural population, they are regularly recorded from various sites in Europe, e.g. Abramenko and Kravchenko [1998], Halačka and Lusková [2000], Halačka et al. [2003], Toth et al. [2005], Liasko et al. [2010], and Mezhzheryn et al. [2012]. This raises the question of what percentage of tetraploid Prussian carps can be of allopolyploid origin, since the genome composition is not usually investigated, and fish with different ploidy levels are morphologically indistinguishable [Vasileva, 1990].

It remains unknown whether such a genome addition mechanism is associated with phylogenetically closely related *Carassius* and *Cyprinus* genomes, or another mechanism is involved, for example that a certain amount of asexual females are available for genome addition as it was described by Choleva et al. [2012], following the idea that polyploidy is not a trigger of clonality, but rather a consequence.

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Carassius gibelio: a cryptic invader arriving to Iberian Peninsula

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Abstract We describe the presence of polyploid biotype of Prussian carp, *Carassius gibelio*, in three large river systems of Iberian Peninsula (Guadalquivir, Guadiana and Tagus Rivers). This represents a recent invasion of this inconspicuous species in Iberian watersheds due to high morphological resemblance with the long established goldfish *Carassius auratus*. Genetic markers were employed since morphological characters failed in species identification. The introduction of *C. gibelio* to these Iberian drainages is discussed in light of the potential vector to the Iberian Peninsula.

Introduction

Non-native fishes (NNF) in aquatic ecosystems continues to rise in Europe (García-Berthou et al. 2005; Rabitsch et al. 2012), being the Mediterranean freshwater ecosystems one of the most invaded areas, with NNF already accounting for 50% of the fish diversity (Clavero and García-Bertou 2006; Leprieur et al. 2008). Not surprisingly, the Mediterranean freshwater ecosystems are invasions hotspots, regardless of an increasing awareness that NNF are a threat to native faunas (Hermoso and Clavero 2011). Iberian Peninsula (Portugal and Spain) is one of the Mediterranean regions with highest number of NNF after Italy (Bianco 2014), a situation largely due to illegal introductions due for recreational fisheries purposes (Elvira and

Almodóvar 2001; Ribeiro et al. 2009a). Unfortunately, the number of NNF species in Portugal and Spain continues to rise (e.g. Ribeiro and Veríssimo 2014; Aparicio et al. 2013), with the previous invasion records for this region outdated (Elvira and Almodóvar, 2001; Ribeiro et al., 2009a).

The goldfish *Carassius auratus* (Linnaeus, 1758) is one of the oldest fish introductions in Iberian Peninsula (Ribeiro et al. 2009a), but morphologically indistinct from other *Carassius auratus* complex species (*sensu* Takada et al. 2010; Hensel 1971; Vasil'eva and Vasil'ev 2000). *Carassius gibelio* (Bloch 1782), native to Central and Eastern Europe, has spread swiftly across Europe in the last decade (e.g. Perdikaris et al. 2012; Verreycken et. al 2007; Wouters et al. 2012). The high morphological resemblance of Prussian carp to other species from the genus *Carassius* contributed to very late detection of this invasive species (Wouters et al. 2012) . Here we confirm the presence of the Prussian carp - *Carassius gibelio* - in Iberian Peninsula using molecular and cytogenetic methods, demonstrating that this species is widespread across the main drainages of Iberia Peninsula.

Materials and Methods

Fish specimens were collected from five locations in Iberian Peninsula (Fig. 1), including four out of the five main rivers (Table I). All specimens from the Guadiana and Tagus estuary were fixed in 4% formalin, followed by preservation in 70% ethanol, and deposited and registered in the collection of Museu Nacional de História Natural e da Ciência (MUHNAC). Measurements of standard length (SL±1 mm) and total weight (TW±0.1 g) of each specimen were determined. Fish were sexed, maturity stages were recorded after preservation and finclips were extracted for analysis. Genomic DNA was isolated from ethanol preserved tissue using DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). The mitochondrial cytochrome *b* gene was amplified using primers Glu L. Ca14337-14359: GAA GAA CCA CCG TTG TTA TTC AA and Thr H. Ca15568-15548: ACC TCC RAT CTY CGG ATT ACA (Šlechtová et al., 2006). PCR amplification was performed in 50µl reaction volumes containing 15,5µl Combi ppp Master Mix (Top-Bio s.r.o., Praha, Czech Republic), 3µl of each primer and template DNA. The PCR profile started with 10 min period of initial denaturation at 95°C, followed by 34 cycles each consisting of denaturation step at 94°C for 30 s, annealing step at 54°C for 30 s and elongation step at 72°C for 1 min. PCR was terminated by final elongation step at 72°C for 10 min. PCR was carried out on MJ Mini

thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea. The raw chromatograms were manually assembled, checked for mistakes using computer software BioEdit 5.0.9 (Hall 1999) and sequence alignment was done using the ClustalW algorithm. Final editing and formatting of the dataset was done by on-line phylogenetic tool FaBox (Villesen 2007). The phylogenetic relationships were estimated from aligned sequences using the method of maximum parsimony (MP) performed in PAUP* version 4.0b10 (Swofford 2000) and Bayesian analyses (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck and Ronquist 2001) as described in Šlechtová et al. (2004). (Table II)

Additionally to the molecular techniques nine individuals of *Carassius sp*. (Table I) were checked for their ploidy level using blood smears. The ploidy level was determined by computer-assisted image analyses using the measurements of mean erythrocyte nuclei area (MENA) as was proposed by Flajšhans (1997). The blood was obtained with a heparinised syringe and blood smears were prepared as for conventional haematological examination, then air-dried, fixed in 90 % ethanol, and stained in 4% Giemsa solution. Blood smears were then processed on a system consisting of microscope (Nikon Eclipse 600, immersion objective 100x), analogue video camera (Hitachi HVC 20) and software (NIS-Elements 3.2, Laboratory Imaging spol. s r. o. – LIM, Czech Republic). The mean area of nuclei and the SD was calculated from 200 erythrocytes for each individual and compared with the karyologically calibrated reference data (Kalous and Petrtýl 2004; Daněk et al. 2012).

Results

The final matrix of the cytochrome *b* sequences consisted of 1084 characters containing 250 variable characters with 154 parsimony informative sites. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted sequences into five well-supported lineages (Fig. 2). The Iberian specimens were pooled together with specimens from *Carassius gibelio* from Odra River, Ukraine and from France. The ploidy level of the nine specimens from Guadiana showed that all individuals were triploids (evolutionary hexaploids) with values of the nuclei area ranging from 19.8 ± 1.9 μm^2 to 21.3 ± 2.3 μm^2, corresponding to Prussian carp.

Discussion

Since 2012, the increasing number of occurrence of the *Carassius sp.* in the Doñana National Park (Guadalquivir drainage) was initially detected. Posteriorly, in 2013 reports of high abundances of *Carassius* spp. were detected in the lower Guadiana drainage and in the Canha river (Tagus drainage) (Fig. 1). These records were further investigated in order to provide scientific evidence of *Carassius gibelio* presence in Iberian Peninsula. Molecular techniques are used to detect *C. gibelio* mtDNA in fish from three different drainages in the Iberian Peninsula.

According to phylogenetic screening made by Rylková et al. (2013) genetic diversity of *C. gibelio* in Europe is not high and it is represented by two main haplotypes. Other haplotypes are not numerous. Our analysis showed that population of *C. gibelio* at/on Iberian Peninsula is very varied (15 samples split into 11 haplotypes). This suggests multiple introduction events.

All values are in agreement with those previously published for triploid *C. gibelio* (Kalous & Petrtýl 2004, Daněk et al., 2012) corresponding to approximately 150 chromosomes they have very different introduction history in Europe (Rylková et al., 2013). While goldfish (*C. auratus sensu stricto*) which is of Yang-tze River origin (Rylková et al., 2010; Wang et al., 2013) and was brought to Europe already in 17th century, the Prussian carp (*C. gibelio*, Bloch 1782) is believed to be native to Central and Eastern Europe (Kalous et al., 2012) but the invasive polyploid biotypes of this species are most likely of Eastern Asian origin (Rylková et al. 2013). The occurrences of invasive triploid unisexual population of Prussian carp in Europe is coincident with the imports of herbivorous fish from Asia like grass carp, silver carp and big head carp (Copp et al., 2005; Tóth et al., 2005). The wave of the invasion is well documented in Danubian basin (Holčík and Žitňan, 1978), where the catches of *Carassius sp.* (most likely feral goldfish) were rather negligible until the 1960s, when another but morphologically very similar *Carassius sp*. later identified as triploid biotype of *C. gibelio* (Peňáz et al., 1979) became more abundant and started to spread (Holčík and Žitňan, 1978). Subsequently, the abundant populations of Prussian carp were observed in other European river basins across different countries (Balon, 1962; Bergerot et al. 2008; Deinherdt, 2009, Halačka et al. 2003; Perdikaris et al. 2012; Rylková et al. 2013, Verreycken et. al 2007;) and even in the Baltic Sea (Pihu et al., 2003; Vetemaa et al., 2005; Wouters et al., 2012).

Possible is also “cryptic” invasion (Mezhzherin et al., 2012; Vetešník et al. 2007, Wouters et al., 2012,).

Prussian carp has impacted ecology of rivers as well as other water bodies (Holčík, 1980; Tarkan et al., 2012a). It has impact to fish species composition (Gaygusuz et al., 2007; Tarkan, 2012b), quality of water (Crivelli, 1995; Navodaru et al., 2002) but also to aquaculture and recreational fisheries (Lusková et al., 2010).

Unfortunately very similar morphological appearance of the fishes from the genus *Carassius* contributed often to very late awareness of the invasive event and the employment of molecular genetic and cytogenetic methods are needed for clear identification.

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Captions for Figures

Figure 1 – Confirmed records of *Carassius gibelio* in the Iberian Peninsula in this study (●); D – Douro river, T – Tagus river, Gdn – Guadiana river, Gdv – Guadalquivir river.

Figure 2 – Reconstructed phylogeny of the cytochrome b sequences of *Carassius* samples included in present study. Numbers at nodes represent statistical supports for BAY and MP analyses respectively.

FIGURE 1

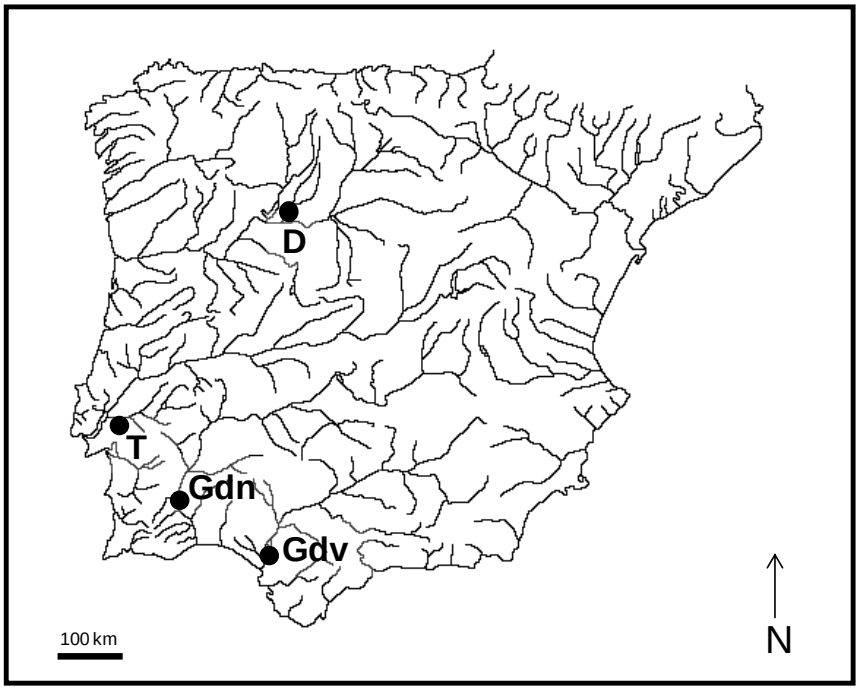

FIGURE 2

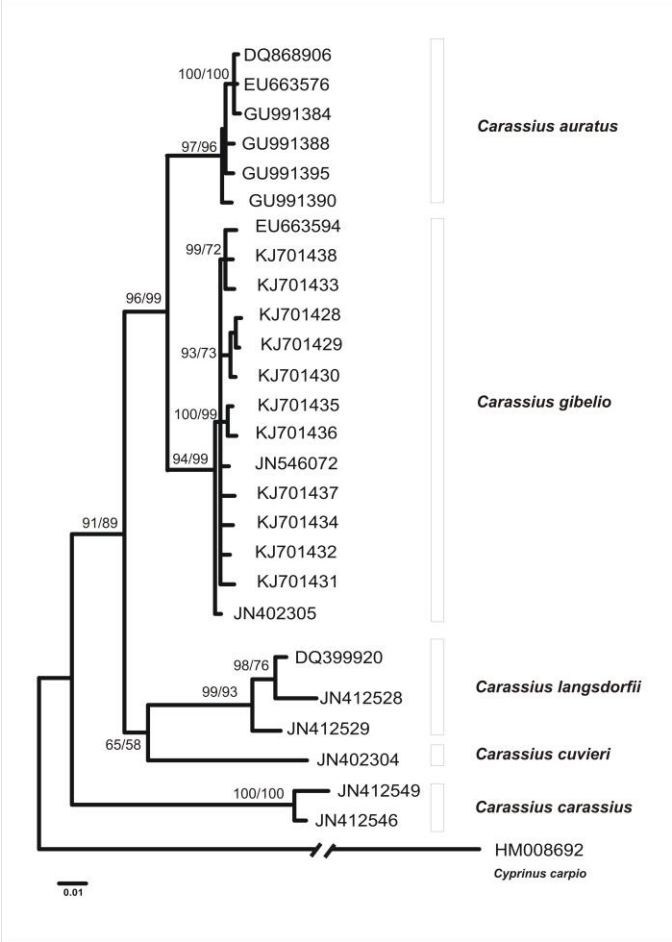

TABLE I – Samples used from Iberian Peninsula of *Carassius* sp., with the respective site location data (River, Basin, Locality, Latitude, Longitude and date), methods used (Tissue for molecular analysis, FC – Flow cytometry). *Specimens deposit in the National Museum of Natural History and Science (Lisbon – Portugal) collection, accession #MB05-003125).

Basin, River	**Locality**	**Latitude**	**Longitude**	**Date**	**Tissue**	**FC**
Douro, Pisuerga	Valladolid	41°40'16.11"N	4°42'55.27"W	LUKAS	1	-
Tagus, Canha	Canha	38°44'29.16"N	8°32'19.18"W	03/12/2013	19	-
Guadiana, Guadiana	Mértola	37º41'22.46"N	7º39'21.58"W	03/02/2013	10*	-
Guadiana, Guadiana	Mértola	37°38'28.91"N	7°39'15.00"W	28/12/2014	9	9
Guadalquivir,	Doñana	37º06'19.70"N	6º15'24.11"W	26/06/2013	10	-

TABLE II – Specimens used to built the parsimonious tree PAUP, with Sample ID, haplotype, origin, taxon and reference

SAMPLE ID	HAP	ORIGIN	TAXON	REFERENCE
DQ868906		Guadiana River, Portugal	*C. auratus*	Rylková et al. (2010)
GU991390		Ishem River, Albania	*C. auratus*	Kalous et al. (2012)
GU991388		Skadar Lake, Montenegro	*C. auratus*	Rylková et al. (2013)
GU991395		Prespa Lake, Greece	*C. auratus*	Rylková et al. (2013)
GU991384		Tejo River, Portugal	*C. auratus*	Rylková et al. (2013)
EU663576		Pet shop, Czech Republic	*C. auratus*	Rylková et al. (2009)
JN402305		Olza River, Odra river basin, Czech Republic	*C. gibeilo*	Kalous et al. (2012)
JN546072		Western Bug River, Bus'k, Ukraine	*C. gibelio*	Rylková et al. (2013)
EU663594		Canal de Fougeres, Loire river basin, France	*C. gibelio*	Rylková et al. (2010)
KJ701428	3	Lisbon, Portugal	*C. gibelio*	This study
KJ701429	1	Lisbon, Portugal	*C. gibelio*	This study
KJ701430	2	Guadiana, Portugal	*C. gibelio*	This study
KJ701431	1	Guadiana, Portugal	*C. gibelio*	This study
KJ701432	2	Guadiana, Portugal / Doňana, Spain	*C. gibelio*	This study
KJ701433	1	Doňana, Spain	*C. gibelio*	This study
KJ701434	1	Doňana, Spain	*C. gibelio*	This study
KJ701435	1	Doňana, Spain	*C. gibelio*	This study
KJ701436	1	Guadiana, Portugal	*C. gibelio*	This study
KJ701437	1	Guadiana, Portugal	*C. gibelio*	This study
KJ701438	1	Pisuerga river, Valladolid, Spain,	*C. gibelio*	This study
DQ399920		Abashiri Lake, Hokkaido, Japan	*C. langsdorfii*	Kalous et al. (2007)
JN412528		ponds at Ridne village, Chorna river basin, Ukraine	*C. langsdorfii*	Kalous et al. (2013)
JN412529		pool at Litvínovice, Czech Republic	*C. langsdorfii*	Rylková et al. (2013)
JN402304		Lake Mikatako, Honsyu, Japan	*C. cuvieri*	Kalous et al. (2012)
JN412549		fish farm Višňová, Czech Republic	*C. carassius*	Rylková et al. (2013)
JN412546		Lake Örlången, Sweden	*C. carassius*	Rylková et al. (2013)
HM008692		Mekong River, Thailand	*Cyprinus carpio*	Kalous et al. (2012)