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Dříve evoluční zatracenci, nyní tvůrci reprodukční strategie: původ a reprodukce samčí linie vodních skokanů *Pelophylax esculentus*

Earlier evolutionary dead-ends, now the creators of a reproductive strategy: the origin and reproduction of the all-male water frog lineage *Pelophylax esculentus*

DISERTAČNÍ PRÁCE

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…….

Marie Doležálková

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SOUHRN

Asexuální způsoby reprodukce jsou obvykle založeny na principu kopírování (klonování) samičí DNA do potomstva. U většiny asexuálně se množících obratlovců se potomstvo vyvíjí z neredukovaného a často neoplozeného vajíčka. Umožňuje to mechanismus partenogenetické a gynogenetické reprodukce. Zatímco v prvním případě se klonální pohlavní buňka vyvíjí spontánně a samostatně, v druhém případě je zapotřebí pohlavní partner, který aktivuje rýhování vajíčka. Ani v jednom případě však nedochází k oplodnění (splynutí spermie s vajíčkem). Klonální potomstvo proto tvoří výhradně dcery a dosavadní výzkum probíhal jen na asexuálních liniích samičího pohlaví. Vzácně mohou vznikat při pravém oplodnění klonálního vajíčka asexuální samci, ti však bývají často neplodní. Na světě jsou známí diploidní zástupci pouze tří rodů obratlovců hybridního původu, kteří disponují plodnými asexuálními samci. Jedním z nich jsou evropští vodní skokani rodu *Pelophylax*, žijící také na území České republiky.

V oblastech horního toku řeky Odry byly nedávno objeveny populace hybridních samců, kteří po vzoru asexuálních samic vytváří vše-samčí linie. Výsledky této studie ukázaly, že samci tvoří klonální spermie procesem hybridogeneze, během níž je z pohlavních buněk vymazán genom matky. Pravou fertilizací dochází k oplodnění rekombinovaného vajíčka od sexuálního druhu klonální spermií, která nese polovinu otcovské genetické informace ve formě hemiklonu. Ten je předáván z generace na generaci. Navíc, hybridní jedinci tento hemiklon sdílejí, což poukazuje na jejich společného předka. V populacích jsou dále přítomni samci schopni vytvářet současně dva typy klonálních spermií, z nichž jedny nesou mateřský a druhé otcovský genom. Po spáření se sexuální samicí vzniká po boku potomstva hybridních hemiklonálních synů také potomstvo sexuálních dcer. Jejich potenciální evoluční role práce rovněž diskutuje.

Studium asexuální linie formující hybridní samce vodních skokanů je prvním krokem k obecnému poznání samčí asexuality, jejího vzniku a evolučního vývoje. Předložená práce diskutuje společné a odlišné rysy samičí a samčí asexuality, studuje princip persistence vše-samčích populací v rodu *Pelophylax* a rozšiřuje obecné poznání o původu a reprodukčních strategiích obratlovců.

KLÍČOVÁ SLOVA

Hybridogeneze, hemiklon, *Pelophylax*, samčí asexualita, *in situ* hybridizace, meióza, mikrosatelity, křížící experiment.

LITERÁRNÍ PŘEHLED

I Úvod

Asexuálně se množící samci už nejsou považováni výhradně za genetický odpad, jakýsi neplodný vedlejší produkt mezidruhového křížení (Mallet 2005). Studie naopak ukazují, že jejich role v populacích, například obratlovců, není nevýznamná, a čím více se o nich dozvídáme, tím více nás přesvědčují o své evoluční vynalézavosti. Po vzoru přírodních vše-samičích klonálních linií si také samci dokázali vytvořit reprodukční strategii, díky které vytváří v čase i prostoru přetrvávající hemiklonální linie samčího potomstva. Nedávno byla na území České republiky objevena jedna z prvních vše-samčích linií tohoto typu, schopna sebe-reprodukce po mnoho generací a současně osídlit geograficky významné území. Dostala se nám tím jedinečná příležitost lépe prozkoumat jejich mechanismus rozmnožování, původ a odpovědět na otázky, jak se dokáže samčí klon šířit mezi populacemi, a přitom si zachovávat mezi generacemi svou identitu.

II Evoluční význam samců

Co bylo dřív, vejce nebo slepice? Tuto otázku si jistě položil každý z nás a nejeden filosof či vědec se nad ní zamýšlel. Ať už je odpověď jakákoliv, jistotou zůstává, že základním kamenem celé podstaty existence života, přinejmenším v říši obratlovců, a tím i reprodukce, je samičí vajíčko. Právě samice jsou oním hnacím motorem populačního růstu, stability a evolučního vývoje. K úspěšnému založení a udržení druhu v přírodě je zapotřebí kromě samic také vhodná reprodukční strategie. To, jakým způsobem se daný druh (taxon) bude rozmnožovat, je dáno jeho životní strategií (Meirmans 2009). Může jim být primitivnější a původnější způsob - klonování genetického materiálu, nebo komplikovanější sexuální rozmnožování – mísení genů dvou jedinců. V prvním případě jedinec přenáší do potomstva

celou svou DNA v bloku, neztrácí nic ze své genetické výbavy a je schopen vyprodukovat za svůj život velké množství genomových kopií. Naopak pohlavně se množící sexuální tvor předává do potomstva jen polovinu své DNA, o druhou polovinu přichází následkem dvou redukčních cyklů během meiózy. Promísením svých genů s geny jiného jedince zajišťuje svým potomkům genetickou identitu - jedinečnou a neopakovatelnou kombinaci genů. Otázkou, která z těchto strategií je evolučně výhodnější, se vědci zabývají řadu let. Přestože se na první pohled může zdát, že klonální typ rozmnožování je vzhledem k typu potomstva výhodnější (neplýtvá se investicí do synů), z hlediska přizpůsobivosti (adaptace) a konkurenceschopnosti potomků je pohlavní rozmnožování bezesporu strategie výhodnější (Hurst and Peck 1996, Jokela et al. 2009, Schurko et al. 2009, Lively and Morran 2014). Teorie Červené královny považuje pohlavní rozmnožování za cestu, která minimalizuje riziko napadení parazity v potomstvu právě vysokou genetickou variabilitou (Johnson et al. 1995, Meirmans 2009, Brockhurst et al. 2014, Vergara et al. 2014). Lze říci, že variabilita potomstva na úrovni genů je zřejmě důsledek majoritního rozšíření pohlavního rozmnožování v živočišné říši (Burt 2000).

Ve světě pohlavního rozmnožování jsou samci nenahraditelnou součástí reprodukčního systému. Bez jejich genetického příspěvku by samice nebyly schopny produkovat potomstvo s rekombinovaným genomem a jejich neoplozená vajíčka by nebyla schopna se vyvíjet. Selháni schoponosti reprodukovat se by vedlo k zániku druhu (populace). U asexuálních organismů s klonální dědičností nemá existence samců praktický prospěch, neboť samice jsou schopny reprodukovat sebe sama bez účasti samce ve formě vše-samičích klonálních linií (Dawley and Bogart 1989, Neaves and Baumann 2011). Samice tedy bez sexu persistovat mohou. Na rozhraní sexuality a klonality existují samice, kterým postačuje přítomnost samce k tomu, aby jejich spermie iniciovaly vývoj vajíčka, a zajistily tak vznik klonálních linií.

V rozmnožovacích procesech mimo sexuální reprodukci hrají samci zjevně druhořadou roli. Dokáží tedy samci pohlavní hendikep neschopnosti vytvořit vajíčko a tím rozmnožit sebe sama obejít? V předkládané práci ukazuji, jak významnou roli v reprodukci může hrát výhradně samčí pohlaví jednoho taxonu v přenosu genetické informace do dalších generací.

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III Asexuální říše a výskyt samců

Asexuální (též označována jako unisexuální či klonální) reprodukce je tradičně rozdělována do tří typů – partenogeneze *sensu stricto*, gynogeneze a hybridogeneze (Tab. 1). U bezobratlých živočichů byly tyto rozmnožovací strategie známy mnohem dříve než u obratlovců (Suomalainen et al. 1987, Cuellar 1987, Dawley and Bogart 1989). Dnes známe sto a více klonálních partenogenetických plazů a gynogenetických či hybridogenetických ryb a obojživelníků (Kearney 2003, Kearney et al. 2009) a další případy fakultativní partenogeneze (Booth et al. 2012, Fields et al. 2015, Siddique et al. 2016, Straube et al. 2016).

Tab. 1: Základní typy nepohlavního rozmnožování

Typ rozmnožování	
Partenogeneze	klonální reprodukce, vývoj neoplodněného samičího vajíčka bez účasti samce, vývoj vajíčka je spouštěn vnějšími faktory prostředí např. teplotou
Gynogeneze	vývoj neoplodněného vajíčka v přítomnosti samce, kde spermie jen aktivuje vývoj vajíčka a její genom se na vzniku nového jedince nepodílí
Hybridogeneze	hemiklonální reprodukce, vývoj oplodněného vajíčka v přítomnosti a za aktivní účasti samce, kde se genom spermie kombinuje s genomem vajíčka a vzniká tak F1 hybrid

V asexuální říši se můžeme setkat se dvěma typy samců, co se týče jejich vzniku. Hybridní samci vznikají z mezidruhového křížení dvou pohlavně se rozmnožujících (rodičovských) druhů a dále se rozmnožují nepohlavně prostřednictvím klonálních či pseudorekombinantních gamet (Alves et al. 1999, Stöck et al. 2002, Sousa-Santos et al. 2007, Pruvost et al. 2015, Morgado-Santos et al. 2016). Samci nehybridního genotypu bývají potomci zpětného křížení hybridů s rodičovskými druhy (hybrid typu AB produkuje A spermie a páří se s AA sexuálním druhem) a dále se rozmnožují pohlavně (Vorburger 2001, Alves et al. 2002, Sousa-Santos et al. 2006, Lamatsch and Stöck 2009).

Androgeneze je typická forma samčího nepohlavního rozmnožování, která byla objevena u několika zástupců asexuální říše; jedná se o uniparentální vývoj, ve kterém se do potomstva přenáší pouze otcovské jaderné geny (McKone and Halpern 2003, Pigneur et al. 2012). Samičí jaderný genom je deaktivován samčími geny a vznikající embryo je tak genetickou kopií svého otce (Zhou et al. 2015). Tento způsob reprodukce byl experimentálně navozen u některých druhů ryb s vnějším oplozením jako např. kapr obecný (*Cyprinus carpio*, Bongers et al. 1999), pstruh duhový (*Oncorhynchus mykiss*, Scheerer et al. 1991), tlamoun nilský (*Oreochromis niloticus*, Ezaz et al. 2004), a piskoř dálnovýchodní (*Misgurnus anguillicaudatus*, Arai et al. 1995). Přirozeně byl pozorován u pakobylek rodu *Bacillus*, kde samci vznikají dvojím typem androgeneze. Celý proces se odehrává v těle samice, která může být oplozena více samci najednou (je tedy polyspermní). Absence syngamie spermie s vajíčkem vede ke splynutí dvou samčích prvojader – vývoj se přepne do módu androgeneze. Vznikne tak diploidní potomstvo samců s čistě samčími jadernými geny a samičí mitochondriální DNA (Mantovani and Scali 1992, Tinti and Scali 1996). Tito samci se však dále rozmnožují sexuálně. U druhého typu androgeneze vzniká čistě samčí potomstvo z diploidní spermie (Tinti and Scali 1995). Další záznamy o vše-samčích výskytech můžeme nalézt u měkkýše rodu *Corbicula*, který vytváří androgenetické linie (Hedtke et al. 2008), či

ibérie ouklejovité (*Squalius alburnoides*). U těchto ryb byla pomocí sekvencí cytochromu *b* a diagnostických alozymových markerů popsána vše-samčí populace nehybridního původu, která vznikla v původně gynogenetické vše-samičí populaci (Alves et al. 2002, Sousa-Santos et al. 2006).

Androgeneze však není jediným mechanismem vzniku vše-samčích populací. První zmínka o vše-samčí partenogenetické linii, označované jako arrhenotokie, byla popsána u peruánského štíra (*Ananteris coineani*, Lourenço 1999). Jedná se o formu partenogeneze, kdy se z neoplodněného vajíčka vyvíjí samčí potomstvo. Také některé partenogenetické samice plazů chované v zajetí mohou vytvářet samčí potomky. Tento případ byl zaznamenán u varana komodského *Varanus komodoensis* s ZW systémem pohlavních chromozómů (samice ZW, samci ZZ), kde při dlouhodobější izolaci samic od samců dokázaly klonální samice tzv. automiktickou partenogenezí vyprodukovat vše-samčí potomstvo ZZ (Watts et al. 2006, Johnson Pokorná et al. 2016). Dále pak obligátně asexuální samice sladkovodního plže *Potamopyrgus antipodarum* jsou rovněž schopny produkovat samčí potomstvo. Výsledky ukázaly dvouleté experimenty, kdy vědci odchovávali v zajetí 45 linií plžů, z nichž zhruba v polovině z nich samice klonálně vytvářely samce (Neiman et al. 2012). Podobný fenomén byl zaznamenán u vše-samičích linií partenogenetické žábronožky *Artemia parthenogenetica* (MacDonald and Browne 1987). Předpokládaným mechanismem vzniku těchto samců je jejich homogametická konstituce (XX), mohou tedy vznikat fúzí dvou haploidních jader nesoucích X chromozóm (Neiman et al. 2012). Jinými laboratorními experimenty bylo docíleno vzniku samčího potomstva u mnohých dalších asexuálních samiček. Například křížením hybridních samiček živoroděnek *Poeciliopsis monacha-lucida* se sexuálními samci živoroděnky hnědé *P. monacha* vzniklo nezávisle u dvou samic čistě samčí potomstvo. Genotypově však odpovídalo sexuálnímu druhu *P. monacha* (Leslie and Vrijenhoek 1978). Dále například křížením samičího androgenního potomstva s karasem obecným (*Carassius*

carassius) s dvěmi Y chromozómy vznikla vše-samčí linie tetraploidních samečků. Když byl stejný samec *C. carassius* pářen s příbuznou samicí *C. auratus* var. Red, vznikalo hybridní potomstvo čistě triploidních samečků (Zhou et al. 2015). Androgeneze je tak využívána jako jedna z chromozómových manipulačních technik k vytváření čistých genetických linií samců (Guerrero 1975) nebo fertilních samců s dvěmi Y chromozómy (Mair et al. 1997). Z komerčních důvodů bývají uměle vytvářeny také vše-samčí linie tlamouna nilského *Oreochromis niloticus* známého jako „tilápie" (Sarder et al. 1999, Alcántar-Vázquez et al. 2014). Vlivem nejrůznějších faktorů jako je např. hormony obohacená strava dochází k řízené změně pohlaví z klonální samice na klonálního samce, tzv. „sex-reversal". Ať už je příčina vzniku samčích linií jakákoliv, žádný z těchto samců není schopen přežívat a množit se nezávisle na ostatních asexuálních či sexuálních samicích.

Shrneme-li dostupná fakta o hybridních klonálních samcích, můžeme říci, že:

- Asexuální samci vznikají náhodně – jako genetický odpad, vedlejší produkt reprodukce
- Jsou velmi často substerilní (vyvinuté gonády s nefunkčními spermiemi), sterilní (bez vyvinutých gonád) nebo jinak reprodukčně omezení
- Pokud jsou plodní, rozmnožují se dále pohlavně (tvorbou rekombinovaných gamet)
- Jsou závislí na pohlavně se množících samicích

IV Reprodukční možnosti a limity hybridních samců

Hybridní samci, ať už je jejich zastoupení v populacích asexuálů jakékoliv, bývají oproti samicím značně limitováni – v míře přežívání, plodnosti nebo reprodukční úspěšnosti (Wu et al. 1996). Fenomén je znám jako Haldanovo pravidlo, které říká, že u hybridních zástupců se

neplodnost nebo neživotaschopnost přednostně vyskytuje u heterogametického pohlaví (XY nebo WZ, Coyne 1984, Hurst and Pomiankowski 1991). U samčích spermatocytů se navíc předpokládá odlišná exprese genů, než je tomu u samičích oocytů, což může vysvětlovat, proč se míra sterility mezi pohlavími liší (Cimino and Schultz 1970, Darevsky et al. 1978, Rasch et al. 1982, Goddard and Dawley 1990). Příkladem jsou pakobylky rodu *Bacillus*, kde hybridní samci mají velmi často sníženou životaschopnost nebo jsou přímo sterilní (Mantovani and Scali 1992). Také klíšťata čeledi *Oribatidae* mohou produkovat sporadicky sterilní samce v populaci běžně tvořené partenogenetickými samicemi (Heethoff et al. 2006). Spermatofory těchto samců jsou nefunkční, protože spermatogeneze není kompletní. Další případy neplodných samců byly objeveny u hybridního sekavce *Cobitis elongatoides-taenia* (Vasil'ev et al. 1989, 2003, Choleva et al. 2012, Janko et al. 2016) nebo kříženců laboratorně odchované linie s divokou formou myši domácí (*Mus musculus*), kteří měli nedokončenou spermatogenezi (Forejt et al. 1974, Turner et al. 2014).

Známe však také příklady fertilních samců, jako je tomu u motýlů rodu *Heliconius* (Naisbit et al. 2002), kde se u hybridních forem objevují fertilní samci. Jejich plodnost lze vysvětlit právě Haldanovým pravidlem, protože tito samci jsou homogametičtí (pohlavní systém ZZ/ZW). Dále se setkáváme s plodnými samci v některých liniích panarktické perloočky hrotnatky obecné (*Daphnia pulex*), kde klonální samci vytváří funkční redukované haploidní spermie, díky nimž se můžou pářit se sexuálními samicemi z normálních cyklicky partenogenetických linií (Innes and Hebert 1988, Wolinska and Lively 2008). V průměru polovina potomstva se dále rozmnožuje klonálně (obligátní partenogenezí), i když životaschopnost potomstva je snížená (Innes and Hebert 1988).

Plodné samce můžeme nalézt také u zástupců jelců rodu *Squalius*. Zde se nachází hybridní samci různých ploidních úrovní. Diploidní samci běžně produkují neredukované klonální spermie a jsou plně plodní (Alves et al. 1999), podobně jako triploidní samci (Sousa-

Santos et al. 2007). U tetraploidních samců dochází ke klasické segregaci a tvorbě funkčních, redukovaných diploidních spermií (Alves et al. 1999, Morgado-Santos et al. 2016). U těchto vyšších ploidních úrovní bývá efekt kumulace mutací způsobujících sterilitu obecně nižší (Mable 2004). Polyploidie tak dokáže neutralizovat negativní efekty hybridizace, jakým je součinnost dvou odlišných genomových sad (Leggatt and Iwama 2003, Choleva and Janko 2013, Madlung 2013).

Kromě běžných mechanismů polyploidizace, jako je zdvojení genomu v průběhu gametogeneze (autopolyploidie) nebo při mezidruhovém křížení (alopolyploidie), se u asexuálních hybridů setkáváme s tzv. „paternal leakage“. Díky „paternal leakage“ se malá část otcovského genetického materiálu dostane do genomu potomstva. A to buďto ve formě malých mikrochromozómů (jako např. u sladkovodní živorodky křížené (*Poecilia formosa*); Schlupp 2005) nebo celého haploidního genomu samce, mechanismem tzv. „genome addition“, kdy se zvýší ploidní úroveň potomstva (Schultz 1969). Tento jev je charakteristický pro populace, kde spolu žijí jedinci různých genotypů nebo ploidních úrovní. Jeden z mála příkladů asexuálních obratlovců, kde můžeme potkat hybridní fertilní samce, navíc různých ploidních úrovní, je již zmiňovaný komplex ibérie ouklejovité (Beukeboom and Vrijenhoek 1998, Morgado-Santos et al. 2016). Triploidní samice tvoří jak redukovaná haploidní, tak ale také diploidní vajíčka meiotickou hybridogenezí (Alves et al. 1998), ze kterých vznikají diploidní i triploidní samci s životaschopnými a plně funkčními spermiemi díky „paternal leakage“ (Sousa-Santos et al. 2007). Navíc zde pozorujeme také tetraploidní samce, kteří vznikají z klonálních spermií diploidních hybridních samců (Alves et al. 1999). Tito tetraploidní samci se ovšem dále rozmnožují pohlavně a vykazují klasickou meiózu (tvorbu rekombinovaných gamet, Cunha et al. 2008). Odlišné reprodukční strategie znamenají nepřetržité přemisťování rodičovských genomů mezi různými ploidními formami hybridů, čímž jsou zachovávány po generace (Morgado-Santos et al. 2015).

Také u gynogenetických samic karase stříbřitého *Carrasius gibelio* byl popsán „paternal leakage“ celé samčí chromozómové sady. Po aktivaci vajíčka se spermie cizího druhu rozplyne v cytoplazmě vyvíjejícího se klonálního embrya, jak je tomu u běžné gynogeneze. Dojde-li však k aktivaci vajíčka spermií téhož druhu, pronukleus samce se spojí se samičím a vznikne triploidní embryo. Z něj je následně odstraněna polovina samičích chromozómů (alogynogeneze, Yigui et al. 1983), čímž dojde ke vzniku diploidního potomstva. Vznikají tak alogynogenetičtí samci, kteří jsou životaschopní a plodní (Lamatsch and Stöck 2009, Liasko et al 2010). Další případy triploidních plodných samců byly objeveny také u našich u vodních skokanů *P. esculentus* (Heppich et al. 1982, Vinogradov et al. 1990, Berger and Günther 1991–1992), hybridních samců skokana *Rana brevipoda-lessonae* (Nishioka and Ohtani 1984), japonských hybridních samců skokana *Rana tsushimensis-japonica* (Sumida and Nishioka 1993), karase zlatého *C. auratus* (Zhaoting and Shaobai 1984) a plodnost se také předpokládá u triploidních samečků ústřice velké *Crassostrea gigas*, kteří tvoří funkční spermatocyty (Guo and Allen 1994, Allen Jr. and Downing 1990).

Občas však zvýšená ploidní úroveň způsobuje přesně opačný efekt a zapříčiní sterilitu. Příkladem jsou tetraploidní samci v klonálních asexuálních liniích rodu *Cobitis*, kteří mají buď zcela nevyvinuté, nebo degenerované gonády a nemohou tvořit normální spermatocyty (Vasil´ev et al. 2003). Triploidní samci některých druhů ryb mají normálně vyvinuté testes, avšak nejsou schopni vytvořit zralé spermie, jako např. u piskořů dálnovýchodních *Misgurnus anguillicaudatus* (Suzuki et al. 1985). V případě úspěšně dokončené spermatogeneze jsou přesto tito samci v podstatě sterilní kvůli produkci aneuploidních nebo abnormálně tvarovaných spermatozoí (Zhang and Arai 1999, Sousa-Santos et al. 2007), jak je známo také u hybridních triploidních samců rodu *Poecilia* (Lamatsch et al. 2001, Lampert et al. 2007).

Jak můžeme vidět na těchto příkladech, také klonální samci si našli cestu, jak se vyhnout neplodnosti, plodit životaschopné potomstvo a vyrovnat se tak asexuálním samicím.

Jsou však tito samci schopni vytvářet také populace po vzoru vše-samičích klonů? Zde si zvláštní pozornost zaslouží skupiny organismů, které stojí na pomyslné hranici mezi pohlavním a nepohlavním rozmnožováním, tedy zkráceně sexualitou a klonalitou. Obecně jsou tito živočichové považováni za asexuály, avšak pravdou je, že využívají přednosti obou typů rozmnožování. Páří se stejně jako sexuální druhy, produkují geneticky odlišné potomstvo s účastí genů otce i matky, ale zároveň umí předávat část své genetické výbavy do potomstva v nezměněné podobě jako klonální tvorové. Hovoříme zde o hemiklonálních jedincích, kde nejen samice ale také samci našli schopnost vytvářet klonální gamety a tím předávat své geny dál do dalších generací. Samci, zdá se tedy, mohou existovat bez sexuálního rozmnožování *sensu stricto*, ačkoli jen napůl.

V Hemiklonální organismy

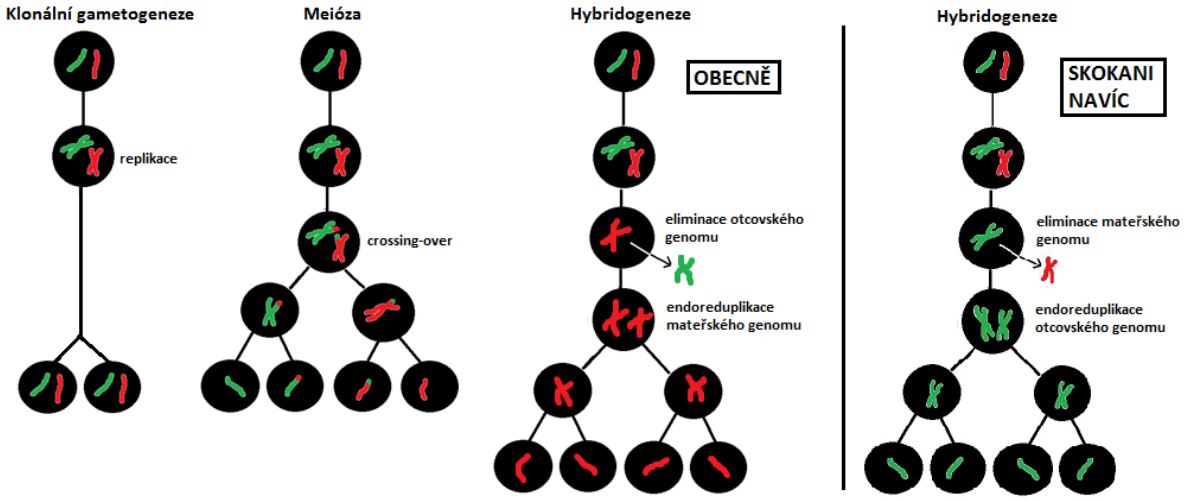
U obratlovců je hemiklonální reprodukce, řazená do kategorií rozmnožování za hranicí klasické sexuální reprodukce, spojena s hybridizací, neboť všechny známé taxony hemiklonálně se množící jsou hybridního původu (Vrijenhoek 1989, Dawley and Bogart 1989, Kearney et al. 2009, Kimura-Kawaguchi et al. 2014). Primární hybridizace probíhají křížením dvou příbuzných sexuálních druhů, čímž vzniká generace potomků hybridních samců a samic, a ti se dále rozmnožují tvorbou klonálních gamet (Choleva and Janko 2013). Protože klonální gameta nese jen polovinu genetické informace rodiče (stejně jako sexuální gameta), je zapotřebí spojení dvou gamet, aby mohlo dojít ke vzniku nového jedince. Náhodným pářením dvou hybridních jedinců se potkávají dvě klonální gamety, což vede k menším či větším komplikacím, ať už ve vývoji embrya, životaschopnosti či reprodukční sterilitě potomků (kumulace mutací, Guex et al. 2002, Vorburger et al. 2009, Reyer et al. 2015, Stelkens et al. 2015). Naopak v případě křížení hybrida s některým ze svých sexuálních

předků, vzniká potomstvo napůl klonální a napůl sexuální (odtud název hemiklonální, Hubbs and Hubbs 1932). Mechanismus společného výskytu ve smíšených populacích se sexuálními druhy a preferenčního páření hybridů se sexuálním partnerem zajišťuje hybridnímu potomstvu jak genetickou variabilitu, tak genetickou uniformitu.

Dosud se až na výjimky většina prací věnovala studiu hemiklonálních samic a jejich reprodukčním vztahům k sexuálních samcům, angl. tzv. „sperm-parasitism“ (Lehtonen et al. 2013) či mechanismům samičí gametogeneze (Dedukh et al. 2015). Nabízí se tímto otázka, co je dosud známo o hemiklonálních samcích. Dokáží se alespoň přiblížit evoluční úspěšnosti samic?

VI Princip hybridogeneze u samců vodních skokanů *Pelophylax esculentus*

Přes všechna výše uvedená omezení existuje systém hemiklonálních samců, kteří dokáží být plodní a evolučně úspěšní – vodní skokani *P. esculentus*. Poprvé se o tyto vodní skokany zajímal Berger v roce 1967. Objevil u nich způsob rozmnožování, který se částečně podobal rozmnožování klonálnímu a částečně pohlavnímu. Hybridní skokani vytvářejí během gametogeneze klonální vajíčka a spermie, ale přitom dochází k pravé fertilizaci, jak je tomu u pohlavně se množících organismů. Stejně jako klonální organismy dokáží také hemiklonální jedinci předávat do svých potomků nezměněný genom, byť ne celý. Současně u nich dochází k mezigenerační ztrátě poloviny genetického materiálu jako u sexuálních organismů. Tento způsob rozmnožování je označován jako hybridogeneze (Obr. 1).

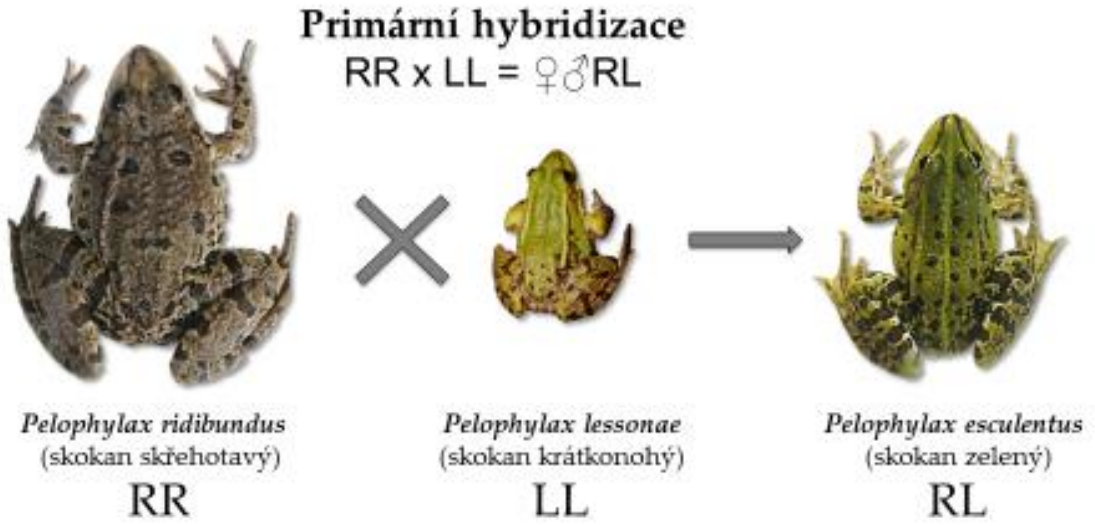


Obr. 1: Porovnání typů gametogeneze u klonálních, sexuálních a hemiklonálních organismů.

Obecná charakteristika tohoto systému je ta, že u hybridogenetických organismů je do gamet (a tedy do potomstva) předáván pouze mateřský genom, přičemž otcovský genom je ze zárodečných buněk eliminován (Ogielska 1994, Lamatsch and Stöck 2009, Kimura-Kawaguchi et al. 2014). Hybridní hybridogeneticky se množící samice spojí své klonální vajíčko se spermií pohlavně se množícího samce a tím se zachovává hybridní genotyp potomstva. Obnova samčího genomu v každé generaci zajišťuje udržení genetické variability a tím se kompenzují nevýhody spojené s klonální reprodukcí (Burt and Trivers 2006 in Kimura-Kawaguchi et al. 2014). Ačkoliv je takovýto mezigenerační mechanismus hybridogeneze evolučně výhodnou strategií, jeho rozšíření mezi organismy je relativně vzácné. Od jejího prvního objevení u mexické sladkovodní živorodky křížené (*Poecilia formosa*) zhruba před 60 lety (Miller and Schultz 1959, Schultz 1961) byla hybridogeneze dále zaznamenána u pakobylky druhu *Bacillus rossius-grandii* (Bullini and Nascetti, 1990), u pyrenejské ibérie ouklejovité (*S. alburnoides*, Carmona et al. 1997), nedávno u australské hlavačkovité ryby rodu *Hypseleotris spp.* (Schmidt et al. 2011) a rybky hřebeníka

maskovaného (*Hexagrammos octogrammus*, Kimura-Kawaguchi et al. 2014) a evropských vodních skokanů rodu *Pelophylax* (Berger 1967).

Hybridní skokan zelený *P. esculentus* vznikl z křížení dvou příbuzných sexuálně se rozmnožujících druhů – skokana skřehotavého (*P. ridibundus*) a skokana krátkonohého (*P. lessonae*), Obr. 2. Pro velikostní rozdíl mezi těmito druhy probíhala primární hybridizace mezi samicí *P. ridibundus* a samcem *P. lessonae* (Berger 1970). Do hybridního potomstva se klonálně přenáší dominantně mateřský *ridibunda* (R) genom a nikoli oba genomy současně. Hybridi dále předávají matčin genom R do svých potomků, ti dále do svých potomků, stále



Obr. 2: Schéma primární hybridizace mezi dvěma rodičovskými druhy, ukázky zástupců všech tří taxonů.

v nezměněné klonální podobě. Tak se tento genom začal označovat jako hemiklon (Uzzell et al. 1977, Günther et al. 1979, Graf and Polls Pelaz 1989). Otcovský genom je každou generaci přijímán „*de novo*“. Aby byl hybridní genotyp RL zachován, musí se hybridní samice pářit se samcem *P. lessonae*, který poskytuje genom L. Spojením klonálního genomu R hybridní matky a genomu L sexuálního otce dává vzniknout hybridním RL potomkům. Mezi těmito

potomky se kromě hybridních samic objevují také hybridní samci. Ti zachovávali hybridní genotyp potomstva křížením se sexuální LL samicí. Populace, ve které spolu žil sexuální druh *P. lessonae* a hybridní samice a samci je označován jako populační systém L-E (Uzzell and Berger 1975). Kromě zmíněného R hemiklonu existuje u skokanů také L hemiklon. Ten je předáván z generace na generaci hybridními samci, kteří se kříží se samicemi *P. ridibundus* v populačním systému R-E (Uzzell and Berger 1975). První zmínka o klonálně přenášeném L hemiklonu v populaci R-E pochází z východního Německa (Uzzell et al. 1977). Napříč Evropou dominují populace L-E s hybridy obého pohlaví tvořící v gametách samičí R hemiklon (Litvinchuk et al. 2015), což silně kontrastuje s výrazně nižší distribucí populací R-E s výhradně diploidními samčími hybridy nesoucí samčí L hemiklon: R-E populace jsou soustředěny například v části Německa, Polska a Česka (Rybacki and Berger 2001, Plötner 2005, Choleva 2004, Doležálková et al. 2016).

Studie prokázaly, že hemiklonálně přenášený genom R nese geny determinující samičí pohlaví, neboť křížením hybridního samce tvořícího spermie s genomem R se samicí *P. lessonae* dává vznik výhradně hybridním samicím (Blankenhorn 1977, Lengagne et al. 2006). Naopak, mnozí samci v populacích R-E přenáší genom L (Doležálková et al. 2016). Absence hybridních samic v tomto typu populace naznačuje, že L hemiklon musí nést geny určující samčí pohlaví a klonální dědičnost – klíčové podmínky přetrvávání samčí asexuální linie.

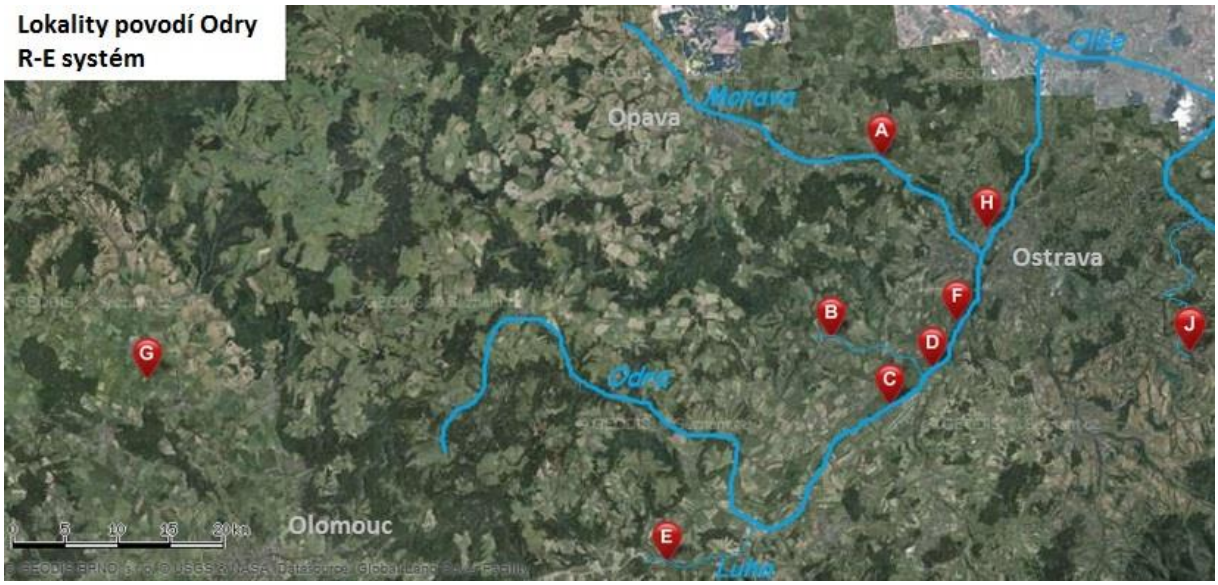
Zdůvodnění výběru samců *P. esculentus* jakožto objektu výzkumného zájmu

Samci *P. esculentus* z povodí Odry představují výborný komparativní model k již po mnoho let studovaným systémům s klonálními liniemi, z angl. tzv. sperm-dependent *sensu* Beukeboom and Vrijenhoek (1998), hybridních samic. Zatímco ty jsou reprodukčně závislé

na samcích parentálních sexuálních druhů, hybridní *P. esculentus* samci jako opositum parazitují na sexuálních samicích *P. ridibundus*. Principy reprodukčních vztahů nezbytných pro udržení a stabilitu hybridních vše-samčí linie zůstávají neznámé. Podobně zatímco u řady klonálních samic známe jejich původ, zde nebylo známo, zda hledat původ v „Adamovi“, tj. jediném hybridovi vzniklém na jednom místě, který založil vše-samčí populace, či zda jde o směs opakovaně vznikajících polyfyletických linií. V širších souvislostech si klade studium vše-samčích populací porozumět podstatě vzniku a udržení obratlovčí asexuality jako takové (jevy narušující konzervativní meiózu, zde během spermatogeneze) a ukázat, jaké průvodní jevy se mohou objevit v přírodních populacích, založil-li například jediný „Adam“ samčí asexuální linie.

CÍLE PRÁCE

V předkládané práci jsem se zaměřila na studium populačně-genetické variability populací vodních skokanů s přítomností samčích linií *P. esculentus* v povodí řeky Odry (Obr. 3). Dále jsem se věnovala komparativní analýze těchto a jiných linií *P. esculentus*, a to jak přírodních, tak uměle vytvořených laboratorních hybridů a porovnávala genetické profily hemiklonů a genomů sexuálních druhů *P. lessonae a P. ridibundus*. Dále jsem studovala mechanizmus tvorby klonálních gamet a samotnou podstatu přenosu klonálních genomů do potomstva.



Obr. 3: Lokality studovaných R-E populací podél Odry.

Hlavní studované otázky:

1. Jaký je průběh hybridogeneze v *P. ridibundus*-*P. esculentus*-samčích populacích povodí Odry? Kdy je mateřský genom eliminován ze zárodečných buněk?

2. Jaký je předpokládaný původ klonálně přenášených genomů u hybridních samců *P. esculentus*? Je mezi jedinci nějaká genetická variabilita klonálně přenášených genomů? Jaký je rozdíl mezi L genomy diploidních samců *P. esculentus* z povodí Odry a sexuálních druhů *P. lessonae* z okolních populací?

3. Jak se hybridní samci rozmnožují, jaký typ gamet vytváří? Existuje vazba mezi klonálně děděným genomem a pohlavím? Jaký potencionální evoluční dopad může mít vznikající potomstvo na stabilitu R-E populací?

Přehled rukopisů

Rukopis I se zabývá studiem tvorby gamet u hybridních samců *P. esculentus*. S využitím cytogenetických metod sleduje strukturu a chování rodičovských genomů v různých fázích gametogeneze. Společně s výsledky laboratorních křížení rukopisu III předkládá hypotézu o alternativním způsobu tvorby gamet, než jaké byly dosud popsány u jiných hybridogenetických organismů.

Rukopis II zkoumá původ pozorované samčí unisexuality. S využitím multilokusových genotypů mikrosatelitových markerů je identifikována genetická variabilita klonálně přenášených genomů, studován typ samčí reprodukce a dále jsou definovány geneaologické vztahy mezi klonálně a sexuálně děděnými genomy.

Rukopis III tematicky navazuje na výsledky Rukopisu II, který odhaluje monofyletický původ populací hybridních samců. V této práci je experimentálně kříženo 16 hybridníh samců původem z dvou přírodních populací s *P. ridibundus* samicemi pro experimentální studium vzoru dědičnosti hybridních samců, včetně vazby přenášených klonálních genomů na pohlaví potomstva.

Metodické nástroje použité v rukopisech zařazených do disertace:

- zpětné křížení s rodičovským druhem *P. ridibundus* (genotyp potomstva, pohlaví potomstva, míra přezívání)
- CGH (detekce rodičovských genomů v premeiotických (mitotických) a meiotických fázích gametogeneze hybrida)

- mikrosatelitové lokusy hybridních samců a jejich potomků

Použité metodiky:

- Základní metody: Izolace DNA, amplifikace mikrosatelitových lokusů a fragmentační analýza mikrosatelitových repetic
- Pokročilejší metody: CGH
- Optimalizované metody pro daný taxon: Získání suspenze meiotických chromozómů z gonád juvenilních i adultních zástupců, multiplexy mikrosatelitových lokusů a zavedení metodiky pro statistické analýzy hemiklonální dědičnosti

K rukopisům zařazeným do disertační práce byl využit materiál:

- svalová tkáň skokana ze sbírky dříve odchycených jedinců (uložených v etanolu při teplotě -20 ^{o}C až -80 ^{o}C) z oblasti Bulharska z roku 2005 (genotypu RR),
- svalové tkáně, jaterní tkáně, kostní dřeně a pohlavních žláz odlovených subadultních a adultních skokanů z přírodních populací z ČR a Slovenska v letech 2010 - 2014 v počtu 226 jedinců (110 LL, 55 RR a 61 RL),
- materiál získaný od zahraničních kolegů (Polsko, Prof. M. Ogielska, University of Wroclaw, Německo Dr. J. Plötner, Humboldt-Universität zu Berlin),
- svalové a jaterní tkáně laboratorně odchovaných juvenilních skokanů z křížících experimentů z roku 2013 v počtu 274 jedinců (mikrosatelitové lokusy, 60 genotypovaných RR jedinců, 88 genotypovaných RL jedinců, alozymově genotypovaných 260 jedinců z jaterní tkáně).

K odchytu zvířat byla udělena Výjimka pro odchyt zvláště chráněných druhů (výpis Výjimek viz. Rukopis I-III), odběr biologického materiálu proběhl v souladu se schválenými Projekty pokusů (PP č. 216/2010 a č. 217/2010, Ústav živočišné fyziologie a genetiky AV ČR, v.v.i).

Laboratorní práce a experimenty probíhaly v molekulárně-genetických laboratořích Ústavu živočišné fyziologie a genetiky AV ČR, v.v.i. v Liběchově, společném pracovišti Katedry zoologie Přírodovědecké fakulty Univerzity Karlovy v Praze a v soukromé výzkumné laboratoři prof. D-G Guexe (Datwil, Švýcarsko).

Na výzkumu jsem spolupracovala s následujícími kolegy:

1. Prof. Dr. Heinz-Ulrich Reyer and Dr. Nicolas Pruvost, Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Švýcarsko. Skupina profesora Reyera se intenzivně věnovala evoluci ekologii a experimentálnímu křížení *P. esculentus*.
2. Dr. Jörg Plötner, Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung, an der Humboldt-Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Německo. Skupina Dr. Jörg Plötnera se věnuje skokanům *P. esculentus* na molekulární a genové úrovni.
3. Prof. G-D Guex, Zoologisches Museum and Zoologisches Institut, Universitdt Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Švýcarsko. Profesor Guex se věnuje řadu let křížícím experimentům a evolučním aspektům životních strategií evropských skokanů rodu *Pelophylax*.

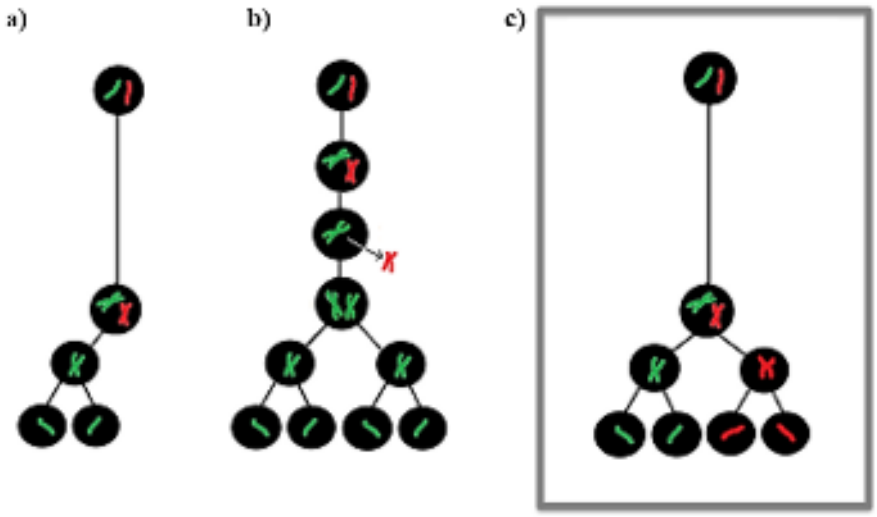
V rámci spolupráce s prof. H.-U. Reyerem a Dr. N. Pruvostem jsme vytvořili a zanalyzovali rozsáhlý soubor mikrosatelitových dat vodních skokanů různých populačních typů. Za spolupráce s Prof. D-G Guexem a Dr. J. Plötnerem jsme rovněž ve Švýcarsku prováděli křížící experimenty a získali potomstvo zpětného křížení hybridních samců s rodičovskými samicemi *P. ridibundus*.

SHRNUTÍ A ZÁVĚR

Předložená práce přináší nové poznatky o dosud neprozkoumané problematice samčí asexuality. Dílčí výstupy se zaměřují na detailnější studium gametogeneze a reprodukčních vzorů dědičnosti hybridních hemiklonálních samců skokana zeleného v populacích v povodí horního toku řeky Odry. Studovaná oblast zahrnuje 6 lokalit, v nichž žijí hybridní samci společně s rodičovským druhem, skokanem skřehotavým, v tzv. populacích R-E. Tyto modelové populace nám přinesly možnost studovat původ samčích hybridních linií a jejich reprodukční strategie s cílem lépe pochopit, jak se asexuální formy obratlovců vyvíjí ze svých sexuálních předků a jak jsou schopny přetrvávat v přírodních populacích.

Hybridní samci klonálně dědí polovinu genomu (jako hemiklon), zatímco druhá polovina genomu je každou generaci přijímána „*de novo*“. Zde nás zajímalo, zda hybridní samci z povodí Odry přenáší do svých gamet hemiklon a o jaký typ genomu se jedná, co se týče původu z rodičovských druhů. V **prvním rukopise** jsme se zaměřili na průběh spermatogeneze a tvorby klonálních gamet. U hybridních skokanů zelených se předpokládá, že jeden rodičovský genom je vyloučen ze zárodečné linie před meiózou, zatímco druhý genom vstupuje do meiózy po endoreduplikaci a následně přechází do gamet (Schultz 1969). Přestože proces hybridogeneze byl u skokanů teoreticky popsán již dříve, nevysvětluje tento princip současný vznik gamet s genomem R a L. S využitím komparativní genomové hybridizace (CGH) jsme fluorescenčně označili genomy obou rodičovských druhů a sledovali jejich přítomnost v různých fázích gametogeneze (Doležálková et al. 2016). Výsledky pozorování nebyly vždy v souladu s obecně rozšířenou hypotézou premeiotické eliminace neboť jsme zachytili přítomnost obou genomů v pozdních meiotických fázích. Na základě těchto výsledků vznikly dvě hlavní hypotézy. První z nich počítá s možností pozměněného průběhu gametogeneze, kdy k eliminaci jednoho z rodičovských genomů dochází až v pozdějších fázích meiózy. Druhá hypotéza připouští existenci nové strategie tvorby gamet,

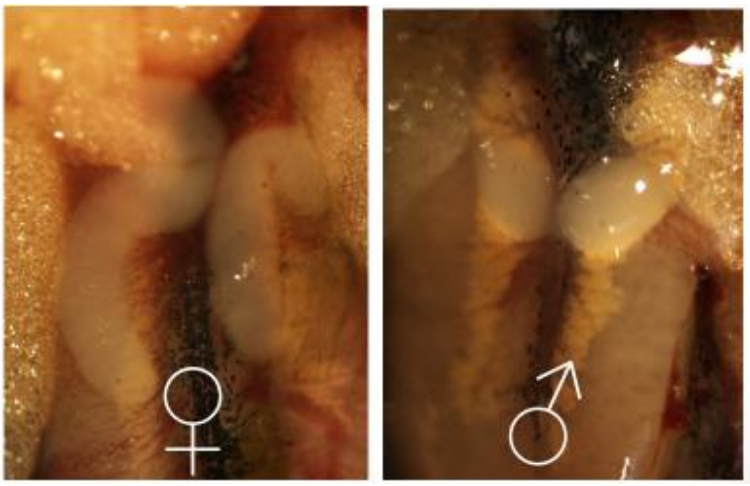
kde nedochází k eliminaci, avšak pouze k separaci genomů, a to v druhém meiotickém dělení (viz. Obr. 4).



Obr. 4: Schéma hybridogeneze u rybek *Poeciliopsis* (a), vodních skokanů rodu *Pelophylax* obecně (b) a nově navržená teorie tvorby gamet u amfispermních samců (c).

V **druhém rukopise** jsme použili jaderné markery, abychom definovali druhovou specifitu hemiklonu u 21 samců z pěti různých lokalit. Analýza multilokusových genotypů na základě specifické kombinace alel odhalila, že se jedná o jeden a tentýž hemiklon, pocházející z druhu *P. lessonae* (L). Ten byl označen jako hemiklon „Oder L1“. Tímto jsme objevili vše-samčí linii monofyletického původu, která vznikla zřejmě jedinou hybridizační událostí před mnoha lety a dodnes přežívá vpopulacích v nivě řeky Odry. K ověření původu této linie jsme využili genotypy jedinců z 11 okolních L-E populací a porovnali jsme alely hemiklonu Oder L1 s L-specifickými alelami rodičovského druhu skokana krátkonohého. Naše data ukazují, že klonální *lessonae* genom hybridních samců nepochází z recentní hybridizace *P. lessonae* jedinců z okolních populací, což naznačuje starší in situ nebo ex-situ původ a přetrvávání hemiklonu po mnoho generací v rámci soběstačných hybridních samců.

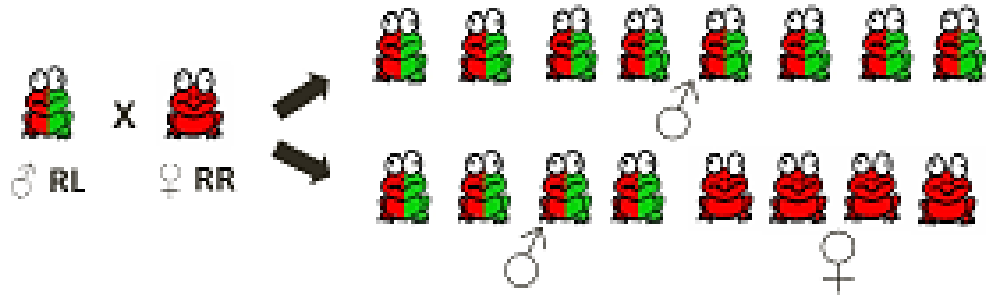
Současně jsme chtěli laboratorně ověřit vzory dědičnosti *P. esculentus* samců pozorované v přírodních populacích. **Třetí rukopis** se proto zaměřil na otázku, zda je hemiklon Oder L1 přenášen do potomstva uniformě tj. zda je potomstvo tvořeno výhradně hybridními samci nesoucími právě hemiklon Oder L1. Experimentálním křížením 12 hybridních samců se samicemi skokana skřehotavého jsme získali potomstvo dvojího genotypu – RR (skokan skřehotavý) a RL (skokan zelený). Analýza pohlavních orgánů juvenilních jedinců prokázala, že všichni genotypovaní RR jedinci byly samice, zatímco RL jedinci byli samci (gonády viz. Obr. 5).



Obr. 5: Pohlavní žlázy juvenilních jedinců skokanů, vaječníky (a) a varlata (b).

Výsledky dále ukáualy, že se v samčí linii vyskytují dva typy samců – jedni produkují pouze gamety nesoucí hemiklon L a druzí umí vytvářet současně gamety s hemiklonem L a gamety s hemiklonem R (Obr. 6). Výsledky tímto prokazují vazbu druhově specifického genomu (hemiklonu) na pohlaví. Genom L je vázán na samčí pohlaví, genom R na samičí. Již dřívější studie (Uzzell et al. 1977, Vinogradov et al. 1991, Berger and Günther 1991-1992, Polls Pelaz 1994) zaznamenaly minoritní výskyt těchto „amfispermních“ samců tvořících gamety L a R, avšak proces vzniku gamet či navazující evoluční souvislosti nebyly studovány.

V kontextu stability a udržení vše-samčí linie to znamená, že: i. hybridní samci přijmou od sexuální samice skokana skřehotavého haploidní R genom, a fůzí s Oder L1 hemiklonem (L spermií) zajistí reprodukci sebe sama (RL samčí potomstvo); ii. někteří samci však R genom po jednu generaci zadrží a poté jej v nerekombinované formě vrátí do genofondu skokana skřehotavého, neboť fertilizace R vajíčka skokana skřehotavého a takovéto R spermie skokana zeleného vytváří samičky skokana skřehotavého. Samice skokana skřehotavého jsou tak zde sexuálními partnerkami jak pro samce skokana skřehotavého, tak pro hybridní samce. Schopnost skokanů zelených plodit také sexuální dcery zvyšuje počet sexuálních samic v populaci a tím také šanci hybridních synů nalézt partnerku pro rozmnožování.



Obr. 6: Ilustrační schéma potomstva ze zpětného křížení hybridních samců (RL) se samicemi skokana skřehotavého (RR).

Souhrnně lze říci, že hybridní samci skokana zeleného z povodí horní Odry

- Tvoří jednu hybridní linii monofyletického původu nesoucí tentýž *lessonae* hemiklon
- Pářením s rodičovským druhem skokanem skřehotavým plodí jednak hybridní syny skokana zeleného a také sexuální dcery skokana skřehotavého, čímž navyšují dostupnost partnerek pro své syny
- Během tvorby gamet nedochází u samců skokanů zelených vždy k premeiotické eliminaci mateřského genomu, neboť u některých jedinců byly přítomny oba

rodičovské genomy i v pozdních meiotických fázích spermatogeneze. Tento fakt společně s předchozími cytologickými a experimentálními důkazy pro tvorbu obou typů spermií (s mateřským R a otcovským L genomem) podporuje hypotézu, že eliminace genomu může být posunuta do pozdějších fází meiózy nebo u těchto samců úplně chybí.

V pokračujícím výzkumu se budeme zabývat otázkou, zda je tvorba gamet podmíněna geneticky, a zda je vzor gametogeneze amfispermních samců dědičný či nikoliv. Dále pak jaký ekologický dopad má vznik dcer skokanů zelených na stabilitu R-E systému a zda jsou tyto dcery schopny tvořit rekombinované gamety jako klasické sexuální druhy. Základní otázkou pro nás zůstává vyřešit původ linie s analýzou nových populací podél celého toku řeky Odry, kde jsme přítomnost R-E populací s hybridními samci rovněž potvrdili.

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PODÍL AUTORA NA RUKOPISECH ZAŘAZENÝCH DO DIZERTACE

Publikace 1: Is premeiotic genome elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus *Pelophylax*? (Doležálková et al. 2016)

Příspěvek autora: Příprava chromozómových suspenzí a preparátů, příprava fluorescenčně značených sond na metodu CGH, *in-situ* hybridizace, pomoc při vyhodnocování mitotických a meiotických figur, sepsání draftu

Rukopis 2: Evolution of unisexuality in reverse order: molecular insights into the origin and persistence of an all-male water frog lineage *Pelophylax esculentus.* (submitován v časopise Evolution)

Příspěvek autora: Odchyt skokanů v terénu, izolace DNA, pomoc při sestavování multiplexů na mikrosatelitové lokusy, optimalizace PCR protokolu, vyhodnocování mikrosatelitových dat (analýzy, fylogenetické stromy, multilokusové genotypy), sepsání draftu

Rukopis 3: When a sexual genome becomes clonal for a single generation: Evidence from Pelophylax water frogs. (submitován v časopise eLife)

Příspěvek autora: Křížící experimenty, určení pohlaví u potomstva, genotypování rodičů i potomků pomocí mikrosatelitových multiplexů, vyhodnocování mikrosatelitových dat, sepsání draftu

Prohlašuji, že příspěvky Marie Doležálkové k jednotlivým rukopisům (Doležálková et al. 2016, Rukopis 2 a Rukopis 3), jak jsou zde uvedeny, jsou pravdivé.

Školitel: RNDr. Lukáš Choleva, PhD.

Kapitola I

Is premeiotic genome elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus *Pelophylax*?

Marie Doležálková, Alexandr Sember, František Marec, Petr Ráb, Jörg Plötner & Lukáš Choleva

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Is premeiotic genome elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus *Pelophylax*?

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Abstract

Background: The ability to eliminate a parental genome from a eukaryotic germ cell is a phenomenon observed mostly in hybrid organisms displaying an alternative propagation to sexual reproduction. For most taxa, the underlying cellular pathways and timing of the elimination process is only poorly understood. In the water frog hybrid *Pelophylax esculentus* (parental taxa are *P. ridibundus* and *P. lessonae*) the only described mechanism assumes that one parental genome is excluded from the germline during metamorphosis and prior to meiosis, while only second genome enters meiosis after endoreduplication. Our study of hybrids from a *P. ridibundus*—*P. esculentus*-male populations known for its production of more types of gametes shows that hybridogenetic mechanism of genome elimination is not uniform.

Results: Using comparative genomic hybridization (CGH) on mitotic and meiotic cell stages, we identified at least two pathways of meiotic mechanisms. One type of *Pelophylax esculentus* males provides supporting evidence of a premeiotic elimination of one parental genome. In several other males we record the presence of both parental genomes in the late phases of meiotic prophase I (diplotene) and metaphase I.

Conclusion: Some *P. esculentus* males have no genome elimination from the germ line prior to meiosis. Considering previous cytological and experimental evidence for a formation of both *ridibundus* and *lessonae* sperm within a single *P. esculentus* individual, we propose a hypothesis that genome elimination from the germline can either be postponed to the meiotic stages or absent altogether in these hybrids.

Keywords: Hybridogenesis, Asexual propagation, Hemiclone, Meiotic cycle, Genomic *in situ* hybridization, *Rana esculenta*

Background

Meiosis is a vital process in all sexual organisms, ensuring fertility and genome stability and encouraging genetic diversity [14, 22]. Sexual reproduction involves the recombination of parental genomes followed by the coordinated segregation of the recombined chromosomes into gametes [57]. Despite the conservative nature of meiotic machinery, a number of anticipated mechanisms, including hybridization, can disrupt the regular cycles and alter the normal course of meiosis [41]. In hybrid animals, these deviations have resulted in a loss of sexual reproduction accompanied by modifications in gametogenesis such as premeiotic endomitosis (duplication of chromosomes), and genome exclusion (the loss of one parental genome) (reviewed in [26, 43]).

Hybridogenesis is a mode of bisexual reproduction characterized by the exclusion of one complete parental genome from the germline, while the remaining genome is endoreduplicated and subsequently transferred clonally (referred to as a hemiclone; [39, 55]). Hybridogenetic

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animals usually mate with the sexual species that contribute the eliminated genome [6, 9, 39]. New hybrids are generated via true fertilization, however, the genome from the sexual mate is discarded again in the next round of gamete formation.

Hybridogenesis has been recorded in the diploid all-female fish of the genus *Poeciliopsis* [39, 40], and Cimino [7, 8] observed the exclusion of *P. lucida* chromosomes during the onset of meiosis, while in *P. monacha* the genome is transferred into a reconstituted nucleus by the unipolar spindle. Apart from these species, very little is known about the cytological processes in other hybridogenetic or hybridogenesis-related animals such as the *Squalius alburnoides* fish [1], the *Misgurnus anguillicaudatus* fish [27], the Asian loach fish of the genus *Cobitis* [23], the carp gudgeon *Hypseleotris* [38], *Ambystoma* salamanders, *Bufotes baturae* toads [44], and *Pelophylax esculentus* water frogs [10, 17, 49].

The European sexual species *Pelophylax lessonae* and *P. ridibundus* hybridize and produce the hybrid form *P. esculentus*, which maintains a permanent F1 (first filial) hybrid state from generation to generation. This hybrid is able to exclude one parental genome from its germline and to duplicate the remaining one. As a result, the hybrid produces unrecombined *ridibundus* or *lessonae* gametes and therefore continues with only one parental species, i.e. the species whose genome has been eliminated (e.g. [2, 18, 47]).

It is generally believed that the exclusion of a parental genome from *P. esculentus* germ cells takes place before the onset of meiotic prophase I, followed by the endoreduplication of the remaining *ridibundus* genome [10, 11, 48]. In females the majority of oogonia have already been transformed into oocytes with 13 diplotene bivalents, usually by the time *P. esculentus* have entered their first hibernation [48]. Similarly, the proliferating spermatozoa in the testes of adult *P. esculentus* contained a diploid set of only *ridibundus* chromosomes [20]. Hence, the process of genome elimination and reduplication seems to occur at an early stage of spermatogenesis [20]. Further evidence comes from Günther [17], who observed in *P. esculentus* males from Eastern Germany a large number of meiotic figures with irregularities such as aneuploidy, univalency and heterologous multivalency. He interpreted his results as evidence contradicting the occurrence of a single cytological mechanism of hybridogenesis. Detailed cytological studies of male meiosis have yet to be carried out.

P. esculentus typically forms two reproductive systems; one with *P. lessonae* and one with *P. ridibundus*. The latter mostly consists of *P. ridibundus* (females and males) and only diploid hybrid males [50, 51]. Such *P. ridibundus—P. esculentus*-male populations have been found in Central Europe, mostly along the Oder River (reviewed by [34]). Here, hybrid males inherit either the *lessonae* or the *ridibundus* genome, or produce a combination of both kinds of sperm [3, 19, 35, 51, 54].

In order to understand the cytogenetic basis of these inheritance patterns, we studied the mitotic and meiotic cell stages of hybrids of a *P. ridibundus—P. esculentus*-male population from the Upper Oder River. Using comparative genomic hybridization (CGH) we discovered that the elimination of one parental genome does not necessarily precede meiotic divisions. In fact, the opposite is often true, where maintaining both parental genomes later in meiotic phases is actually relatively common.

Methods

Animals

We examined 14 adult and 4 subadult male individuals of *P. esculentus* from three different *P. ridibundus—P. esculentus* male populations along the Upper Oder River (49.914498, 18.091502; 49.705486, 18.092624; 49.735014, 18.152479). For genomic probes, we used two adult *P. lessonae* males (50.043063, 13.441079; 49.761259, 18.597399) and two adult *P. ridibundus* males from surrounding localities (49.705293, 18.081609). Specimens were genotyped using three polymorphic allozyme loci: Aspartate aminotransferase (*Aat*; EC 2.6.1.1), *Glucose-6-phosphate isomerase* (*Gpi*; EC 5.3.1.9) and Lactate dehydrogenase (*Ldh-1*; EC 1.1.1.27) [50]. All experimental procedures were conducted with the approval, and under the supervision of the Ethical Committee of the Faculty of Science,

Charles University, Prague, according to the directives of the State Veterinary Administration of the Czech Republic, permit number 34711/2010-30 from the Ministry of Agriculture of the Czech Republic. Specimens were deposited in the frog collection of the Laboratory of Fish Genetics, IAPG CAS, Liběchov. Permissions 358/2011 required for the field work collection of the frogs were obtained from the Agency for Nature Conservation and Landscape Protection of the Czech Republic.

Chromosome preparations

We employed two different protocols to obtain chromosome spreads from gonadal tissues. In the majority of adult and subadult individuals we adapted the protocol of Zaleśna et al. [56], originally designed for chromosome preparation from bone marrow. In juvenile specimens with small gonads we applied a spreading technique previously used for spiders [25] with slight modifications. Briefly: after the dissection of a juvenile specimen the gonads were removed and hypotonized in 0.075 M KCl for 8 min, followed by three rounds (15, 30, 60 min) of fixation in 3:1 methanol / acetic acid solution. The fixed gonadal tissue was then suspended in 60 % acetic acid and spread on a hot-plate (40 °C).

For conventional cytogenetic analysis, chromosomes were stained with 5 % Giemsa solution (pH 6.8) (Merck, Darmstadt, Germany). Selected slides were destained in methanol / acetic acid fixative, dehydrated in an ethanol series (70, 80, and 96 %, 3 min each) and stored in a freezer (-20 °C) for subsequent cytogenetic experiments.

DNA extraction and probe preparation

Whole genomic DNAs (gDNAs) from *P. ridibundus* and *P. lessonae* were extracted from muscle tissue using the conventional phenol-chloroform-isoamylalcohol method [13]. Probes prepared from both parental species were differentially labelled either with biotin-16-dUTP (2'-Deoxyuridine, 5'-Triphosphate, Roche, Mannheim, Germany) or digoxigenin-11-dUTP (Roche) using Nick Translation Mix (Abbott Molecular, Illinois, USA or Roche Diagnostics, Mannheim, Germany). For each slide, 1 μg of *P. ridibundus* gDNA, 1 μg of *P. lessonae* gDNA and 50 μg of sonicated salmon sperm DNA (Sigma-Aldrich) were added and the resulting probe was precipitated in 96 % ethanol, washed in 70 % ethanol, air-dried and re-dissolved in 25 μl of hybridization buffer (50 % formamide, 10 % dextran sulphate, 2× SSC (Standard saline buffer), 0.04 M $NaPO_4$ (Sodium Phosphate) buffer, 0.1 % SDS, Denhardt's reagent, see [29]). In some experiments, the final probe also included 15–30 μg of unlabelled species-specific competitive DNA prepared from *P. esculentus* gDNA using a Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK), followed by sonication of the amplified product (40 cycles, 10 pulses, 100 % power) to approximate fragment size of 100–200 bp using the ultrasonic homogenizer Sonopuls HD 2070 (Bandelin Electric, Berlin, Germany).

Comparative genomic hybridization (CGH)

In order to identify the chromosome sets of particular parental species within a hybrid genome throughout the meiotic phases we performed the CGH method according to Bi and Bogart [4] with several modifications. After thermal aging (3–4 h at 37 °C and 1 h at 60 °C) the chromosomes were treated with RNase A (Sigma-Aldrich) (200 μg/ml in 2× SSC, 90 min, 37 °C) and then pepsin (50 μg/ml in 10 mM HCl, 3 min, 37 °C). The slides were denatured in 75 % formamide (pH 7.0) (Sigma-Aldrich) in 2× SSC at 74 °C for 3 min, and then immediately cooled and dehydrated in 70 % (cold), 80 % and 96 % (RT) ethanol. The hybridization mixture was denatured at 86 °C for 6 min. Hybridization was performed at 37 °C for 48–72 h. Post-hybridization washes were applied twice in 50 % formamide in 2× SSC (pH 7.0) at 42 °C for 5 min and three times in 1× SSC at 42 °C (7 min each). In order to block non-specific binding sites for streptavidin and anti-digoxigenin, the slides were incubated with 500 μl of 3 % BSA (Vector Labs, Burlington, Canada) in 4× SSC in 0.01 % Tween 20 at 37 °C for 20 min. The hybridization signal was detected using Anti-Digoxigenin-Rhodamine (Roche) and Streptavidin-FITC (fluorescein isothiocyanate; Invitrogen Life Technologies, San Diego, CA, USA) or alternatively with Anti-Digoxigenin-Fluorescein (Roche) and Streptavidin-Cy3 (Invitrogen Life Technologies), to exclude any influence of antibodies and/or fluorochromes. The slides were incubated with antibodies at 37 °C for 60 min in a dark humid chamber. Finally, the slides were washed four times (7 min each) in 4× SSC in 0.01 % Tween (pH 7.0) at 42 °C and mounted in antifade containing 1.5 μg/ml DAPI (4', 6-diamidino-2-phenylindole; Cambio, Cambridge, United Kingdom).

Image processing

Chromosomal preparations were inspected using a Provis AX70 (Olympus) fluorescence microscope equipped with standard fluorescence filter sets. Selected images for each fluorescent dye were captured separately with a black and white CCD camera (DP30BW Olympus) using Olympus Acquisition Software. The digital images were then pseudocoloured (blue for DAPI, red for Rhodamine or Cy3, green for FITC) and superimposed using MicroImage software (Olympus, version 4.0). The images were optimized for brightness and contrast using Adobe Photoshop, version CS5.

Results

We obtained chromosomal preparations from the gonads of 18 male individuals. The preparations contained different phases of meiotic division as well as spermatogonial mitotic metaphases. Giemsa-stained karyotypes (not shown) confirmed the previous description of Zaleśna et al. [56], with all species of the *Pelophylax* hybridogenetic complex having 26 metacentric and submetacentric chromosomes. Moreover, in line with the findings from the mentioned study, the homologous chromosomes in *P. esculentus* differed slightly in size. Along with spermatogonial metaphases, we also observed stages with haploid or diploid chromosome numbers corresponding to particular meiotic and/or pre-meiotic phases (Fig. 1a-e). Haploid chromosome complements appeared to correspond to either a premeiotic stage after the elimination of one parental genome (Fig. 1b) or to chromosomes in the first meiotic division (Fig. 1d). Diploid chromosome complements represented either mitotic metaphases (Fig. 1a) or stages of the first meiotic division with bivalents (Fig. 1c).

We examined the mitotic and meiotic spreads further by means of CGH in four hybrid males (M1-M4). Although chromosome spreads were successfully obtained from all individuals, the hybridization procedure was only successful in four of them. Some examples of unsuccessful

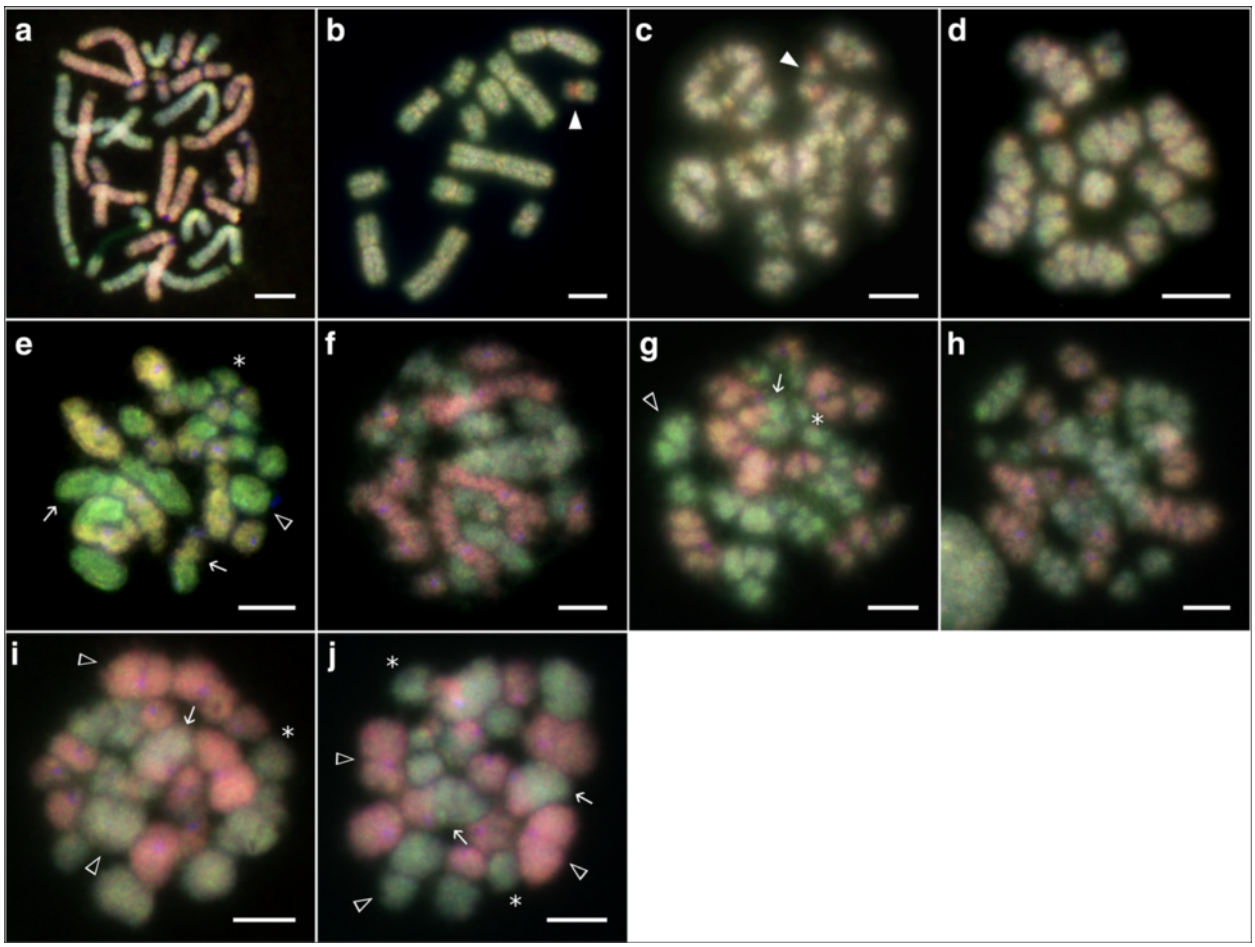

Fig. 1 Comparative genomic hybridization (CGH) in mitotic and meiotic chromosomes of four water frog *Pelophylax esculentus* males. M1 (**a**), M2 (**b-d**), M3 (**e-g, j**) and M4 (**h, i**). CGH clearly distinguished chromosomes of the parental species, *P. ridibundus* (*red*) and *P. lessonae* (*green*). **a** Mitotic prometaphase. **b** Haploid mitotic metaphase after elimination of the *ridibundus* genome. **c** Diplotene. **d** Meiotic metaphase I. **e, f, g, h** Late meiotic prophase I. **i, j** Meiotic metaphase I showing bivalent-like configurations and univalents. *Solid arrowheads* indicate the smallest submetacentric chromosome pair with marked *ridibundus*-specific repetitive DNA in the *lessonae*-derived chromosome set, *arrows* indicate bivalent-like configurations between two different parental genomes, *open arrowheads* indicate bivalent-like configurations within one parental genome, asterisks indicate univalents. Scale bars equal 10 μm

hybridization patterns are shown in Additional file 1: Figure S1-S3. A possible explanation for the general failure of CGH could be its high sensitivity in respect to experimental conditions [45, 46]. Multiple successful repetitions of the CGH experiments did however confirm that the chromosomal patterns observed in germinal cells of four *esculentus* males (M1-M4) were not artefacts. CGH provided a clear discrimination between the chromosomes of *P. lessonae* and *P. ridibundus* (Fig. 1a). The observed differential hybridization pattern of chromosome complements containing both parental genomes most probably resulted from the presence of species-specific repetitive sequences [24], very likely including some sort of transponable elements (TEs) and microsatellites [33]. Both experimental approaches (either with- or without the specific competitive DNA prepared from *P. esculentus*) yielded the same resulting hybridization pattern (Fig. 2a, b).

Two groups of males were distinguishable by their differences in hybridization patterns. In the first group (male M2), nearly all chromosomes, with the exception of the smallest submetacentrics, were predominately highlighted with the *lessonae*-derived probe (Fig. 1b-d). The smallest submetacentric chromosome pair displayed a marked *ridibundus*-specific repetitive DNA region, even in the homologous *lessonae*-specific chromosomes (Fig. 1b, c, *solid arrowheads*). The number and morphology of the chromosomes indicated the presence of both mitotic (Fig. 1b) and meiotic stages (Fig. 1c, d). In the second group, 89 out of 122 chromosome complements (49 out of 55 in male M1, 18/22 in male M3, and 22/45 in male M4) showed a mixture of chromosomes

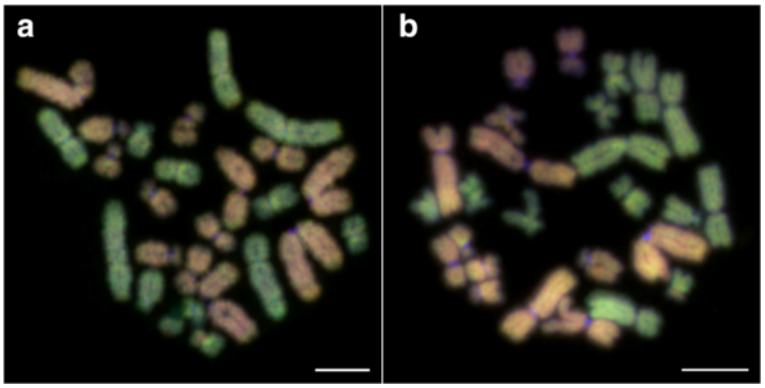

Fig. 2 Mitotic metaphases of a *Pelophylax esculentus* male after comparative genomic hybridization (CGH). **a** CGH with specific competitive DNA prepared from *P. esculentus*. **b** CGH without specific competitive DNA. *P. ridibundus* chromosomes are visible as *red* signals, *P. lessonae* chromosomes as *green* signals. Scale bars equal 10 μm

with two different hybridization patterns, i.e. with strong hybridization signals of the *lessonae*-derived probe and the *ridibundus*-derived probe (Fig. 1a, e-j). All chromosomal complements showing both parental genomes were classified as diploid sets, either composed of mitotic chromosomes (Fig. 1a) or meiotic chromosomes in a late meiotic prophase I (Fig. 1e, f, g, h) or in a metaphase I (Fig. 1i, j).

Based on the accurate identification of meiotic stages and on the scheme of hybridogenesis (Fig. 3) we tried to provisionally reconstruct the process of hybrid spermatogenesis. From 170 observed figures we identified five different mitotic or meiotic stages i.e. (i) mitotic metaphase with either diploid (Fig. 1a) or haploid (Fig. 1b) chromosome numbers, (ii) meiotic diplotene with regular bivalents (Fig. 1c) and (iii) meiotic metaphase MI (Fig. 1d) where 1c and 1d are composed of only one parental genome, (iv) late meiotic prophase I (Fig. 1e, f, g, h) and (v) meiotic metaphase MI (Fig. 1i, j) where chromosomes of both parental species formed bivalent-like configurations. More specifically, while male M2 exhibited only the *lessonae*-derived chromosomes in meiotic prophase I and metaphase I with 13 bivalents (each of them presumably composed of a pair of endoreduplicated identical chromosomes), the males M3 and M4 displayed chromosomes apparently derived from both parental genomes in their meiotic prophase I. These males formed bivalent-like configurations from non-homologous chromosomes that paired randomly either within (Fig. 1e, g, i, j, *open arrowheads*) or between parental genomes (Fig. 1e, g, i, j, *arrows*). Moreover, some chromosomes did not form a bivalent-like configuration, but instead remained unpaired as univalents (Fig. 1e, g, i, j, asterisks).

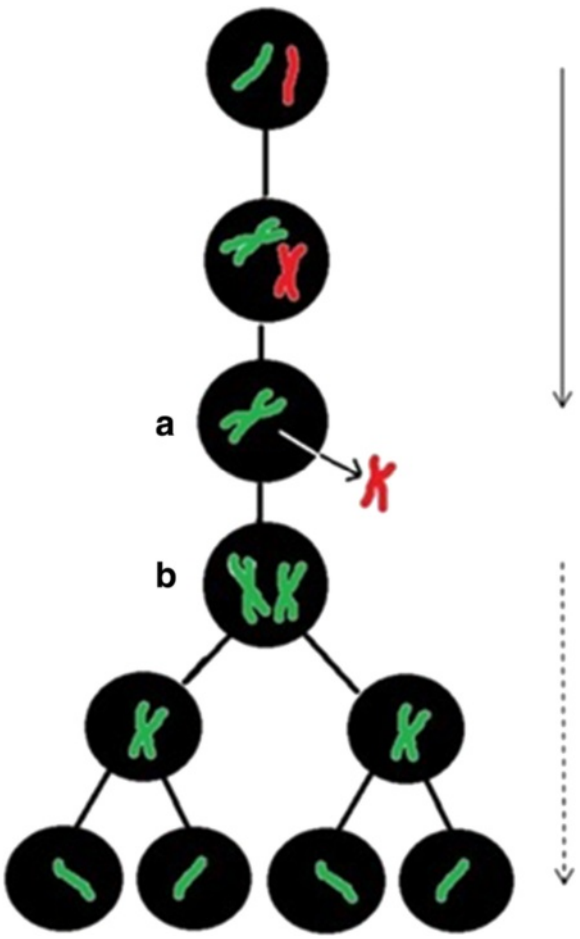

Fig. 3 Schema of hybridogenesis assumed for maintenance of diploid hybrid male M2 (this study) in mixed populations with *P. ridibundus*. **a** elimination of the *P. ridibundus* genome (*red*); **b** reduplication of the *P. lessonae* genome (*green*). As a result haploid *P. lessonae* gametes are produced. The vertical solid arrow shows spermatogonia, the dashed arrow spermatocytes. Meiotic cycle starts after **b**

Discussion

Our analysis of the meiotic mechanism of *Pelophylax esculentus* males provides supporting evidence of premeiotic

genome elimination. In addition to this observation, we record the presence of both parental genomes in the late phases of meiotic prophase I (diplotene) and metaphase I in several other males. Our results suggest that some males have no genome elimination from the germ line prior to meiosis.

The formation of clonal gametes during hybridogenetic spermatogenesis depends on a range of coordinated molecular and cytogenetic processes that are not yet fully understood. It is generally believed that in the germ cells of diploid hybrids one parental chromosome set is eliminated before entering the meiotic cycle, while the remaining set is endoreduplicated (e.g., [20]). This pattern was observed in at least one hybrid male (M2; Fig. 1b-d). The meiotic divisions obtained from this male contained only green coloured *lessonae* chromosomes either in a haploid set, after the elimination of the red coloured *ridibundus* chromosomes, Fig. 1b), or in a diploid number, after genome duplication (Fig. 1c-d). Such an inheritance mode would lead to sperm with a *lessonae* genome, which would mean that after fertilization of the *P. ridibundus* egg the F1 hybrid state would be restored. As the meiotic chromosomes treated with comparative genomic hybridization (CGH) did not display any recombination between the *lessonae* and *ridibundus* chromosomes such as crossing-over or other types of recombination, this male must have transferred its *lessonae* genome clonally into its sperm as assumed for hybrid males from *P. ridibundus—P. esculentus*-male populations [19, 51].

A completely different pattern of spermatogenesis was found in males M3 and M4 where the majority of nuclei in the first meiotic division contained both *ridibundus* and *lessonae* chromosome sets. Most of the nuclei were in the late meiotic prophase I, probably corresponding to diplotene (Fig. 1e, f, g, h) with some of them even reaching metaphase I (Fig. 1i, j). This finding clearly suggests that the majority of spermatocytes did not carry out genome elimination prior to meiosis. Previous studies based on protein electrophoresis have indicated that in the germ line of *P. esculentus* genome elimination takes place before meiosis [12, 20, 52], likely during the last mitotic division [48] in the so called "E" (Elimination) phase [53]. There are two principle hypotheses concerning genome exclusion: 1) an exclusion takes place during the mitotic phase whereby the excluded genome is enzymatically degraded [31, 54], or 2) the elimination of whole chromosomes, or at least parts of them, takes place during mitosis of the gametogonia [31]. The latter hypothesis seems less likely as no irregularities in the spindle apparatus or in the heterochromatization have been observed (see pp. 91–92 of [34]). It is not yet clear whether genome elimination is a one-step or a gradual process during mitotic division [31]. Within vertebrates, only the all-female fish of the genus *Poeciliopsis* eliminate one chromosome set as late as in meiosis but even in this fish it occurs during prophase I [7, 8].

The occurrence of both parental genomes in the proliferating spermatozoa of *P. esculentus* investigated in this study conflicts with our expectation of observing only one parental genome in the meiotic cells of adult males [20]. It further suggests that the elimination phase (if present) is not restricted to the period around metamorphosis.

Using conventional cytogenetic techniques, the absence of genome exclusion has been assumed in some hybrids from *P. ridibundus—P. esculentus*-male populations [17, 21] and in just a single laboratory-synthesized *P. esculentus* male [36]. The related observations of numerous aberrations during meiosis in *P. esculentus* males such as aneuploidy, degenerated chromosomes and heterologous multivalents [17, 32] and of fertility disorders in many *P. esculentus* males (e.g. [15, 16, 30]) can be considered as evidence for selection processes acting during pregametic and/or gametic stages [19]. As well as cell lineages in which one parental genome is excluded premeiotically, lineages (spermatogonia, spermatocytes) with both parental genomes may undergo cellular selection during meiosis. As a result, lineages with balanced genomes (probably with the chromosomes of only one parental species) may yield fertile sperm while those with unbalanced haploid genomes (a mixture of *lessonae* and *ridibundus* chromosomes) would result in infertile sperm [19].

Indeed, irregular diplotene stages (Fig. 1e, g, i, j) with bivalent-like configurations and univalents, and the fact that most *ridibundus* chromosomes paired with non-homologous *ridibundus* chromosomes rather than with homologous *lessonae* chromosomes and vice-versa, may indicate malfunctions in the process of genome haploidization and meiosis in general. But in terms of the number of chromosomes, meiotic prophase I with 13 *ridibundus* and 13 *lessonae* chromosomes (Fig. 1i, j) did not differ from regular meiotic phases with 13 bivalents. More thorough analyses are necessary to understand whether such cells may or not produce functional sperm. Currently, two alternative hypotheses remain open. First, such cells may still result in dysfunctional sperms [19]. It was already observed that many *P. esculentus* males exhibit degenerated testes, low numbers of sperm, high numbers of immobilized and/or inhibited sperm [19, 30, 37]. Second, the cells may yield both unrecombined *lessonae* and *ridibundus* sperm [19, 51, 54]. Vinogradov et al. [54] recorded "so-called hybrid amphispermy" in 14–17 % of *P. esculentus* males. Although the underlying cytogenetic mechanisms were not identified, in principle, two mechanisms are conceivable: 1) genome exclusion is unspecific and takes place during meiosis leading to

clonal cell lineages with only *lessonae* or *ridibundus* chromosomes, or 2) the chromosomes are segregated non-randomly during meiosis, probably in anaphase I, i.e. without interchromosomal recombination, resulting in both *lessonae* and *ridibundus* spermatids and sperms.

Chromosomal studies of deviations from canonical gametogenesis in *P. esculentus* females have shown observations of very rare oocytes in which elimination has not occurred [5, 10] resembling the mechanism of premeiotic endoreplication in automictic parthenogenesis [28, 42]. Dedukh et al. [10] also observed aneuploid oocytes suggesting a partial loss of chromosomes during gametogenesis. Together with our observations that some diploid *P. esculentus* males have no genome elimination from the germ line prior to meiosis, the phenomenon of no chromosome elimination may be more common than previously thought.

Conclusions

The central finding of this study is that genome elimination in *P. esculentus* males is not always restricted to larval or juvenile stages, as both parental genomes were discovered to still be present in the germline of the adult specimens. We propose the following three hypotheses about the fate of homologous and non-homologous bivalent-like configurations of *lessonae* and *ridibundus* chromosomes observed in the first meiotic division: 1) such bivalents represent a process leading to unviable gametes; 2) the elimination phase is postponed to later stages of the meiotic cell cycle; 3) there is no genome elimination, homologous *lessonae* and *ridibundus* chromosomes segregate in anaphase I resulting in both haploid *lessonae* and *ridibundus* sperm.

Overall, our data provide new information about the behavior of two species-specific genomes in the meiotic cycle which will help us understand the underlying cytogenetic mechanisms regulating the formation of clonal gametes. As the molecular mechanisms leading to genome exclusion and subsequent gamete formation are still unclear, not only in water frogs but also in other asexuals, further research should focus on the mechanisms of homologous chromosome pairing and segregation in later meiotic phases.

Additional file

Additional file 1: Figure S1-S3. Comparative genomic hybridization (CGH) on mitotic (1) and meiotic (2, 3) chromosomes of *Pelophylax esculentus* males showing several types of experimental artefacts and failures. 1) Unsuccessful differentiation of parental chromosomes: note the apparent accumulation of probes on the edges/surface of chromosomes, possibly due to over fixed gonadal tissues used for chromosome spreads. 2) Inconclusive hybridization pattern: note equal hybridization intensity of both genome-derived probes. 3) Week hybridization pattern, insufficient for differentiation of parental chromosomes. *Lessonae*-derived genomic probes were labelled with biotin-16-dUTP and hybridization signals detected with Streptavidin-FITC (*green*) (**1a**, **2a**, **3a**), *ridibundus*-derived genomic probes (**b**) with digoxigenin-11-dUTP and Anti-Digoxigenin-Rhodamine (*red*) (**1b**, **2b**, **3b**). Figures **1c**, **2c**, **3c** show merged images of both genomic probes, figures 1d, 2d, 3d merged images of both probes and DAPI staining of chromosomes (*blue*). Scale bar = 10 μm. (TIF 2427 kb)

Abbreviations

Aat, aspartate aminotransferase; Cy3, cyanine dye; CGH, comparative genomic hybridization; DAPI, 4′, 6-diamidino-2-phenylindole; dUTP, 2′-Deoxyuridine, 5′-Triphosphate; E, elimination; F1, first filial generation; FITC, fluorescein isothiocyanate; gDNA, whole genomic DNA; *Gpi*, Glucose-6-phosphate isomerase; HCl, hydrogen chloride; IAPG CAS, v.v.i., Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, v.v.i.; KCl, kalcium chloride; *Ldh-1*, lactate dehydrogenase; $NaPO_4$, sodium phosphate; SDS, sodium dodecyl sulfate used as Denhardt's reagent; SSC, Standard saline buffer; TEs, transposable elements

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Availability of data and materials

All important data are provided in the Results and Figures. The dataset includes all the figures used to reach the conclusions drawn in the manuscript, and any additional data required to replicate the reported study findings in their entirety.

Authors' contributions

MD participated in the design of the study, collected samples, made chromosomal preparations, participated in the *in situ* hybridization analysis and wrote the initial draft of the manuscript. AS performed the *in situ* hybridization analysis and drafted the manuscript. FM, PR and JP participated in the data interpretation and helped to draft the manuscript. LC conceived of the study, and participated in its design, sampling, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

All authors declare no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All experimental procedures involving water frogs were performed in agreement with directives and under the supervision of the Ethical Committee of the Faculty of Science, Charles University, Prague, according to the directives of the State Veterinary Administration of the Czech Republic, permit number 34711/2010-30 from the Ministry of Agriculture of the Czech Republic. All institutional and national guidelines for the care and use of laboratory animals were followed.

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Kapitola II

Evolution of unisexuality in reverse order: molecular insights into the origin and persistence of an all-male water frog lineage *Pelophylax esculentus*

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Evolution of unisexuality in reverse order: molecular insights into the origin and persistence of an all-male water frog lineage *Pelophylax esculentus*

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Abstract

As opposed to sperm-dependent parthenogens, populations of some taxa consist only of males. It is therefore necessary to investigate their origin and perpetuation in order to understand how asexuals operate under reversal reproductive mode. Following this, we studied *Pelophylax* water frogs along the upper Oder River in Central Europe and found that hybrid forms, collectively named *P. esculentus*, live syntopically with their parental species *P. lessonae* or *P. ridibundus*. Specifically, we investigated the latter case where *P. ridibundus* occurs in both sexes and *P. esculentus* occurs only as diploid males, in order to identify: i. the variability of individual genomes, ii. the type of all-male reproduction, and iii. the genealogic relationships between the hybrid and sexual genomes. Our microsatellite data revealed that *P. esculentus* males bear Mendelian-inherited *ridibundus* genomes while the *lessonae* genome is a clone (hybridogenetic reproduction). The clonal *lessonae* genome is of a single origin across all-male populations and did not recently originate from the adjacent *P. lessonae*, suggesting an older *in situ* or *ex-situ* origin. This study shows that unisexual *P. esculentus*, although very rarely formed, perpetuates over many generations as self-persisting all-male animals, and successfully compete with *P. ridibundus* males for eggs from its conspecific females.

Keywords: *Pelophylax*, water frog, hemiclone, hybridogenesis, sexual parasites, asexual, all-male lineage

Introduction

Unisexual animals are model systems for understanding the evolution and maintenance of sex and recombination despite the cost when compared to asexual reproduction (Smith and Maynard-Smith 1978; Charney 2012). In vertebrates, interspecific hybridization is a well-established cause of unisexuality (Dawley 1989). In 46 described cases of squamate reptiles, 51 cases of fish and 23 cases of amphibians, unisexuality is almost exclusively linked to the female sex (Vrijenhoek et al. 1989; Kearney et al. 2009; Lamatsch and Stöck 2009). As a result, current theories dealing with the evolution and maintenance of sexual reproduction, e.g. Red Queen models and host-parasite mode coevolution (Lively 2010), two-fold numerical cost of sex relative to unisexual reproduction ((Smith and Maynard-Smith 1978), stochastic and deterministic theories of mutation clearance (Howard and Lively 2002), and male mate choice in sexual/unisexual mating complexes (Schlupp and Plath 2005), are mostly derived from interactions between female unisexuals and their male and female sexual relatives. Non-sexual males are frequently inviable or sterile (e.g. (Choleva et al. 2012) but interestingly a few cases of viable and likely fertile unisexual males have been documented e.g. in water frogs of the genus *Pelophylax* (Uzzell et al. 1977, Günther 1983) and fish of the genus *Squalius* (Alves et al. 2001) and *Hypseleotris* (Schmidt et al. 2011), although little is known about their origin and maintenance.

These occurrences of male asexuals offer a unique opportunity to obtain valuable and relevant information regarding the evolution of sex and reproductive systems. Inclusion of unisexual male populations into models would provide opposite sex parameters to the aforementioned and other studies (Foran and Ryan 1994; Neiman et al. 2009, 2014; Janko et al. 2011) exploring the paradox of sex on all-female asexuals. Other recent projects also highlight the importance of hybrid males not only in vertebrate speciation i.e. research into a bisexually reproducing all-triploid vertebrate (Stöck et al. 2002), the origin of an allotetraploid

vertebrate species (Cunha et al. 2008), and the general role of polyploid evolution (Choleva and Janko 2013). Clearly, the study of asexuality in general brings us deeper insights concerning the question of what it means to be a eukaryote (Bengtsson 2009).

Vertebrate asexuality is linked to sexual reproduction through different reproductive modes based on whether hybrid males participate in mating; parthenogenesis, gynogenesis and hybridogenesis. Parthenogenetic squamate reptiles reproduce clonally with eggs developing in the absence of sperm. This gives rise to genetically uniform all-female offspring that are genetically identical to their mother, except for somatic mutations. Sperm-dependent parthenogenetic fish and amphibian reproduce via gynogenesis. In this reproductive mode, females produce unreduced oocytes but need sperm from a sexual male to trigger the onset of embryonic development. Usually, the sperm does not contribute any genetic material to the all-female progeny, but if a clonal egg from a parthenogenetic or gynogenetic female is fertilized by a sperm from a heterogametic sexual male, polyploid hybrid females and also males are formed (Alves et al. 2001; Choleva et al. 2012; Janko et al. 2012). However, the most promising reproductive mode for the maintenance of male asexuality is hybridogenesis and its modifications, which combines elements of both clonal and sexual reproduction.

In hybridogenesis, hybrids usually discard one complete parental genome from their germ line prior to meiosis and clonally transmit the remaining one. Thus, the gametes contain unrecombined genomes from one of the two parental species (Schultz 1969; Dawley 1989). Hybridity is restored in each generation via fertilization with gametes of the sexual species, whose genome has been eliminated in the germline of the hybrid. Hence, the soma of such hybrids consists of both clonal and sexual inherited genomes, and are referred to as "hemiclones" (Vrijenhoek 1979). A number of modifications of this basic hybridogenetic reproduction pattern exist. In the "meiotic hybridogenesis" of some triploid lineages of fish

and amphibians, the elimination of one complete haploid set of chromosomes is not followed by the clonal transmission of the remaining diploid set. Instead, the two chromosomes are recombined during normal meiosis and haploid gametes are produced e.g. in *Squalius alburnoides* fish (Alves et al. 2001). In the "pre-equalizing hybrid meiosis" found in *Bufotes baturae* toads diploid gametes combining one clonal and one meiotic chromosome set are produced (Stöck et al. 2012). In kleptogenesis, a mode that is similar to hybridogenesis, unisexual *Ambystoma* salamander females incorporate the full or partial genomes of their mates into their clonally transmitted genomes, which can lead to the replacement of the original maternal set (Bogart et al. 2007).

Hybridogenetic systems vary in the degree to which unisexuality is observed (Schmidt et al. 2011). Diploid hybridogenetic fish of the genus *Poeciliopsis* are invariably all-female (Schultz 1969, 1977), whereas in other hybridogenetic systems males also exist, and both hybrid sexes may propagate clonal or hemiclonal genomes. The latter holds for the cyprinid fish *Squalius alburnoides* (Alves et al. 2001), the carp gudgeon *Hypseleotris* (Schmidt et al. 2011), the pond loach *Misgurnus anguillicaudatus* (Morishima et al. 2004; Fujimoto et al. 2008), the water frog *Pelophylax esculentus* (Graf and Polls Pelaz 1989; Plötner 2005), and for *B. baturae* toads which have a hybridogenesis-related reproductive mode (Stöck et al. 2002, 2012).

Perpetuation of hybrid males in populations usually depends on highly complex reproductive mechanisms, often supported by various gamete production patterns and the presence of polyploid animals in a mating system. An example comes from the *Squalius alburnoides* complex (Alves et al. 2001). Here, hybrid females are sperm-dependent sexual parasites on the males of the host species which reproduces sexually. In this system hybrid males are rather a by-product of crosses between hybrid females and sexual males. In contrast to sperm-dependent parthenogenetic systems, in some populations of European water frogs (*Pelophylax esculentus* complex) a reversal reproductive mode can be observed. In this case,

only male hybrids coexist with both sexes of the parental species (Uzzell et al. 1977; Günther and Plötner 1988; Tunner and Heppich-Tunner 1992). So far, it is not known whether one, a few, or multiple hybridization events led to the formation of hybrid all-male populations. Consequently, there has been, and still might be, the possibility for *de novo* formation of *P. esculentus* males via ongoing primary hybridizations between the two sexual species, thus leaving open the question of their stable persistence in natural populations.

The *Pelophylax esculentus* complex consists of two sexual species and a hybrid form: the pool frog *Pelophylax lessonae* (genomic composition LL), the marsh frog *P. ridibundus* (RR), and the edible frog *P. esculentus* (RL) which originated from matings between *P. lessonae* and *P. ridibundus*. In most parts of Western and Central Europe *P. esculentus* lives in sympatry with *P. lessonae*, in what is known as the "L-E system" (Uzzell and Berger 1975). In this system, both male and female hybrids usually exclude the haploid *lessonae* (L) genome and transmit the *ridibundus* (R) genome to their gametes. Hybridity is restored in each generation by heterospecific crosses between *P. esculentus* and *P. lessonae*, the donor of the L genome. Moreover, in some populations of the L-E system many hybrid females produce diploid RL eggs, which lead to triploid RLL genotypes and RRL genotypes when fertilized by L and R sperm, respectively.

Conversely, in the R-E system most *P. esculentus* exclude the *ridibundus* genome, transmit their *lessonae* genome and mate with *P. ridibundus* to perpetuate the hybrid lines (Graf and Polls Pelaz 1989; Plötner 2005). A special case are populations which consist of *P. ridibundus* and only *P. esculentus* males. Such populations have been reported in the Czech Republic (Doležálková et al. 2016), Denmark (Fog 1994), Germany (Günther and Hähnel 1976; Berger and Günther 1991), Hungary (Tunner and Heppich-Tunner 1992) and Poland (Rybacki 1994a, 1994b; Rybacki and Berger 2001). In contrast to the L-E system, some *P. esculentus* males from this population type transmit only the *lessonae* genome, others only the

ridibundus genome, and some males even produce both *ridibundus* and *lessonae* gametes (Uzzell et al. 1977; Günther and Plötner 1988, Doležálková et al. 2016). Moreover, some of the hybrid males from a population in the Oder River possessed recombined genomes (Uzzell et al. 1977). Artificial crossing experiments with female *P. ridibundus* and male *P. esculentus* from such R-E systems suggest that *ridibundus* sperm produce female *P. ridibundus* when fertilized by *ridibundus* sperm but give rise to *P. esculentus* males when fertilized by *lessonae* sperm. This offers a potential explanation for the existence and perpetuation of male-only *P. esculentus* in natural populations (Uzzell et al. 1977; Günther 1983).

In this study we sampled water frogs from 16 populations along the upper Oder River valley in Central Europe with two main aims. Firstly, to investigate the origin of male asexuality in order to better understand how it evolved from their sexual ancestors. Secondly, to test the hypothesis that all-male hybrids are able to persist in natural populations. In pursuing these goals, we used 17 microsatellite markers to identify a) the genetic variability of clonally transmitted genomes, b) the mode of asexual reproduction, and c) the putative origin of clonally inherited genomes.

Methods

Study species and sampling

In 2002 and 2008, a total of 249 individuals from all three *Pelophylax* taxa were collected. Frogs were caught with a hand net at 16 locations in the upper Oder River drainage (Czech Republic; Fig. 1, Tab. 1). *Pelophylax kurtmuelleri* from Greece, a sister species of *P. ridibundus*, was used to test a power of microsatellite data in a phylogenetic tree construction. Males were distinguished from females by the presence of vocal sacs and nuptial pads. Taxon affiliation was determined on the basis of external morphological characters (Günther 1990;

Plötner 2005), and identification was later verified genetically with allozyme markers. Tissue samples obtained from finger tips or muscles were stored at -20°C for allozyme analyses and in 96% ethanol for DNA analyses.

Taxon assignment and composition

For genotype determination, six allozyme loci previously identified to be diagnostic were used (Uzzell and Berger 1975). Approximately 1g of skeletal muscle (or gonads) was homogenized on crushed ice for 20 s in an equal volume of Tris NaCl extraction buffer (pH 8.5; (Valenta et al. 1971) using an Ultra-Turrax homogenizator (IKA-WERK). The homogenate was then centrifuged at 11,000 rpm at 4°C for 20 min. Enzymes obtained from these tissues, namely Aspartate aminotransferase (*Aat*; EC 2.6.1.1), Glucose-6-phosphate isomerase (*Gpi*; EC 5.3.1.9), Glycerol-3-phosphate dehydrogenase (*G3pdh*; EC 1.1.1.8), L-lactate dehydrogenase (*Ldh-1*; EC 1.1.1.27), Phosphoglucomutase (*Pgm-2*; EC 5.4.2.2), and Phosphogluconate dehydrogenase (*6-Pgd*; EC 1.1.1.44), were analysed by horizontal potato starch gel electrophoresis (Valenta et al. 1971; Uzzell and Berger 1975). Subsequently, gels were cut into three 2 mm thick slices and stained with appropriate allozyme chromogenic detection methods according to (Harris and Hopkinson 1976; Buth and Murphy 1980; Pasteur et al. 1987). Stained gel slices were photographed and the agar layers were transferred to filter paper, dried and stored as part of the protocol. The visualised allele products were designated from „*a*“ fastest to „*e*“ slowest according to their mobility. Samples which revealed unclear patterns were reprocessed.

DNA extraction, microsatellite genotyping

Genomic DNA was extracted using a NucleoSpin commercial kit (Macherey-Nagel GmbH and Co.) with the epMotion 5075 automated pipetting system (Eppendorf). We amplified 17 microsatellite loci: Ga1a19, Re2caga3, Re1Caga10, RICA1b6 (Arioli et al. 2010), RlCA1b5,

RlCA5, RlCA18 (Garner et al. 2000), RlCA2a34, GA1a23, Rrid169A, Rrid059A, RlCA1a27, Rrid135A (Christiansen and Reyer 2009), Res16, Res20, Res22 (Zeisset et al. 2000), Rrid013A (Garner et al. 2000; Hotz et al. 2001), using the redesigned primer sets described in (Hermaniuk et al. 2013). We followed (Pruvost et al. 2013) for species-specific marker characterization of the aforementioned markers. Polymerase chain reaction (PCR) was performed according to (Christiansen and Reyer 2009). Fragment-lengths were determined using an ABI 3730 Avant capillary sequencer (Applied Biosystems, Zug, Switzerland) and an internal size standard (GeneScan-500 LIZ); alleles were scored with GeneMapper v. 3.7 (Applied Biosystems, Zug, Switzerland).

Preparing data sets

Raw microsatellite genotypes of *P. lessonae P. ridibundus* and *P. esculentus* were checked for potential genotyping errors due to the presence of null alleles with Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). This method estimates frequencies of null alleles with the Brookfield 2 null allele estimator, which treats nonamplifications as data and regards them as null homozygotes when calculating null allele frequencies (Brookfield 1996). As this method cannot be applied to diploid hybrids, we inspected *lessonae* and *ridibundus* genomes in hybrids visually and considered the absence of an allele as evidence for a null allele. We found one locus (Res20) in *P. lessonae* and four loci (Res16, Rrid069A, RICA5 and Re1Caga10) in *P. ridibundus* with a potential presence of null alleles, and therefore applied a manual correction for null alleles at these loci following (Wagner et al. 2006). The after-correction analysis in Micro-Checker did not detect any locus with a presence of null alleles. The software MSA v. 4.05 (Dieringer and Schlötterer 2003) was used to determine mean microsatellite allele numbers (*AN*), observed heterozygosities (H_O) and expected heterozygosities (H_E) within populations. In each population, every locus was tested for departure from the Hardy-Weinberg (*HW*) genotypic equilibrium.

Based on the definition of hybridogenetic reproduction, diploid hybrid water frogs from Central Europe transmit one haploid set clonally to gametes (therefore termed a "hemiclone" (Vrijenhoek 1979), whereas the other set is discarded and regained for the next generation by mating with an individual of the sexual parental species. As our aim was to test the origin of a particular hemiclone in this vertebrate species, we analysed sexual and clonal genomes of hybrid individuals separately. First, we sorted *lessonae* and *ridibundus* genomes according to the allele species-specificity know from the literature. Then, the correctness of allele separation was tested visually assuming that one allele per locus was received from a sexual mate, and therefore such allele has to be present (and always was in our study) in the gene pool of a sympatric sexual population. Our approach of separating sexually- and clonally-inherited alleles in hybrid genomes was 100% successful.

While for hybrids from R-E system populations both *ridibundus* and *lessonae* genomes were included in our analyses, we used only *lessonae* genomes of hybrids from the L-E system because we were not interested in the origin of *ridibundus* hemiclones in this system. We determined a hemiclone by a multilocus genotype (MLG), defined by the identical combination of alleles found in our microsatellite analyses. A minimum of three samples exhibiting the same allele composition was a clear indication that the genome was inherited clonally and did not originate from a sexual donor (Pruvost et al. 2015). As most statistical programs dealing with microsatellite data - including those used in this study - are not designed to compare haploid and diploid data, we doubled the haploid data sets separately for *lessonae* and *ridibundus*.

Flow cytometry

In order to determine ploidy levels, all individuals were analysed by flow cytometry on blood samples. A drop of blood was added into 70% ethanol and immediately shaken to prevent

clotting. Chicken blood was used as a reference standard for cell size measurement. Relative nuclear DNA content was measured with DAPI fluorochrome using the Cystain two Step High Resolution DNA Staining commercial kit (Partec GmbH, Münster, Germany). Fluorescence intensity of 5,000 stained nuclei was measured with a Partec PAII flow cytometer at a speed of 0.5 µl/s. Flow cytometric histograms were evaluated using FloMax 2.52 (Partec GmbH, Münster, Germany).

Statistical analysis of microsatellite data

In oreder to determine the origin of all-male *P. esculentus* populations we analysed genetic relationships between hybrids and their parental species based on allele frequencies of 17 microsatellite loci using STRUCTURE v. 2.3.1 (Pritchard et al. 2000), GenAlEx v. 6.41 (Peakall and Smouse 2012), Arlequin v. 3.1 (Excoffier et al. 2007), Populations v. 1.2.32 (Langella 1999) and GeneClone v.2.0 (Arnaud-Haond and Belkhir 2007).

Hybrid origin. In order to analyse the hybrid origin of *P. esculentus* a model-based clustering method in STRUCTURE was used that infers population structure based on genotype data consisting of unlinked markers. The analysis was carried out using a burn-in period of 20,000 iterations followed by 200,000 Markov Chain Monte Carlo (MCMC) repeats. The probability of the used admixture model was tested for clusters $K = 1 - 7$. The most probable number of K populations was estimated using log-likelihood ln $P(D)$ according to (Evanno et al. 2005).

Hemiclonal reproduction. In order to distinguish whether individual genomes are of sexual or clonal origin, we estimated P_{SEX} values using the GeneClone program. When the same genotype is detected more than once, P_{SEX} express the probability of these MLG being derived from distinct reproductive events (Arnaud-Haond et al. 2005). We applied the P_{SEX} probability taking into account the F_{IS} estimated in the dataset (Young et al. 2002). F_{IS} was

also estimated on the basis of the round-robin method, and further used to estimate a slightly corrected P_{GEN}, calculated as the unique MLG probability (Arnaud-Haond et al. 2005), which in turn provides a better estimate of the probability of clonal identity P_{SEX} (Arnaud-Haond and Belkhir 2007). Considering that the presence of missing data precludes the use of P_{SEX} in the program, we considered only those loci where both genomes were amplified i.e. individuals with missing alleles were eliminated (Tab. S1).

Hemiclone and their genetic relatedness. In order to estimate the genetic relatedness between individual genomes we compared allele frequencies, heterozygosity and polymorphism estimates with the program GenAlEx. A centred principal component analysis (PCA) was applied to examine clustering of individuals based on total variation of microsatellite allele frequencies without scaling of alleles. For the PCA, we converted a list of 278 MLG´s from 17 loci (Tab. S1) into a genetic distance matrix (Covariance matrix with data standardization) and then used standard PCA analysis to visualize the results.

The UPGMA trees were constructed for 205 MLGs based on 10 loci using resulting genetic Nei's *DA* distance (Takezaki and Nei 2008) matrices and with the program Populations. Support for internal branches was evaluated with 7,000 replicates. Distinct genetic groups of individual genomes were identified with PCA on the basis of Nei's *DA*. The degree of genetic difference among populations was estimated using Wright's *F*-statistics (pairwise F_{ST}) (Weir and Cockerham 1984) and analysis of molecular variance (AMOVA) using Arlequin. We ran locus-by-locus AMOVA which is preferred when datasets contain missing data up to the rate of 5 %. Therefore, all loci that amplified only in one parental species were excluded from analysis. In total, we tested three datasets, each containing two different genomic groups. Groups were arranged in the same way as for PCA analysis. The first group contained 43 *ridibundus*-specific MLG obtained from *P. ridibundus* individuals and 27 *ridibundus*-specific MLG from *P. esculentus* individuals, the second group was composed of 108 *lessonae*-

specific MLG from *P. lessonae* individuals and 27 *lessonae*-specific MLGs from *P. esculentus* individuals, and the third group comprised 43 MLGs received from *P. ridibundus* individuals and 108 MLG from *P. lessonae* individuals. Pairwise AMOVA was run under the H_O hypothesis that both tested groups belong to a single large population; runs were counted with 10,100 permutations. Pairwise F_{ST} calculations were performed using the distances and 10,000 permutations.

Results

Taxon determination

In total, we identified 108 individuals of *P. lessonae*, 43 individuals of *P. ridibundus* and 98 individuals of *P. esculentus* using allozyme diagnostic loci (Tab. S2) and microsatellite loci (Tab. S1). In *P. lessonae*, there were four monomorphic (*Aat, Gpi, Pgm, G3pdh*) and two polymorphic allozyme loci (*Ldh-1, 6-Pgd*); in *P. ridibundus*, there were two monomorphic (*Aat, G3pdh*) and four polymorphic loci (*Ldh-1, Gpi, 6-Pgd, Pgm*). In *P. esculentus*, all six loci were polymorphic. The allelic products of three loci (*G3pdh, Pgm and Gpi*) displayed an atypical expression in 12 individuals. Here, four *P. ridibundus* possessed alleles characteristic for *lessonae* genome in *G3pdh* and *Pgm*, one *P. lessonae* possessed alleles characteristic for *ridibundus* genome in *Pgm*, and seven *P. esculentus* possessed either two alleles characteristic for *lessonae* or two alleles characteristic for *ridibundus* in both *Gpi* and *Pgm*. Detailed information is shown in Tab. S2. Whether the shared alleles represent introgression or equal inheritance from a common ancestor is not clear and difficult to score in these allozyme data.

Genetic diversity

Three loci (Res20, RICA1a27 and RICA18) were specific for the *P. ridibundus* genome, one locus was monomorphic, and 13 loci were polymorphic. The loci Re2caga3, Res22 and Rrid169A were specific for the *P. lessonae* genome, where three loci were monomorphic and 10 loci were polymorphic. Two loci (RICA5 andGa1a23), that are known as species-specific for *P. lessonae* (Garner et al. 2000; Christiansen and Reyer 2009), and one locus (Rrid135A), that is known as species-specific for *P. ridibundus* (Christiansen and Reyer 2009), could be amplified in both the *P. lessonae* and the *P. ridibundu*s genomes. Overall, six out of 17 microsatellite loci were polymorphic among 249 *Pelophylax* individuals.

A total of 187 alleles were detected, with 6 - 17 alleles per locus (Tab. S1, S3 and S4). The allele called 83 in RICA1b6 was marked as a non-specific because it was amplified in the genomes of both parental species (12 *P. lessonae* and four *P. ridibundus*). The presence of one or two alleles per locus supported the flow cytometric data that all individuals were diploid. In addition, allele-dosage effects as indications of polyploidy were not observed.

Detection of distinct genetic groups using microsatellite data

STRUCTURE, AMOVA and Populations analyses hereinafter mentioned were run on 177 individuals (27 hybrid males from the R-E male system, 108 *P. lessonae*, 42 *P. ridibundus*; *P. kurtmuelleri* was included only in Populations). PCA analysis included also 71 male and female hybrids from the L-E system. Bayesian cluster analysis estimated the maximal log-likelihood value ($LnP(D)$) for $K = 2$. Admixture clustering for $K = 2$ clearly indicated that the posterior distribution of allele frequencies is best explained by three clusters. Individuals assigned to group 1 represent *P. lessonae*, individuals assigned to group 2 represent *P. esculentus* and individuals assigned to group 3 represent *P. ridibundus* (Fig. 2a).

As a result of PCA analyses three groups of microsatellite MLGs were identified, the first two principle components accounted for 60.19 % (axis 1) and 12.92 % (axis 2) of genetic variation, respectively (Figure 2b). Cluster 1 (green and dark blue symbols) grouped *P. lessonae* and *lessonae*-specific MLGs of *P. esculentus* sampled in L-E system populations. Cluster 2 (yellow symbols) included *lessonae*-specific MLGs obtained from *P. esculentus* of R-E male populations while cluster 3 (red and light blue symbols) grouped *P. ridibundus* and *ridibundus*-specific MLGs of *P. esculentus* from R-E male populations.

Genealogical analyses made with program POPULATIONS revealed two distinct clusters of *lessonae*-specific MLGs, one is characteristic of *lessonae*-specific MLGs found in *P. esculentus* from R-E male populations, the second comprises genotypes of *P. lessonae* individuals (Fig 2c and Fig. S1). Contrary to the previous pattern, *ridibundus*-specific MLGs found in *P. esculentus* from R-E male populations did not represent a separate lineage but instead clustered with *P. ridibundus*.

Identification of MLGs and hemiclones. In the data set which contained missing data for some loci, GenAlex estimated a total of 186 MLGs among all *Pelophylax* individuals investigated. Among 151 *P. lessonae* and *P. ridibundus* individuals, the program generated 151 MLGs. Considering the 27 *P. esculentus* from R-E male populations, *ridibundus* genomes were represented by 27 MLGs, whereas the *lessonae* genomes exhibited only eight MLGs (Fig. 3, Tab. S5. One of the *lessonae*-specific MLGs was found among 14 *lessonae* genomes, while the remaining eight MLGs were discovered in one to three *lessonae* genomes. In contrast to MLGs obtained in *ridibundus* genomes, MLGs found in *lessonae* genomes shared allelic variation in full and their division arose from the variation of missing data for some loci (Tab. S5). Therefore, we consider the nine MLGs detected in the hybrids' *lesonae* genomes as a single MLG. This MLG is further suggested to represent a clone (or a hemiclone from an individual level) designated as ODERL1.

Considering only MLGs with complete allelic data, GenClone generated 122 MLGs among 143 genotypes. 21 *lessonae* genomes, all originating from *P. esculentus* of R-E male populations, were represented by only a single MLG. The P_{SEX} value of this MLG ranged from 0.15 to 1.50E-41, indicating a non-sexual inheritance.

AMOVA. A single comparison of MLGs obtained from the genomes of *P. lessonae* and *P. ridibundus* based on Wright's *F*-statistics partitioned total variation into variation between the two species (53 %; F_{ST} = 0.537), variation within individuals (32 %), and variation among individuals (15 %). When *ridibundus* genomes of *P. esculentus* and *P. ridibundus* were compared, 3 % of the total variation in MLGs could be attributed to the difference between the hybrid and the parental species (F_{ST} = 0.032), while a comparison of the *lessonae* genomes from *P. esculentus* (ODERL1) and *P. lessonae* partitioned 32 % of total MLG variation between hybrid and parental individuals (F_{ST} = 0.321, see Tab. S6. The low F_{ST} value of 0.032 is in line with the null hypothesis that *ridibundus* genomes in hybrid *P. esculentus* and *ridibundus* genomes in sexual *P. ridibundus* share the same alleles, meaning that shared genetic variation seems to be maintained within random-breeding populations (Fig. 4).

Discussion

I. Detection of all-male *P. esculentus* populations

The upper Oder River valley is mainly inhabited by *P. ridibundus* of both sexes and only males of *P. esculentus*, whereas populations of the L-E system (typically represented by *P. lessonae* and *P. esculentus* of both sexes) are found outside the valley. Since 2001, no hybrid female was detected among 132 hybrid individuals in localities where *P. ridibundus* co-

occurred with *P. esculentus* males (Tab. 1 and unpublished data), which makes this region a newly discovered area for the coexistence of sexual *P. ridibundus* with diploid hybrid *P. esculentus* males. Similar populations have been found in Germany (Günther 1975; Uzzell et al. 1977; Plötner and Grunwald 1991), Poland (lower Oder River) (Rybacki 1994b) and Bornholm Island (Fog 1994). Our data emphasize the role of the Oder River drainage basin in the existence of the population type with *P. esculentus* males and points to a potential area of its geographic origin.

II. *P. esculentus* males are active in maintaining their own all-male hybrid populations

Hybrid males are usually infertile (Wu et al. 1996) and therefore considered as a by-product of hybridizing sexual species or a sexual male species with a unisexual female (Vasil'ev et al. 2003; Choleva et al. 2012). In the typical L-E system in Europe, *P. esculentus* males result only from crosses between *P. esculentus* females and heterogametic *P. lessonae* males. Backcrosses between such hybrid males and *P. lessonae* females result exclusively in *P. esculentus* females, due to the presence of clonal *ridibundus* genomes in the sperm that contain female determining factors (Graf and Polls Pelaz 1989; Plötner 2005).

Two lines of evidence support our finding that *P. esculentus* males from the upper Oder River valley are fertile and maintain their own all-male hybrid populations, meaning that they sexually parasitize sympatric *P. ridibundus* (female hosts within hybridogenesis) and do not originate directly from primary hybridization between the two sexual parental taxa, or between sexual males and hybrid females.

First, our extensive sampling did not reveal the presence of *P. lessonae* or a single hybrid female, necessary for the origin of diploid hybrid males (Graf and Polls Pelaz 1989). The second line of evidence comes from independent statistical tests of allelic variation in the nuclear loci. The individually-specific MLGs and the presence of both sexes indicate that *P.*

ridibundus and *P. lessonae* are randomly mating sexual populations. *Ridibundus* alleles from *P. esculentus* males gather together with *P. ridibundus* males and females in two different clustering approaches (Figs 2a and b). Simultaneously, 27 *ridibundus* genomes from *P. esculentus* males represent 27 MLGs, i.e. again all combinations are unique. It is therefore reasonable to assume that *P. esculentus* received haploid *ridibundus* genomes from sympatric *P. ridibundus* females. On the other hand, we suggest that *lessonae* genomes found in *P. esculentus* from the Oder River are transmitted by hybrids themselves between generations because *lessonae* alleles from *P. esculentus* males are positioned separately not only from sympatric *P. ridibundus* individuals but also from *P. lessonae* (Figs 2a and b). The statistical test for the p_{sex} value of these 27 *lessonae* genomes ($1.50E^{-41}$) supports its inheritance without sex.

III. Single hemiclone-bearing all-male hybrids

Hemiclonal hybrids are genetically identical for half of the diploid parental genome (Abbott and Morrow 2011) which they clonally pass on to the next generation. Analyses of *ridibundus* and *lessonae* allelic richness in all-male *P. esculentus* revealed that 13 loci within the *ridibundus* genome were polymorphic, which correlates with the observed low allelic frequencies (0.035714 - 0.842105). Together with the above mentioned observation that *ridibundus* MLGs were not shared among individuals at all, it is reasonable to assume that *ridibundus* genomes come from recombinant eggs of sexually reproducing *P. ridibundus*. In the *lessonae* genome, however, we observed only monomorphic loci with allele frequencies of 1.00. Therefore, it is proven that the *lessonae* genome is clonally inherited and represents a single hemiclone (Figs 2 and 3, Tab. S1).

The consequence of the *lessonae* genome only producing male hybrids is in agreement with the XX/XY type of sex determination proposed for the R-E system much earlier (Uzzell et al. 1977; Günther 1983). However, our finding that all hybrid males belong to the same single

hemiclone (ODERLI1) is new and surprising. Most animal systems with female unisexuality include multiple clonal MLGs, as shown in spined loach fishes of the genera *Cobitis* (Janko et al. 2012) and *Poeciliopsis* (Angus and Schultz 1979) and in the *Phoxinus eos-neogaeus* complex (Angers and Schlosser 2007). Even where female hybrids form a monophyletic group e.g. *Poecilia formosa*, the populations show a fairly high level of clonal diversity (Stöck et al. 2010). In *Pelophylax* populations of the L-E system where both hybrid males and females coexist, diversity of typically transmitted *ridibundus* hemiclones is high (Mikulíček et al. 2014; Pruvost et al. 2015).

IV. Origin of the hemiclonal line

Apparently, the existence and maintenance of all-male unisexuality among animals in general, and in *Pelophylax* water frogs in particular, hinges on the formation of a specific genome from a particular sexual species. In water frogs, the hemiclone (ODERL1) determining diploid all-male unisexuality originally comes exclusively from the sexual species *P. lessonae*.

The genome of *P. lessonae* seems to play a key role in the formation of all-male *P. esculentus* lines. Supporting evidence come from rare Central- and West-European mixed populations, where triploid *P. esculentus* males originate from clonal diploid *lessonae* sperm and haploid *ridibundus* eggs (Graf and Polls Pelaz 1989; Pruvost et al. 2015).

Despite the evidence for a single origin of the ODERL1 hemiclone and the fact that it has evolved from *P. lessonae*, the separation of the ODERL1 alleles from the *P. lessonae* cluster (Fig. 2a) and clade (Fig. 2c) indicates significant differences between the *lessonae* hemiclone and *P. lessonae*. Compared to the difference between *ridibundus* alleles (from *P. esculentus* males) and *P. ridibundus*, a considerably higher F_{ST} value (0.321) was obtained between the ODERL1 hemiclone and *P. lessonae* from 11 surrounding L-E populations. Due to high allele

variability (32 %) between the two data sets of *lessonae* alleles, we hypothesise that current *P. lessonae* populations from the L-E system adjacent to the R-E system in the Oder River valley are not direct ancestors of all-male hybrid lineages bearing the unique *lessonae* hemiclone. It also seems unlikely that the *lessonae* hemiclone recently originated from primary *P. lessonae* in the region. Although age estimates of the ODERL1 hemiclone and comparison with more distant *P. lessonae* populations are yet to be obtained, we hypothesise either an older *in situ* origin or a possible *ex situ* origin of the ODERL1 hemiclone, i.e. some distance away from the local *P. lessonae*. Maybe the source lies in similar *P. ridibundus – P. esculentus* all-male populations downstream in the Oder River in Germany (Uzzell et al. 1977) and Poland (Rybacki and Berger 2001). At present, however, the existence and nature of *lessonae* hemiclones along the whole Oder River remain unclear.

Wherever the origin was for the formation of the ODERL1 hemiclone from hybridizing *P. lessonae* and *P. ridibundus*, the "Balance hypothesis" (Wetherington et al. 1987; Moritz et al. 1989) proposes that a certain phylogenetic distance between the hybridizing sexual species is required to affect meiosis and produce an unisexual lineage with a non-sexual heredity. However, this hypothesis does not sufficiently explain its rarity and single origin. Beyond this precondition, our data support the propositions by Vrijenhoek (Vrijenhoek 1989) that formation of some unisexual lineages faces several constraints (e.g. genetic, developmental and ecological), and by Stöck et al. (Stöck et al. 2010) that sometimes solely the combination of very specific genotypes might lead to the successful formation of an asexual lineage.

V. Evolutionary implications and conclusions

By studying natural populations of *Pelophylax* water frogs along the upper Oder River valley we discovered a clonal *lessonae* genome represented by a single hemiclone that is spread over

all-male *P. esculentus* populations living in sympatry with sexual *P. ridibundus*. This reproductive mode mirrors the one that previously has been identified in some all-female hybrid animals (Beukeboom and Vrijenhoek 1998; Kearney et al. 2009; Lamatsch and Stöck 2009). Therefore, we name this mode "unisexuality in reverse". This hybridogenetic system in which all-male hybrids coexist with sexual males and females offers intriguing opportunities to compare evolutionary forces forming mating systems that may reverse those operating in all-female systems. These include phenomena like egg-dependent instead of sperm-dependent reproduction or male-male rather than female-female competition over the gamete donors. Additionally, this natural system provides a comparative model to a hemiclonal laboratory system developed in *Drosophila melanogaster* (Rice et al. 2005) for estimating quantitative genetic parameters in hemiclonal analyses (Abbott and Morrow 2011). Elucidating the mechanisms underlying these peculiarities might shed more light on the general processes of evolution of sex or mate-choice theory, and contribute to understanding the origin and maintenance of host–parasite dynamics.

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Competing Interests

All authors declare no competing interests.

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Tables, Figures and Legends

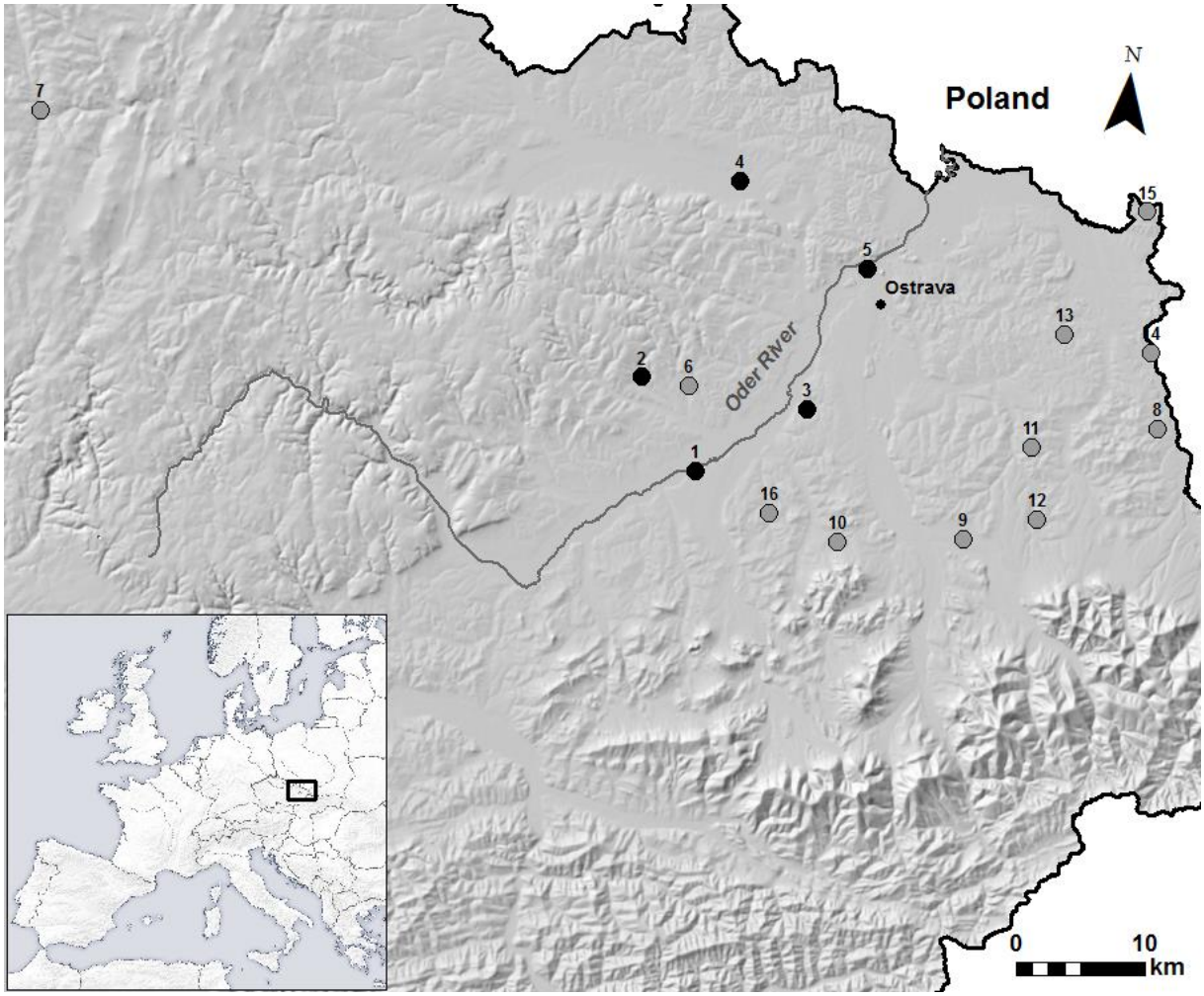

Fig. 1: Map of the investigated water frog populations in the upper Oder River. Numbers inside the symbols correspond with numbers of localities given in Tab. 1. Light grey dots refer to the R-E male system, dark grey dots to the L-E system. The inset indicates the position of the study region in Europe.

Tab. 1: Information on the investigated 16 populations of *Pelophylax* taxa from the upper Oder River valley. Given are population types, names and coordinates for sampling sites, genotypes and numbers of collected females, males and juveniles (juv) in each population, types and numbers of the created multilocus genotype (MLG) and IDs of found hemiclones. For more details see Tab. S1.

No.*	Pop. type	Sampling site	Latitude Longitude	Genotype	No. of females/males	MLG type	No. of MLGs	Hemiclone ID
1	R-E male	Albrechtičky	49.708056 18.098889	RR	8/9 + 4juv	RR	21	

				RL	-/10	R	10	
						L	1	OderL1
2		Bílovec	49.769722	RR	1/-	RR	1	
			18.032222	RL	-/2	R	2	
						L	1	OderL1
3		Darkovice	49.757778	RR	3/5	RR	8	
			18.213056	RL	-/4	R	4	
						L	1	OderL1
4		Dolní Benešov	49.912222	RR	4/2	RR	6	
			18.120000	RL	-/4	R	4	
						L	1	OderL1
5		Ostrava	49.858889	RR	5/4	RR	9	
			18.265278	RL	-/5	R	5	
						L	1	OderL1
6	L-E	Bravantice	49.766944	LL	1/-	LL	1	
			18.084444	RL	7/-	R	NA	
						L	7	
7		Břidličná	49.916389	LL	-/4	LL	4	
			17.359444					
8		Český Těšín	49.764444	LL	3/7	LL	10	
			18.591667	RL	5/-	R	NA	
						L	5	
9		Dobrá	49.676667	LL	7/22 + 2juv	LL	31	
			18.392222					
10		Důl Staříč	49.667222	LL	-/2	LL	2	
			18.258611	RL	13/-	R	NA	
						L	13	
11		Horní Bludovice	49.744444	LL	5/15	LL	20	
			18.457500	RL	2/2	R	NA	
						L	4	
12		Horní Domaslavice	49.694722	LL	3/4	LL	7	
			18.470000					
				RL	7/1	R	NA	
						L	8	
13		Karviná-Doly	49.825000	LL	-/2	LL	2	
			18.483056	RL	10/3	R	NA	
						L	13	
14		Louky	49.817222	LL	1/-	LL	1	
			18.576944	RL	10/4	R	NA	
						L	14	
15		Prstná	49.915000	LL	3/5	LL	8	
			18.560556	RL	3/-	R	NA	
						L	3	
16		Trnávka	49.683333	LL	8/10 + 3juv	LL	21	
			18.181389	RR	juv	RR	1	
				RL	4/-	R	NA	
						L	4	

OG	PK	Greece	39.873166 22.732813	PK	1/-	PK	1

* The localities are numbered according to Fig. 1; R-E male, *P. ridibundus* - *P. esculentus* male populations; L-E, *P. lessonae* - *P. esculentus* populations; OG, outgroup; RR, *P. ridibundus*; RL, *P. esculentus*; LL, *P. lessonae*; PK, *P. kurtmuelleri*; NA, not analysed.

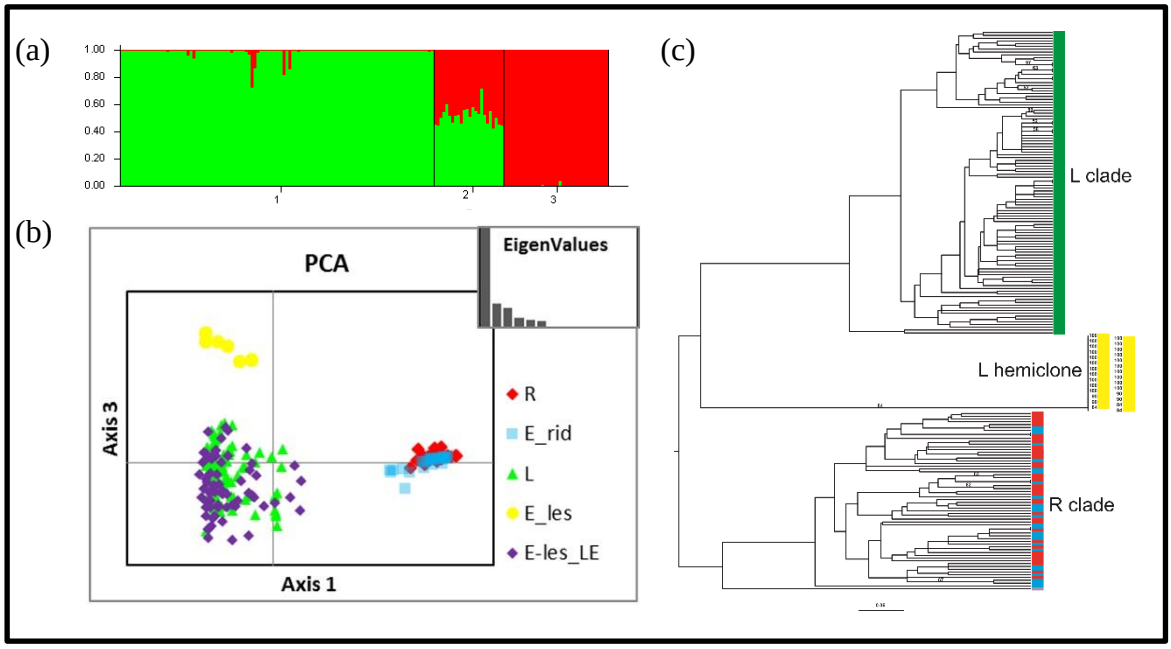

Fig. 2 Clustering analyses performed on MLGs of *Pelophylax* individuals based on microsatellite loci.

(a) Bar plot of 17 microsatellite loci from Bayesian clustering analysis performed in STRUCTURE (K = 2). Each vertical line represents one individual, each colour represents a species-specificity of allele to one parental genome (green = *P. lessonae* genome, red = *P. ridibundus* genome), each cluster represents a different genotype (cluster 1 = LL, cluster 2 = RL, cluster 3 = RR).

(b) Principal component analysis (PCA) of 17 microsatellite loci performed in GenAlex. Each point represents an individual MLG, each colour and symbol a group of related MLGs (according to allele-sharing). Group R (red diamonds) – *P. ridibundus* individuals, group E_rid (blue squares) – R genome from *P. esculentus* from R-E system, group L (green triangles) – *P. lessonae* individuals, group E_les (yellow circles) – L genome from *P. esculentus* from the R-E system, group E_les_LE (violet diamonds) - L genome from *P. esculentus* from the L-E system. Inset screenshot shows the eigenvalues for each axis as principle components of the analysis.

(c) Phylogenetic tree of DAS distance of 10 microsatellite loci reconstructed in program POPULATIONS (method UPGMA, 7 000 replicates; shown are only bootstraps above 50 %, distance scale). One terminal branch represents one individual: Green colour – *P. lessonae* (L clade), yellow colour - L genome from *P. esculentus* (L hemiclone), red colour – *P.*

ridibundus (R clade), blue colour – R genome from *P. esculentus* (R clade), violet colour – *P. kurtmuelleri*. Detailed information is listed in Fig. S1.

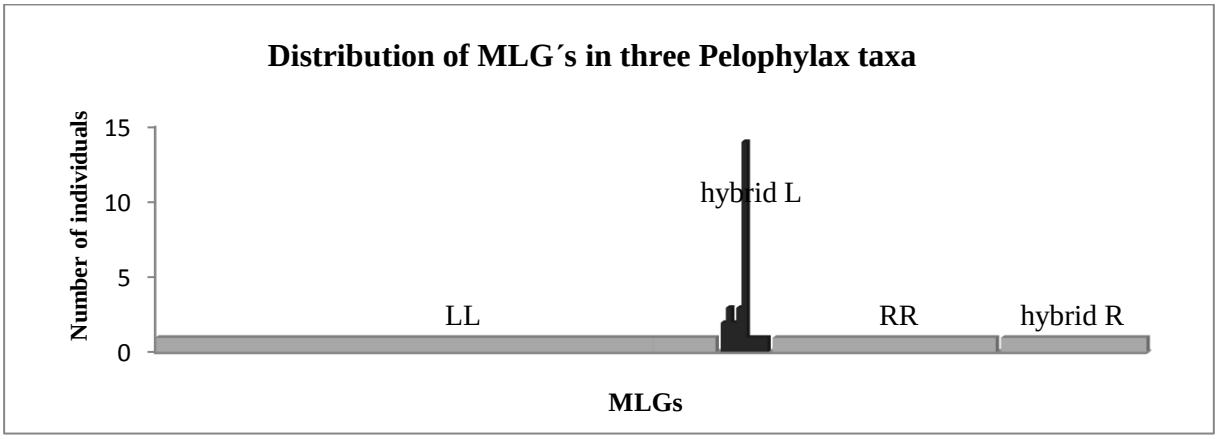

Fig. 3: Distribution of 188 distinct microsatellite multilocus genotypes (MLGs) in three *Pelophylax* taxa generated in GenAlex (LL, *P. lessonae*; hybrid L, *lessonae* genome from hybrid *P. esculentus*; hybrid R, *ridibundus* genome from hybrid *P. esculentus*; RR, *P. ridibundus*). For details see Tab. S6

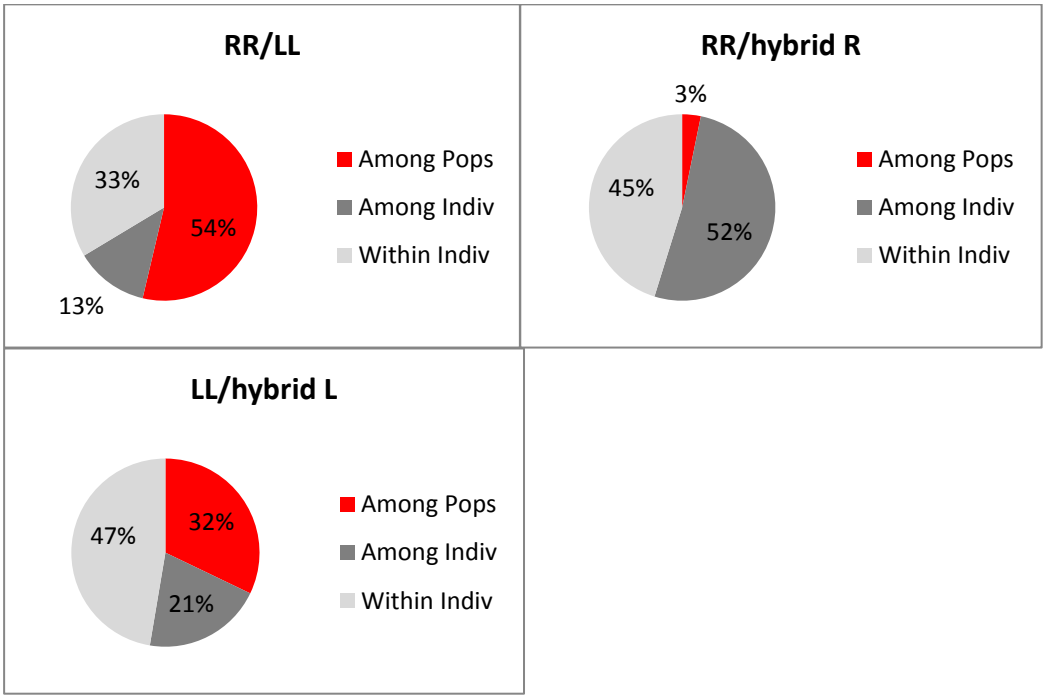

Fig. 4: Locus-by-locus analysis of molecular variance (AMOVA) among and within genomes in three *Pelophylax* taxa generated in ARLEQUIN. RR = *P. ridibundus;* LL= *P. lessonae*; hybrid R = *ridibundus* genome from *P. esculentus*; hybrid = *lessonae* genome from *P. esculentus*.

Appendixes

Tab. S1: DNA microsatelitte data file of 249 water frog individuals for the 17 loci used in the study.

			RlCA1b6		RlCA1b5		Ga1a19		RlCA5		Res16		Res20		RICA2a34		Re2Caga3		Res22		Ga1a23		Rrid169A		Rrid013A		Rrid059A		Re1Caga10		CA1a27		RlCA18		Rrid135A	
Sample	**Population**	**Taxon**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**	**Al1**	**Al2**
BI-6-LL	Bravantice Horní	LL	78	78	118	118	195	195	260	260	121	121	124	0	145	145	0	0	0	0	113	119	0	0	0	0	0	0	97	97	95	111	177	186	0	0
BT-10-LL	Bludovice Horní	LL	80	80	118	118	195	195	256	256	121	121	120	126	145	147	0	0	0	0	119	127	0	0	296	296	278	278	97	97	95	111	177	181	236	236
BT-4-LL	Bludovice Horní	LL	80	80	118	118	195	195	256	260	121	121	120	126	145	147	0	0	0	0	119	121	0	0	296	296	278	278	97	97	111	125	177	181	0	0
BT-5-LL	Bludovice Horní	LL	80	80	118	118	195	195	256	256	121	121	110	116	145	147	0	0	0	0	119	119	0	0	296	296	278	278	97	97	111	111	177	181	0	0
BT-6-LL	Bludovice Horní	LL	78	80	118	118	195	195	256	264	121	121	128	0	145	150	0	0	0	0	119	121	0	0	296	296	278	278	97	97	111	119	177	181	236	236
BT-7-LL	Bludovice Horní	LL	80	80	118	118	195	195	256	256	121	121	126	0	145	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BT-8-LL	Bludovice Horní	LL	80	80	118	118	195	195	252	260	121	121	116	126	145	147	0	0	0	0	119	121	0	0	296	296	278	278	97	97	95	111	181	181	0	0
BT-9-LL	Bludovice	LL	80	80	118	118	195	195	256	260	121	121	116	126	145	147	0	0	0	0	121	121	0	0	296	296	278	278	97	97	95	111	181	181	0	0
CT-10-LL	Český Těšín	LL	80	80	118	118	195	195	256	262	121	121	120	0	138	147	0	0	110	0	119	119	0	0	296	296	278	278	97	97	111	119	177	186	236	236
CT-11-LL	Český Těšín	LL	80	80	118	118	195	195	256	256	121	121	120	0	145	152	0	0	0	0	117	121	0	0	293	296	278	278	97	97	111	111	181	181	0	0
CT-1-LL	Český Těšín	LL	80	80	118	118	195	195	256	256	121	121	116	0	147	150	0	0	0	0	113	131	0	0	0	0	0	0	97	97	111	111	186	186	0	0
CT-2-LL	Český Těšín	LL	80	80	118	118	195	195	256	256	121	121	108	116	145	147	0	0	0	0	131	131	0	0	296	296	278	278	97	97	111	111	177	186	236	236
CT-3-LL	Český Těšín	LL	80	80	118	118	195	195	256	256	121	121	120	142	145	147	0	0	0	0	123	131	0	0	293	299	278	278	97	97	111	119	186	186	0	0
CT-4-LL	Český Těšín	LL	80	80	118	118	195	195	0	0	121	121	110	120	145	150	0	0	0	0	113	121	0	0	0	0	278	278	97	97	111	119	177	181	0	0
CT-5-LL	Český Těšín	LL	78	80	118	118	195	195	256	256	121	121	110	120	147	150	0	0	0	0	117	119	0	0	296	296	278	278	97	97	111	113	177	179	236	236
CT-6-LL	Český Těšín	LL	78	78	118	118	195	195	256	262	121	121	108	110	147	154	0	0	0	0	123	123	0	0	296	296	278	278	97	97	111	119	181	186	236	236
CT-7-LL	Český Těšín	LL	78	80	118	118	195	195	260	260	121	121	108	110	112	130	0	0	0	0	117	119	191	0	296	296	278	278	97	97	111	113	177	181	0	0
CT-8-LL	Český Těšín	LL	78	80	118	118	195	195	256	256	121	121	110	0	145	145	0	0	0	0	119	119	0	0	296	299	278	278	97	97	111	111	177	177	0	0
DS-1-LL	Důl Staříč	LL	80	80	118	118	195	195	260	262	121	121	120	0	112	138	0	0	0	0	119	123	0	0	296	296	278	278	97	97	95	119	181	186	0	0
DS-2-LL	Důl Staříč Horní	LL	78	78	118	118	195	195	0	0	121	121	116	120	112	145	0	0	0	0	119	119	0	0	0	0	0	0	97	97	95	95	0	0	0	0
HB-10-LL	Bludovice Horní	LL	80	83	118	118	195	195	256	256	121	121	120	126	145	152	0	0	110	0	121	125	0	0	0	0	0	0	97	97	95	111	177	184	0	0
HB-11-LL	Bludovice Horní	LL	80	80	118	118	195	195	260	260	121	121	116	126	145	147	0	0	0	0	119	131	0	0	0	0	0	0	97	97	111	111	177	186	0	0
HB-12-LL	Bludovice Horní	LL	80	80	118	118	195	195	0	0	0	0	0	0	0	0	0	0	0	0	119	121	0	0	0	0	0	0	97	97	95	125	181	181	0	0
HB-13-LL	Bludovice	LL	78	80	0	0	0	0	0	0	121	121	120	0	145	145	0	0	83	110	119	129	0	0	0	0	0	0	97	97	111	111	188	188	0	0

HB-14-LL	Horní Bludovice	LL	80	80	0	0	195	195	256	256	121	121	110	120	145	147	0	0	0	0	119	121	0	0	0	0	0	0	97	97	95	119	0	0	0	0
HB-15-LL	Horní Bludovice	LL	80	82	118	134	195	195	0	0	121	121	120	0	140	145	0	0	110	0	125	131	0	0	0	0	0	0	97	97	119	119	0	0	0	0
HB-1-LL	Horní Bludovice	LL	80	80	118	118	195	195	252	256	121	121	120	0	140	145	0	0	0	0	121	129	0	0	296	296	278	278	97	97	111	121	177	188	0	0
HB-2-LL	Horní Bludovice	LL	78	80	118	118	195	195	256	256	121	121	116	120	145	145	0	0	0	0	119	119	0	0	0	0	0	0	97	97	111	121	181	186	0	0
HB-3-LL	Horní Bludovice	LL	80	80	118	118	195	195	256	256	121	121	110	116	145	145	0	0	0	0	125	131	0	0	296	296	278	278	97	97	111	111	177	188	236	236
HB-4-LL	Horní Bludovice	LL	78	78	118	118	195	195	256	256	121	121	120	0	145	145	0	0	0	0	119	121	0	0	0	0	0	0	97	97	111	111	177	181	0	0
HB-5-LL	Horní Bludovice	LL	78	78	118	118	195	195	0	0	121	121	116	120	147	162	0	0	0	0	119	121	0	0	0	0	0	0	97	97	111	119	181	186	0	0
HB-6-LL	Horní Bludovice	LL	78	80	118	118	195	195	252	256	121	121	124	0	145	147	0	0	0	0	119	119	0	0	296	296	0	0	97	97	95	121	177	177	0	0
HB-7-LL	Horní Bludovice	LL	78	78	118	118	195	195	252	260	121	121	110	0	145	147	0	0	0	0	119	119	0	0	296	296	278	278	97	97	125	125	177	177	236	236
HB-8-LL	Horní Bludovice	LL	78	80	118	118	195	195	256	258	121	121	108	110	145	145	0	0	0	0	119	121	0	0	296	296	0	0	97	97	95	119	181	186	236	236
HD-12-LL	Horní Domaslavice	LL	78	80	118	118	195	195	256	256	121	121	120	126	140	143	0	0	0	0	119	121	0	0	296	296	278	278	97	97	95	95	186	186	236	236
HD-13-LL	Horní Domaslavice	LL	78	80	118	118	195	195	256	256	121	121	112	120	140	145	0	0	0	0	119	119	0	0	293	296	278	278	97	97	111	121	186	186	236	236
HD-14-LL	Horní Domaslavice	LL	78	80	118	118	195	195	256	256	121	121	104	110	112	145	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HD-15-LL	Horní Domaslavice	LL	80	83	118	118	195	195	256	260	121	121	120	124	145	152	0	0	0	0	119	119	0	0	291	296	278	278	97	97	111	121	177	181	236	236
HD-4-LL	Horní Domaslavice	LL	78	83	118	118	195	195	256	256	121	121	110	120	145	152	0	0	110	0	119	131	187	0	296	296	278	278	97	97	119	125	186	188	236	236
HD-6-LL	Horní Domaslavice	LL	78	80	118	118	195	195	256	256	121	121	110	0	140	145	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HD-9-LL	Horní Domaslavice	LL	80	80	118	118	195	195	256	260	121	121	120	0	140	152	0	0	0	0	113	121	0	0	296	296	278	278	97	97	95	111	181	186	236	236
Ka-13-LL	Karviná-Doly	LL	80	80	118	118	195	195	256	256	121	121	110	120	147	156	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ka-9-LL	Karviná-Doly	LL	80	80	118	118	195	195	256	260	121	121	108	110	136	145	0	0	0	0	119	131	0	0	296	299	278	278	97	97	95	119	181	197	236	236
LO-9-LL	Louky	LL	80	80	118	118	195	195	256	260	121	121	108	110	140	145	0	0	110	0	119	125	191	234	296	296	278	278	97	97	95	111	183	183	0	0
PR-11-LL	Prstná	LL	78	80	118	118	195	195	256	260	121	121	110	120	145	152	0	0	110	0	98	131	227	0	296	296	278	278	97	97	95	95	181	186	236	236
PR-2-LL	Prstná	LL	78	78	118	134	195	201	252	256	121	121	116	126	140	145	0	0	110	0	98	115	187	0	296	296	278	278	97	106	111	121	179	181	199	0
PR-3-LL	Prstná	LL	80	80	118	118	195	195	256	256	121	121	110	0	123	147	0	0	110	0	98	125	187	227	291	296	278	278	97	116	95	115	181	186	236	236
PR-4-LL	Prstná	LL	78	80	118	118	195	195	256	256	121	121	120	122	150	164	0	0	0	0	119	133	187	0	296	296	278	278	97	97	119	121	184	186	236	236
PR-5-LL	Prstná	LL	78	80	118	118	195	195	256	260	121	121	110	116	147	152	0	0	0	0	119	139	0	0	296	296	278	278	97	97	117	119	177	181	236	236
PR-6-LL	Prstná	LL	80	80	118	118	195	195	256	256	121	121	110	120	140	145	0	0	0	0	125	125	0	0	296	299	278	278	97	97	111	119	179	188	236	236
PR-7-LL	Prstná	LL	78	80	118	118	195	195	256	260	121	121	110	131	147	147	0	0	0	0	115	131	0	0	296	296	278	278	97	97	111	119	181	195	236	236
PR-8-LL	Prstná	LL	80	80	118	118	195	195	256	256	121	121	110	116	147	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TR-10-LL	Trnávka	LL	78	78	118	118	195	195	260	260	121	121	142	144	145	147	0	0	0	0	115	127	0	0	296	296	278	278	97	97	95	111	181	202	0	0
TR-11-LL	Trnávka	LL	80	83	118	118	195	195	256	256	121	121	110	124	145	145	0	0	0	0	123	135	0	0	296	296	278	278	97	97	95	95	183	183	236	236
TR-12-LL	Trnávka	LL	78	80	118	118	195	195	256	256	121	121	110	0	138	145	0	0	0	0	123	127	0	0	296	296	278	278	97	97	95	121	181	186	236	236
TR-13-LL	Trnávka	LL	78	80	118	118	195	195	260	260	121	121	110	120	145	152	0	0	0	0	119	123	0	0	296	296	278	278	97	97	119	121	177	202	236	236
TR-14-LL	Trnávka	LL	80	83	118	118	195	195	256	260	121	121	110	120	112	140	235	0	0	0	131	141	0	0	296	296	278	278	97	116	95	115	181	181	199	0
TR-15-LL	Trnávka	LL	78	80	118	118	195	195	252	260	121	121	124	142	154	154	0	0	0	0	0	0	0	0	0	0	0	0	97	97	95	121	177	181	236	236
TR-3-LL	Trnávka	LL	78	80	118	118	195	195	260	260	121	121	110	146	152	154	231	0	110	0	115	119	191	227	296	296	278	278	97	106	95	119	181	181	236	236
TR-4-LL	Trnávka	LL	78	78	118	118	195	195	256	260	121	121	146	0	145	152	0	0	0	0	123	123	0	0	296	296	278	278	97	97	95	119	177	181	0	0
TR-5-LL	Trnávka	LL	78	80	118	118	195	195	260	260	121	121	120	0	140	147	0	0	110	0	127	131	0	0	296	296	278	278	97	97	95	95	181	186	236	236
TR-8-LL	Trnávka	LL	78	80	118	118	195	195	260	260	121	121	120	0	145	147	0	0	110	0	127	131	191	0	296	296	278	278	97	97	95	95	181	186	236	236
TR-9-LL	Trnávka	LL	78	80	118	118	195	195	256	260	121	121	142	0	150	152	0	0	0	0	127	127	0	0	296	296	278	278	97	97	113	119	177	181	236	236
DO-91-LL	Dobrá	LL	78	80	118	118	195	195	247	256	121	121	111	0	136	145	0	0	0	0	121	131	0	0	296	296	278	278	97	97	95	111	184	186	0	0
DO-92-LL	Dobrá	LL	78	80	118	118	195	195	256	260	152	0	117	0	145	162	0	0	0	0	119	121	0	0	296	296	278	278	97	97	111	115	186	190	0	0
DO-93-LL	Dobrá	LL	80	80	118	118	195	195	256	256	121	121	111	0	138	150	0	0	0	0	123	127	0	0	293	296	278	278	97	97	111	111	175	190	0	0
DO-94-LL	Dobrá	LL	78	80	118	118	195	195	256	256	121	121	121	0	136	140	0	0	0	0	121	131	0	0	296	296	278	278	97	97	111	119	179	181	0	0
DO-95-LL	Dobrá	LL	78	80	118	118	195	195	256	260	121	121	121	0	145	162	0	0	0	0	127	131	0	0	296	296	278	278	97	97	111	121	179	181	0	0
DO-01-LL	Dobrá	LL	80	80	118	118	195	195	256	256	121	121	102	110	145	145	0	0	0	0	121	121	0	0	296	296	278	278	97	97	111	121	181	186	0	0
DO-02-LL	Dobrá	LL	78	80	118	118	195	195	256	256	121	121	121	0	145	145	0	0	0	0	119	121	0	0	296	296	278	278	97	97	111	119	186	186	0	0
DO-03-LL	Dobrá	LL	78	80	118	118	195	195	256	260	121	121	102	110	145	145	0	0	0	0	121	125	0	0	296	296	278	278	97	97	111	119	184	186	0	0
DO-24-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	128	0	140	145	0	0	0	0	119	123	0	0	296	296	278	278	97	97	95	118	177	190	0	0
DO-25-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	110	123	145	145	0	0	0	0	125	131	0	0	296	299	278	278	97	97	118	127	184	186	0	0
DO-26-LL	Dobrá	LL	78	80	118	118	195	195	260	260	121	121	110	115	145	147	0	0	0	0	131	131	0	0	296	296	278	278	97	97	119	121	186	190	0	0
DO-27-LL	Dobrá	LL	78	80	118	118	195	195	256	256	121	121	117	128	136	145	0	0	0	0	119	125	0	0	296	296	278	278	97	97	111	118	181	186	0	0
DO-28-LL	Dobrá	LL	80	80	118	118	195	195	260	260	121	121	123	128	145	145	0	0	0	0	119	123	0	0	296	299	278	278	97	97	111	119	190	195	0	0
DO-29-LL	Dobrá	LL	78	83	118	118	195	195	256	260	0	0	110	123	140	140	0	0	0	0	131	131	0	0	296	299	278	278	97	97	95	127	175	179	0	0
DO-30-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	110	128	145	152	0	0	0	0	119	125	0	0	296	296	278	278	97	97	111	111	181	188	0	0
DO-31-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	110	123	140	162	0	0	0	0	119	131	0	0	296	296	278	278	97	97	111	111	184	188	0	0
DO-32-LL	Dobrá	LL	78	80	118	118	195	195	256	264	121	121	110	0	136	147	0	0	0	0	119	125	0	0	296	296	278	278	97	97	118	127	186	190	0	0
DO-33-LL	Dobrá	LL	78	78	113	118	195	195	256	264	121	121	110	0	140	143	0	0	0	0	121	131	0	0	296	296	278	278	97	97	95	111	186	190	0	0
DO-34-LL	Dobrá	LL	80	83	118	118	195	195	256	256	121	121	128	0	145	147	0	0	0	0	121	131	0	0	296	296	278	278	97	97	95	111	179	186	0	0

DO-35-LL	Dobrá	LL	78	80	118	118	195	195	256	256	121	121	110	0	147	154	0	0	0	0	119	131	0	0	296	296	278	278	97	97	119	121	177	195	0	0
DO-36-LL	Dobrá	LL	78	83	113	118	195	195	256	260	121	121	110	125	140	145	0	0	0	0	123	131	0	0	296	296	278	278	0	0	111	127	177	186	0	0
DO-37-LL	Dobrá	LL	80	83	118	118	195	195	256	260	121	121	117	0	145	147	0	0	0	0	121	123	0	0	296	296	278	278	97	97	111	111	184	184	0	0
DO-38-LL	Dobrá	LL	78	78	118	118	195	195	256	256	121	121	110	0	140	145	0	0	0	0	119	131	0	0	296	296	278	278	97	97	95	111	186	190	0	0
DO-39-LL	Dobrá	LL	78	80	118	118	195	195	260	260	121	121	117	0	145	147	0	0	0	0	131	131	0	0	296	296	278	278	97	97	119	121	186	195	0	0
DO-40-LL	Dobrá	LL	80	83	118	118	195	195	256	256	0	0	117	128	145	147	0	0	0	0	121	121	0	0	296	296	278	278	97	97	95	115	186	190	0	0
DO-41-LL	Dobrá	LL	78	80	118	118	195	195	260	260	121	121	110	115	150	152	0	0	0	0	119	121	0	0	296	296	278	278	97	97	119	121	175	181	0	0
DO-42-LL	Dobrá	LL	80	83	118	118	195	195	256	260	121	121	121	0	145	150	0	0	0	0	115	123	0	0	296	296	278	278	97	97	119	121	177	177	0	0
DO-44-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	110	123	140	147	0	0	0	0	119	131	0	0	296	296	278	278	97	97	95	119	181	186	0	0
DO-46-LL	Dobrá	LL	78	80	118	118	195	195	247	256	121	121	110	117	147	162	0	0	0	0	121	131	0	0	296	296	278	278	97	97	118	118	179	186	0	0
DO-47-LL	Dobrá	LL	80	80	118	118	195	195	256	264	121	121	110	0	136	145	0	0	0	0	125	131	0	0	296	296	278	278	97	97	111	127	184	190	0	0
DO-48-LL	Dobrá	LL	80	80	118	118	195	195	256	260	121	121	121	123	136	136	0	0	0	0	125	131	0	0	296	296	278	278	97	97	113	118	181	186	0	0
TR-26-LL	Trnávka	LL	78	83	118	118	195	195	256	260	121	121	113	121	145	150	0	0	0	0	125	131	0	0	296	296	278	278	97	97	111	119	181	195	0	0
TR-29-LL	Trnávka	LL	80	80	118	118	195	195	256	260	121	121	111	0	147	150	0	0	0	0	121	123	0	0	296	296	278	278	97	97	95	95	181	181	0	0
TR-30-LL	Trnávka	LL	80	80	118	118	195	195	260	260	121	121	111	0	145	150	0	0	0	0	131	131	0	0	296	296	278	278	97	97	111	111	177	181	0	0
TR-40-LL	Trnávka	LL	78	80	118	118	195	195	256	260	121	121	142	144	145	152	0	0	0	0	119	131	0	0	296	296	278	278	97	97	119	127	181	186	0	0
TR-49-LL	Trnávka	LL	80	80	118	118	195	195	256	256	121	121	0	0	145	152	0	0	0	0	119	131	0	0	296	296	278	278	97	97	95	95	181	186	0	0
TR-53-LL	Trnávka	LL	78	80	118	118	195	195	256	256	121	121	0	0	145	145	0	0	0	0	123	131	0	0	296	299	278	278	97	97	95	119	186	186	0	0
TR-66-LL	Trnávka	LL	78	80	118	118	195	195	256	260	121	121	113	115	136	147	0	0	0	0	121	131	0	0	296	296	278	278	97	97	95	95	181	181	0	0
TR-67-LL	Trnávka	LL	78	80	118	118	195	195	256	260	121	121	110	0	112	145	0	0	0	0	115	131	0	0	296	296	278	278	97	97	111	111	181	190	0	0
TR-68-LL	Trnávka	LL	78	80	118	118	195	195	260	260	121	121	110	0	145	145	0	0	0	0	121	131	0	0	296	296	278	278	97	97	95	119	181	186	0	0
TR-69-LL	Trnávka	LL	78	80	118	118	195	195	256	260	121	121	121	0	145	147	0	0	0	0	123	125	0	0	296	296	278	278	97	97	95	119	181	181	0	0
BD-95-LL	Břidličná	LL	80	80	118	118	195	195	260	260	121	133	119	0	112	145	0	0	0	0	119	121	0	0	293	299	278	278	97	97	119	124	181	184	0	0
BD-01-LL	Břidličná	LL	78	78	118	118	195	195	252	256	121	121	119	123	143	145	0	0	98	110	121	121	0	0	293	296	278	278	97	97	95	111	181	184	0	0
BD-08-LL	Břidličná	LL	78	80	118	118	195	195	260	260	121	121	111	125	145	145	0	0	0	0	129	129	0	0	293	287	278	278	97	97	95	119	186	186	0	0
BD-16-LL	Břidličná	LL	78	80	118	118	195	195	258	260	121	121	123	125	145	145	0	0	0	0	119	119	0	0	296	296	278	278	97	97	95	111	186	186	0	0
BI-10-RL	Bravantice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	143	NA	0	NA	0	NA	127	NA	0	NA	0	NA	0	NA	97	NA	107	NA	181	NA	0	NA
BI-12-RL	Bravantice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	115	NA	0	NA	0	NA	0	NA	97	NA	119	NA	181	NA	0	NA
BI-2-RL	Bravantice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	112	NA	0	NA	0	NA	125	NA	0	NA	0	NA	0	NA	97	NA	119	NA	186	NA	0	NA
BI-3-RL	Bravantice	RL	80	NA	118	NA	195	NA	0	NA	121	NA	110	NA	120	NA	0	NA	0	NA	121	NA	0	NA	0	NA	0	NA	97	NA	107	NA	0	NA	0	NA

BI-4-RL	Bravantice	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	145	NA	0	NA	0	NA	125	NA	0	NA	0	NA	0	NA	97	NA	95	NA	186	NA	0	NA
BI-5-RL	Bravantice	RL	78	NA	118	NA	195	NA	256	NA	0	NA	110	NA	145	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	97	NA	95	NA	181	NA	0	NA
BI-9-RL	Bravantice Horní	RL	78	NA	118	NA	195	NA	0	NA	121	NA	110	NA	112	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	97	NA	95	NA	186	NA	0	NA
BT-1-RL	Bludovice Horní	RL	78	NA	118	NA	195	NA	256	NA	121	NA	120	NA	154	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	181	NA	0	NA
BT-2-RL	Bludovice Horní	RL	80	NA	118	NA	195	NA	260	NA	121	NA	120	NA	145	NA	0	NA	0	NA	113	NA	0	NA	296	NA	278	NA	97	NA	111	NA	181	NA	0	NA
BT-3-RL	Bludovice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	113	NA	0	NA	296	NA	278	NA	97	NA	95	NA	186	NA	236	NA
CT-12-RL	Český Těšín	RL	80	NA	118	NA	195	NA	0	NA	0	NA	116	NA	145	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	97	NA	95	NA	181	NA	0	NA
CT-13-RL	Český Těšín	RL	78	NA	118	NA	195	NA	256	NA	121	NA	0	NA	138	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	97	NA	111	NA	181	NA	0	NA
CT-14-RL	Český Těšín	RL	78	NA	118	NA	195	NA	262	NA	0	NA	122	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	186	NA	0	NA
CT-15-RL	Český Těšín	RL	80	NA	118	NA	195	NA	256	NA	121	NA	114	NA	140	NA	0	NA	0	NA	121	NA	0	NA	0	NA	0	NA	116	NA	111	NA	181	NA	0	NA
CT-9-RL	Český Těšín	RL	78	NA	118	NA	195	NA	256	NA	121	NA	0	NA	145	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	111	NA	0	NA	0	NA
DS-10-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	111	NA	0	NA	0	NA
DS-11-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	154	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	111	NA	177	NA	236	NA
DS-12-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	264	NA	0	NA	146	NA	147	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	115	NA	186	NA	0	NA
DS-13-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	123	NA	0	NA	0	NA	0	NA	0	NA	95	NA	0	NA	0	NA
DS-14-RL	Důl Staříč	RL	78	NA	118	NA	195	NA	260	NA	121	NA	110	NA	145	NA	0	NA	0	NA	125	NA	0	NA	296	NA	0	NA	97	NA	95	NA	181	NA	0	NA
DS-15-RL	Důl Staříč	RL	80	NA	0	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	97	NA	111	NA	0	NA	0	NA
DS-3-RL	Důl Staříč	RL	83	NA	0	NA	195	NA	0	NA	121	NA	0	NA	147	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	111	NA	0	NA	0	NA
DS-4-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	260	NA	121	NA	120	NA	145	NA	0	NA	0	NA	139	NA	0	NA	0	NA	0	NA	97	NA	119	NA	186	NA	0	NA
DS-5-RL	Důl Staříč	RL	80	NA	118	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	111	NA	0	NA	0	NA
DS-6-RL	Důl Staříč	RL	80	NA	118	NA	195	NA	234	NA	121	NA	108	NA	145	NA	0	NA	0	NA	125	NA	0	NA	296	NA	278	NA	97	NA	111	NA	186	NA	236	NA
DS-7-RL	Důl Staříč	RL	78	NA	118	NA	195	NA	260	NA	121	NA	142	NA	147	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	195	NA	0	NA
DS-8-RL	Důl Staříč	RL	83	NA	118	NA	195	NA	260	NA	121	NA	120	NA	147	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	95	NA	0	NA	0	NA
DS-9-RL	Důl Staříč Horní	RL	80	NA	118	NA	195	NA	256	NA	121	NA	144	NA	147	NA	0	NA	0	NA	119	NA	0	NA	0	NA	0	NA	97	NA	95	NA	181	NA	0	NA
HB-9-RL	Bludovice Horní	RL	80	NA	118	NA	0	NA	0	NA	121	NA	120	NA	145	NA	0	NA	0	NA	131	NA	0	NA	0	NA	0	NA	106	NA	0	NA	0	NA	0	NA
HD-10-RL	Domaslavice Horní	RL	83	NA	118	NA	195	NA	256	NA	121	NA	110	NA	152	NA	0	NA	0	NA	131	NA	0	NA	293	NA	278	NA	97	NA	119	NA	181	NA	236	NA
HD-11-RL	Domaslavice Horní	RL	80	NA	118	NA	195	NA	256	NA	121	NA	126	NA	152	NA	0	NA	0	NA	131	NA	0	NA	293	NA	278	NA	97	NA	119	NA	181	NA	236	NA
HD-1-RL	Domaslavice Horní	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	162	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	236	NA
HD-2-RL	Domaslavice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	150	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	236	NA

HD-3-RL	Horní Domaslavice	RL	78	NA	118	NA	195	NA	256	NA	121	NA	120	NA	143	NA	0	NA	0	NA	119	NA	0	NA	291	NA	278	NA	97	NA	119	NA	177	NA	236	NA
HD-5-RL	Horní Domaslavice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	126	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	181	NA	0	NA
HD-7-RL	Horní Domaslavice	RL	80	NA	118	NA	195	NA	256	NA	121	NA	116	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	181	NA	236	NA
HD-8-RL	Horní Domaslavice	RL	83	NA	118	NA	195	NA	256	NA	121	NA	126	NA	145	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	0	NA
Ka-10-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	136	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	177	NA	236	NA
Ka-11-RL	Karviná-Doly	RL	78	NA	118	NA	195	NA	256	NA	121	NA	108	NA	145	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	95	NA	186	NA	236	NA
Ka-12-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	147	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	177	NA	0	NA
Ka-14-RL	Karviná-Doly	RL	78	NA	118	NA	195	NA	256	NA	121	NA	120	NA	156	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	177	NA	236	NA
Ka-15-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	124	NA	147	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	95	NA	177	NA	236	NA
Ka-1-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	150	NA	0	NA	0	NA	119	NA	227	NA	296	NA	278	NA	97	NA	111	NA	177	NA	236	NA
Ka-2-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	147	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	121	NA	186	NA	236	NA
Ka-3-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	150	NA	0	NA	0	NA	119	NA	0	NA	291	NA	278	NA	97	NA	111	NA	186	NA	236	NA
Ka-4-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	145	NA	0	NA	0	NA	117	NA	0	NA	296	NA	278	NA	97	NA	111	NA	186	NA	0	NA
Ka-5-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	140	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA
Ka-6-RL	Karviná-Doly	RL	78	NA	118	NA	195	NA	256	NA	121	NA	120	NA	134	NA	0	NA	0	NA	133	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	236	NA
Ka-7-RL	Karviná-Doly	RL	80	NA	118	NA	195	NA	256	NA	121	NA	116	NA	150	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	177	NA	236	NA
Ka-8-RL	Karviná-Doly	RL	78	NA	118	NA	195	NA	256	NA	121	NA	120	NA	154	NA	0	NA	0	NA	133	NA	0	NA	296	NA	278	NA	97	NA	111	NA	183	NA	236	NA
LO-10-RL	Louky	RL	80	NA	118	NA	195	NA	260	NA	121	NA	131	NA	147	NA	0	NA	0	NA	121	NA	0	NA	296	NA	278	NA	97	NA	113	NA	175	NA	0	NA
LO-11-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	112	NA	150	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	111	NA	179	NA	236	NA
LO-12-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	186	NA	0	NA
LO-13-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	0	NA
LO-14-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	108	NA	145	NA	0	NA	0	NA	121	NA	0	NA	296	NA	278	NA	97	NA	95	NA	186	NA	0	NA
LO-15-RL	Louky	RL	78	NA	118	NA	195	NA	256	NA	121	NA	110	NA	138	NA	0	NA	0	NA	113	NA	0	NA	291	NA	278	NA	97	NA	111	NA	181	NA	0	NA
LO-1-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	128	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	195	NA	236	NA
LO-2-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	147	NA	0	NA	0	NA	119	NA	0	NA	293	NA	278	NA	97	NA	119	NA	173	NA	236	NA
LO-3-RL	Louky	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	145	NA	0	NA	0	NA	127	NA	0	NA	296	NA	278	NA	97	NA	111	NA	186	NA	0	NA
LO-4-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	150	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	177	NA	236	NA
LO-5-RL	Louky	RL	78	NA	118	NA	195	NA	256	NA	121	NA	110	NA	145	NA	0	NA	0	NA	113	NA	0	NA	299	NA	278	NA	97	NA	111	NA	186	NA	0	NA
LO-6-RL	Louky	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	145	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	111	NA	186	NA	0	NA

LO-7-RL	Louky	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	147	NA	0	NA	0	NA	115	NA	0	NA	296	NA	278	NA	97	NA	119	NA	177	NA	236	NA
LO-8-RL	Louky	RL	80	NA	118	NA	195	NA	256	NA	121	NA	126	NA	150	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	195	NA	236	NA
PR-10-RL	Prstná	RL	80	NA	118	NA	195	NA	256	NA	121	NA	120	NA	140	NA	0	NA	0	NA	125	NA	0	NA	296	NA	278	NA	97	NA	119	NA	181	NA	0	NA
PR-1-RL	Prstná	RL	80	NA	118	NA	195	NA	0	NA	121	NA	120	NA	147	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	119	NA	169	NA	0	NA
PR-9-RL	Prstná	RL	80	NA	118	NA	195	NA	260	NA	121	NA	118	NA	147	NA	0	NA	0	NA	131	NA	0	NA	296	NA	278	NA	97	NA	111	NA	177	NA	236	NA
TR-1-RL	Trnávka	RL	80	NA	118	NA	195	NA	260	NA	121	NA	110	NA	147	NA	0	NA	0	NA	121	NA	0	NA	296	NA	278	NA	97	NA	95	NA	181	NA	236	NA
TR-2-RL	Trnávka	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	147	NA	0	NA	0	NA	121	NA	0	NA	296	NA	278	NA	97	NA	95	NA	181	NA	236	NA
TR-6-RL	Trnávka	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	147	NA	0	NA	0	NA	119	NA	0	NA	296	NA	278	NA	97	NA	95	NA	177	NA	236	NA
TR-7-RL	Trnávka	RL	80	NA	118	NA	195	NA	256	NA	121	NA	110	NA	147	NA	0	NA	0	NA	123	NA	0	NA	296	NA	278	NA	97	NA	125	NA	181	NA	236	NA
PO-75-RL	Albrechtičky	RL	78	92	118	134	195	201	260	0	121	127	121	0	145	0	0	196	0	84	123	98	0	187	296	281	278	313	0	108	115	0	186	0	0	203
PO-82-RL	Albrechtičky	RL	78	94	118	134	195	223	260	0	121	0	121	0	145	106	0	169	0	110	123	98	0	191	296	281	278	313	0	122	115	0	186	0	0	203
PO-84-RL	Albrechtičky	RL	78	92	118	134	195	205	260	0	121	0	121	0	145	110	0	204	0	98	123	0	0	191	296	281	278	313	97	114	115	0	186	0	0	203
BI-7-RL	Bílovec	RL	78	92	118	134	195	205	260	256	121	127	120	0	145	0	0	0	0	110	123	98	0	187	0	0	0	0	97	106	115	0	186	0	0	0
BI-8-RL	Bílovec	RL	78	92	118	134	195	201	0	0	121	0	120	0	145	106	0	0	0	110	123	98	0	191	0	0	0	0	97	106	95	115	186	0	0	0
Da-10-RL	Darkovice	RL	78	92	118	134	195	201	260	232	121	127	120	0	145	0	0	212	0	83	123	0	0	198	296	281	278	311	97	112	115	0	186	0	236	183
Da-11-RL	Darkovice	RL	78	85	118	134	195	205	260	234	121	127	120	0	145	106	0	208	0	133	123	98	0	198	296	281	278	315	97	116	115	0	186	0	236	199
Da-12-RL	Darkovice	RL	78	92	118	134	195	247	260	232	121	115	120	0	145	106	0	231	0	83	123	0	0	187	296	281	278	0	97	114	115	0	186	0	236	203
Da-9-RL	Darkovice	RL	78	92	118	134	195	201	260	0	121	115	120	0	145	0	0	212	0	83	123	98	0	198	296	281	278	311	97	112	115	0	186	0	236	183
DB-10-RL	Dolní Benešov	RL	78	85	118	134	195	205	260	0	121	127	120	0	145	106	0	208	0	110	123	98	0	189	296	287	278	319	97	108	115	0	186	0	236	203
DB-1-RL	Dolní Benešov	RL	78	92	118	134	195	201	260	0	121	127	120	0	145	110	0	235	0	110	123	98	0	179	296	281	278	321	97	110	115	0	186	0	236	199
DB-4-RL	Dolní Benešov	RL	78	92	118	134	195	243	260	0	121	0	120	0	145	0	0	169	0	110	123	98	0	191	296	281	278	313	97	110	115	0	186	0	236	205
DB-5-RL	Dolní Benešov	RL	78	92	118	134	195	223	260	0	121	0	120	0	145	0	0	169	0	110	123	98	0	191	296	281	278	313	97	110	115	0	186	0	236	169
OS-10-RL	Ostrava	RL	78	92	118	134	195	205	260	236	121	127	120	0	145	110	0	212	0	110	123	98	0	198	296	281	278	313	97	116	115	0	186	0	236	199
OS-11-RL	Ostrava	RL	78	92	118	134	195	201	260	0	121	0	120	0	145	106	0	196	0	110	123	98	0	191	296	281	278	315	97	116	115	0	186	0	236	0
OS-12-RL	Ostrava	RL	78	85	118	134	195	201	260	0	121	0	120	0	145	106	0	196	0	110	123	98	0	191	296	287	278	315	97	116	115	0	186	0	236	0
OS-14-RL	Ostrava	RL	78	92	118	136	195	205	260	0	121	127	120	0	145	110	0	169	0	104	123	0	0	191	296	281	278	313	97	114	115	0	186	0	236	203
OS-6-RL	Ostrava	RL	78	83	118	134	195	201	260	0	121	115	120	0	145	106	0	200	0	110	123	98	0	214	296	0	278	319	97	116	115	0	186	0	236	0
PO-10-RL	Albrechtičky	RL	78	94	118	134	195	201	260	0	121	0	120	0	145	110	0	169	0	110	123	98	0	0	296	281	278	313	97	0	115	0	186	0	0	199
PO-14-RL	Albrechtičky	RL	78	92	118	134	195	243	260	0	121	127	120	0	145	106	0	0	0	104	123	98	0	0	0	0	0	0	97	116	115	0	186	0	0	0

PO-15-RL	Albrechtičky	RL	78	83	0	0	195	0	0	0	121	0	120	0	145	0	0	176	0	110	123	98	0	0	0	0	0	0	97	112	115	0	186	0	0	0
PO-1-RL	Albrechtičky	RL	78	85	118	134	195	209	260	0	121	0	120	0	145	106	0	169	0	110	123	98	203	0	296	281	278	315	97	112	115	0	186	0	236	169
PO-2-RL	Albrechtičky	RL	78	92	118	134	195	201	260	0	121	129	120	0	145	110	0	0	0	110	123	98	187	0	296	281	278	313	97	116	115	0	186	0	0	199
PO-3-RL	Albrechtičky	RL	78	92	118	132	195	201	0	0	121	0	120	0	145	110	0	0	0	110	123	98	0	0	0	0	0	0	97	108	115	0	186	0	0	0
PO-7-RL	Albrechtičky	RL	78	92	118	0	0	0	0	0	121	127	120	0	145	106	0	169	0	110	123	98	0	0	0	0	0	0	97	108	115	0	186	0	0	203
PO-8-RL	Albrechtičky	RL	78	94	118	134	195	201	260	0	121	127	120	0	145	106	0	169	0	110	123	98	191	0	296	287	0	0	97	108	115	0	186	0	0	203
PO-9-RL	Albrechtičky	RL	78	85	118	134	195	201	260	0	121	127	120	0	145	106	0	169	0	110	123	98	203	0	0	0	0	0	97	106	115	0	186	0	0	203
BI-11-RR	Bílovec	RR	92	94	134	134	201	259	234	0	127	0	0	0	106	106	220	235	110	110	98	98	187	0	281	281	0	0	106	0	0	0	0	0	169	199
Da-1-RR	Darkovice	RR	85	92	134	134	201	247	232	0	119	123	0	0	106	106	212	231	87	129	98	98	187	191	281	281	313	313	106	114	0	0	0	0	169	169
Da-2-RR	Darkovice	RR	92	98	134	134	205	205	232	250	127	0	0	0	106	110	208	235	83	110	98	98	191	0	281	287	303	313	110	0	0	0	0	0	169	183
Da-3-RR	Darkovice	RR	92	92	134	134	201	247	232	0	117	123	0	0	106	106	212	231	87	110	98	98	187	0	287	296	313	315	114	0	0	0	0	0	169	199
Da-4-RR	Darkovice	RR	85	92	134	134	201	247	232	260	117	123	0	0	106	106	192	212	87	129	98	98	187	0	287	296	307	313	106	114	0	0	0	0	169	169
Da-5-RR	Darkovice	RR	85	92	134	134	201	247	232	0	119	123	0	0	106	106	192	212	83	129	98	98	187	0	281	281	307	313	93	106	0	0	0	0	169	169
Da-6-RR	Darkovice	RR	92	92	134	134	201	247	232	0	119	0	0	0	106	106	169	231	83	129	98	98	187	0	287	296	313	313	93	106	0	0	0	0	169	203
Da-7-RR	Darkovice	RR	92	92	134	134	201	247	232	0	119	0	0	0	106	106	169	231	83	129	98	98	187	191	281	296	307	313	93	106	0	0	0	0	169	203
Da-8-RR	Darkovice Dolní	RR	85	92	134	134	201	201	0	0	127	0	0	0	106	110	169	235	110	110	98	98	187	195	281	281	313	317	106	0	0	0	0	0	199	203
DB-2-RR	Benešov Dolní	RR	92	92	134	138	201	243	232	248	0	121	0	0	106	106	169	196	110	110	98	98	191	203	275	281	313	317	110	114	0	0	0	0	169	203
DB-3-RR	Benešov Dolní	RR	85	92	134	134	205	205	232	248	127	0	0	0	0	0	169	169	110	110	98	98	191	203	281	281	313	317	106	108	0	0	0	0	169	203
DB-6-RR	Benešov Dolní	RR	92	92	134	134	243	243	232	260	127	0	0	0	106	106	169	227	110	110	98	98	191	0	281	281	311	313	114	137	0	0	0	0	203	203
DB-7-RR	Benešov Dolní	RR	0	0	134	134	0	0	0	0	0	0	0	0	0	0	0	0	110	110	98	98	187	191	281	281	313	315	114	137	0	0	0	0	0	0
DB-8-RR	Benešov Dolní	RR	74	92	134	134	201	243	232	0	115	0	0	0	106	110	169	220	110	110	98	98	187	0	281	287	311	313	106	108	0	0	0	0	169	199
DB-9-RR	Benešov	RR	85	85	134	134	201	243	232	250	127	0	0	0	106	106	169	231	110	110	98	98	191	207	281	281	311	313	106	114	0	0	0	0	169	203
OS-13-RR	Ostrava	RR	83	85	134	136	201	217	234	236	115	127	0	0	106	106	204	208	110	129	98	98	189	191	281	287	313	313	106	0	0	0	0	0	169	203
OS-1-RR	Ostrava	RR	83	92	134	134	205	209	232	0	127	0	0	0	106	106	169	200	83	83	98	98	191	198	281	281	0	0	108	116	0	0	0	0	199	203
OS-2-RR	Ostrava	RR	85	92	134	134	201	205	234	0	127	0	0	0	110	110	200	208	110	129	0	0	187	203	281	287	313	321	116	0	0	0	0	0	169	203
OS-3-RR	Ostrava	RR	78	92	134	134	201	253	232	0	127	0	0	0	106	106	169	200	110	116	98	98	195	214	281	281	313	317	106	0	0	0	0	0	203	203
OS-4-RR	Ostrava	RR	92	92	134	134	201	205	234	0	115	0	0	0	106	106	212	220	110	110	98	98	191	203	281	281	315	321	97	116	0	0	0	0	169	199
OS-5-RR	Ostrava	RR	92	98	134	134	201	255	232	0	127	0	0	0	106	106	220	235	106	110	98	98	187	198	281	281	303	313	112	114	0	0	0	0	199	203
OS-7-RR	Ostrava	RR	92	92	134	134	201	205	232	250	127	0	0	0	106	110	200	220	83	110	98	98	187	0	281	281	315	317	108	116	0	0	0	0	199	205

OS-8-RR	Ostrava	RR	78	92	134	134	205	253	232	0	115	117	0	0	106	106	231	255	110	110	98	98	187	198	281	281	303	315	114	116	0	0	0	0	199	203
OS-9-RR	Ostrava	RR	85	92	134	136	201	205	232	234	127	0	0	0	106	106	212	235	83	110	98	98	191	0	287	287	307	313	112	118	0	0	0	0	0	0
PO-11-RR	Albrechtičky	RR	94	94	134	134	201	243	232	0	0	121	0	0	106	110	200	231	110	110	0	0	191	0	281	281	313	319	108	114	0	0	0	0	169	203
PO-12-RR	Albrechtičky	RR	92	94	134	134	239	247	232	0	127	0	0	0	106	110	0	0	106	110	98	98	189	203	0	0	313	313	125	140	0	0	0	0	0	0
PO-13-RR	Albrechtičky	RR	92	98	134	134	201	201	0	0	127	0	0	0	106	106	169	220	110	127	98	98	187	195	281	281	311	313	106	137	0	0	0	0	199	199
PO-4-RR	Albrechtičky	RR	83	92	134	134	201	217	0	0	127	0	0	0	106	106	169	192	104	110	98	98	189	0	0	0	0	0	106	122	0	0	0	0	203	203
PO-5-RR	Albrechtičky	RR	92	92	134	134	205	205	232	0	0	121	0	0	110	110	169	200	83	108	98	98	191	207	281	281	311	313	118	0	0	0	0	0	199	203
TR-65-RR	Trnávka	RR	92	94	134	134	201	243	232	0	0	121	0	0	106	110	169	169	110	110	98	98	207	0	281	287	313	313	118	0	0	0	0	0	203	203
PO-70-RR	Albrechtičky	RR	92	92	134	136	201	201	0	0	0	121	0	0	106	110	169	235	110	116	98	98	195	207	281	281	313	313	116	122	0	0	0	0	203	203
PO-71-RR	Albrechtičky	RR	92	92	134	134	205	217	0	0	127	0	0	0	106	106	169	169	110	110	98	98	212	0	281	281	313	313	108	106	0	0	0	0	203	203
PO-72-RR	Albrechtičky	RR	85	94	132	134	223	223	0	0	127	0	0	0	106	106	208	220	98	108	0	0	191	0	281	287	307	313	114	118	0	0	0	0	199	205
PO-73-RR	Albrechtičky	RR	85	92	134	134	205	217	0	0	0	121	0	0	106	106	169	169	114	116	98	98	191	0	281	287	313	315	106	0	0	0	0	0	203	203
PO-74-RR	Albrechtičky	RR	94	94	134	134	205	247	0	0	0	121	0	0	106	106	212	231	83	106	98	98	191	203	281	287	313	313	108	110	0	0	0	0	199	203
PO-76-RR	Albrechtičky	RR	85	98	134	134	243	243	0	0	127	0	0	0	106	110	169	169	104	110	98	98	203	0	281	281	313	319	114	0	0	0	0	0	199	199
PO-77-RR	Albrechtičky	RR	85	85	134	134	205	223	0	0	0	121	0	0	106	106	169	231	110	124	98	98	191	0	281	287	311	313	108	116	0	0	0	0	199	199
PO-78-RR	Albrechtičky	RR	94	94	134	134	205	205	0	0	119	0	0	0	110	110	169	192	110	114	98	98	0	0	281	281	313	313	108	114	0	0	0	0	169	169
PO-79-RR	Albrechtičky	RR	92	92	134	134	201	205	232	0	0	121	0	0	106	106	192	200	110	116	98	98	191	212	281	281	307	313	106	114	0	0	0	0	203	203
PO-80-RR	Albrechtičky	RR	92	92	134	134	201	201	0	0	127	0	0	0	106	106	169	231	110	110	98	98	191	0	281	287	311	313	108	106	0	0	0	0	199	203
PO-81-RR	Albrechtičky	RR	85	92	134	134	217	249	0	0	127	0	0	0	110	110	169	169	110	110	98	98	191	0	281	281	317	317	116	0	0	0	0	0	199	203
PO-83-RR	Albrechtičky	RR	83	92	134	134	201	205	0	0	115	0	0	0	106	106	169	192	110	116	98	98	212	0	281	287	313	317	108	0	0	0	0	0	205	205
GR07--R1	Greece	RR	87	104	132	132	197	205	246	258	115	115	0	0	110	110	200	208	81	91	98	98	0	0	279	281	298	311	83	83	0	0	0	0	169	185

RR, *P. ridibundus;* RL, *P. esculentus;* LL, *P. lessonae*; NA, not analysed.

Tab. S2: Details for six allozyme loci from somatic (soma) and gonadal (gonad) tissue from analysed *Pelophylax* individuals.

		Ldh-1		*Gpi*		*Aat*		*Pgm*		*G3pdh*	*6-Pgd*	
Sample	Taxon	soma	gonad	soma	gonad	soma	gonad	soma	gonad	soma	soma	gonad
BI-11-RR	RR	aa	aa	aa	aa	aa	aa	cc	cc	aa	cc	cc
Da-1-RR	RR	ac	ac	aa		aa	aa	cc		aa	bb	
Da-2-RR	RR	aa		bb		aa		cc		aa	bb	
Da-3-RR	RR	ac	ac	ab	ab	aa	aa	cc		aa	bc	
Da-4-RR	RR	aa		aa		aa		cc		aa	bc	
Da-5-RR	RR	ac	ac	ab	ab	aa	aa	cc		ab	bc	bc
Da-6-RR	RR	aa	aa	aa	aa	aa	aa	cc		aa	bb	Bb
Da-7-RR	RR	aa		ab		aa		cc	cc	aa	bc	
Da-8-RR	RR	ac	ac	aa	aa	aa	aa	cc	cc	aa	bb	
DB-2-RR	RR	ac	ac	ab	ab	aa	aa	cc	cc	bb	bb	
DB-3-RR	RR	aa	aa	bb	bb	aa	aa	cc	cc	aa	bb	
DB-6-RR	RR	ac		ab		aa		cc		aa	bc	
DB-7-RR	RR	aa		aa		aa		cc		aa	bb	
DB-8-RR	RR	aa		aa		aa		aa		aa	bb	
DB-9-RR	RR	ac		aa		aa		cc		aa	bc	
OS-13-RR	RR	aa		aa		aa		cc		aa	cc	ab
OS-1-RR	RR	aa	aa	bb	bb	aa	aa	cc		aa	bc	
OS-2-RR	RR	cc	cc	ab	ab	aa	aa	bb	bb	aa	bc	bc
OS-3-RR	RR	aa	aa	ab	ab	aa	aa	cc	cc	aa	bb	bb
OS-4-RR	RR	cc	cc	ab	ab	aa	aa	cc	cc	aa	bb	bb
OS-5-RR	RR	aa	aa	ab	ab	aa	aa	cc	cc	aa	bc	bc
OS-7-RR	RR	aa	aa	aa	aa	aa	aa	cc	cc	aa	bb	bb
OS-8-RR	RR	aa		ab		aa		cc		aa	bc	
OS-9-RR	RR	cc		aa		aa		bc		aa	bc	ab
PO-11-RR	RR	aa	aa	ab	ab	aa	aa	cc	cc	aa	bb	bb
PO-12-RR	RR	aa	aa	ab	ab	aa	aa	cc	cc	aa	bb	bb
PO-13-RR	RR	ac	ac	aa	aa	aa	aa	cc	cc	aa	bc	bc
PO-4-RR	RR	aa		bb		aa		cc		aa	bb	
PO-5-RR	RR	cc		ab		aa		ac		aa	bc	
BI-6-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
BT-10-RL	LL	dd		bb		bb		bb		bb	aa	
BT-4-RL	LL	dd	dd	bb	bb	bb	bb	bb	bb	bb	aa	aa
BT-5-RL	LL	dd		bb		bb		bb		bb	aa	
BT-6-RL	LL	bd		bb		bb		bb		bb	aa	
BT-7-RL	LL	dd		bb		bb		bb		bb	aa	
BT-8-RL	LL	dd		bb		bb		bb		bb	aa	
BT-9-RL	LL	dd		bb		bb		bb		bb	aa	
CT-10-RL	LL	bd		bb		bb		bb		bb	aa	
CT-11-RL	LL	bd		bb		bb		bb		bb	aa	
CT-1-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
CT-2-RL	LL	dd		bb		bb		bb		bb	aa	
CT-3-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	
CT-4-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	
CT-5-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
CT-6-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
CT-7-RL	LL	dd		bb		bb		bb		bb	aa	
CT-8-RL	LL	dd		bb		bb		bb		bb	aa	
DS-1-RL	LL	dd		bb		bb		bb		00	aa	
DS-2-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa

HB-10-RL	LL	dd	dd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-11-RL	LL	dd		bb		bb		bb		bb	aa	
HB-12-RL	LL		dd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-13-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-14-RL	LL	dd		bb		bb		bb		bb	aa	
HB-15-RL	LL	dd		bb		bb		bb		00	aa	
HB-1-RL	LL	dd		bb		bb		bb		00	aa	
HB-2-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	00	aa	aa
HB-3-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-4-RL	LL	dd		bb		bb		bb		bb	aa	
HB-5-RL	LL	dd		bb		bb		bb		bb	aa	
HB-6-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-7-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
HB-8-RL	LL	bb		bb		bb		bb		bb	aa	
HD-12-RL	LL	bd	bd	bb	bb	bb	bb	bb		bb	aa	
HD-13-RL	LL	bd		bb		bb		bb		bb	aa	
HD-14-RL	LL	bd	bd	bb	bb	bb	bb	bb		bb	aa	
HD-15-RL	LL	bd	bd	bb	bb	bb	bb	bb		bb	aa	
HD-4-RL	LL	bd	bd	bb	bb	bb	bb	bb		bb	aa	
HD-6-RL	LL	bd		bb		bb		bb		bb	aa	
HD-9-RL	LL	bd		bb		bb		bb		bb	aa	
Ka-13-RL	LL	bd		bb		bb		bb		bb	aa	
Ka-9-RL	LL	dd	dd	bb	bb	bb	bb	bb	bb	bb	aa	
LO-9-RL	LL	dd	dd	bb	bb	bb	bb	bb		bb	aa	
PR-11-RL	LL	bd	bd	bb	bb	bb		bb		bb	aa	
PR-2-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	
PR-3-RL	LL	bd	bd	bb	bb	bb	bb	bb		bb	aa	aa
PR-4-RL	LL	dd		bb		bb		bb		bb	aa	
PR-5-RL	LL	dd		bb		bb		bb		bb	aa	
PR-6-RL	LL	dd		bb		bb		bb		bb	aa	
PR-7-RL	LL	bd	bd	bb	bb	bb	bb	ab	ab	bb	aa	
PR-8-RL	LL	bb	bb	bb	bb	bb	bb	bb	bb	bb	aa	
TR-10-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	
TR-11-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	aa
TR-12-RL	LL	bb		bb		bb		bb		bb	aa	
TR-13-RL	LL	bb		bb		bb		bb		bb	aa	
TR-14-RL	LL	dd		bb		bb		bb		bb	aa	
TR-15-RL	LL	bd	bd	bb	bb	bb	bb	bb	bb	bb	aa	
TR-3-RL	LL	dd		bb		bb		bb		bb	aa	
TR-4-RL	LL	bb		bb		bb		bb		bb	aa	
TR-5-RL	LL	bd	bd	bb		bb	bb	bb		bb	aa	
TR-8-RL	LL	dd		bb		bb		bb		bb	aa	
TR-9-RL	LL	bd	bd	bb		bb	bb	bb	bb	bb	aa	
BI-7-RL	RL	cd	dd	bb	bb	ab	bb	bc	bb	ab	ab	bb
BI-8-RL	RL	cd	dd	ab	bb	ab	bb	bc	bb	ab	ab	bb
Da-10-RL	RL	ad		ab		ab		bb		ab	ab	
Da-11-RL	RL	ad		bb		ab		bb		ab	ab	
Da-12-RL	RL	ad		ab		ab		bc		ab	ab	
Da-9-RL	RL	ad		bb		ab		bb		ab	ac	
DB-10-RL	RL	cd		ab		ab		bc		ab	ac	
DB-1-RL	RL	ad	dd	ab	bb	ab	bb	bc		ab	ab	
DB-4-RL	RL	ad		ab		ab		bb		ab	ab	
DB-5-RL	RL	ad		ab		ab		cc		ab	ab	
OS-10-RL	RL	cd		bb		ab		bc		ab	bc	ab

OS-11-RL	RL	cd		bb		ab		bc		ab	bb	
OS-12-RL	RL	cd		bb		ab		bc		ab	bb	ab
OS-14-RL	RL	cd		ab		ab		bc		bb	bb	
OS-6-RL	RL	cd	dd	ab	ab	ab	bb	bc		ab	ac	ac
PO-10-RL	RL	cd		ab		ab		ab		ab	bb	
PO-14-RL	RL	cd	dd	ab	bb	ab	bb	ab	bb	ab	ac	aa
PO-15-RL	RL	ad	dd	ab	bb	ab	bb	ab		ab	bb	
PO-1-RL	RL	cd	dd	ab	bb	ab	bb	bc		ab	ac	
PO-2-RL	RL	cd		ab		ab		bc		ab	bb	
PO-3-RL	RL	cd				ab		bc		ab	ab	
PO-7-RL	RL	cd		ab		ab		bc		ab	bb	
PO-8-RL	RL	cd		ab		ab		bc		ab	ac	
PO-9-RL	RL	cd	dd	ab	bb	ab	bb	bc	bb	ab	bb	
BI-7-RL	RL	cd	dd	bb	bb	ab	bb	bc	bb	ab	ab	bb
BI-8-RL	RL	cd	dd	ab	bb	ab	bb	bc	bb	ab	ab	bb
Da-10-RL	RL	ad		ab		ab		bb		ab	ab	
Da-11-RL	RL	ad		bb		ab		bb		ab	ab	
Da-12-RL	RL	ad		ab		ab		bc		ab	ab	
Da-9-RL	RL	ad		bb		ab		bb		ab	ac	
DB-10-RL	RL	cd		ab		ab		bc		ab	ac	
DB-1-RL	RL	ad	dd	ab	bb	ab	bb	bc		ab	ab	
DB-4-RL	RL	ad		ab		ab		bb		ab	ab	
DB-5-RL	RL	ad		ab		ab		cc		ab	ab	
OS-10-RL	RL	cd		bb		ab		bc		ab	bc	ab
OS-11-RL	RL	cd		bb		ab		bc		ab	bb	
OS-12-RL	RL	cd		bb		ab		bc		ab	bb	ab
OS-14-RL	RL	cd		ab		ab		bc		bb	bb	
OS-6-RL	RL	cd	dd	ab	ab	ab	bb	bc		ab	ac	ac
PO-10-RL	RL	cd		ab		ab		ab		ab	bb	
PO-14-RL	RL	cd	dd	ab	bb	ab	bb	ab	bb	ab	ac	aa
PO-15-RL	RL	ad	dd	ab	bb	ab	bb	ab		ab	bb	
PO-1-RL	RL	cd	dd	ab	bb	ab	bb	bc		ab	ac	
PO-2-RL	RL	cd		ab		ab		bc		ab	bb	
PO-3-RL	RL	cd				ab		bc		ab	ab	
PO-7-RL	RL	cd		ab		ab		bc		ab	bb	
PO-8-RL	RL	cd		ab		ab		bc		ab	ac	
PO-9-RL	RL	cd	dd	ab	bb	ab	bb	bc	bb	ab	bb	
BI-10-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
BI-12-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
BI-2-RL	RL	cd		ab		ab		bc		ab	bb	
BI-3-RL	RL	cd		ab		ab		bc		ab	ab	
BI-4-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
BI-5-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
BI-9-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
BT-1-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
BT-2-RL	RL	cd		ab		ab		bc		ab	ab	
BT-3-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
CT-12-RL	RL	ad	aa	bb	bb	ab	aa	bc	cc	ab	ab	bb
CT-13-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
CT-14-RL	RL	ab	aa	bb	bb	ab	aa	bc	cc	ab	ab	bb
CT-15-RL	RL	ac	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
CT-9-RL	RL	cd		ab		ab		bc		ab	ab	
DS-10-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc		ab	
DS-11-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	

DS-12-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc		ab	
DS-13-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc		ab	
DS-14-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc		ab	
DS-15-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc		ab	
DS-3-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc		bb	ab
DS-4-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc		ab	
DS-5-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	
DS-6-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	
DS-7-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	
DS-8-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc		ab	
DS-9-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	
HB-9-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bc	bb
HD-10-RL	RL	cd		ab		ab		bc		ab	ab	
HD-11-RL	RL	cd		ab		ab		bc			ab	
HD-1-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	bb	cc
HD-2-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
HD-3-RL	RL	cd	cc	ab	aa	ab	aa	bc			ab	cc
HD-5-RL	RL	bc		ab		ab		bc		ab	bb	
HD-7-RL	RL	cd		aa		ab		bc		ab	ab	
HD-8-RL	RL	cd	cc	ab	aa	ab	aa	bc		ab	ab	
Ka-10-RL	RL	cd		ab		ab		cc		ab	ab	
Ka-11-RL	RL	bc		ab		ab		bc		ab	ab	
Ka-12-RL	RL	cd		ab		ab		bc		ab	ab	
Ka-14-RL	RL	cd		ab		ab		bc		ab	ab	
Ka-15-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
Ka-1-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-2-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-3-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-4-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-5-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-6-RL	RL	cd	cc	ab	aa	ab	aa	bc		ab	ab	
Ka-7-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	
Ka-8-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
LO-10-RL	RL	ab		ab		ab		bc		ab	bb	
LO-11-RL	RL	cd		ab		ab		bc		ab	bb	
LO-12-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-13-RL	RL	bc		ab		ab		bc		ab	bb	
LO-14-RL	RL	cd		bb		ab		bc		ab	bb	
LO-15-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-1-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-2-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-3-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-4-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-5-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	
LO-6-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-7-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
LO-8-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb
PR-10-RL	RL	bc		ab		ab		bc			ab	
PR-1-RL	RL	bc	cc	ab	aa	ab	aa	bc	cc	ab	ab	bb
PR-9-RL	RL	cd		ab		ab		bc		ab	ab	
TR-1-RL	RL	cd	cc	ab	aa	ab	aa	bc		ab	bb	bb
TR-2-RL	RL	cd	cc	ab	aa	ab		bc		ab	bb	
TR-6-RL	RL	bc	cc	ab		ab	aa	bc	cc	ab	bb	bb
TR-7-RL	RL	cd	cc	ab	aa	ab	aa	bc	cc	ab	bb	bb

RR, *P. ridibundus;* RL, *P. esculentus;* LL, *P. lessonae*.

Tab. S3: Microsatellite loci used in this study and information on species-specificity of alleles.

Locus	L specific alleles	R specific alleles	unspecified alleles
101RlCA1b6	78/80/82	74/85/92/94/98	83
102RlCA1b5	113/118	132/134/136	
103Ga1a19	195	195/201/205/209/217/223/239/243/247/249/253/255/259	
105RlCA5	247/252/256/258/260/262/264	232/234/236/250	
106Res16	121/133/152	115/117/119/123/127	
107Res20	102/104/108/110/111/112/113/115/116/117/119/120/121/122/123/124/125/126/128/131/142/144/146	__	
108RICA2a34	112/123/130/136/138/140/143/145/147/150/152/154/156/162/164	106/110	
109Re2Caga3	__	169/192/200/204/208/212/220/227/231/235/239/255	
110Res22	__	83/84/85/87/98/104/106/108/110/114/116/124/127/129/133	
111Ga1a23	113/115/117/119/121/123/125/127/129/131/133/135/139/141	98	
112Rrid169A	__	187/189/191/193/195/198/203/207/212/214/227/234	
113Rrid013A	291/293/296/299	281/287	
114Rrid059A	278	303/307/311/313/315/317/319/321	
115Re1Caga10	97/98	93/106/108/110/112/114/116/118/122/125/137/140	
116CA1a27	95/111/113/115/117/118/119/121/124/125/127	__	
117RlCA18	175/177/179/181/183/184/186/188/190/195/197/202	__	
118Rrid135A	236	169/183/199/203/205	

L, *P. lessonae*; R, *P. ridibundus*.

Tab. S4: Microsatellite variability and allele frequency in 17 loci in four population groups of three *Pelophylax* taxa generated in MSA. Population name, locus name, heterozygosity observed (Het obs) and expected (Het exp), number of alleles (NA) determined in each population and frequencies of alleles in each loci.

Pop. name	Locus name	Het obs	Het exp	NA	Frequencies								
LL	RlCA1b6	0.527778	0.519854	3	0.333333	0.606481	0.060185						
LL	RlCA1b5	0.037736	0.037378	3	0.009434	0.981132	0.009434						
LL	Ga1a19	0.009346	0.009346	2	0.995327	0.004673							
LL	RlCA5	0.480392	0.555057	7	0.009804	0.034314	0.578431	0.009804	0.333333	0.014706	0.019608		
LL	Res16	0.009524	0.019093	3	0.990431	0.004785	0.004785						
LL	Res20	0.580952	0.835853	17	0.012048	0.006024	0.036145	0.301205	0.018072	0.018072	0.114458	0.012048	0.222892
					0.018072	0.084337	0.054217	0.042169	0.006024	0.030120	0.012048	0.012048	
LL	RICA2a34	0.803738	0.770392	15	0.032710	0.004673	0.004673	0.042056	0.018692	0.093458	0.014019	0.425234	0.172897
					0.065421	0.070093	0.023364	0.004673	0.023364	0.004673			
LL	Re2Caga3	0.000000	1.000000	2	0.500000	0.500000							
LL	Res22	0.153846	0.257143	3	0.066667	0.066667	0.866667						
LL	Ga1a23	0.764706	0.829615	15	0.014706	0.019608	0.029412	0.014706	0.284314	0.176471	0.088235	0.078431	0.044118
					0.019608	0.210784	0.004902	0.004902	0.004902	0.004902			
LL	Rrid169A	0.333333	0.772727	4	0.333333	0.333333	0.250000	0.083333					
LL	Rrid013A	0.179775	0.198946	5	0.005618	0.011236	0.039326	0.893258	0.050562				
LL	Rrid059A	0.000000	0.000000	1	1.000000								
LL	Re1Caga10	0.039216	0.038829	3	0.980392	0.009804	0.009804						
LL	CA1a27	0.737864	0.764433	9	0.252427	0.344660	0.019417	0.019417	0.004854	0.218447	0.082524	0.025642	0.029126
LL	RlCA18	0.750000	0.800704	11	0.015000	0.165000	0.040000	0.305000	0.075000	0.265000	0.040000	0.055000	0.025000
					0.005000	0.010000							
LL	Rrid135A	0.000000	0.065537	2	0.033333	0.966667							
E_les	RlCA1b6	0.000000	0.000000	1	1.000000								
E_les	RlCA1b5	0.000000	0.000000	1	1.000000								
E_les	Ga1a19	0.000000	0.000000	1	1.000000								

E_les	RlCA5	0.000000	0.000000	1	1.000000									
E_les	Res16	0.000000	0.000000	1	1.000000									
E_les	Res20	0.000000	0.000000	1	1.000000									
E_les	RICA2a34	0.000000	0.000000	1	1.000000									
E_les	Re2Caga3	n.d.	n.d.	n.d.										
E_les	Res22	n.d.	n.d.	n.d.										
E_les	Ga1a23	0.000000	0.000000	1	1.000000									
E_les	Rrid169A	n.d.	n.d.	n.d.										
E_les	Rrid013A	0.000000	0.000000	1	1.000000									
E_les	Rrid059A	0.000000	0.000000	1	1.000000									
E_les	Re1Caga10	0.000000	0.000000	1	1.000000									
E_les	CA1a27	0.000000	0.000000	1	1.000000									
E_les	RlCA18	0.000000	0.000000	1	1.000000									
E_les	Rrid135A	0.000000	0.000000	1	1.000000									
RR	RlCA1b6	0.585366	0.648299	7	0.012195	0.024390	0.048780	0.195122	0.548780	0.121951	0.048780			
RR	RlCA1b5	0.119048	0.115318	4	0.011905	0.940476	0.035714	0.011905						
RR	Ga1a19	0.731707	0.788317	12	0.365854	0.243902	0.012195	0.060976	0.036585	0.012195	0.109756	0.097561	0.012195	
					0.024390	0.012195	0.012195							
RR	RlCA5	0.333333	0.574603	6	0.638889	0.138889	0.027778	0.055556	0.083333	0.055556				
RR	Res16	0.146341	0.745606	6	0.106383	0.063830	0.106383	0.191489	0.085106	0.446809				
RR	Res20	n.d.	n.d.	n.d.										
RR	RICA2a34	0.225000	0.338924	2	0.787500	0.212500								
RR	Re2Caga3	0.850000	0.825949	12	0.362500	0.075000	0.012500	0.087500	0.012500	0.050000	0.087500	0.087500	0.012500	
					0.125000	0.075000	0.012500							
RR	Res22	0.619048	0.665519	12	0.119048	0.035714	0.011905	0.023810	0.035714	0.023810	0.559524	0.023810	0.059524	
					0.011905	0.011905	0.083333							
RR	Ga1a23	0.000000	0.000000	1	1.000000									
RR	Rrid169A	0.487805	0.804918	9	0.245902	0.049180	0.344262	0.065574	0.049180	0.114754	0.065574	0.049180	0.016393	
RR	Rrid013A	0.400000	0.418671	4	0.012500	0.737500	0.200000	0.050000						
RR	Rrid059A	0.743590	0.657010	8	0.038462	0.076923	0.089744	0.564103	0.076923	0.102564	0.025641	0.025641		

RR	Re1Caga10	0.690476	0.861569	13	0.042254	0.014085	0.253521	0.154930	0.042254	0.028169	0.197183	0.112676	0.056338
					0.028169	0.014085	0.042254	0.014085					
RR	CA1a27	n.d.	n.d.	n.d.									
RR	RlCA18	n.d.	n.d.	n.d.									
RR	Rrid135A	0.589744	0.699634	5	0.269231	0.012821	0.256410	0.410256	0.051282				
E_rid	RlCA1b6	0.000000	0.572650	4	0.074074	0.185185	0.629630	0.111111					
E_rid	RlCA1b5	0.000000	0.156667	3	0.040000	0.920000	0.040000						
E_rid	Ga1a19	0.000000	0.683333	6	0.520000	0.240000	0.040000	0.080000	0.080000	0.040000			
E_rid	RlCA5	0.000000	0.900000	4	0.400000	0.200000	0.200000	0.200000					
E_rid	Res16	0.000000	0.425000	3	0.187500	0.750000	0.062500						
E_rid	Res20	n.d.	n.d.	n.d.									
E_rid	RICA2a34	0.000000	0.478947	2	0.650000	0.350000							
E_rid	Re2Caga3	0.000000	0.813853	9	0.409091	0.045455	0.136364	0.045455	0.045455	0.090909	0.136364	0.045455	0.045455
E_rid	Res22	0.000000	0.492877	5	0.148148	0.037037	0.074074	0.703704	0.037037				
E_rid	Ga1a23	0.000000	0.000000	1	1.000000								
E_rid	Rrid169A	0.000000	0.787879	7	0.045455	0.181818	0.045455	0.409091	0.181818	0.090909	0.045455		
E_rid	Rrid013A	0.000000	0.280702	2	0.842105	0.157895							
E_rid	Rrid059A	0.000000	0.712418	5	0.111111	0.500000	0.222222	0.111111	0.055556				
E_rid	Re1Caga10	0.000000	0.858462	7	0.115385	0.192308	0.115385	0.153846	0.115385	0.269231	0.038462		
E_rid	CA1a27	0.000000	n.d.	1	1.000000								
E_rid	RlCA18	n.d.	n.d.	n.d.									
E_rid	Rrid135A	0.000000	0.719298	5	0.105263	0.105263	0.263158	0.473684	0.052632				

LL, *P. lessonae;* E_les, L genome from *P. esculentus;* RR, *P. ridibundus;*E_rid, R genome from *P. esculentus;* n.d., not determined.

Tab. S5: Multilocus Genotypes (MLGs) derived from representative samples. Listed are IDs, types of MLGs and number of individuals per sample in which each MLG was found.

Sample ID	Multilocus Genotypes	MLG´s ID	Type of MLG´s	No. of ind.
DO-33-LL	7878113118195195256264121121110110140143000012113100296296278278979795111186190000	1	L	1
DS-2-LL	787811811819519500121121116120112145000011911900000097979595000	2	L	1
HB-5-LL	78781181181951950012112111612014716200001191210000009797111119181186000	3	L	1
BD-01-LL	787811811819519525225612112111912314314500981101211210029329627827897989511118118400	4	L	1
HB-7-LL	787811811819519525226012112111011014514700001191190029629627827897971251251771772362360	5	L	1
DO-38-LL	787811811819519525625612112111011014014500001191310029629627827897979511118619000	6	L	1
HB-4-LL	787811811819519525625612112112012014514500001191210000009797111111177181000	7	L	1
TR-4-LL	787811811819519525626012112114614614515200001231230029629627827897979511917718100	8	L	1
CT-6-LL	7878118118195195256262121121108110147154000012312300296296278278979711111918118623623600	9	L	1
BI-6-LL	787811811819519526026012112112412414514500001131190000009797951111771860000	10	L	1
TR-10-LL	787811811819519526026012112114214414514700001151270029629627827897979511118120200	11	L	1
PR-2-LL	7878118134195201252256121121116126140145001100981151870296296278278971061111211791811990	12	L	1
HB-13-LL	7880000000121121120120145145008311011912900000097971111111818818800	13	L	1
DO-46-LL	7880118118195195247256121121110117147162000012113100296296278278979811811817918600	14	L	1
DO-91-LL	7880118118195195247256121121111111113614500001211310029629627827897979511118418600	15	L	1
HB-6-LL	7880118118195195252256121121124124145147000011911900296296009797951211771770000	16	L	1
TR-15-LL	7880118118195195252260121121124142154154000000000000097979512117718123623600	17	L	1
TR-53-LL	7880118118195195256256121121001451450000123131002962992782789798951191861860000	18	L	1
HD-14-LL	7880118118195195256256121121104110112145000000000000000000000	19	L	1
TR-12-LL	7880118118195195256256121121110110138145000012312700296296278278979795121181186236236	20	L	1
HD-6-LL	7880118118195195256256121121110110140145000000000000000000000	21	L	1
CT-8-LL	7880118118195195256256121121110110145145000011911900296299278278979711111117717700	22	L	1
DO-35-LL	7880118118195195256256121121110110147154000011913100296296278278979711912117719500	23	L	1
CT-5-LL	7880118118195195256256121121110120147150000011711900296296278278979711111317717923623600	24	L	1
HD-13-LL	7880118118195195256256121121112120140145000011911900293296278278979711112118618623623600	25	L	1

HB-2-LL	78801181181951952562561211211161201451450000119119000000979711112118118600	26	L	1
DO-27-LL	788011811819519525625612112111712813614500001191250029629627827897971111818118600	27	L	1
PR-4-LL	7880118118195195256256121121120122150164000011913318702962962782789797119121184186236236	28	L	1
HD-12-LL	788011811819519525625612112112012614014300001191210029629627827897979595186186236236	29	L	1
DO-94-LL	78801181181951952562561211211211211361400000121131002962962782789797111111917918100	30	L	1
DO-02-LL	788011811819519525625612112112112145145000011912100296296278278979711111918618600	31	L	1
HB-8-LL	78801181181951952562581211211081101451450000119121002962960097979511919181186236236	32	L	1
DO-03-LL	788011811819519525626012112110211014514500001211250029629627827897971111118418600	33	L	1
TR-67-LL	788011811819519525626012112111011011214500001151310029629627827897971111111118119000	34	L	1
PR-5-LL	78801181181951952562601211211101161471520000119139002962962782789797117119177181236236	35	L	1
PR-11-LL	788011811819519525626012112111012014515200110098131227029629627827897979595181186236236	36	L	1
PR-7-LL	788011811819519525626012112111013114714700001151310029629627827897971111119181195236236	37	L	1
TR-66-LL	78801181181951952562601211211131151361470000121131002962962782789797959518118100	38	L	1
TR-69-LL	788011811819519525626012112112112114514700001231250029629627827897979511918118100	39	L	1
DO-95-LL	788011811819519525626012112112112114516200001271310029629627827897971111217918100	40	L	1
TR-9-LL	78801181181951952562601211211421421501520000127127002962962782789797113119177181236236	41	L	1
TR-40-LL	788011811819519525626012112114214414515200001191310029629627827897971912718118600	42	L	1
DO-92-LL	78801181181951952562601520117117145162000011912100296296278278979711111518619000	43	L	1
DO-32-LL	788011811819519525626412112111011013614700001191250029629627827897971812718619000	44	L	1
BT-6-LL	78801181181951952562641211211281281451500000119121002962962782789797111119177181236236	45	L	1
BD-16-LL	78801181181951952582601211211231251451450000119119002962962782789789511118618600	46	L	1
CT-7-LL	788011811819519526026012112110811011213000001171191910296296278278979711111317718100	47	L	1
TR-68-LL	78801181181951952602601211211101101451450000121131002962962782789797511918118600	48	L	1
DO-26-LL	788011811819519526026012112111011514514700001311310029629627827897971912118619000	49	L	1
DO-41-LL	78801181181951952602601211211101151501520000119121002962962782789797119121175181 00	50	L	1
TR-13-LL	788011811819519526026012112111012014515200001191230029629627827897971191211772022362 36	51	L	1
TR-3-LL	78801181181951952602601211211101461521542312311100115119191227296296278278971069511918118123623 6	52	L	1
BD-08-LL	788011811819519526026012112111112514514500001291290029328727827897979511918618600	53	L	1
DO-39-LL	788011811819519526026012112111711714514700001311310029629627827897971912118619500	54	L	1
TR-5-LL	78801181181951952602601211211201201401470011001271310029629627827897979595181186236236	55	L	1

TR-8-LL	78801181181951952602601211211201201451470011001271311910296296278278979795951811862362 36	56	L	1
DO-36-LL	78831131181951952562601211211101251401450000123131002962962782780011112717718600	57	L	1
HD-4-LL	78831181181951952562561211211101201451520011001191311870296296278278979711912518618823 6236	58	L	1
DO-29-LL	78831181181951952562600011012314014000001311310029629927827897979512717517900	59	L	1
TR-26-LL	78831181181951952562601211211131211451500000125131002962962782789797111119181195 00	60	L	1
HB-14-LL	80800019519525625612112111012014514700001191210000009797951190000	61	L	1
HB-12-LL	80801181181951950000000000001191210000009797951251811810 0	62	L	1
CT-4-LL	80801181181951950012112111012014515000001131210000278278979711111917718100	63	L	1
HB-1-LL	80801181181951952522561211211201201401450000121129002962962782789797111121177188 00	64	L	1
BT-8-LL	80801181181951952522601211211161261451470000119121002962962782789797951111811810 0	65	L	1
TR-49-LL	80801181181951952562561211210014515200001191310029629627827897975951811860 0	66	L	1
DO-01-LL	80801181181951952562561211211021101451450000121121002962962782789797111121186118600	67	L	1
CT-2-LL	80801181181951952562561211211081161451470000131131002962962782789797111111177186236236	68	L	1
PR-3-LL	80801181181951952562561211211011012314700110098125187227291296278278971169511518118623 6236	69	L	1
HB-3-LL	80801181181951952562561211211011614514500001251310029629627827897971111111771882362 36	70	L	1
BT-5-LL	80801181181951952562561211211011614514700001191190029629627827897971111111771810 0	71	L	1
PR-8-LL	80801181181951952562561211211011614715000000000000000000000	72	L	1
PR-6-LL	80801181181951952562561211211012014014500001251250029629927827897971111191791882362 36	73	L	1
Ka-13-LL	80801181181951952562561211211012014715600000000000000000000	74	L	1
DO-93-LL	80801181181951952562561211211111113815000001231270029329627827897971111111751900 0	75	L	1
CT-1-LL	80801181181951952562561211211161161471500000113131000000979711111118618600	76	L	1
CT-11-LL	80801181181951952562561211211201201451520000117121002932962782789797111111181181 00	77	L	1
BT-10-LL	80801181181951952562561211211201261451470000119127002962962782789797951111771812362 36	78	L	1
CT-3-LL	80801181181951952562561211211201421451470000123131002932992782789797111119186186 00	79	L	1
BT-7-LL	80801181181951952562561211211261261451500000000000000000000	80	L	1
Ka-9-LL	80801181181951952562601211211081101361450000119131002962992782789797951191811972362 36	81	L	1
LO-9-LL	80801181181951952562601211211081101401450011001191251912342962962782789797951111831830 0	82	L	1
DO-44-LL	80801181181951952562601211211101231401470000119131002962962782789797951191811860 0	83	L	1
DO-31-LL	80801181181951952562601211211012314016200001191310029629627827897981111111841880 0	84	L	1
DO-25-LL	80801181181951952562601211211012314514500001251310029629927827897971181271841860 0	85	L	1

DO-30-LL	8080118118195195256260121121110128145152000011912500296296278278979711111118118800	86	L	1
TR-29-LL	8080118118195195256260121121111111147150000012112300296296278278979759518118100	87	L	1
BT-9-LL	8080118118195195256260121121116126145147000012112100296296278278979511118118100	88	L	1
HD-9-LL	8080118118195195256260121121120120140152000011312100296296278278979511118118623623 6	89	L	1
BT-4-LL	8080118118195195256260121121120126145147000011912100296296278278979711112517718100	90	L	1
DO-48-LL	8080118118195195256260121121121123136136000012513100296296278278979711311818118600	91	L	1
DO-24-LL	8080118118195195256260121121128128140145000011912300296296278278979511817719000	92	L	1
CT-10-LL	8080118118195195256262121121120120138147001100119119002962962782782979111119177186236236	93	L	1
DO-47-LL	8080118118195195256264121121110110136145000012513100296296278278979711112718419000	94	L	1
TR-30-LL	8080118118195195260260121121111111145150000013113100296296278278979711111117718100	95	L	1
HB-11-LL	8080118118195195260260121121116126145147000011913100000097971111117718600	96	L	1
DO-28-LL	8080118118195195260260121121123128145145000011912300296299278278979711111919019500	97	L	1
BD-95-LL	8080118118195195260260121133119119112145000011912100293299278278979711912418118400	98	L	1
DS-1-LL	8080118118195195260262121121120120112138000011912300296296278278979511918118600	99	L	1
HB-15-LL	8082118134195195001211211201201401450011001251310000009797119119000 0	100	L	1
DO-40-LL	8083118118195195256256001171281451470000121121002962962782789797511518619000	101	L	1
TR-11-LL	8083118118195195256256121121110124145145000012313500296296278278979595183183236236	102	L	1
HB-10-LL	8083118118195195256256121121120126145152001100121125000000979795111117718400	103	L	1
DO-34-LL	8083118118195195256256121121128128145147000012113100296296278278979511117918600	104	L	1
TR-14-LL	8083118118195195256260121121110120112140235235001311410029629627827897116951151811811990	105	L	1
DO-37-LL	8083118118195195256260121121117117145147000012112300296296278278979711111118418400	106	L	1
HD-15-LL	8083118118195195256260121121120124145152000011911900291296278278979711112117718123623 6	107	L	1
DO-42-LL	8083118118195195256260121121121121145150000011512300296296278278979711912117717700	108	L	1
PO-3-L	7878118118195195001211211201201451450000123123000000979711511518618600	109	hybrid L	2
PO-9-L	7878118118195195260260121121120120145145000012312300000097971151151861860 0	110	hybrid L	3
PO-82-L	7878118118195195260260121121120120145145000012312300296296278278001151151861860 0	111	hybrid L	2
PO-2-L	7878118118195195260260121121120120145145000012312300296296278278979711511518618600	112	hybrid L	3
PO-1-L	7878118118195195260260121121120120145145000012312300296296278278979711511518618623623 6	113	hybrid L	14
PO-15-L	7878001951950012112112012014514500001231230000009797115115186186000 0	114	hybrid L	1

PO-6-L	7878118118000000000000001231230000009797115115000 0	115	hybrid L	1
PO-7-L	787811811800001211211201201451450000123123000000979711511518618600	116	hybrid L	1
PO-8-L	7878118118195195260260121121120120145145000012312300296296009797115115186186 00	117	hybrid L	1
DB-7-RR	00134134000000000000110110989818719128128131331511413700000 0	118	R	1
DB-8-RR	749213413420124323223211511500106110169220110110989818718728128731131310610800001691 99	119	R	1
OS-3-RR	789213413420125323223212712700106106169200110116989819521428128131331710610600002032 03	120	R	1
OS-8-RR	789213413420525323223211511700106106231255110110989818719828128130331511411600001992 03	121	R	1
OS-13-RR	838513413620121723423611512700106106204208110129989818919128128731331310610600001692 03	122	R	1
PO-83-RR	839213413420120500115115001061061691921101169898212212281287313317108108000020520 5	123	R	1
PO-4-RR	83921341342012170012712700106106169192104110989818918900001061220000203203	124	R	1
OS-1-RR	839213413420520923223212712700106106169200838398981911982812810010811600001992 03	125	R	1
DB-9-RR	858513413420124323225012712700106106169231110110989819120728128131131061140000169203	126	R	1
PO-77-RR	85851341342052230001210010610616923111012498981911912812873113131081160000199199	127	R	1
Da-8-RR	859213413420120100127127001061101692351101109898187195281281313317106116000019920 3	128	R	1
OS-2-RR	859213413420120523423412712700110110200208110129001872032812873133211161160000169203	129	R	1
Da-5-RR	859213413420124723223211912300106106192212831299898187187281281307313931060000169169	130	R	1
Da-1-RR	859213413420124723223211912300106106212231871299898187191281281313313106114000016916 9	131	R	1
Da-4-RR	859213413420124723226011712300106106192212871299898187187287296307313106114000016916 9	132	R	1
DB-3-RR	859213413420520523224812712700001691691101109898191203281281313317106108000016920 3	133	R	1
PO-73-RR	859213413420521700012100106106169169114116989819119128128731331510610600002032 03	134	R	1
PO-81-RR	859213413421724900127127001101101691691101109898191191281281317317116116000019920 3	135	R	1
OS-9-RR	859213413620120523223412712700106106212235831109898191191287287307313112118000000	136	R	1
PO-72-RR	859413213422322300127127001061062082209810800191191281287307313114118000019920 5	137	R	1
PO-76-RR	859813413424324300127127001061101691691041109898203203281281313319114114000019919 9	138	R	1
PO-80-RR	929213413420120100127127001061061692311101109898191191281287311313108106000019920 3	139	R	1
PO-79-RR	92921341342012052320012100106106192200110116989819121228128130731310611400002032 03	140	R	1
OS-7-RR	929213413420120523225012712700106110200220831109898187187281281315317108116000019920 5	141	R	1
OS-4-RR	92921341342012052342341151150010610621222011011098981912032812813153219711600001691 99	142	R	1
Da-3-RR	92921341342012472322321171230010610621223187110989818718728729631331511411400001691 99	143	R	1

Da-6-RR	92921341342012472322321191190010610616923183129898187187287296313313931060000169203	144	R	1
Da-7-RR	92921341342012472322321191190010610616923183129898187191281296307313931060000169203	145	R	1
DU-5-RR	92921341342012512322341271270010610619623111011098981891912812813133171061089500016920 3	146	R	1
PO-5-RR	92921341342052052322320121001101101692008310898981912072812813113131181180000199203	147	R	1
PO-71-RR	92921341342052170012712700106106169169110110989821221228128131331310810600002032 03	148	R	1
DB-6-RR	92921341342432432322601271270010610616922711011098981911912812813113131141370000203203	149	R	1
PO-70-RR	92921341362012010001210010611016923511011698981952072812813133131161220000203203	150	R	1
DB-2-RR	92921341382012432322480121001061061691961101109898191203275281313317110114000016920 3	151	R	1
PO-65-RR	92941341342012432320012100106110169169110110989820720728128731331311811800002032 03	152	R	1
BI-11-RR	92941341342012592342341271270010610622023511011098981871872812810010610600001691 99	153	R	1
PO-12-RR	92941341342392472322321271270010611000106110989818920300313313125140000000	154	R	1
PO-13-RR	92981341342012010012712700106106169220110127989818719528128131131310613700001991 99	155	R	1
OS-5-RR	92981341342012552322321271270010610622023510611098981871982812813033131121140000199203	156	R	1
Da-2-RR	92981341342052052322501271270010611020823583110989819119128128730331311011000001691 83	157	R	1
PO-11-RR	94941341342012432322320121001061102002311101100019119128128131331910811400001692 03	158	R	1
PO-78-RR	94941341342052050011911900110110169192110114989800281281313313108114000016916 9	159	R	1
PO-74-RR	94941341342052470001210010610621223183106989819120328128731331310811000001992 03	160	R	1
PO-15-R	83830000000000001761761101109898000000112112000000	161	hybrid R	1
OS-6-R	83831341342012010011511500106106200200110110989821421400319319116116000000	162	hybrid R	1
OS-12-R	85851341342012010000001061061961961101109898191191287287315315116116000000	163	hybrid R	1
PO-9-R	85851341342012010012712700106106169169110110989820320300001061060000203203	164	hybrid R	1
DB-10-R	85851341342052050012712700106106208208110110989818918928728731931910810800002032 03	165	hybrid R	1
Da-11-R	85851341342052052342341271270010610620820813313398981981982812813153151161160000199199	166	hybrid R	1
PO-1-R	85851341342092090000001061061691691101109898203203281281315315112112000016916 9	167	hybrid R	1
PO-7-R	92920000001271270010610616916911011098980000001081080000203203	168	hybrid R	1
PO-3-R	92921321322012010000001101100011011098980000001081080000000	169	hybrid R	1
BI-8-R	92921341342012010000001061060011011098981911910000106106959500000	170	hybrid R	1
OS-11-R	92921341342012010000001061061961961101109898191191281281315315116116000000	171	hybrid R	1
Da-9-R	92921341342012010011511500002122128383989819819828128131131111211200001831 83	172	hybrid R	1

PO-75-R	92921341342012010012712700001961968484989818718728128131331310810800002032 03	173	hybrid R	1
DB-1-R	92921341342012010012712700110110235235110110989817917928128132132111011000001991 99	174	hybrid R	1
PO-2-R	92921341342012010012912900110110001101109898187187281281331311611600001991 99	175	hybrid R	1
Da-10-R	92921341342012012322321271270000212212838300198198281281311311112112000018318 3	176	hybrid R	1
PO-84-R	92921341342052050000001101102042049898001911912812813133131141140000203203	177	hybrid R	1
OS-10-R	92921341342052052362361271270011011021221211011098981981982812813133131161160000199199	178	hybrid R	1
BI-7-R	92921341342052052562561271270000001101109898187187000010610600000	179	hybrid R	1
DB-5-R	92921341342232230000000016916911011098981911912812813133131101100000169169	180	hybrid R	1
DB-4-R	92921341342432430000000016916911011098981911912812813133131101100000205205	181	hybrid R	1
PO-14-R	92921341342432430012712700106106001041049898000000116116000000	182	hybrid R	1
Da-12-R	92921341342472472322321151150010610623123183830018718728128100114114000020320 3	183	hybrid R	1
OS-14-R	92921361362052050012712700110110169169104104001911912812813133131141140000020320 3	184	hybrid R	1
PO-6-R	94941341340000127127000000858598980000001141140000000	185	hybrid R	1
PO-10-R	94941341342012010000001101101691691101109898002812813133130000000199199	186	hybrid R	1
PO-8-R	94941341342012010012712700106106169169110110989819119128728700108108000020320 3	187	hybrid R	1
PO-82-R	94941341342232230000001061061691691101109898191191281281331312212200002032 03	188	hybrid R	1

P. ridibundus type (R), *P. lessonae* type (L), *P. esculentus* R genome (hybrid R), *P. esculentus* L genome (hybrid L).

Tab. S6: Locus-by-locus analysis of molecular variance (AMOVA) among and within genomes in three *Pelophylax* taxa generated in ARLEQUIN.

	RR/LL		**RR/hybrid R**		**LL/hybrid L**	
	df	**%**	**df**	**%**	**df**	**%**
Variation among populations	1	54 %	1	3 %	1	32 %
Variation among individuals	149	13 %	69	52 %	134	21 %
Variation within individuals	151	34 %	71	45 %	136	47 %
F_{ST}	**0,537**		**0,032***		**0,321**	
P (rand > = data)	0,010		0,010		0,010	

**P*-value < 0,05; *P. ridibundus* (RR), *P. lessonae* (LL) and *P. esculentus* (hybrid R; hybrid L).

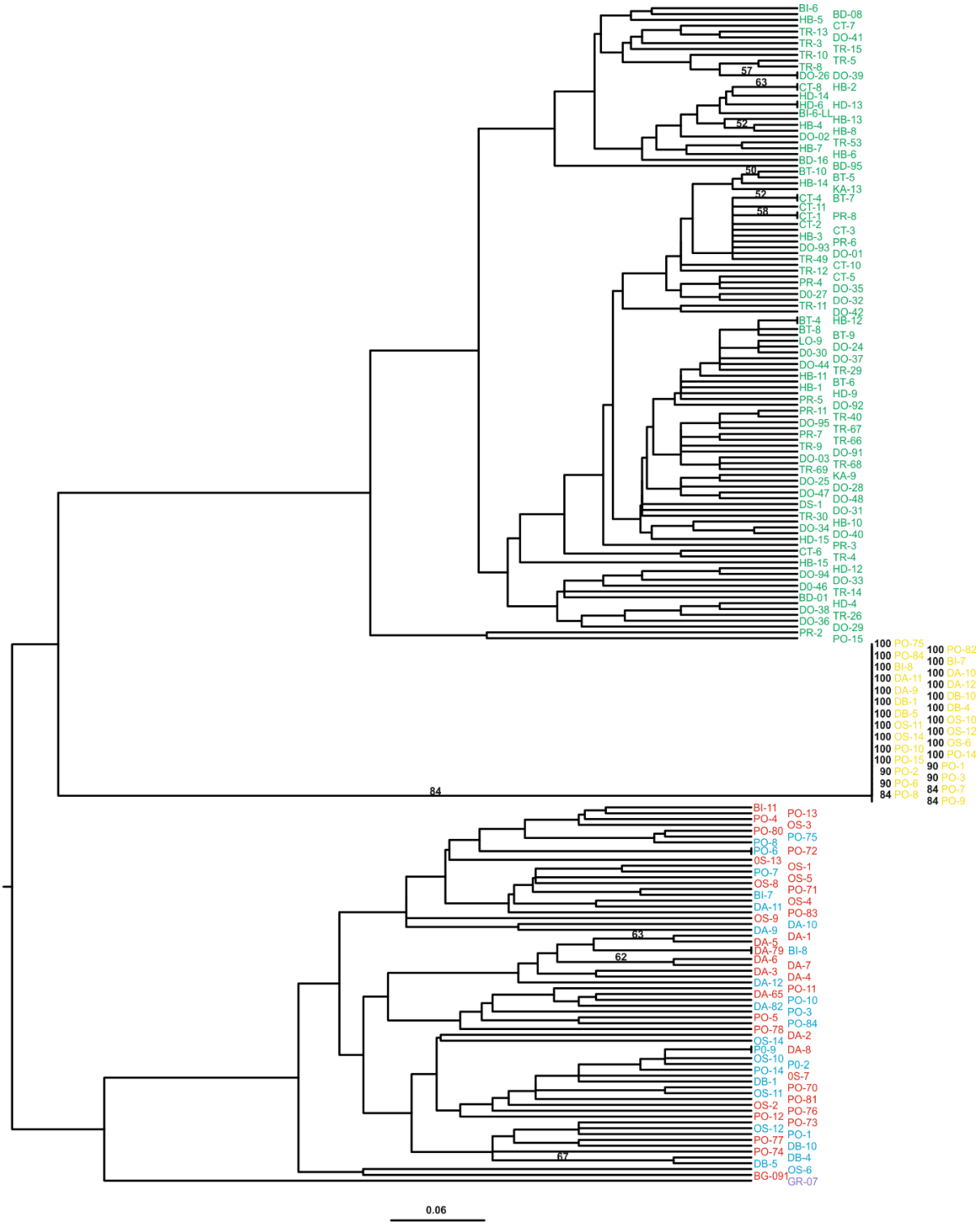

Fig. S1: Phylogenetic tree of DAS distance of 10 microsatellite loci reconstructed in Populations (method UPGMA, 7 000 replicates, shown only bootstraps above 50 %, distance scale). Each coloured point represents one individual: Green points – *P. lessonae* individuals, yellow points - L genome from *P. esculentus*, red points – *P. ridibundus* individuals, blue points – R genome from *P. esculentus*, violet colour – *P. kurtmuelleri*.

Kapitola III

When a sexual genome becomes clonal for a single generation: Evidence from *Pelophylax* water frogs

Marie Doležálková, Marcela Doležalová, Jörg Plötner, Gaston-Denis Guex & Lukáš Choleva

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When a sexual genome becomes clonal for a single generation: Evidence from *Pelophylax* water frogs

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Abstract

Asexual reproduction is one way vertebrates, mostly of female sex, realize to compensate genomic incompatibilities caused by hybridization. To understand how asexuality operates in the opposite system, we studied unisexual *Pelophylax esculentus* water frog males (parentals *P. lessonae* and *P. ridibundus*) that have to compete with sexual *P. ridibundus* males for eggs from its conspecific females. Using experimental and microsatellite data, we found that some hybrid males produced clonal *lessonae* sperm (hybridogenesis), resulting in hybrid sons while others formed also *ridibundus* sperm resulting in *P. ridibundus* daughters. Clonally transmited *lessonae* genome thus determines male sex, while *ridibundus* genome female sex. Moreover, *P. esculentus* males receive *ridibundus* genome as a sexual gamete from its mother, freeze it in a clonal form for one generation and return it back to sexual population (*P. ridibundus* daughters). This strategy **increases a number of females, yet a chance of hybrid males to find a mate.**

Keywords: *Pelophylax*, hemiclone, hybridogenesis, microsatellites, recombination, asexual,crossing experiment.

Introduction

Asexual reproduction is one way vertebrates realize to compensate genomic incompatibilities caused by hybridization (Dawley 1989, Kearney 2005, Lamatsch and Stöck 2009, Neaves and Baumann 2011). Hybrid females use several reproducing strategies in order to form new generations of clonal female progeny (Schultz 1969, Mantovani and Scali 1992, Avise 2008, Neaves and Baumann 2011). On the contrary the occurrence of hybrid males in natural populations is rarer. One of the main reasons for hybrid male rarity, next to lower viability, is the high level of sterility observed in heterogametic sex (Mallet 2005, Landry et al. 2007). The low reproductive potential of such males, however, can not explain the stable presence of males in natural populations over generations as seen in some taxa (Uzzell et al. 1977, Günther 1983, Alves et al. 2001, Schmidt et al. 2011). Still a little is know how such males perpetuate themselves and the role they play in natural populations.

Only few asexual taxa reproduce via backcrosses with sexually reproducing species (Schultz 1969, Beukeboom and Vrijenhoek 1998, Lamatsch and Stock 2009, Neaves and Baumann 2011). Such hybrids are referred to as sexual parasites (e.g. Choleva 2004, Lehtonen 2013). While hybrid females behave as sperm-dependent parasites, ie. are dependent on a male as a sperm donor (Schultz 1977, Beukeboom and Vrijenhoek 1998, Echelle et al. 1989, Vrijenhoek 1994, Bogart et al. 2007, Choleva et al. 2008), hybrid males can be referred as "egg-dependent parasites". They need a sexual female in order to self-reproduce via hybridogenesis (Uzzell et al. 1977, Doležálková et al. 2016). From definition, hybridogenetic animals usually discard the complete genome of one parental species, and only the second genome is transferred clonally, referred to as hemiclonal reproduction (Schultz 1969). The eliminated half of an individual's genome is usually is the one received as a recombinant gamete from previous generation (Avise and Vrijenhoek 1987, Mantovani and

Scali 1992). Fusion of a clonal gamete with a recombinant gamete from a bisexual species results in new generation of hemiclonal progeny (Dawley 1989, Schmidt et al. 2011). The elimination process maintains the integrity of both parental genomes, which is the main prerequisite for a hemiclonal inheritance (Zaleśna et al. 2011).

It is to note that the elimination process is not always precise as in hybridogenetic *Squalius*, *Poeciliopsis*, *Hypseleotris* or *Hexagrammos* (Avise and Vrijenhoek 1987, Alves et al. 2001, Schmidt et al. 2011, Kimura-Kawaguchi et al. 2014). The hemiclone can bear some incorporated genes from the eliminated parental genome as a consequence of occasional recombination, or incomplete elimination phase known e.g. in *Pelophylax* water frogs (Uzzell et al. 1977, Schmeller et al. 2005, Plötner et al. 2008, Mikulíček et al. 2014).

The *Pelophylax esculentus* complex consists of two parental species *P. lessonae* (LL) and *P. ridibundus* (RR) and their interspecies hybrid *P. esculentus. P. esculentus* comprises diploid individuals with one *lessonae* (L) and one *ridibundus* (R) genome (genotype LR) and triploid individuals that possess either two L genomes and one R genome (genotype LLR) or *vice versa* (genotype RRL). *P. esculentus* reproduces hybridogenetically; that is, they exclude one of their parental genomes during gametogenesis (either the R or the L genome) and pass the remaining one clonally to their gametes. Hybridogenesis enables hybrids to reproduce via back-crossing with the parental species that provides the genome excluded from the hybrid germline. Because the genome provided by the syntopic parental species undergoes Mendelian inheritance (e.g. Schmeller et al. 2001), reproduction of *P. esculentus* is typically hemiclonal.

Hybrid lineages of *P. esculentus* vary in a type of gamete production and the population type. In principle, there are two basic types in which *P. esculentus* parasitize a sexual species. In the so-called L-E system *P. esculentus* is present of both sex. Diploid (LR) hybrids eliminate the L genome from their germline and transmit the R genome clonally to

their gametes. Mating between hybrids and syntopic *P. lessonae* generate a new generation of *P. esculentus*. In some cases, matings between two LR hybrids resulted in a *P. ridibundus* progeny (Tunner 1978, Kotlík 1996, Vorburger 2001, Christiansen 2009). However, this progeny is mostly inviable or sterile due to accumulation of deleterious mutations in clonal genomes (Berger 1976, Graf and Müller 1979, Uzzell 1982, Vorburger et al. 2001a, Guex et al. 2002).

In the R-E system hybrids live together with *P. ridibundus* that serves as the host species. In some populations the hybrids are of male sex only and clonally transmit mostly the L genome (Uzzell et al. 1977, Günther and Plötner 1988, Plötner and Grunwald 1991, Doležálková et al. 2016).

Compared to other hemiclonal taxa (*Poeciliopsis, Squalius*) that form clonal gametes using mitotic division with unipolar spindle (Schultz 1961, Cimino 1972), water frog hybrids undergo meiotic division. In some cases, however, both parental genomes enter meiosis (Doležálková et al. 2016). The occasional recombination between parental genomes in hybrids gave it the name "*leaky*" hybridogenetic system (Uzzell et al. 1977). Still, the phenomenon remains controversial as no recombination events have been reported in experimental crosses so far (Schmeller 2004).

In this study we focused on the R-E populations from the Oder River drainage. In this system, where a hybrid taxon is present in a male sex only, unisexual *P. esculentus* has to compete with *P. ridibundus* males for eggs from its conspecific females. We used crossing experiments and microsatellite data to study the reproductive patterns of hybrid males. Specifically, we studied: i) the type of produced gametes; and ii) the genotype and sex of progeny to understand the potential evolutionary impact of the progeny on the stability and maintenance of the R-E populations.

Material and Methods

Sample sites

The *P. esculentus* males used for the crosses originated from two populations of the R-E system near the upper Oder River (Albrechtičky, Košatka; Figure 1). The *P. ridibundus* females and one *P. ridibundus* male were collected in two R-E system populations (Dolní Benešov and Košatka; Figure 1) and in two pure *ridibundus*-populations (Cítov and Liběchov; Figure 1). Frogs were sampled during spring 2013, at night, by hand and using a flashlight. Sex and taxon idendity were determined according to phenotypic characters (e.g. Berger 1988, Plötner 2005). A tissue sample for DNA analysis was taken from all frogs by toe clipping. Individuals suitable for crossings were selected on the basis of their size, health condition, and, in case of females, the presence of eggs. All females were individually tagged with transponders (micro transponder ID 111 ISO from Animal ID, s.r.o.) and released into outdoor cages after the crossing experiments. Permits for crossing frogs and rearing tadpoles were obtained from Swiss authorities (Gaston Denis-Guex, experimental permit 119/2013: TV 5113 and TH 103).

Design of crossing experiments

In order to determine the type of gametes produced by LR males we crossed each hybrid male with two RR females. To estimate the impact of the mitochondrial genome on the development of progeny we crossed RR females with either species-specific (*ridibundus*,) or introgressed (*lessonae* or *kurtmuelleri*-specific) mtDNA. As a control, a single RR female was crossed with a RR male to estimate the effects of the maternal genome on the peformance of progeny (the crossing scheme is presented in Figure S1, for labelling of adult frogs see Table S2).

Crossing experiments

Crosses were performed using the artificial fertilization procedure according to Berger et al. (1994) with some modifications (Pruvost et al. 2013). Males were euthanized in a buffered (*p*H = 7.0) 2 mg/l MS-222 solution (Sigma A-5040, St. Gallen, Switzerland); their testes were removed and stored in a Petri dish with Holtfreter's solution (pH = 7.4) until usage. Then females were triggered to ovulate by an injection of salmon luteinizing releasing hormone (LHRH, Sigma L4897, Prague, Czech Republic). For this purpose 2 mg hormone were diluted in 100 ml Holtfreter's solution; per 10 g of body mass 0.1 ml of this solution were injected into the abdominal cavity. After 16-18 hours, ovulation was checked by pressing the female's belly carefully between the thumb and the index finger of the left hand and opening the cloaca with a curved forceps. Ovulation was indicated when some eggs came out of the cloaka. Females that did not ovulate received a second hormone injection.

To produce sperm solutions, testes were sliced and crushed in a new Petri dish containing aged tap water. Eggs from one female were gently stripped into the sperm solutions and covered with aged tap water. Fertilization success (FS), was indicated by ~~a~~ rotation of eggs that turned the black animal hemisphere to the top within 10-30 min after fertilization. The next day, eggs were checked again under a microscope for a presence of the fertilization membrane (FM) that indicates successful penetration of a sperm into the egg. All eggs were photographed and transferred to 1.5 l plastic boxes (20x12x7cm), whichcontained 0.8 litres of aged tap water. After 2 days, unfertilized eggs, egg yolk, and/or aborted embryos were carefully removed and this process repeated every second day to avoid bacterial and fungal development causing embryonic mortality (EM). Some malformed embryos were stored in 70% or 96% ethanol for later analyses. After about 12 days, when the embryos started to reach the free swimming stage (stage 25, Gosner 1960), boxes were photographed and tadpoles were transfered to 6000 l outdoor containers filled with pond water and steamed

straw. Crosses with more than 60 tadpoles were evenly distributed to two boxes to reduce inter-individual competition during development. After about 50 days, when tadpoles started to metamorphose, individuals that exhibited at least one forelimb were caught and measured, weighted and transferred to Petri dishes, which contained a piece of damp cotton, where they completed metamorphosis. Tadpole mortality (TM) was estimated from the number of tadpoles that died after stage 25. Fully metamorphosed frogs that completely resorbed the tail were measured, weighted again and later sexed and genotyped. Individuals of the two crosses that produced most progeny, were toe-clipped and released to outdoor cages for further rearing.

Sex determination in juveniles

Three to ten days after completing metamorphosis, juveniles were anesthetized in a 2 mg/l MS-222 solution and dissected. Sex was determined by the morphology of both, the left and the right gonads. If a gonad was malformed, or insufficently developed, the juvenile was marked as "0" sex. For all individuals a photo documentation of their gonads was issued.

DNA extraction and analysis of mtDNA and microsatellites

DNA was extracted from a piece of web, or muscle tissue, using a commercial kit (NucleoSpin, Macherey-Nagel GmbH and Co.) using automated pipetting system epMotion 5075 (Eppendorf). Amplification and sequencing of the mitochondrial ND3 gene was done as described in Plötner et al. (2008). We also amplified 15 microsatellite loci in two multiplexes. Multiplex 1: Res20 (Zeisset et al. 2000), RlCA1b5, RlCA5 and RICA18 (Garner et al. 2000), Ga1a19 (Arioli et al. 2010), RICA2a34 (Christiansen and Reyer 2009), Rrid013A (Hotz et al. 2001, Garner et al. 2000), Res14. Multiplex 2: Res22 (Zeisset et al. 2000), Rrid059A and Rrid169A (Christiansen and Reyer 2009), Re1Caga10 and Re2Caga3 (Arioli et al. 2010),

RlCA1b6 (Arioli et al. 2010), Rrid082A (Table S3). We followed Pruvost et al. (2013) for species-specific characterization of these markers. PCR was done according to the protocol of Christiansen and Reyer (2009). Fragment-length analyses were performed on an ABI 3730 Avant capillary sequencer (Applied Biosystems, Foster City, California, USA) with an internal size standard (GeneScan-500 LIZ, Thermo Fisher Scientific); the alleles were scored with GeneMapper v. 3. 7 (Applied Biosystems, Zug, Switzerland).

Enzyme electrophoresis

For enzyme electrophoresis livers were homogenized on crushed ice for 20 s in an equal volume of Tris NaCl extraction buffer (pH 8.5, Valenta et al. 1971) using the homogenizator Ultra-Turrax (IKA-WERK). The homogenate was then centrifugated at 13,000 rpm at 4°C for 30 min. Enzymes obtained from these tissues were analysed by horizontal starch gel electrophoresis (Uzzell and Berger, 1975, Valenta et al. 1971). After electrophoresis was stoped, gels were sliced into three 2 mm thick layers and stained with allozyme-specific procedures similar to those described by Harris and Hopkinson, 1976, Buth and Murphy, 1980, Pasteur et al. 1987. Stained gels were photographed, enzyme patterns were recorded onpaper and the agar layers were transfered to a filter paper, dried and stored as a part of the protocol. The following polymorphic enzymes were visualized: aspartate aminotransferase (*Aat*, EC 2.6.1.1.), Glucose-6-phosphate isomerase (*Gpi*, EC 2.7.5.1.) and Lactate dehydrogenase (Ldh, EC 1.1.1.27.). Allele products were designated from „*a*“ to „*d*“ according to the electrophoretic mobility of the enzyme, i.e. the allele product with the highest anodal mobility was designated “*a*”. Samples with unclear results were reprocessed.

Microsatellite analysis and Genotyping

Raw microsatellite genotypes of *P. ridibundus* and *P. esculentus* parents were checked for homozygosity, genotyping errors and null alleles with Micro-Checker version 2. 2. 3 (Van Oosterhout et al. 2004). Hybrid genotypes were converted to separate L and R genotypes, following the procedure of Doležálková et al. (*in prep*.). To distinguish between clonal inherited and recombined genomes in F1 individuals we ran multilocus analysis to separate mulilocus genotypes (MLGs) using GeneClone v. 2. 0 (Arnaud-Haond and Belkhir 2007). To reveal a potential gene flow between L and R genomes we analyzed allele frequencies, heterozygosity, and polymorphism using GenAlEx v. 6. 41 (Peakall and Smouse 2006).

Genotypes of adult males and juveniles were identified using three polymorphic allozyme loci *Aat*, *Gpi,* and *Ldh-B* (Uzzell and Berger 1975) and additionally, on the basis of 17 species-specific mircosatellite loci.

Results

For interspecific backcrosses (B1) 26 adult frogs were used - 16 *P. esculentus* and 10 *P. ridibundus* individuals. Sample sites, taxon composition and sex are listed in Table 1.

Fertility of parents

All *P. esculentus* males had different sized and shaped testes; the right one was usually smaller than the left one. One male from Košatka (M7) was sterile, almost no spermatozoa was found in its small testes. Another male (M8) had only the left testis. Mating success of the other fertile *P. esculentus* males, one control *P. ridibundus* male, and *P. ridibundus* females were varied.

Mortality of B1 progeny

We crossed 16 *P. esculentus* and one *P. ridibundus* male (Figure 1) with nine *P. ridibundus* females. In total, we did 43 crosses (34 RR x RL crosses, nine control RR x RR crosses) involving 18,498 eggs (14,416 eggs in RR x RL crosses, 4,082 eggs in control crosses), which resulted in 514 B1 individuals. In RR x RL crosses, the number of embryos decreased from 14,088 to 1,653 during the development. In most cases, the eggs cleaved, but the zygotes died during the blastula stage.

Fertilization success (FS) was significantly lower in males M3, M6, M7, M8 and M15 compared to progeny of male M1 which reached the highest 90 % fertilization success. In female F8, no eggs were fertilized as indicated by the absence of a FM. The EM ranged from 36 to 100 % with an average of 88.5 %.

Families from two individuals, female F4 and male M7, showed the highest EM (100 %), in other families EM ranges between 36 % and 99 %. In RRxRL crosses, only 514 tadpoles successfully completed metamorphosis; the total TM was 68.9 %. The single surviving juvenile from cross 64-2013 escaped from a Petri dish before he completed metamorphosis, the progeny of males M2 and M6 died (100 % TM), as well as the progeny from a female F9 (detailed informations about mortality are given in Table 2).

The average weight of metamorphosed froglets ranged from 0.2 g to 0.9 g. In crosses 63-2013, 49-2013, and 39-2013 we observed abnormal size of metamorphosed froglets. Four hybrid froglets from crosses 63-2013 and 49-2013 weighted 1.2723g, 1.3481g, 1.2445g and 1.7781g, respectively, and three *P. ridibundus* forglets from crosses 49-2013 and 39-2013 weighted 2.0070 g, 2.6061 g and 3.4694 g, respectively.

Sex ratio in B1 progeny

The distribution of males and females in various crosses was uneven (Table 3). We took into consideration only those juveniles for which genetic data of taxon determination were available. Most crosses resulted in only male progeny. The only females were observed in crosses 59-2013 and 39-2013, while in crosses 27-2013, 30-2013, and 49-2013 males and females originated with sex ratios of approximately 1:1 (Table 3). Nine out of 12 hybrid males had only sons (male progeny), two males had mixed progeny of daughters and sons (M4, M11) and hybrid male M12 had two offsprings, both females.

Electrophoretic genotypes of B1 progeny

We genotyped 274 froglets, which were also sex-determined. According to the electropherograms of two diagnostic loci (*Ldh-B* and *Aat*) RR and LR genotypes were detected (Table 3). Genotype of *P. ridibundus* with a presence of pure R-specific alleles „*a*“ and „*c*“ and RL genotype of *P. esculentus* with a combination of R-specific „*a*“ and „*c*“ and L-specific alleles „*b*“ and „*d*“. In 24 juveniles belonging to RL genotypes, only the *lessonae*-specific “*b*” allele was expressed at the *Aat* locus. As a result of all of them being a progeny from one female (F1) they obtained the second L-specific allele from its mother. Data about the genotypes are presented in Table 3.

Inheritance of genomes and sex

Based on genotyped B1 progeny we distinguished three types of *P. esculentus* males producing different clonal gametes. While the majority of hybrid males (M1, M3, M5, M8, M9, M10, M13, M14, and M16) produced L gametes, two males (M4, M11) produced both L and R gametes, whereby the latter dominated quantitatively. From cross no. 59-2013 where male M12 was involved, only three RR genotypes originated. It cannot be excluded, however,

that this male also produced L gametes beside R gametes. The combination of L sperm with R eggs resulted in only RL males, while the R sperm combined with R eggs exclusively yielded in RR females (Table 3). In four juveniles, gonads were not fully developed, therefore it was difficult to determine their sex. Informations about sex of B1 progeny are presented in Table 3.

Multi locus genotypes

Among 220 juveniles and 25 adult individuals in 17 families, we have found alleles representing RR and RL genotypes. Species specificity of amplified alleles are listed in Table 4. We detected two types of multilocus genotypes (MLGs) in progeny. The first type of MLGs was observed in R genomes of *P. ridibundus* adult females and *P. esculentus* sons, where various alleles amplified throughout 15 analysed loci, suggesting sexual recombinant gametes. The second type of MLGs was observed in L genomes of *P. esculentus* and R genome of *P. ridibundus* daughters, suggesting clonal inheritance. Detailed data are presented in Table S4.

Discussion

An old view regarding the asexual organisms as the evolutionary death ends has been overcome as soon as the ability of asexuals to create a genetic variability was discovered (Alves et al. 2002, Christiansen, 2009). Here we present another reproductive strategy of a hemiclonal hybrid lineage, playing a key role i a dynamics of a mixed population system they live in.

Development of B1 progeny

From 14088 RRxRL fertilized eggs only 514 froglets finished the metamorphosis successfully. Mortality of progeny fathered by R-E males was significantly higher (88,3 %) in the initial phases of embryogenesis (EM) than in latter phases of tadpole development (TM, 3.6 %). This high mortality of cleaved embryos overcame observed mortality rate of 26 % (Uzzell et al. 1977) in *P. esculentus* males from R-E system in Germany. B1 progeny of hybrid males from western Germany used in crossing experiments by Kawamura and Nishioka (1986) showed, in one case, similar mortality to our results (90 %), the other ones varied in mortality between 8 % and 73 %. In hybrid males from Osternienburg and Ullnitz, Berger and Gunther (1991) noticed quite a low EM 13 - 32% compared to higher TM 53 - 99%. We separated mortality of EM and TM to take into consideration that factorscausing mortality might vary during development. During the initial stages of early embryonic development only maternal genes (mRNAs and ribosomes) are expressed whereas the expression of paternal genes starts in late blastula (Stick and Dreyer 1989 in Schatten 2012). The outcoming incompatibility of L and R genome in blastula stage may explain observed interrupted or malformed development of cleaved embryos and high EM. Later, when embryo finishes neurulation and arising tadpole reaches stage 25, rather ecological than genetic factors influence the rate of mortality (TM). Omitting the biotic factors like infections (Semlitsch and Reyer 1992, Tietje and Reyer 2004, Pruvost et al. 2013) observed in their seminatural experiments, factors like competition, density of tadpoles, temperature and water level can influence the amount of succesfully metamorphosed froglets. Hotz and collegues (1999) added a food supply as an imporatnt factor influencing the rearing of tadpoles. Even though rearing conditions can influence the size of metamorhed froglets, they would not explain seven oversized tadpoles that we found. Normally, after finishing the metamorphosis weight ranged from 0.2 to 0.8g in both *P. ridibundus* and *P. esculentus* froglets. In four giant

P. esculentus (1.2 – 1.8 g) and four giant *P. ridibundus* (2.0 – 3.5 g) individuals we got at least four-times heavier weights that we expected. Guex and colleques (2001) noticed 11 giant tadpoles (out of 3293 reared ones) in total in their two-year long experiment, all belonging to *P. esculentus* genotype. Contrary to our tadpoles, these gigants were trapped in stage 36 and did not reach the metamorphosis. Similar cases of giant *P. ridibundus* tadpoles were occasionally found in natural populations (Borkin et al. 1982). Even though it is known that the size of tadpoles is physiologically regulated by growth hormones and other cooperating gene products of hypothalamus and thyroid glands (Guex et al. 2001) it is difficult to identify a single cause of tadpole gigantism.

As to the mitochondrial genome, in most families the EM was lower in progeny that possesed *P. ridibundus* mtDNA (crosses 30-2013, 32-2013, 39-2013, 40-2013, 41-2013, 42-2013, 70-2013). This result does not correlate with the findings of Plenet et al. (2000), who proved better performance of juveniles with *P. lessonae* mtDNA in hypoxic conditions. Plötner et al. (2008) screened European populations of PEC and found that *P. lessonae* mtDNA pre-dominate in hybrid individuals and can work successfully in *P. ridibundus* too. Nevertheless, our data did not confirm the expected better effectiveness of *P. lessonae* mtDNA in larval development.

Inheritance of L and R genomes

Inheritance patterns of hybrid males in our crossing experiments matched the expected results of described inheritance in R-E system. We found 10 hybrid males producing L sperm, one producing R sperm (male M12) and two males producing both L and R sperm. Male M12 was crossed with three different females, but only three juveniles developped succesfully. All three froglets presented *P. ridibundus* genotype, but with respect to a low portion of cleaved embryos and high mortality it is difficult to classify reproductive mode its father. The only

case of R-E hybrid male producing R gametes was noticed by Berger and Günther (1991-1992). According to phenotype, they observed 100 % of *P. ridibundus* progeny from backcrossing with *P. ridibundus* female. Many other laboratory experiments mentioned production of both types of gametes (Vinogradov et al. 1991, Christiansen 2009) but those hybrids came from populations where triploid individuals coexist. Only four studies found such amphispermic males in R-E populations with diploid unisexual male hybrids namely atat German localities near the Alte Oder and one locality near the Elbe River (Uzzell et al. 1977, Berger and Günther 1991-1992, Günther and Plötner 1988, Ragghianti et al. 2007). A close proximity of these populations with similar reproductive mode in the region of Central Europe may suggest their shared origin, however, no phylogenetic data are yet available.

As in sperm-dependent female parthenogens, one would assume a selective evolutionary preference of only those *P. esculentus* males able to produce 100 % L gametes leading to 100 % hybrid-male progeny. Indeed, males usually transmit preferentially L genome to the gametes (Uzzell et al. 1977, Berger and Günther 1991-1992, Vinogradov et al. 1991, Ragghianti et al. 2007). Still, at least a part of arising progeny was represented by a sexual *P. ridibundus* daughters. Based on our results, one quarter of hybrids (25%) can form both types of progeny, *P. esculentus* and *P. ridibundus*, The expectation also does not correlate with our findings at amphispermic males related to a ratio of a gamete production (3L : 7R on average; 33 *P. esculentus* sons and 69 *P. ridibundus* daughters). The data show that amphispermic males are not as rare as it was thought. Moreover, we hypothesize that a production of both types of gametes is not a failure during gametogenesis but a reproductive strategy of unisexual males. Unlike sperm-dependent asexual females having relatively unlimited source of sexual males able to repeatedly reproduce during a reproductive season, *Pelophylax* females do reproduce once *per* season. Production of *P. ridibundus* daughters thus

may increase i. a number of females in mixed populations and ii. success of hybrid males to find a female mate.

Multilocus genotype analysis of microsatellite data confirmed that both types of genomes, L and R, were inherited clonally. In 2011, Schmidt *et al*. showed a similar pattern of inheritance we know from *Pelophylax* water frogs on carp gudgeon of the genus *Hypseleotris* based on a field data. Here, two groups of hybrids exhibit two different types of hybridogenetic elimination. The first group, largely female-biased, eliminate paternal recombined genome and by backcrossing with sexual male restore hybrid progeny. The second group, largely male-biased, eliminate maternal recombined genome and form male hybrid progeny. Crossing experiments of water frogs allowed us to detect another phenomenon of genome inheritance between generations. The amphispermic *P. esculentus* males receive R genome from recombinant egg of sexual *P. ridibundus*. Because R and L genome do not recombine during hybrid spermatogenesis, these males retain such R genome for a single generation and return it in a clonal form back to a sexual *P. ridibundus* population (through *P. ridibundus* daughter progeny).

Schmidt *et al*. (2011) further suggested that paternal genome includes Y chromosome. Coupling of hemiclonal genome with sex chromosome (X or Y) is also expected in *Pelophylax* water frogs (Graf and Polls-Pelaz 1989). Determination of sex through dissection of 278 froglets from 17 families indicated male sex determination system (XX-XY) with the binding of parental genomes to sex. With the exception of four froglets with undeveloped gonads we determined 207 *P. esculentus* juveniles as males and 67 *P. ridibundus* juveniles as females, linking the L genome to male sex and R genome to female sex. Inheritance of L hemiclonal genome to sons and R genome to daughters was first revealed by Uzzell et al. (1977). Since then, few examples of ambiguous results in sex-determined progeny were detected. Three hybrid females received the L genome from their R-E hybrid fathers (Uzzell

et al. 1977), four hybrid males received the R genome from their R-E hybrid father and one hybrid male received the R genome from their L-E hybrid father (Ragghianti et al. 2007). Such exceptional causes might be explained by sex reversal (Wallace et al. 1999, Ogata et al. 2003).

Introgression of nuclear genes in hybrids

To detect introgression of nuclear genes in any of the two parental genomes (R, L) we used three allozyme and 15 microsatellite loci as species-specific markers. In allozymes, approximately half of the *P. esculentus* progeny of three different families (25-2013, 26-2013, 27-2013) expressed two L-specific alleles in *AaT* locus. One "*b*" allele belonged to hybrid father as was confirmed by the allozyme analysis and the other "*b*" allele should be inheritted from the sexual mother. It is obvious, that this *P. ridibundus* mother had introgressed the L allele because all juveniles with "*bb*" profile were her sons. In microsatellites, out of 245 analyzed animals, only three *P. esculentus* juveniles possesed recombined genotypes at a given locus (one R-specific allele from *P. ridibundus* mother one R-specific allele from *P. esculentus* father). Previous studies showed that introgression of nuclear markers between *P. lessonae* and *P. ridibundus* can be bi-directional (Uzzell et al. 1977, Spasic-Boskovic et al. 1999, Schmeller et al. 2005, Mikulíček et al. 2014). Based on allozyme loci, a level of recombination ranged between 2-3% (Schmeller et al. 2005, Plötner et al. 2008) with a higher rate of introgression into *P. ridibundus* genome (Uzzell et al. 1977, Hotz 1983, Vorburger et al. 2001b, Guex et al. 2002, Choleva 2004, Mezhzherin et al. 2004). Additionally, some rare introgression of R-specific alleles between two R hemiclonal genomes in homospecific crosses was documented (Vorburger et al. 2001b). Other method using AFLP confirmed higher introgression of L-specific markers into *P. ridibundus* (10%) than vice versa (6%) (Mikulíček et al. 2014).

If we consider that such horizontal transfer of nuclear genes between two genomes is linked with a disturbed meiotic division of *P. esculentus* hybrids (Pagano and Schmeller 1999, Abbott et al. 2013, Doležálková et al. 2016) we might guess if any other asexual hybrid can recombine. Barbiano *et al*. 2013 documented high levels of genetic variation among asexual individuals of *P. formosa*, which was attributed to recombination. Turner and collegues (1980) came with the hypothesis that the ancestral *P. formosa* might have been a sexually reproducing hybrid for some time before becoming gynogenetic. Barbiano *et al*. (2013) alternatively explained it as some form of asexual recombination, most likely mitotic gene conversion that may have caused such genetic variation, as similarly proposed in unisexual lizards of the genus *Darevskia* (Kupryianova 2009). Other example was documented in sexual-asexual complexes of American ants, which are characterised by social hybridogenesis. Laboratory experiments demonstrated that any way sterile hybrid females with meiotic oogenesis are able to, in certain circumstances, produce fertile hybrid males with recombined genomes. Backcrossing with a sexual queen might lead to introgression of foreign genes to a pure sexual lineage and compromise a formation of a new colony (Eyer et al. 2013).

Coclusion

In general, clonal hybrids are considered genetically conservative organisms with a tendency to accumulate deleterious. Hemiclonal hybrids partialy solve the problem of genetic uniformity by mating backwards with a sexual species. Incorporation of sexual recombined gamete to a hybrid genotype provides a sufficient degree of genetic variability in progeny, which ensures them a long-term existence. In this way they became existentially dependent on sexual partners and used to behave as sexual parasites (Avise 2008). Despite the hundreds of crossing experiments based on hybrid inheritance done in the past, the hybrid life-strategies

are not fully understood. A common feature for hybridogenetic taxa is that one parental genome is trapped in hybrids as a hemiclone, while the other is renewed each generation from sexual species. In this regard, hybrid males from the Oder River basin exhibit a new evolutionary strategy of inheritance as they can return the borrowed sexual genome back to the sexual population in one generation, in a form of viable and fertile sexual females. In a newly arised sexual genotype, R genome frozen as a hemiclone changes the role again and most likely undergoes recombination in *P. ridibundus* gametogenesis again. Up-to-date there existed some indications that clonal genome can be returned back to sexual population, but nobody expected it to be such a common phenomenon. Whether the *P. esculentus* males´ ability to form new sexual females is beneficial for hybrids or not, remains unclear, because females are produced at the expense of hybrid genotype. On the other hand, hybrid males produce potencial sexual partners for themselves and for the future generation of hybrid males in this way. Our experimental data illustrate a new manner by which asexuals maintain themselves bringing us, as pointed by Bengtsson (2009) deeper insights concerning the question of what it means to be a eukaryote.

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Tables

Table 1. Information on sampling locations, sex, genotype, mitochondrial (mt) DNA and number (N) of sampled individuals of two *Pelophylax* taxa.

Population type	Sample site	Latitude, longitude	Sex	Genotype	MtDNA	N
R-E	Albrechtičky	49°42'29"N, 18°5'56"E	M	RL	rid	1
	Dolní Benešov	49°54‘44‘‘N, 18°07‘12‘‘E	F	RR	kurt	1
	Košatka	49°44'10"N, 18°9'15"E	F	RR	rid	3
					kurt	1
			M	RR	rid	1
				RL	rid	13
					kurt	2
R	Cítov	50°21'59"N, 14°26'49"E	F	RR	les	3
	Liběchov	50°24'37"N, 14°27'12"E	F	RR	les	1

R-E, *P. ridibundus-P. esculentus* population; R, pure *P. ridibundus* population; RR, *P. ridibundus*; RL, *P. esculentus*; rid, *P. ridibundus* like mtDNA; kurt, *Rana kurmuelleri* like mtDNA; les, *P. lessonae* like mtDNA.

Table 2. Mortality of B1 progeny sorted by males. Cross ID = identification number for a cross, ID male = identification number for a male, MtDNA = mitochondrial DNA of the male, N = the amount of juveniles, 1st day = the first day after fertilization, 3rd day = the third day after fertilization, stage 25 = free swimming stage of the juvenile, Met. = metamorphosed froglets, Fert. Rate = a fertilization rate, EM = an embryonic mortality, TM = a tadpole mortality.

			N					**EM**		**TM**	
Cross ID	**Male**	**MtDNA**	**1st day**	**3rd day**	**stage 25**	**Met.**	**Fert. Rate %**	**N**	**%**	**N**	**%**
25-2013	M1	rid	675	**633**	253	**137**	90	422	62	116	46
32-2013			672	**672**	433	**32**	70	239	36	401	93
40-2013	M2	rid	363	**363**	6	**0**	<10	357	98	6	100
44-2013			351	**321**	0	**0**	50	351	100		
56-2013	M3	rid	398	**393**	0	**0**	0	398	100		
65-2013			685	**684**	3	**0**	<1	682	99	2	67
39-2013	M4	rid	413	**369**	216	**23**	20	197	47	193	89
49-2013			220	**220**	48	**2**	<10	172	78	46	96
52-2013			266	**263**	0	**0**	0	266	100		
42-2013	M5	rid	396	**396**	96	**66**	<10	300	76	30	31
46-2013			134	**133**	0	**0**	50	134	100		
28-2013	M6	rid	436	**417**	3	**0**	<1	433	99	3	100
29-2013			393	**390**	0	**0**	0	393	100		
61-2013	M7	kurt	331	**330**	0	**0**	0	331	100		
71-2013			312	**311**	0	**0**	<1	312	100		
53-2013	M8	rid	309	**308**	3	**2**	<1	306	99	1	33
62-2013			435	**382**	0	**0**	0	435	100		
41-2013	M9	rid	287	**287**	150	**64**	50	137	48	76	51

45-2013			378	**352**	0	**0**	0	378	100		
57-2013	M10	rid	394	**393**	8	**3**	<1	386	98	5	62
66-2013			489	**485**	57	**33**	1-5	432	88	24	42
27-2013	M11	rid	476	**476**	48	**29**	<10	428	90	19	39
30-2013			601	**599**	83	**76**	<1	518	86	7	8
59-2013	M12	rid	451	**451**	7	**3**	<1	444	98	4	57
68-2013			29	**29**	0	**0**	0	29	100		
69-2013			508	**483**	1	**1**	<1	507	99	0	0
60-2013	M13	kurt	541	**534**	45	**20**	50	496	92	25	56
70-2013			521	**517**	139	**0**	20	382	73	139	100
54-2013	M14	rid	227	**226**	5	**2**	<1	222	98	3	60
63-2013			578	**578**	23	**3**	<10	555	96	20	87
55-2013	M15	rid	476	**466**	0	**0**	<1	476	100		
64-2013			556	**526**	3	**1**	<1	553	99	2	67
26-2013	M16	rid	612	**598**	23	**17**	30	575	96	5	22
31-2013			503	**503**	0	**0**	<1	503	100		

Table 3. Inheritance of gametes and sex in B1 progeny. Genotype of progeny was determined using allozymes. Cross ID = identification number for a cross, Undev = undeveloped gonads, juveniles with unknown sex.

			RR			**RL**		
Cross ID	**Male**	**Gamete**	♂	♀	**Undev**	♂	♀	**Undev**
25-2013	M1	**L**				20		
32-2013		**L**				3		
65-2013	M3	**L**				1		
49-2013	M4	**L, R**		1		1		
39-2013		**R**		22				
42-2013	M5	**L**				64		
53-2013	M8	**L**				2		
41-2013	M9	**L**				20		
57-2013	M10	**L**				3		
66-2013		**L**				20		2
27-2013	M11	**L, R**		14	1	11		
30-2013		**L, R**		28		21		
59-2013	M12	**R**		2	1			
60-2013	M13	**L**				19		
54-2013	M14	**L**				2		
63-2013		**L**				3		
26-2013	M16	**L**				17		

L, *P. lessonae* specific genome;R, *P. ridibundus*specific genome.

Table 4. Polymorphic microsatellite loci used in this study

Locus	L specific alleles	R specific alleles	unspecified alleles
RICA2a34	144	—	
RICA5	262	—	
Rrid013A	301	287/293	
Ga1a19	197	203/207/211/224/245/249/251	
RICA18	188	—	176
RICA1b5	120	134/136/140	
Res14	140	146/150	
Res20	124	—	
Re1Caga10	—	106/108/110/113/115/117/119/122/127/133/137	
Re2Caga3	—	173/204/212/223/234/236/239	
RICA1b6	—	80/83/86/90/96/98	87
Res22	—	85/90/106/108/113/120/130	
Rrid059A	—	129/131/135/137/142	
Rrid082A	—	163/139/178/182/184	
Rrid169A	—	187/189/190/192/195/197/204/208	

Supplementary material

F/M	RR 1	RL 1	RL 2	RL 3	RL 4	RL 5	RL 6	RL 7	RL 8	RL 9	RL 10	RL 11	RL 12	RL 13	RL 14	RL 15	RL 16
RR 1	10-2013	25-2013	26-2013	27-2013	28-2013												
RR 2	16-2013	32-2013	31-2013	30-2013	29-2013												
RR 3	47-2013					39-2013	40-2013	41-2013	42-2013								
RR 4	48-2013					49-2013											
RR 5	50-2013					52-2013											
RR 6	51-2013						44-2013	45-2013	46-2013								
RR 7	58-2013									53-2013	54-2013	55-2013	56-2013	57-2013	59-2013	60-2013	61-2013
RR 8	67-2013									62-2013	63-2013	64-2013	65-2013	66-2013	68-2013		
RR 9	72-2013														69-2013	70-2013	71-2013

Figure S1: Crossing design scheme with noted crosses ID (i. a. 10-2013). F = female, M = male, RR = *P. ridibundus* male, red colour = *P. ridibundus* like mtDNA, green colour = *P. lessonae* like mtDNA, grey colour = *P. kurtmuelleri* like mtDNA.

Table S2. A list of abbreviations representing adult frogs used for crosses.

Male	ID	Female	ID
RR 1	13CZ3WF21M	**RR 1**	13CZ1WF3F
RL 1	13CZ3WF28M	**RR 2**	13CZ3WF10F
RL 2	13CZ5WF1M	**RR 3**	13CZ3WF11F
RL 3	13CZ3WF40M	**RR 4**	13CZ1WF4F
RL 4	13CZ3WF33M	**RR 5**	54F3 (2012)
RL 5	13CZ3WF31M	**RR 6**	13CZ2WF1F
RL 6	13CZ3WF29M	**RR 7**	13CZ4WF1F
RL 7	13CZ3WF38M	**RR 8**	13CZ3WF3F
RL 8	13CZ3WF32M	**RR 9**	13CZ3WF2F
RL 9	13CZ3WF36M		
RL 10	13CZ3WF44M		
RL 11	13CZ3WF45M		
RL 12	13CZ3WF30M		
RL 13	13CZ3WF39M		
RL 14	13CZ3WF42M		
RL 15	13CZ3WF43M		
RL 16	13CZ3WF35M		

RR = *Pelophylax ridibundus*, RL = *P. esculentus*.

Table S3. Informations about microsatellite loci used. Repeat = minimum and maximum number of repeats in each locus, specifity = species specific amplification of alleles in loci known from literature, RR = *Pelophylax ridibundus*, LL = *P. lessonae*.

			Repeat		Specifity	
	Locus	**Label**	**Min.**	**Max.**	**RR**	**LL**
Multiplex 1	Res20	red	106	146	nonamplifying	polymorfic
	RlCA1b5	yellow	118	138	polymorfic	polymorfic
	Ga1a19	blue	199	255	polymorfic	monomorfic
	RlCA18	yellow	169	188	nonamplifying	polymorfic
	RlCA5	green	232	264	polymorfic	polymorfic
	RICA2a34	green	106	160	polymorfic	polymorfic
	Rrid013A	red	275	299	polymorfic	polymorfic
	Res14	blue	133	150	polymorfic	unknown
Multiplex 2	Rrid082A	yellow	161	184	polymorfic	unknown
	Res22	yellow	83	133	polymorfic	nonamplifying
	Rrid059A	green	111	139	polymorfic	monomorphic
	Rrid169A	green	181	214	polymorfic	nonamplifying
	Re1Caga10	blue	97	140	polymorfic	polymorfic
	RlCA1b6	red	74	108	polymorfic	polymorfic
	Re2Caga3	red	161	251	polymorfic	nonamplifying

Table S4. Microsatellite data of 245 individuals divided into 17 families and two multiplexes (Multiplex 1 and 2). Each line represents one individual (ID), its sex and genotype (Gen). A single locus has alleles separated to two columns, the first allele is R-specific, the second one is L-specific. Each family starts with two lines of crossed adult frogs (i. a. 13CZ3WF28M as a male and 13CZ1WF3F as a female) and continue with juveniles (i. a. 25_2013JUV1).

Multiplex 1

ID	Sex	Gen	RICA2a34		RICA5		Rrid013A		Ga1a19		RICA18		RICA1b5		Res14		Res20	
13CZ3WF28M	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	150	140	0	124
13CZ1WF3F	F	RR	0	0	0	0	287	287	207	207	0	0	136	136	146	146	0	0
25_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
25_2013JUV10	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV11	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV12	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
25_2013JUV13	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
25_2013JUV14	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
25_2013JUV15	M	RL	0	144	0	262	287	301	207	197	176	188	136	120	0	140	0	0
25_2013JUV16	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
25_2013JUV17	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
25_2013JUV18	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
25_2013JUV19	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
25_2013JUV2	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV20	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
25_2013JUV3	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
25_2013JUV4	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV5	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV6	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV7	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV8	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
25_2013JUV9	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
13CZ5WF1M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
13CZ1WF3F	F	RR	0	0	0	0	287	287	207	207	0	0	136	136	146	146	0	0
26_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV2	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV3	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV10	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV11	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
26_2013JUV12	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
26_2013JUV13	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
26_2013JUV14	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
26_2013JUV15	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	0
26_2013JUV16	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
26_2013JUV17	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26_2013JUV4	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124

26_2013JUV5	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
26_2013JUV6	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
26_2013JUV7	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV8	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
26_2013JUV9	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
13CZ3WF40M	M	RL	0	144	0	262	293	301	245	197	0	188	136	120	146	140	0	124
13CZ1WF3F	F	RR	0	0	0	0	287	287	207	207	0	0	136	136	146	146	0	0
27_2013JUV10	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	0	140	0	124
27_2013JUV11	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV12	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV13	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV14	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV15	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV16	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV17	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
27_2013JUV18	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	1WF46	146	0	0
27_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
27_2013JUV21	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV22	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV23	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV24	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV25	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV26	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV27	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV28	F	RR	0	0	0	0	287	293	245	207	0	0	136	136	146	146	0	0
27_2013JUV2	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
27_2013JUV3	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
27_2013JUV4	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
27_2013JUV5	M	RL	0	0	0	0	0	0	207	197	0	188	0	0	0	0	0	124
27_2013JUV6	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
27_2013JUV7	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	0	140	0	124
27_2013JUV8	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
27_2013JUV9	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
13CZ3WF40M	M	RL	0	144	0	262	293	301	245	197	0	188	136	120	146	140	0	124
13CZ3WF10F	F	RR	0	0	0	0	287	293	207	224	0	0	136	136	150	150	0	0
30_2013JUV1	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV10	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV10B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV11	F	RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30_2013JUV11B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV12B	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30_2013JUV13	F	RR	0	0	0	0	287	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV13B	M	RL	0	144	0	262	293	301	0	0	0	0	136	120	150	140	0	124
30_2013JUV14	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124

30_2013JUV14B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV17	F	RR	0	0	0	0	287	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV18	F	RR	0	0	0	0	287	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV19	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV1B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV2	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV20	F	RR	0	0	0	0	293	293	0	0	0	0	0	0	150	146	0	0
30_2013JUV21	F	RR	0	0	0	0	287	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV22	M	RL	0	144	0	262	293	301	0	0	0	0	0	0	150	140	0	124
30_2013JUV23	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV24	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV25	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV2B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV3	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV32	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV33	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30_2013JUV38	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	0	146	0	0
30_2013JUV3B	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV4	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV4B	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV5	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV5B	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV6	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV6B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV7	F	RR	0	0	0	0	287	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV7B	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV8	F	RR	0	0	0	0	293	293	0	0	0	0	136	136	150	146	0	0
30_2013JUV8B	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV9B	M	RL	0	144	0	262	293	301	0	0	0	188	136	120	150	140	0	124
30_2013JUV12	F	RR	0	0	0	0	293	293	245	207	0	0	136	136	150	146	0	0
30_2013JUV15	F	RR	0	0	0	0	293	293	245	224	0	0	136	136	150	146	0	0
30_2013JUV16	F	RR	0	0	0	0	293	293	245	224	0	0	136	136	150	146	0	0
30_2013JUV9	F	RR	0	0	0	0	293	293	245	224	0	0	136	136	150	146	0	0
13CZ3WF28M	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	150	140	0	124
13CZ3WF10F	F	RR	0	0	0	0	287	293	207	224	0	0	136	136	150	150	0	0
32_2013JUV1B	M	RL	0	144	0	262	293	301	224	197	0	188	136	120	150	140	0	0
32_2013JUV1	M	RL	0	144	0	262	287	301	224	197	0	188	136	120	150	140	0	0
32_2013JUV2	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	150	140	0	0
13CZ3WF31M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	0
13CZ3WF11F	F	RR	0	0	0	0	287	293	207	251	0	0	140	136	150	146	0	0
39_2013JUV10	F	RR	0	0	0	0	287	293	251	207	0	0	136	136	150	150	0	0
39_2013JUV11	F	RR	0	0	0	0	287	293	251	207	0	0	140	136	150	150	0	0
39_2013JUV13	F	RR	0	0	0	0	287	293	0	207	0	0	140	136	150	150	0	0
39_2013JUV14	F	RR	0	0	0	0	287	287	0	207	0	0	136	136	150	146	0	0

39_2013JUV15	F	RR	0	0	0	0	287	287	251	207	0	0	136	136	150	146	0	0
39_2013JUV16	F	RR	0	0	0	0	287	293	0	207	0	0	140	136	150	150	0	0
39_2013JUV17	F	RR	0	0	0	0	287	287	0	207	0	0	136	136	150	146	0	0
39_2013JUV18	F	RR	0	0	0	0	287	287	251	207	0	0	140	136	150	150	0	0
39_2013JUV19	F	RR	0	0	0	0	287	287	251	207	0	0	140	136	150	150	0	0
39_2013JUV1B	F	RR	0	0	0	0	287	293	0	207	0	0	136	136	150	150	0	0
39_2013JUV1	F	RR	0	0	0	0	287	293	251	207	0	0	136	136	150	146	0	0
39_2013JUV20	F	RR	0	0	0	0	287	293	251	207	0	0	136	136	150	150	0	0
39_2013JUV2B	F	RR	0	0	0	0	287	293	0	207	0	0	140	136	150	146	0	0
39_2013JUV2	F	RR	0	0	0	0	287	293	251	207	0	0	140	136	150	150	0	0
39_2013JUV3B	F	RR	0	0	0	0	287	293	251	207	0	0	140	136	150	150	0	0
39_2013JUV4B	F	RR	0	0	0	0	287	287	0	207	0	0	136	136	150	146	0	0
39_2013JUV5B	F	RR	0	0	0	0	287	287	0	207	0	0	136	136	150	150	0	0
39_2013JUV6B	F	RR	0	0	0	0	287	287	0	207	0	0	136	136	150	150	0	0
39_2013JUV7B	F	RR	0	0	0	0	287	287	0	207	0	0	140	136	150	146	0	0
39_2013JUV8B	F	RR	0	0	0	0	287	287	251	207	0	0	140	136	150	150	0	0
39_2013JUV9B	F	RR	0	0	0	0	287	293	0	207	0	0	136	136	150	150	0	0
13CZ3WF38M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
13CZ3WF11F	F	RR	0	0	0	0	287	293	207	251	0	0	140	136	150	146	0	0
41_2013JUV10	M	RL	0	144	0	262	293	301	207	197	0	188	140	120	146	140	0	124
41_2013JUV11	M	RL	0	144	0	262	287	301	251	197	0	188	136	120	150	140	0	124
41_2013JUV12	M	RL	0	144	0	262	287	301	251	197	0	188	136	120	150	140	0	124
41_2013JUV13	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	150	140	0	124
41_2013JUV14	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	146	140	0	124
41_2013JUV15	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	124
41_2013JUV16	M	RL	0	144	0	262	0	0	0	197	0	0	0	120	150	140	0	0
41_2013JUV17	M	RL	0	144	0	262	287	301	207	197	0	0	136	120	150	140	0	0
41_2013JUV18	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	150	140	0	0
41_2013JUV19	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	146	140	0	0
41_2013JUV20	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	150	140	0	0
41_2013JUV9	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	150	140	0	124
41_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	150	140	0	124
41_2013JUV2	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	150	140	0	124
41_2013JUV3	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	146	140	0	124
41_2013JUV4	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	146	140	0	124
41_2013JUV5	M	RL	0	144	0	262	287	301	251	197	0	188	136	120	146	140	0	124
41_2013JUV6	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	150	140	0	124
41_2013JUV7	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	124
41_2013JUV8	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	150	140	0	124
13CZ3WF32M	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	0	140	0	124
13CZ3WF11F	F	RR	0	0	0	0	287	293	207	251	0	0	140	136	150	146	0	0
42_2013JUV1	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	150	140	0	124
42_2013JUV1B	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	150	140	0	124
42_2013JUV2	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	150	140	0	124

42_2013JUV2B	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	146	140	0	124
42_2013JUV3	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
42_2013JUV3B	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	146	140	0	124
42_2013JUV4	M	RL	0	144	0	262	287	301	207	197	0	188	140	120	150	140	0	124
42_2013JUV4B	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	146	140	0	124
42_2013JUV5	M	RL	0	144	0	262	293	301	207	197	0	188	136	120	146	140	0	124
42_2013JUV5B	M	RL	0	144	0	262	287	301	251	197	0	188	136	120	146	140	0	124
42_2013JUV6	M	RL	0	144	0	262	293	301	207	197	0	188	140	120	150	140	0	124
42_2013JUV6B	M	RL	0	144	0	262	293	301	251	197	0	188	136	120	150	140	0	124
42_2013JUV7B	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	150	140	0	124
42_2013JUV8B	M	RL	0	144	0	262	293	301	251	197	0	188	140	120	150	140	0	124
42_2013JUV9B	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	150	140	0	0
13CZ3WF31M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	124
13CZ1WF4F	F	RR	0	0	0	0	287	287	207	224	0	0	136	136	150	146	0	0
49_2013JUV1	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	146	140	0	124
49_2013JUV2	F	RR	0	0	0	0	287	287	224	207	0	0	136	136	150	146	0	0
13CZ3WF36M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	124
13CZ4WF1F	F	RR	0	0	0	0	287	287	203	249	0	0	140	136	150	146	0	0
53_2013JUV1	M	RL	0	144	0	262	287	301	249	197	0	0	136	120	146	140	0	124
53_2013JUV2	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	150	140	0	124
13CZ3WF44M	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	146	140	0	124
13CZ4WF1F	F	RR	0	0	0	0	287	287	203	249	0	0	140	136	150	146	0	0
54_2013JUV1	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
54_2013JUV2	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13CZ3WF39M	M	RL	0	144	0	262	287	301	207	197	0	188	134	120	150	140	0	124
13CZ4WF1F	F	RR	0	0	0	0	287	287	203	249	0	0	140	136	150	146	0	0
57_2013JUV1	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
57_2013JUV2	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
57_2013JUV3	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
13CZ3WF42M	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	150	140	0	124
13CZ4WF1F	F	RR	0	0	0	0	287	287	203	249	0	0	140	136	150	146	0	0
59_2013JUV1	F	RR	0	0	0	0	287	287	211	203	0	0	136	136	150	150	0	0
59_2013JUV2	F	RR	0	0	0	0	287	287	211	203	0	0	136	136	150	150	0	0
59_2013JUV3	F	RR	0	0	0	0	287	287	0	211	0	0	140	136	150	150	0	0
13CZ3WF43M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	150	140	0	124
13CZ4WF1F	F	RR	0	0	0	0	287	287	203	249	0	0	140	136	150	146	0	0
60_2013JUV10	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
60_2013JUV11	M	RL	0	144	0	262	287	301	249	197	0	188	140	120	150	140	0	124
60_2013JUV12	M	RL	0	144	0	262	287	301	249	197	0	188	136	120	146	140	0	124
60_2013JUV13	M	RL	0	144	0	262	287	301	203	197	0	188	140	120	146	140	0	124

60_2013JUV14	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	146	140	0	124
60_2013JUV16	M	RL	0	144	0	262	287	301	203	197	0	188	140	120	150	140	0	124
60_2013JUV17	M	RL	0	144	0	262	287	301	249	197	0	188	140	120	150	140	0	124
60_2013JUV18	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	146	140	0	0
60_2013JUV19	M	RL	0	144	0	262	287	301	249	197	0	188	136	120	150	140	0	0
60_2013JUV2	M	RL	0	144	0	262	287	301	249	197	0	188	140	120	150	140	0	124
60_2013JUV20	M	RL	0	144	0	262	287	301	203	197	0	188	140	120	150	140	0	124
60_2013JUV3	M	RL	0	144	0	262	287	301	203	197	0	188	140	120	150	140	0	124
60_2013JUV4	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	150	140	0	124
60_2013JUV5	M	RL	0	144	0	262	287	301	249	197	0	0	136	120	146	140	0	124
60_2013JUV6	M	RL	0	144	0	262	287	301	203	197	0	0	0	120	146	140	0	124
60_2013JUV8	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	150	140	0	124
60_2013JUV9	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	146	140	0	124
60_2013JUV15	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	146	140	0	124
60_2013JUV1	M	RL	0	144	0	262	287	301	249	197	0	188	140	120	146	140	0	124
13CZ3WF44M	M	RL	0	144	0	262	287	301	203	197	0	188	136	120	146	140	0	124
13CZ3WF3F	F	RR	0	0	0	0	287	287	207	211	0	0	136	136	146	146	0	0
63_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
63_2013JUV2	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
63_2013JUV3	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
13CZ3WF30M	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
13CZ3WF3F	F	RR	0	0	0	0	287	287	207	211	0	0	136	136	146	146	0	0
65_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
13CZ3WF39M	M	RL	0	144	0	262	287	301	207	197	0	188	134	120	150	140	0	124
13CZ3WF3F	F	RR	0	0	0	0	287	287	207	211	0	0	136	136	146	146	0	0
66_2013JUV1	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	124
66_2013JUV2	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	0
66_2013JUV3	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV4	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV5	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV6	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV7	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
66_2013JUV10	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV11	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
66_2013JUV12	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	0
66_2013JUV13	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
66_2013JUV14	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV15	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV16	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
66_2013JUV17	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV18	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV19	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV20	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0

66_2013JUV21	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	0
66_2013JUV22	M	RL	0	144	0	262	287	301	207	197	0	188	136	120	146	140	0	0
66_2013JUV8	M	RL	0	144	0	262	287	301	211	197	0	188	136	120	146	140	0	124
66_2013JUV9	M	RL	0	144	0	262	287	301	0	0	0	188	136	120	146	140	0	124

F = female, M = male,RR = *Pelophylax ridibundus*, RL = *P. esculentus*.

Multiplex 2

ID	Sex	Gen	Re1Caga10		Re2Caga3		RICA1b6		Res22		Rrid059A		Rrid082A		Rrid169A	
13CZ3WF28M	M	RL	127	0	173	0	83	0	113	0	137	0	163	0	192	0
13CZ1WF3F	F	RR	117	115	223	223	96	83	113	113	135	131	178	178	197	187
25_2013JUV1	M	RL	115	0	223	0	83	0	113	0	131	0	178	0	187	0
25_2013JUV10	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV11	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV12	M	RL	117	0	223	0	83	0	113	0	0	0	178	0	187	0
25_2013JUV13	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV14	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV15	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV16	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV17	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV18	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV19	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV2	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV20	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25_2013JUV3	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV4	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV5	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV6	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	187	0
25_2013JUV7	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV8	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
25_2013JUV9	M	RL	117	0	223	0	83	0	113	0	131	0	178	0	187	0
13CZ5WF1M	M	RL	106	0	173	0	83	0	106	0	135	0	163	0	195	0
13CZ1WF3F	F	RR	117	115	223	223	96	83	113	113	135	131	178	178	197	187
26_2013JUV1	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	187	0
26_2013JUV2	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
26_2013JUV3	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	187	0
26_2013JUV10	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
26_2013JUV11	M	RL	115	0	223	0	83	0	113	0	0	0	178	0	197	0
26_2013JUV12	M	RL	117	0	223	0	83	0	113	0	0	0	178	0	197	0
26_2013JUV13	M	RL	115	0	223	0	83	0	113	0	0	0	178	0	187	0
26_2013JUV14	M	RL	115	0	223	0	83	0	113	0	0	0	178	0	187	0
26_2013JUV15	M	RL	117	0	223	0	83	0	113	0	0	0	178	0	187	0

26_2013JUV16	M	RL	115	0	223	0	83	0	113	0	0	0	178	0	197	0
26_2013JUV17	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26_2013JUV4	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	187	0
26_2013JUV5	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
26_2013JUV6	M	RL	115	0	223	0	83	0	113	0	131	0	178	0	187	0
26_2013JUV7	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	187	0
26_2013JUV8	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
26_2013JUV9	M	RL	115	0	223	0	83	0	113	0	131	0	178	0	197	0
13CZ3WF40M	M	RL	127	0	239	0	83	0	85	0	135	0	178	0	189	0
13CZ1WF3F	F	RR	117	115	223	223	96	83	113	113	135	131	178	178	197	187
27_2013JUV10	M	RL	117	0	223	0	83	0	113	0	131	0	178	0	187	0
27_2013JUV11	F	RR	127	115	239	223	96	0	113	85	135	131	178	178	189	187
27_2013JUV12	F	RR	127	117	239	223	96	0	113	85	135	131	178	178	197	189
27_2013JUV13	F	RR	127	117	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV14	F	RR	127	115	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV15	F	RR	127	115	0	0	96	0	113	85	0	0	178	178	197	189
27_2013JUV16	F	RR	127	115	239	223	96	0	113	85	0	0	178	178	197	189
27_2013JUV17	M	RL	117	0	223	0	83	0	113	0	0	0	178	0	197	0
27_2013JUV18	F	RR	127	115	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV1	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
27_2013JUV21	F	RR	127	115	0	0	96	0	113	85	0	0	178	178	189	187
27_2013JUV22	F	RR	127	115	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV23	F	RR	127	117	0	0	96	0	113	85	0	0	178	178	189	187
27_2013JUV24	F	RR	127	117	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV25	F	RR	127	117	239	223	96	0	113	85	0	0	178	178	189	187
27_2013JUV26	F	RR	127	115	0	0	96	83	113	85	0	0	178	178	189	187
27_2013JUV27	F	RR	127	115	0	0	96	83	113	85	0	0	178	178	189	187
27_2013JUV28	F	RR	127	115	0	0	96	0	113	85	0	0	178	178	189	187
27_2013JUV2	M	RL	117	0	223	0	83	0	113	0	131	0	178	0	197	0
27_2013JUV3	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
27_2013JUV4	M	RL	117	0	223	0	83	0	113	0	131	0	178	0	197	0
27_2013JUV5	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
27_2013JUV6	M	RL	117	0	223	0	83	0	113	0	135	0	178	0	197	0
27_2013JUV7	M	RL	115	0	223	0	83	0	113	0	131	0	178	0	187	0
27_2013JUV8	M	RL	115	0	223	0	83	0	113	0	131	0	178	0	187	0
27_2013JUV9	M	RL	115	0	223	0	83	0	113	0	135	0	178	0	197	0
13CZ3WF40M	M	RL	127	0	239	0	83	0	85	0	135	0	178	0	189	0
13CZ3WF10F	F	RR	115	106	232	173	83	90	113	113	135	135	182	178	204	197
30_2013JUV1	M	RL	115	0	173	0	83	0	113	0	135	0	182	0	189	197
30_2013JUV10	M	RL	115	0	232	0	83	0	0	0	135	0	178	0	204	0
30_2013JUV10B	M	RL	106	0	0	0	83	0	0	0	135	0	0	0	204	0
30_2013JUV11	F	RR	0	115	0	0	83	83	0	0	135	135	0	0	0	0
30_2013JUV11B	M	RL	115	0	232	0	83	0	0	0	135	0	182	0	204	0
30_2013JUV12B	M	RL	106	0	173	0	83	0	0	0	135	0	0	0	197	0

30_2013JUV13	F	RR	127	106	0	0	83	83	0	0	135	135	0	0	204	189
30_2013JUV13B	M	RL	115	0	0	0	83	0	0	0	135	0	0	0	204	0
30_2013JUV14	M	RL	115	0	232	0	83	0	0	0	135	0	182	0	197	0
30_2013JUV14B	M	RL	115	0	173	0	83	0	113	0	135	0	178	0	197	0
30_2013JUV17	F	RR	127	106	0	0	90	83	0	85	135	135	0	0	197	189
30_2013JUV18	F	RR	127	106	0	0	83	83	113	85	135	135	0	0	197	189
30_2013JUV19	F	RR	127	115	0	0	83	83	0	85	135	135	0	0	197	189
30_2013JUV1B	M	RL	106	0	232	0	83	0	113	0	135	0	0	0	204	0
30_2013JUV2	F	RR	127	106	239	173	83	83	113	85	135	135	182	178	204	189
30_2013JUV20	F	RR	127	115	0	0	83	83	0	0	135	135	0	0	204	189
30_2013JUV21	F	RR	127	106	0	0	83	83	113	85	135	135	0	0	204	189
30_2013JUV22	M	RL	115	0	234	0	83	0	0	0	135	0	0	0	197	0
30_2013JUV23	F	RR	127	106	0	0	83	90	0	85	135	135	0	0	204	189
30_2013JUV24	F	RR	127	115	0	0	83	83	0	0	135	135	0	0	197	189
30_2013JUV25	F	RR	127	106	0	0	83	90	113	85	135	135	0	0	204	189
30_2013JUV2B	M	RL	115	0	232	0	83	0	0	0	135	0	182	0	204	0
30_2013JUV3	F	RR	115	106	239	173	83	83	113	85	135	135	182	178	204	189
30_2013JUV32	M	RL	106	0	232	0	83	0	113	0	135	0	182	0	204	0
30_2013JUV33	M	RL	115	0	0	0	83	0	113	85	135	0	0	0	0	0
30_2013JUV38	F	RR	127	115	239	232	83	83	0	0	135	135	0	0	197	189
30_2013JUV3B	M	RL	106	0	173	0	83	0	113	0	135	0	182	0	204	0
30_2013JUV4	F	RR	115	106	239	173	83	83	113	85	135	135	182	178	204	189
30_2013JUV4B	M	RL	115	0	0	0	83	0	0	0	135	0	0	0	197	0
30_2013JUV5	M	RL	106	0	173	0	83	0	0	0	135	0	182	0	204	0
30_2013JUV5B	M	RL	106	0	234	0	83	0	113	0	135	0	0	0	197	0
30_2013JUV6	M	RL	106	0	232	0	83	0	0	0	135	0	182	0	197	0
30_2013JUV6B	M	RL	106	0	173	0	83	0	113	0	135	0	182	0	204	0
30_2013JUV7	F	RR	127	106	239	232	83	0	0	0	135	135	0	0	197	189
30_2013JUV7B	M	RL	115	0	173	0	83	0	0	0	135	0	178	0	197	0
30_2013JUV8	F	RR	127	115	239	173	83	0	0	0	135	135	0	0	204	189
30_2013JUV8B	M	RL	115	0	232	0	83	0	113	0	135	0	0	0	197	0
30_2013JUV9B	M	RL	115	0	232	0	83	0	113	0	135	0	182	0	204	0
30_2013JUV12	F	RR	127	115	0	239	0	98	113	85	135	135	182	178	204	187
30_2013JUV15	F	RR	127	115	0	239	90	98	113	85	0	0	178	178	204	187
30_2013JUV16	F	RR	127	106	239	232	90	98	113	85	0	0	182	178	204	187
30_2013JUV9	F	RR	127	106	239	173	0	98	113	85	135	135	182	178	204	187
13CZ3WF28M	M	RL	127	0	173	0	83	0	113	0	137	0	163	0	192	0
13CZ3WF10F	F	RR	115	106	232	173	83	90	113	113	135	135	182	178	204	197
32_2013JUV1B	M	RL	106	0	173	0	83	0	113	0	0	0	182	0	204	0
32_2013JUV1	M	RL	115	0	173	0	83	0	113	0	0	0	182	0	204	0
32_2013JUV2	M	RL	115	0	173	0	83	0	113	0	0	0	182	0	204	0
13CZ3WF31M	M	RL	117	0	173	0	83	0	113	0	142	0	182	0	189	0
13CZ3WF11F	F	RR	115	115	173	204	0	80	113	85	135	135	182	169	192	192
39_2013JUV10	F	RR	0	117	173	173	98	80	113	85	0	0	182	182	192	190

39_2013JUV11	F	RR	117	115	173	204	86	80	113	113	0	0	182	169	192	190
39_2013JUV13	F	RR	117	115	173	204	98	80	113	113	0	0	182	169	192	190
39_2013JUV14	F	RR	0	117	173	204	98	80	113	85	0	0	182	182	192	190
39_2013JUV15	F	RR	0	117	173	204	98	80	113	85	0	0	182	169	192	190
39_2013JUV16	F	RR	0	117	173	204	98	80	113	113	0	0	182	182	192	190
39_2013JUV17	F	RR	0	117	0	0	98	80	113	113	0	0	182	182	192	190
39_2013JUV18	F	RR	0	117	0	0	98	80	113	113	0	0	182	169	192	190
39_2013JUV19	F	RR	0	117	0	0	83	80	113	113	0	0	182	169	192	190
39_2013JUV1B	F	RR	117	115	173	173	98	80	113	85	142	135	182	182	192	190
39_2013JUV1	F	RR	117	115	173	204	98	80	113	85	142	135	182	169	192	190
39_2013JUV20	F	RR	0	117	173	204	98	80	113	113	0	0	182	182	192	190
39_2013JUV2B	F	RR	0	117	173	204	98	80	113	85	142	135	182	182	192	190
39_2013JUV2	F	RR	0	117	173	173	98	80	113	85	142	135	182	182	192	190
39_2013JUV3B	F	RR	0	117	173	173	98	80	113	113	142	135	182	182	192	190
39_2013JUV4B	F	RR	0	117	173	173	98	80	113	85	142	135	182	182	192	190
39_2013JUV5B	F	RR	117	115	173	204	0	80	113	85	0	0	182	182	192	190
39_2013JUV6B	F	RR	0	117	173	204	98	80	113	113	0	0	182	182	192	190
39_2013JUV7B	F	RR	117	115	173	204	98	80	113	113	142	135	182	169	192	190
39_2013JUV8B	F	RR	117	115	173	204	98	80	113	85	142	135	182	169	192	190
39_2013JUV9B	F	RR	0	117	173	173	98	80	113	85	142	135	182	169	192	190
13CZ3WF38M	M	RL	106	0	223	0	83	0	106	0	137	0	163	0	197	0
13CZ3WF11F	F	RR	115	115	173	204	80	80	113	85	135	135	182	169	192	192
41_2013JUV10	M	RL	0	0	173	0	0	0	113	0	135	0	169	0	192	0
41_2013JUV11	M	RL	0	0	173	0	0	0	113	0	135	0	182	0	192	0
41_2013JUV12	M	RL	115	0	204	0	0	0	85	0	135	0	182	0	192	0
41_2013JUV13	M	RL	0	0	204	0	0	0	0	0	135	0	169	0	192	0
41_2013JUV14	M	RL	115	0	173	0	0	0	0	0	135	0	182	0	192	0
41_2013JUV15	M	RL	0	0	204	0	0	0	0	0	135	0	169	0	192	0
41_2013JUV16	M	RL	0	0	173	0	0	0	85	0	0	0	169	0	192	0
41_2013JUV17	M	RL	0	0	173	0	0	0	85	0	0	0	182	0	192	0
41_2013JUV18	M	RL	0	0	204	0	0	0	85	0	0	0	169	0	192	0
41_2013JUV19	M	RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41_2013JUV20	M	RL	115	0	204	0	0	0	113	0	0	0	169	0	192	0
41_2013JUV9	M	RL	115	0	204	0	0	0	85	0	135	0	169	0	192	0
41_2013JUV1	M	RL	115	0	204	0	0	83	113	0	135	0	182	0	192	0
41_2013JUV2	M	RL	0	0	173	0	0	83	113	0	135	0	182	0	192	0
41_2013JUV3	M	RL	115	0	173	0	0	83	85	0	135	0	182	0	192	0
41_2013JUV4	M	RL	115	0	204	0	0	83	113	0	135	0	182	0	192	0
41_2013JUV5	M	RL	115	0	204	0	0	83	85	0	135	0	182	0	192	0
41_2013JUV6	M	RL	0	0	173	0	0	83	85	0	135	0	182	0	192	0
41_2013JUV7	M	RL	115	0	173	0	0	83	85	0	135	0	182	0	192	0
41_2013JUV8	M	RL	0	0	204	0	0	83	113	0	135	0	182	0	192	0
13CZ3WF32M	M	RL	119	0	0	0	83	0	130	0	135	0	178	0	189	0
13CZ3WF11F	F	RR	115	115	173	204	80	80	113	85	135	135	182	169	192	192

42_2013JUV1	M	RL	115	0	173	0	0	0	113	0	135	0	169	0	192	0
42_2013JUV1B	M	RL	115	0	173	0	0	0	113	0	135	0	182	0	192	0
42_2013JUV2	M	RL	115	0	204	0	0	0	113	0	135	0	169	0	192	0
42_2013JUV2B	M	RL	115	0	173	0	0	0	113	0	135	0	182	0	192	0
42_2013JUV3	M	RL	115	0	204	0	0	0	85	0	135	0	182	0	192	0
42_2013JUV3B	M	RL	115	0	173	0	0	0	113	0	135	0	182	0	192	0
42_2013JUV4	M	RL	115	0	173	0	0	0	85	0	135	0	169	0	192	0
42_2013JUV4B	M	RL	115	0	173	0	0	0	85	0	135	0	182	0	192	0
42_2013JUV5	M	RL	0	0	204	0	0	0	113	0	135	0	182	0	192	0
42_2013JUV5B	M	RL	115	0	173	0	0	0	0	0	135	0	169	0	0	0
42_2013JUV6	M	RL	115	0	204	0	0	0	85	0	135	0	169	0	192	0
42_2013JUV6B	M	RL	115	0	173	0	0	0	113	0	135	0	169	0	192	0
42_2013JUV7B	M	RL	115	0	173	0	0	0	85	0	135	0	182	0	192	0
42_2013JUV8B	M	RL	115	0	173	0	0	0	113	0	135	0	169	0	192	0
42_2013JUV9B	M	RL	115	0	204	0	0	0	85	0	135	0	182	0	192	0
13CZ3WF31M	M	RL	117	0	173	0	83	0	113	0	142	0	182	0	189	0
13CZ1WF4F	F	RR	133	113	212	173	83	88	113	113	135	135	178	163	192	192
49_2013JUV1	M	RL	113	0	173	0	83	0	0	0	135	0	178	0	192	0
49_2013JUV2	F	RR	133	117	173	173	83	83	113	113	142	135	182	163	192	190
13CZ3WF36M	M	RL	117	0	236	0	83	0	85	0	137	0	184	0	208	0
13CZ4WF1F	F	RR	115	106	204	173	83	83	113	108	137	137	182	169	208	204
53_2013JUV1	M	RL	115	0	173	0	83	0	85	0	137	0	182	0	208	0
53_2013JUV2	M	RL	106	0	204	0	83	0	108	0	137	0	182	0	208	0
13CZ3WF44M	M	RL	108	0	173	0	83	0	130	0	129	0	182	0	192	0
13CZ4WF1F	F	RR	115	106	204	173	83	83	113	108	137	137	182	169	208	204
54_2013JUV1	M	RL	106	0	204	0	83	0	0	0	137	0	169	0	208	0
54_2013JUV2	M	RL	106	0	204	0	83	0	113	0	137	0	0	0	208	0
13CZ3WF39M	M	RL	110	0	173	0	83	0	130	0	137	0	182	0	187	0
13CZ4WF1F	F	RR	115	106	204	173	83	83	113	108	137	137	182	169	208	204
57_2013JUV1	M	RL	106	0	204	0	83	0	0	0	137	0	0	0	204	0
57_2013JUV2	M	RL	106	0	173	0	83	0	108	0	137	0	182	0	208	0
57_2013JUV3	M	RL	106	0	204	0	83	0	0	0	137	0	0	0	0	0
13CZ3WF42M	M	RL	113	0	204	0	83	0	113	0	137	0	163	0	189	0
13CZ4WF1F	F	RR	115	106	204	173	83	83	113	108	137	137	182	169	208	204
59_2013JUV1	F	RR	113	106	204	204	83	83	113	113	137	137	182	163	189	208
59_2013JUV2	F	RR	115	113	204	204	83	83	113	108	0	0	182	163	189	204
59_2013JUV3	F	RR	113	115	204	204	0	96	113	113	0	0	182	163	189	208
13CZ3WF43M	M	RL	115	0	173	0	83	0	85	0	135	0	163	0	204	0
13CZ4WF1F	F	RR	115	106	204	173	83	83	113	108	137	137	182	169	208	204
60_2013JUV10	M	RL	115	0	173	0	83	0	108	0	137	0	169	0	204	0

60_2013JUV11	M	RL	106	0	173	0	83	0	113	0	137	0	169	0	204	0
60_2013JUV12	M	RL	106	0	204	0	83	0	108	0	137	0	169	0	204	0
60_2013JUV13	M	RL	106	0	204	0	83	0	113	0	135	0	182	0	208	0
60_2013JUV14	M	RL	106	0	204	0	83	0	113	0	137	0	182	0	208	0
60_2013JUV16	M	RL	106	0	173	0	83	0	113	0	137	0	169	0	208	0
60_2013JUV17	M	RL	106	0	204	0	83	0	108	0	137	0	169	0	204	0
60_2013JUV18	M	RL	106	0	204	0	83	0	113	0	0	0	182	0	208	0
60_2013JUV19	M	RL	115	106	173	0	83	0	113	108	0	0	182	0	204	0
60_2013JUV2	M	RL	115	0	173	0	0	0	113	0	137	0	169	0	204	0
60_2013JUV20	M	RL	106	0	204	0	83	0	113	0	0	0	182	0	208	0
60_2013JUV3	M	RL	115	0	173	0	0	0	113	0	137	0	169	0	204	0
60_2013JUV4	M	RL	115	0	173	0	0	0	113	0	137	0	169	0	204	0
60_2013JUV5	M	RL	106	0	173	0	0	0	113	0	137	0	182	0	208	0
60_2013JUV6	M	RL	106	0	204	0	0	0	113	0	137	0	182	0	208	0
60_2013JUV8	M	RL	106	0	173	0	0	0	113	0	137	0	182	0	208	0
60_2013JUV9	M	RL	115	0	204	0	0	0	108	0	137	0	182	0	208	0
60_2013JUV15	M	RL	106	0	173	0	83	0	113	0	137	0	182	0	208	0
60_2013JUV1	M	RL	106	0	173	0	83	0	113	0	137	0	182	0	204	0
13CZ3WF44M	M	RL	108	0	173	0	83	0	85	0	129	0	182	0	192	0
13CZ3WF3F	F	RR	122	106	239	173	96	83	106	85	142	137	178	163	204	189
63_2013JUV1	M	RL	122	0	239	0	83	0	85	0	0	0	178	0	189	0
63_2013JUV2	M	RL	122	0	239	0	83	0	106	0	0	0	178	0	189	0
63_2013JUV3	M	RL	122	0	239	0	83	0	85	0	0	0	163	0	204	0
13CZ3WF30M	M	RL	137	0	204	0	83	0	113	0	135	0	163	0	192	0
13CZ3WF3F	F	RR	122	106	239	173	96	83	106	85	142	137	178	163	204	189
65_2013JUV1	M	RL	106	0	239	0	83	0	106	0	0	0	178	0	189	0
13CZ3WF39M	M	RL	110	0	173	0	83	0	130	0	137	0	182	0	189	0
13CZ3WF3F	F	RR	122	106	239	173	96	83	106	85	142	137	178	163	204	189
66_2013JUV1	M	RL	122	0	239	0	96	83	106	0	137	0	178	0	189	0
66_2013JUV2	M	RL	122	0	173	0	83	0	85	0	0	0	163	0	189	0
66_2013JUV3	M	RL	106	0	173	0	83	0	106	0	137	0	163	0	204	0
66_2013JUV4	M	RL	106	0	173	0	96	0	106	0	137	0	178	0	189	0
66_2013JUV5	M	RL	122	0	239	0	96	83	120	0	137	0	178	0	189	0
66_2013JUV6	M	RL	106	0	239	0	87	0	90	0	137	0	163	0	204	0
66_2013JUV7	M	RL	122	0	239	0	83	0	106	0	0	0	178	0	189	0
66_2013JUV10	M	RL	106	0	239	0	83	0	106	0	137	0	178	0	189	0
66_2013JUV11	M	RL	122	0	239	0	83	0	106	0	0	0	178	0	189	0
66_2013JUV12	M	RL	122	0	173	0	96	83	85	0	0	0	178	0	189	0
66_2013JUV13	M	RL	122	0	173	0	83	0	85	0	0	0	163	0	204	0
66_2013JUV14	M	RL	122	0	173	0	83	0	106	0	137	0	178	0	189	0
66_2013JUV15	M	RL	122	0	173	0	83	0	106	0	137	0	178	0	189	0
66_2013JUV16	M	RL	122	0	173	0	96	83	106	0	0	0	178	0	189	0
66_2013JUV17	M	RL	122	0	173	0	96	83	106	0	137	0	163	0	189	0

66_2013JUV18	M	RL	122	0	239	0	96	83	106	0	137	0	178	0	204	0
66_2013JUV19	M	RL	106	0	239	0	96	83	85	0	137	0	163	0	204	0
66_2013JUV20	M	RL	106	0	239	0	96	83	85	0	137	0	178	0	189	0
66_2013JUV21	M	RL	122	0	239	0	83	0	106	0	0	0	178	0	189	0
66_2013JUV22	M	RL	122	0	173	0	83	0	85	0	137	0	163	0	204	0
66_2013JUV8	M	RL	122	0	239	0	96	83	85	0	137	0	163	0	189	0
66_2013JUV9	M	RL	122	0	173	0	0	0	106	0	137	0	178	0	189	0

F = female, M = male,RR = *Pelophylax ridibundus*, RL = *P. esculentus*.