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Evoluce způsobů určování pohlaví a genomů u šupinatých plazů (Reptilia: Squamata)

Evolution of sex determining mechanisms in squamate reptiles (Reptilia: Squamata)

disertační práce

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Obsah

Abstrakt

Disertační práce se zabývá evolucí způsobů určování pohlaví a genomů u šupinatých plazů. Jejím základem jsou tři publikované práce a dva rukopisy. Evoluce pohlavně determinačních mechanismů, pohlavních chromosomů a genomů a jejich uspořádání byla studována na širší fylogenetické úrovni všech šupinatých plazů se začleněním dalších linií plazů a také na menší škále jako podrobné srovnání uvnitř vybraných linií. Problematika byla studována klasickými cytogenetickými metodami a metodami molekulární cytogenetiky, zejména s použitím fluorescenční *in situ* hybridizace na různých úrovních. Výsledky byly interpretovány v rámci fylogenetických postupů. Práce přispívá k hlubšímu porozumění principů určování pohlaví a evoluce tohoto fenoménu nejen u šupinatých plazů, ale i u amniotických obratlovců. Na základě výsledků získaných při řešení problematiky je možné konstatovat, že pohlavní chromosomy vznikaly v evoluci v jednotlivých skupinách amniotických obratlovců nezávisle. Tento vznik je v některých případech následován akumulací mikrosatelitových sekvencí na pohlavních chromosomech, jejichž charakter však není společný pro pohlavní chromosomy u různých fylogenetických linií. Uspořádání genomů šupinatých plazů vykazuje značnou míru konzervatismu a chromosomové přestavby mohou být posuzovány jako synapomorfie jednotlivých skupin. Ze srovnání vyplývá, že konzervativní uspořádání genomů je pravděpodobně společným rysem celé skupiny Sauropsida, nejen šupinatých plazů.

Abstract

This Ph.D. thesis is focused on the evolution of sex determining mechanisms and genomes in squamate reptiles. It is based on three published articles and two manuscripts. The evolution of sex determining mechanisms, sex chromosomes and genomes, and their organisation, was studied on a wide phylogenetic scale of the whole group of squamate reptiles and some lineages of other Sauropsids, as well as on the small phylogenetic range as a detailed comparative study inside individual lineages of squamates. This thesis is based upon the use of classical cytogenetic methods, methods of molecular cytogenetic (especially fluorescent *in situ* hybridisation) and the results were analysed using phylogenetic approaches. The results and outputs of this study represent an important contribution to the general knowledge of the principals of sex determination and the evolution of these phenomena not only in squamate reptiles but also in the whole group of amniotes. Using the results obtained during the work on this thesis we can conclude that sex chromosomes evolved in particular lineages of amniotes independently. This origin was in some cases followed by accumulation of microsatellite sequences on sex chromosomes, but their identity is not shared between sex chromosomes of individual lineages across the phylogenetic distribution. Comparison of genome organisation indicates the high degree of conservatism in squamates. A particular chromosome rearrangement could be considered as a synapomorhies of individual phylogenetic groups. Conservatism of genomes thus seems to be the common characteristic of the whole group of sauoropsids and not only the squamates.

Úvod

Disertační práce se zabývá evolucí způsobů určování pohlaví u šupinatých plazů a s tím spojenou evolucí uspořádání genomů dané skupiny. Studovaná problematika byla řešena na různých fylogenetických škálách, od srovnání na široké fylogenetické úrovni skupiny Sauropsida, zahrnující ptáky, krokodýli, želvy, hatérie a šupinaté plazy, až po detailní srovnání uvnitř vybraných linií šupinatých plazů. Základním metodickým přístupem bylo použití metod klasické i molekulární cytogenetiky a metod fylogenetické analýzy. Výsledky disertační práce mohou přispět k hlubšímu porozumění evoluce určování pohlaví, pohlavních chromosomů a uspořádání genomu do karyotypu a to nejen u šupinatých plazů, ale obecněji i u amniotických obratlovců.

Způsob určení pohlaví je pro gonochoristy základním biologickým procesem, který je bezpodmínečně nutný pro vývin jedince a pro ustanovení požadovaného poměru pohlaví v populaci (Fisher 1958). U živočichů se setkáváme se dvěma základními systémy determinace pohlaví. Prvním z nich je environmentálně určené pohlaví (ESD; z angl. „environmental sex determination“), v němž to, zda se vyvine ze zygoty samec či samice, není predikováno pohlavně specifickým genotypem zygoty, ale závisí na vnějších podmínkách. Druhým způsobem je genotypicky určené pohlaví (GSD; z angl. „genotypic sex determination“), kde o pohlaví rozhodují pohlaví determinující geny, jež jsou vázány na pohlavní chromosomy (Ohno 1967, Bull 1983).

Uvnitř každého z těchto základních typů rozlišujeme ještě několik možností, kterými může být o pohlaví rozhodnuto. V souvislosti s environmentálně určeným pohlavím u plazů většinou hovoříme o teplotně určeném pohlaví (TSD; z angl. „temperature-dependent sex determination“), protože z vnějších vlivů je to právě inkubační teplota během kritické periody po oplození, která nejčastěji rozhoduje o pohlaví vyvíjejícího se jedince (Bull 1983, Valenzuela et al. 2003).

Pohlavní chromosomy v genotypickém systému určení pohlaví mohou být morfologicky nerozlišené (homomorfní) nebo rozlišené (heteromorfní) (Ohno 1967, Bull 1983, Charlesworth 2002). I morfologicky nerozlišené chromosomy jsou definovány jako pohlavní, jestliže nesou geny určující pohlaví (Valenzuela et al. 2003). Od autosomů se tedy neodlišují tvarem ani velikostí, ale přítomností pohlaví determinujících genů.

Největší zájem je věnován otázkám možného přechodu mezi jednotlivými pohlavně determinačními mechanismy a také jejich významu pro živé organismy. Na

ESD a GSD je možné nahlížet jako na dva konce jednoho kontinua (Sarre et al. 2004) a v takovém pohledu tedy neexistují ostré hranice mezi jednotlivými systémy. Ty pak mohou volně přecházet jeden v druhý a dokonce mohou existovat intermediátní stavy. Druhou možností je přistupovat k ESD a GSD jako k relativně ostře vymezeným nezávislým stavům. Jsou popsané hypotézy a teoretické mechanismy vzniku a udržování genotypicky určeného pohlaví z původnějšího environmentálního systému (Ohno 1973, Bull 1983, Charlesworth & Charlesworth 2000, Charlesworth 2002). Existují ale i modely o přechodu mezi základními systémy určení pohlaví v opačném směru (Charnov & Bull 1977, Bull 1983, Conover 1984). Na ESD je možné nahlížet jako na vhodný způsob využití vývinové plasticity. V takovém případě by v daných podmínkách mohl vznikat fenotyp výhodný pro jedno pohlaví (Crews 2003). Spekuluje se také o tom, že ESD samice by mohly snáze manipulovat poměrem pohlaví svých potomků (Valenzuela et al. 2003, Kratochvíl et al. 2008). Na rozdíl od toho GSD zaručuje stabilní poměr pohlaví (odolný vůči klimatickým změnám prostředí) a vyrovnaný poměr pohlaví. O GSD se také uvažuje jako o vhodném nástroji k rozřešení intralokusového sexuálního konfliktu a vzniku pohlavně specializovaných genomů (Valenzuela et al. 2003).

Cílem předkládané práce bylo shrnout dosavadní znalosti o pohlavně determinačních mechanismech šupinatých plazů, tato data dále analyzovat za pomoci fylogenetických přístupů. Z takto získaných výsledků vysledovat vhodné kandidátní linie na různé fylogenetické škále, důležité pro podrobnější studii evoluce uspořádání genomu a pohlavně determinačních mechanismů a s použitím vhodných metodických nástrojů klasické a molekulární cytogenetiky získat nová experimentální data.

Práce se zabývají přednostně šupinatými plazy (Squamata) a to zejména z toho důvodu, že je to skupina, která reprezentuje většinu biodiverzity všech plazů, a je zároveň variabilní v počtech chromosomů a ve způsobech determinace pohlaví. Šupinatí plazi tak představují ideální modelovou skupinu pro výzkum obecnějších evolučních zákonitostí souvisejících se studovanou problematikou.

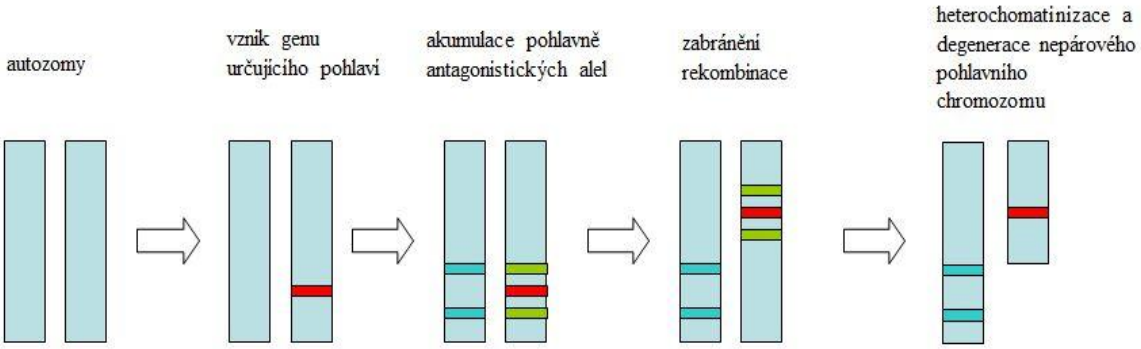
V první publikaci zařazené do disertační práce se zaměřujeme zejména na fenomén evoluce pohlavně determinačních mechanismů. Odpovědi na otázky, který ze systémů určení pohlaví je v evoluci původnější a jakým směrem se u jednotlivých skupin ubíraly přechody mezi jednotlivými pohlavně determinačními systémy, mohou přinést zejména fylogenetické analýzy. Jednu z prvních analýz věnující se studiu evoluce pohlavně determinačních mechanismů vytvořili Janzen & Paukstis (1991a) a

výsledkem bylo konstatování, že ESD spíše než GSD je ancestrálním stavem pro želvy. Ve stejném roce pak publikovali výsledky fylogenetické analýzy pro ještěry se stejným závěrem (Janzen & Paukstis 1991b). Výsledkem pozdější analýzy vytvořené pro všechny obratlovce (Janzen & Phillips 2006) bylo, že v rámci obratlovců je ancestrálním stavem GSD a ESD vznikalo v jednotlivých skupinách nezávisle, např. u společného předka želv. V rámci skupiny Squamata však nebyla ancestrální situace rozřešena.

Data o mechanismech určujících pohlaví nebyla v citovaných publikacích kompletní a to zejména pro šupinaté plazy. Rozhodli jsme se tedy shromáždit všechny dostupné informace o způsobech determinace pohlaví pro jednotlivé druhy šupinatých plazů a vytvořit vlastní fylogenetickou analýzu, která by mapovala ancestrální stavy těchto znaků. Z literatury jsme získali informace o způsobech určení pohlaví pro více než čtyři sta druhů šupinatých plazů. Informace jsme nejprve podrobili kritickému zhodnocení a vyloučili jsme data, u kterých bylo danému druhu přisouzeno GSD nebo TSD na základě nepříliš přesvědčivých experimentálních výsledků. Data jsme potom vynesli na fylogenetický strom studované skupiny a testovali jsme pravděpodobnost, s jakou je pro jednotlivé linie daný stav znaku (TSD, GSD, XY, ZW) ancestrální. Představa o fylogenetických vztazích šupinatých plazů nebyla v době přípravy publikace a není ani v současné době jednoznačně ustálená. V naší práci jsme tedy pracovali se třemi odlišnými fylogenetickými stromy. První z nich je založený převážně na kombinaci morfologických znaků (Estes et al. 1988), další dva jsou molekulární a ve většině větvení jsou ve shodě (Townsend et al. 2004, Vidal & Hedges 2005). Z výsledků naší analýzy a v zásadě už jen z nashromážděných dat vyplývá, že způsoby determinace pohlaví jsou v jednotlivých skupinách šupinatých plazů poměrně konzervativní. Toto zjištění je do určité míry v rozporu s obecně přijímanou avšak nepodloženou představou, že mechanismy určování pohlaví jsou u šupinatých plazů i v rámci jednotlivých čeledí značně variabilní. Z našich zjištění vyplývá, že celé velké skupiny, jako například leguáni (Iguanidae *sensu lato*), hadi, nebo ještěrkovití jsou v rámci své skupiny uniformní ve způsobu determinace pohlaví. Dobře podpořenou variabilitu v tomto znaku jsme pak pozorovali jen v rámci dvou skupin šupinatých plazů a to u agam (Agamidae) a gekonů (Gekkota). Po vyhodnocení výsledků fylogenetických analýz založených na všech alternativních fylogenetických stromech jsme dospěli k závěru, že je pro šupinaté plazi ancestrálním stavem pravděpodobně TSD a GSD tak vznikalo v jednotlivých skupinách nezávisle a pravděpodobně nedocházelo

k opakovaným návratům k TSD. Výsledky fylogenetických analýz nám umožnili formulovat hypotézu, podle které pohlavní chromosomy mohou plnit roli jakési evoluční pasti: jakmile v evoluci jednou vzniknou a jsou dostatečně diferencovány, již není možné je zcela ztratit a ony v takovém případě plní roli bariéry zabraňující návratu ke stavu vyznačujícího se jejich absencí, tedy i k ESD. Tato hypotéza poměrně dobře odpovídá obecně uznávané představě o vzniku a evoluci pohlavních chromosomů, jak je znázorněna na schématu (Obr. 1). Podle tohoto modelu vznikají pohlavní chromosomy z páru autosomů tak, že na jednom chromosomu vznikne alela genu, který je primárním spouštěčem kaskády vedoucí k diferenciaci gonád. K tomu může dojít například duplikací či jinou změnou genu, který se podílel na diferenciaci gonád před vznikem GSD, a který už pouhou změnou genové dávky převezme funkci v determinaci pohlaví. Obdobný mechanismus byl popsán např. u živorodých savců, kde roli genu, jehož přítomnost rozhoduje o samčí determinaci, převzal gen *SRY*, odvozený z genu *SOX3*, tedy genu, který je i u jiných obratlovců součástí genové rodiny účastnící se diferenciace gonád (Berta et al. 1990). Předpokládá se, že v této fázi diferenciace pohlavních chromosomů je stále ještě poměrně snadný návrat zpět k autosomům. V dalším kroku vývoje pohlavních chromosomů pak dochází k akumulaci pohlavně antagonistických alel na nově vzniklých pohlavních chromosomech (Rice 1984). Poté, co se na pohlavních chromosomech začnou akumulovat pohlavně antagonistické alely, je návrat ke stavu bez pohlavních chromosomů poměrně komplikovaný (Valenzuela et al. 2003). Budeme-li na příklad uvažovat alely zvýhodňující samce a znevýhodňující samice, je zřejmé, že tyto alely budou mít tendenci vázat se přednostně na pohlavní chromosom Y. Je tedy pravděpodobné, že při případném přechodu od pohlavních chromosomů zpět k ESD bude mít samec s ESD nutně nižší fitness v porovnání s XY samcem. Kvůli akumulaci pohlavně specifických alel pak dochází k potlačení rekombinace mezi párovým a nepárovým pohlavním chromosomem. Roli v této pokračující diferenciaci mohou mít delece, inverze nebo jiné přestavby suprimující rekombinaci. Nejčastěji navrhovaným mechanismem je pericentrická inverze uvnitř nepárového pohlavního chromosomu. Dojde-li k rekombinaci uvnitř invertovaného segmentu, budou produkovány gamety, jejichž chromosomy ponesou delece nebo duplikace. Po pericentické inverzi následuje takřka zpravidla degenerace a heterochromatinizace Y a W chromosomů, zatímco většina struktur na X a Z chromosomech zůstává zachována (Charlesworth 2002). Geny, které leží na X a Z chromosomech tvoří nepostradatelnou část genomu a jejich ztráta by mohla mít vážné

následky pro životaschopnost, proto se diferenciace vznikajícího heteromorfního páru týká pouze Y a W chromosomů. Ty jsou potom z velké části tvořeny vysoce repetitivními DNA sekvencemi (Ohno 1967). Rozsáhlá degenerace nepárového pohlavního chromosomu však není obecným jevem pro všechny skupiny živočichů; u některých zůstává Y nebo W chromosom stejně velký jako X a Z a nese řadu genů (Charlesworth 2002). Může však také docházet k akumulaci různých mikrosatelitových elementů na nepárovém pohlavním chromosomu, což může v některých případech vést i k tomu, že se tento chromosom stane největším chromosomem v karyotypu (Kubát et al. 2008)



Obr. 1 Obecný model vzniku a diferenciace pohlavních chromosomů.

Naše představa o pohlavních chromosomech jako evoluční pasti se tedy do značné míry shoduje s popsaným modelem vzniku pohlavních chromosomů. Abychom však mohli jednoznačně prokázat, že hypotéza je relevantní a abychom mohli říci víc o možnosti jednotlivých vzájemných přechodů pohlavně determinačních mechanismů, museli bychom vědět víc o homologii pohlavních chromosomů mezi jednotlivými skupinami šupinatých plazů. Pokud by se totiž experimentálně prokázalo, že pohlavní chromosomy jsou syntenní, jsou tvořeny shodným genetickým materiálem a nesou shodné geny napříč fylogenetickým spektrem u šupinatých plazů, museli bychom naši hypotézu o evoluční pasti opustit. Naším úsilím pro další studie se tedy stalo získat více informací o syntenii a homologii pohlavních chromosomů napříč skupinami šupinatých plazů a posoudit tak oprávněnost naší hypotézy.

Porovnáním syntenie nebo homologie pohlavních chromosomů mezi hlavními liniemi amniotických obratlovců se do současnosti zabývalo pouze několik prací. Bylo zjištěno, že některé geny, které leží na pohlavním chromosomu Z u kura domácího (*Gallus gallus*) a dalších ptáků, jsou přítomné na pohlavním chromosomu Z u gekona

Gekko hokouensis (Kawai et al. 2009). Při studiu pozoruhodného systému pohlavních chromosomů u ptakopyska (*Ornithorhynchus anatinus*) bylo také zjištěno, že homology genů z ptačího Z chromosomu jsou přítomné na několika jeho pohlavních chromosomech a také na autosomech (Rens et al. 2007). Tato zjištění pak vedla Graves (2009) k formulaci hypotézy, že pohlavní chromosomy mohou mít společný původ u předka všech amniotických obratlovců a tedy že ancestrálním typem určení pohlaví byl pro všechny amniotické obratlovce „ptačí ZW“ způsob determinace pohlaví. Tato představa pak byla stejnou skupinou autorů propagována v mnoha pracích, přestože některé studie přinášely výsledky, které se s touto hypotézou neshodovaly (Matsubara et al. 2006, Kawai et al. 2007), a získala si značnou popularitu. Zjištění, která nebyla zcela ve shodě s představou ancestrálního „ptačího ZW“ způsobu určení pohlaví u amniotických obratlovců pocházela i ze stejné laboratoře, kde se tato představa zrodila (Ezaz et al. 2009). Bylo prokázáno, že pohlavní chromosomy vznikly v jediné čeledi ještěrů, u agamovitých (Agamidae), minimálně dvakrát nezávisle (Ezaz et al. 2009). Homologií pohlavních chromosomů mezi ptáky a několika druhy hadů se také zabývala práce Matsubary et al. (2006), která dokázala, že pohlavní chromosomy ptáků a fylogeneticky odvozených hadů vznikly z různých párů autosomů. Nicméně ucelenější srovnávací studie o homologii a syntenii pohlavních chromosomů u obratlovců nebyla dosud publikována. Rozhodli jsme se tedy rozšířit spektrum informací o homologii pohlavních chromosomů na širší fylogenetické škále a testovat tak hypotézu o ancestrálním ZW způsobu determinace pohlaví a zároveň i naší hypotézu o pohlavních chromosomech jako evoluční pasti. Naše studie je podrobně popsána ve druhé kapitole disertační práce. Nezabývá se výhradně tématy spjatými s evolucí pohlavních chromosomů, ale řeší i otázku dynamiky karyotypových přestaveb u plazů. Metodicky je práce založena na jednoduchém principu, technické provedení bylo ale obtížnější. Pomocí sortovacího průtokového cytometru jsme měli vyizolovanou DNA z pohlavního chromosomu Z kura domácího a z ní připravenou sondu pro fluorescenční *in situ* hybridizaci (FISH). Sondu jsme aplikovali na metafáze 28 druhů šupinatých plazů celkově ze 17 čeledí představujících hlavní fylogenetické linie. Karyotyp se mezi těmito liniemi výrazně liší. Nicméně sonda z pohlavního chromosomu hybridizovala velmi přesvědčivě s dobře rozlišitelnou částí genomu u všech studovaných druhů. U těch druhů, kde jsou v karyotypu zastoupeny velké metacentrické chromosomy, hybridizovala sonda z ptačího Z vždy s kratším raménkem druhého největšího chromosomu v karyotypu. U těch druhů, kde je v karyotyp složen převážně, nebo

výhradně z akrocentrických chromosomů, hybridizovala sonda s párem středně velkých akrocentrických chromosomů. Z toho vyplývá, že část genomu homologická s ptačím Z chromosomem je napříč sauropsidními liniemi velice konzervativní. Pokud se ale vrátíme zpět k pohlavním chromosomům, z našich výsledků vyplývá, že u žádného z osmi studovaných druhů se známými a rozpoznatelnými pohlavními chromosomy, jsme nepozorovali žádnou homologii mezi těmito a ptačími pohlavními chromosomy. Jediným druhem se zmámou syntenií pohlavních chromosomů s ptačím Z pohlavním chromosomem tedy zůstává výše zmíněný *G. hokouensis* (Kawai et al. 2009). Z našeho pohledu se tedy hypotéza o ancestrálním „ptačím ZW" způsobu determinace pohlaví nesetkává s experimentální podporou, naopak výsledky naší práce a dalších výše popsaných studií zapadají do rámce naší představy, že pohlavní chromosomy vznikaly v jednotlivých liniích nezávisle. Hypotéza o ancestrálním „ptačím ZW" způsobu determinace pohlaví pro amniotické obratlovce je přinejmenším méně parsimonní, než kdybychom uvažovali jako ancestrální stav ESD a nezávislý vznik příslušných typů pohlavních chromosomů v jednotlivých liniích.

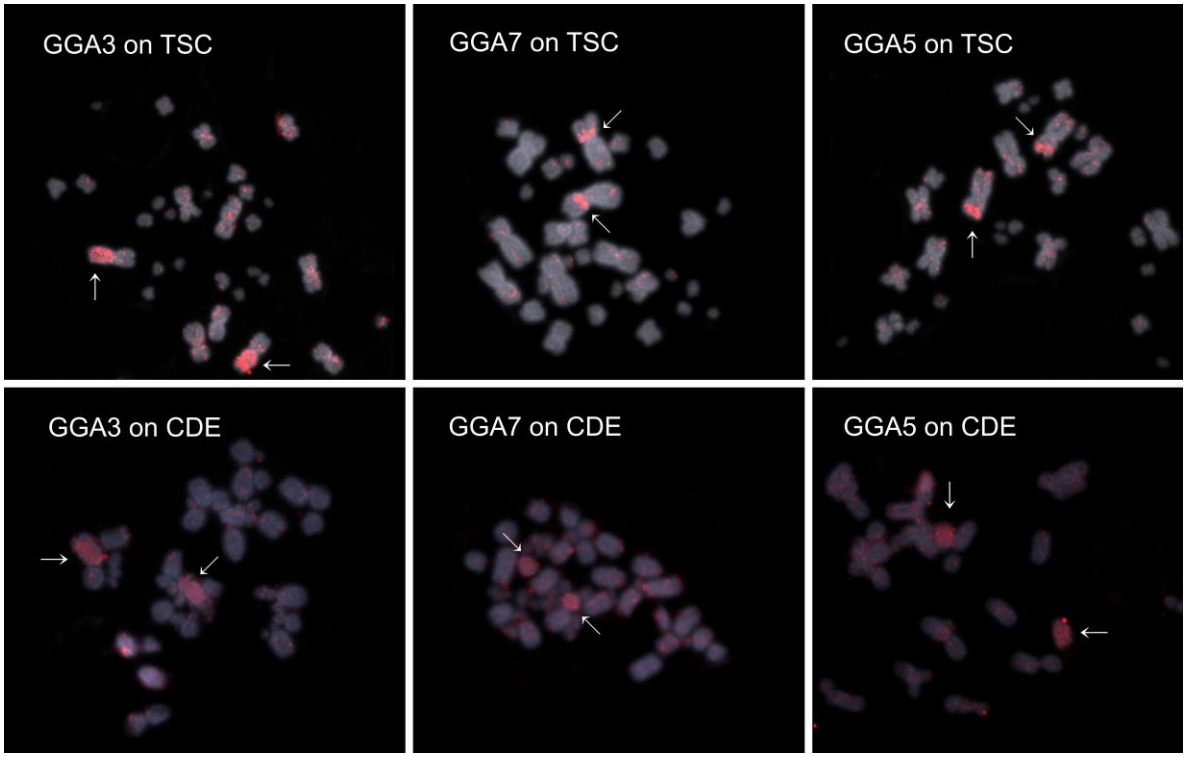
Podrobněji je naše představa evoluce pohlavně determinačních mechanismů u amniotických obratlovců popsána ve třetí kapitole této práce. Podnět pro její sepsání vznikl při čtení publikace o koevolučním vztahu mezi mechanismy určení pohlaví a vejcorodostí či živorodostí (Organ et al. 2009). Autoři používají tohoto vztahu k rekonstrukci počátku GSD před vznikem živorodosti u tří vyhynulých mořských linií plazů. Závěrem jejich práce je tvrzení, že GSD bylo klíčovou vlastností umožňující adaptivní radiaci těchto tří linií v mořském prostředí. Největší úskalí zmíněné práce jsme shledali v tom, že autoři předpokládali GSD u třech vymřelých mořských linií plazů bez dostatečné podpory. Nerespektovali také, že jako ancestrální pohlavně determinační mechanismus bylo pro recentní želvy, krokodýli, hatérie a pro šupinaté plazy fylogenetickými analýzami určeno TSD (Cree et al. 1995, Deeming 2004, Janzen & Phillips 2006, kapitola I. této práce). Závěry jejich práce jsou založené na předpokladu, že tři zmíněné linie vymřelých mořských plazů zdědili GSD od svého společného předka, což by v takovém případě znamenalo od společného předka všech amniotických obratlovců. Jak jsem již uvedla, z našeho pohledu a na základě našich dosavadních výsledků se jeví představa, že ancestrálním systémem určení pohlaví bylo u amniotických obratlovců ESD jako pravděpodobnější a jednodušší. Není totiž možné jednoduše predikovat minulé či budoucí evoluční události pouze na základě jejich statistické pravděpodobnosti, tak jak bylo predikováno GSD pro vymřelé plazí linie na

základě odhadovaného vztahu mezi GSD a vejcorodostí či živorodostí u recentních zástupců. Navíc, živorodost v rámci amniotických obratlovců vznikala jen u předka živorodých savců a v některých liniích šupinatých plazů. U obou skupin převládá GSD. Koevoluční vztah mezi GSD a živorodostí je pak založen především na chování jedné skupiny, tedy šupinatých plazů, která inklinuje k živorodosti.

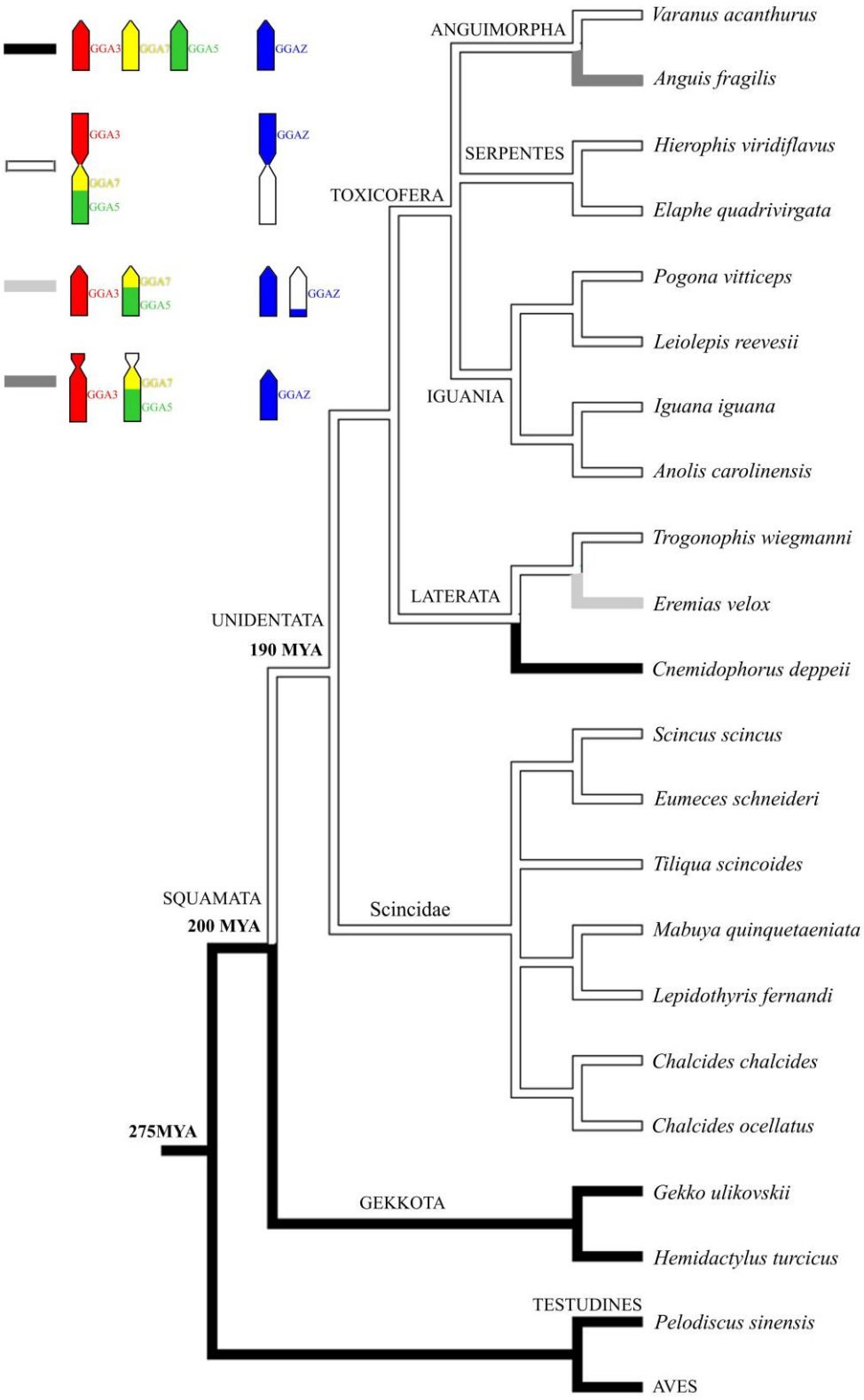
Při revizi publikace tvořící druhou kapitolu předkládané disertační práce, jsme se zapojili do širší diskuse o tom, že ptačí pohlavní chromosom Z hybridizoval tak dobře u ostatních fylogeneticky velice vzdálených linií šupinatých plazů právě z toho důvodu, že je to pohlavní chromosom. Podle této představy by právě skutečnost, že se jedná o pohlavní chromosom, měla propůjčovat tomuto chromosomu zcela specifické postavení v genomu a tedy i specifické vlastnosti jako sondy pro FISH. Jeden z oponentů naší publikace přirovnával pohlavní chromosom Z k pohlavnímu chromosomu X u savců, kde sonda připravená z izolovaného X chromosomu poskytuje daleko lepší výsledky při FISH mezi jednotlivými druhy savců než sondy z autosomů. Oponent naší práce tedy tvrdil, že námi doložený konzervatismus plazích karyotypů by mohla vykazovat jen část genomu plazů homologická s ptačím Z chromosomem. Náš argument proti tomuto tvrzení se opírá o skutečnost, že u živorodých savců je pohlavní chromosom X homologický napříč fylogenetickou distribucí a všude tedy plní funkci pohlavního chromosomu. U dosud zkoumaných plazů naopak ptačí pohlavní chromosom Z vykazuje syntenii s autosomálními částmi genomu (s výjimkou *G. hokouensis*; Kawai et al. 2007). Ptačí pohlavní chromosom Z ale skutečně může vykazovat určité specifické vlastnosti, díky kterým se uplatňuje jako výjimečně dobrá sonda. Rozhodli jsme se otestovat tuto možnost trochu podrobněji a naším cílem bylo zároveň dozvědět se podrobnější informace o evoluci uspořádání genomu ve studované skupině.

Během naší práce na publikaci o homologii ptačího Z pohlavního chromosomu s částí genomu u šupinatých plazů byly zveřejněny výsledky dlouhodobého projektu sekvenování genomu druhu *Anolis carolinensis*, patřícího do skupiny Iguania (http://www.ensembl.org/Anolis_carolinensis). Tento projekt přinesl značné množství informací; mimo jiné i možnost porovnat syntenii chromosomů tohoto druhu leguána s chromosomy kura domácího. Informace z databáze ENSEMBL nejen podpořily naše předchozí výsledky o syntenii části genomu *A. carolinensis* s ptačím Z, ale umožnily nám také nahlédnout do syntenie ptačích autosomů a chromosomů u tohoto druhu. Z databáze vyplývá, že první, největší pár chromosomů *A. carolinensis* vykazuje výraznou syntenii se třemi autosomy kura domácího. Jedno raménko je téměř

kompletně syntenní s chromosomem 3 kura domácího (GGA3) a druhé, delší raménko je syntenní s chromosomem 5 a 7 (GGA5, GGA7). Použili jsme tedy sondy z těchto tří autosomů, abychom testovali, zda podobná syntenie může být nalezena u ostatních fylogeneticky odlišných skupin šupinatých plazů. Celkem jsme hybridizovali GGA3, GGA5, GGA7 sondy na metafáze zástupců deseti čeledí šupinatých plazů pokrývající většinu hlavních fylogenetických linií (Obr. 3). Výsledky našich experimentů ukázaly, že u bazálních linií šupinatých plazů jsou chromosomy syntenní s těmito třemi autosomy kura domácího stále samostatné akrocentrické chromosomy (Obr. 2) stejně jako bylo prokázáno dříve u želvy *Pelodiscus sinensis* (Matsuda et al. 2005) a také v rekonstruovaném ancestrálním ptačím karyotypu (Griffin et al. 2007). U linií, kde jsou v karyotypu přítomné velké metacentrické chromosomy, jsme pozorovali syntenii třech ptačích autosomů s prvním největším párem autosomů (Obr. 2), stejně jako jsou tyto chromosomy syntenní u *A. carolinensis* (ENSEMBL). Fúze těchto tří chromosomů může být datována do období svrchní jury a můžeme jí považovat za synapomorfii skupiny Unidentata, zahrnující skupiny jako jsou Serpentes, Iguania *sensu lato*, Anguimorpha, Scinciformata a skupinu Laterata zahrnující čeledi Lacertidae, Amphisbaenidae a Teiidae (Obr. 3). Z výsledků tedy vyplývá, že nízká dynamika chromosomové evoluce může být chápána jako obecná charakteristika skupiny Sauropsida. A i přesto, že výsledky hybridizací se sondou z ptačího pohlavního chromosomu byly vizuálně kvalitnější, nemůžeme tvrdit, že hybridizace s autosomálními sondami by byly na stejnou fylogenetickou vzdálenost nemožné a že by ptačí pohlavní chromosom Z byl z tohoto pohledu výjimečný.



Obr. 2 Hybridizace sondy derivované z chromosomu 3, 7 a 5 (GGA3, GGA7, GGA5) kura domácího s metafázními chromosomy dvou vybraných zástupců šupinatých plazů. V prvním řádku, metafáze TSC - *Tiliqua scincoides* (Scincidae) s metacentrickými chromosomy v karyotypu; ve druhém řádku CDE – *Cnemidophorus deppei* (Teidae) s akrocentrickými chromosomy. Šipky ukazují FISH signály.



Obr. 3 Diagram znázorňující fylogenetickou distribuci homologie mezi GGA3 (červená barva), GGA5 (zelená barva), GGA7 (žlutá barva) a GGAZ (modrá barva) a částmi genomu uvnitř skupiny Sauropsida. Schéma kombinuje naše výsledky s výsledky převzatými z několika publikací (Matsuda et al. 2005, Griffin et al. 2007, Giovannotti et al. 2009, Sriculnath et al. 2009).

Čtvrtou kapitolu této práce tvoří publikace soustřeďující se na rozdíl od předchozích na mnohem užší fylogenetickou škálu. Jedná se o podrobné karyologické prostudování situace uvnitř jedné čeledi šupinatých plazů a to v čeledi Eublepharidae. Tato skupina je součástí vyšší fylogenetické linie Gekkota a je podle některých fylogenetických studií sesterskou skupinou všech zbývajících linií tohoto taxonu (Han et al. 2004). Jedná se o nevelkou čeleď zahrnující 28 popsaných druhů (Seufer et al. 2005). Druhy této čeledi vykazují variabilitu ve velikosti těla, klimatických nárocích (Seufer et al. 2005), ve velikosti genomu (Starostová et al. 2005) a také ve způsobu determinace pohlaví. Z předchozích studií a pozorování vyplývá, že některé druhy mají pohlaví určeno genotypicky a jiné teplotně (Viets et al. 1994, Bragg et al. 2000, Kratochvíl et al. 2008). A to je jedním z důvodů, proč je tato čeleď velmi vhodnou skupinou ke studiu pohlavních chromosomů a obecných evolučních principů souvisejících s určováním pohlaví. Primárním cílem práce bylo dozvědět se co nejvíce o pohlavních chromosomech druhů, u kterých bylo pokusy s inkubačními teplotami zjištěno GSD a porovnat karyotyp těchto druhů s karyotypem zástupců čeledi s TSD. Mimo to nás ale zajímala obecně karyotypová evoluce v této čeledi a chtěli jsme získat co nejvíce informací o uspořádání genomu jednotlivých zástupců této čeledi. V rámci čeledi Eublepharidae byl před vznikem naší práce karyotyp popsán pouze pro čtyři druhy (Matthey 1933, Gorman 1973, Murphy 1974, Ota et al. 1987). Rozhodli jsme se tedy rozšířit znalosti o karyotypech, jejich uspořádání a o pohlavních chromosomech pro širší spektrum druhů (celkem jsme studovali 12 druhů) této čeledi tak, aby výběr zahrnoval zástupce všech popsaných rodů studované skupiny. Metodický přístup zahrnoval techniky klasické cytogenetiky, přes metody kultivace buněk až po metody molekulární cytogenetiky. Získaná data jsme mapovali na fylogenetický strom čeledi a na základě tohoto posouzení můžeme pozorovat poměrně dlouhou evoluční stázi v uspořádání genomů v rámci čeledi. U většiny druhů je totiž karyotyp sestaven z akrocentrických postupně se zmenšujících chromosomů. U některých zástupců však došlo k výrazným chromosomovým přestavbám sestávajících z centrických fúzí a pak tedy v karyotypu nalézáme veliké metacentrické chromosomy. Tyto masivní fúze postihující téměř celý karyotyp se v evoluci skupiny udály nejméně dvakrát nezávisle a jedná se tedy pravděpodobně o apomorfie těchto dvou linií. Co se týče pohlavních chromosomů, během práce na tomto tématu jsme si čím dál tím více uvědomovali, že v této čeledi se setkáváme s fenoménem, který je pravděpodobně u šupinatých plazů poměrně hojný: a to s existencí homomorfních a tedy běžnými cytogenetickými metodami nerozlišitelných pohlavních chromosomů. Pohlavní chromosomy byly samozřejmě popsány u celé řady druhů šupinatých plazů, nicméně poměrně často se v literatuře setkáváme s faktem, že přestože

daný druh vykazuje GSD (především vyrovnaný poměr pohlaví při inkubaci v různých teplotách) a cytogeneticky byla vyšetřena obě pohlaví, pohlavní chromosomy nebyly nalezeny. V čeledi Eublepharidae tak existenci homomorfních pohlavních chromosomů předpokládáme nejméně u čtyř druhů. Pohlavní chromosomy jsme detekovali u jediného druhu a tím je *Coleonyx elegans*. Jejich rozpoznání nám umožnil samotný charakter vzniku těchto pohlavních chromosomů. Jedná se totiž o neopohlavní chromosomy, kde nepárový pohlavní chromosom Y prodělal centrickou fúzi s jedním z autosomů a nyní ho pozorujeme jako jediný metacentrický chromosom v karyotypu samce. Samičí karyotyp sestává pouze z akrocentrických chromosomů. U tohoto druhu se tedy jedná o pohlavní chromosomy typu $X_1X_1X_2X_2/X_1X_2Y$. Po tomto zjištění jsme podrobili chromosomy tohoto druhu dalším cytogenetickým vyšetřením a zjistili jsme, že během fúze původního Y chromosomu s autozomem nebo po ní došlo ke ztrátě telomerických sekvencí v oblasti centromery nově vzniklého metacentrického chromosomu Y. Dalším zjištěním pak bylo, že jedno raménko metacentrického Y a jeden X u samce a jeden pár X chromosomů u samice nesou geny pro organizátor jadérka. Díky hybridizaci sondy z Y chromosomu získanou sortováním na průtokovém cytometru na metafázi samce stejného druhu se ukázalo, že pohlavní chromosom Y a oba X chromosomy jsou do značné míry shodné ve svých DNA sekvencích. Výsledkem naší práce je nejen rozšíření vědomostí o počtech a uspořádáních chromosomů v karyotypu dalších druhů šupinatých plazů, ale i zjištění, že v jedné čeledi je možné sledovat druhy s TSD i druhy, u kterých není možné detekovat rozdíl mezi karyotypy samce a samice, přestože jejich pohlaví je určováno genotypicky, až po druhy u nichž je možné najít morfologicky značně diversifikované chromosomy. To vše vytváří z této skupiny výjimečný model pro studium evoluce pohlavně determinačních mechanismů a také evoluce pohlavních chromosomů v rané fázi jejich diferenciace. Tato fáze je jedna z nejzajímavějších a také nejméně prostudovaných z celého modelu evoluce pohlavních chromosomů popsaného výše.

A naše zvědavost a touha dozvědět se ještě něco bližšího o tom, co se děje s pohlavními chromosomy v rané fázi jejich diferenciace nás přivedla k projektu, jehož výsledky popsané ve formě rukopisu tvoří poslední kapitolu disertační práce. Fakt, že u různých druhů plazů můžeme nalézt pohlavní chromosomy v různých fázích jejich diferenciace, otevřela cestu studii, ve které jsme se rozhodli porovnat pohlavní chromosomy nově vzniklé a pohlavní chromosomy, které se pravděpodobně nacházejí v již pokročilé fázi své diferenciace. Významným krokem souvisejícím s degenerací nepárového pohlavního chromosomu je akumulace repetitivních sekvencí. Studie zabývající se identitou repetitivních sekvencí na pohlavních chromosomech u

šupinatých plazů jsou velice omezené. Bylo zjištěno, že u fylogeneticky odvozených hadů došlo k akumulaci GATA tandemových repetic na pohlavním chromosomu W (Singh et al. 1976, O'Meally et al. 2010). Přítomnost GATA repetitivních sekvencí na pohlavních chromosomech byla pozorována i u jiných druhů obratlovců (Jones and Singh 1981, Arnemann et al. 1986, Schäfer et al. 1986, Nanda et al. 1990) a spekulovalo se tedy o tom, že GATA sekvence by mohly být s pohlavními chromosomy úzce provázané a jejich přítomnost charakteristická pro pohlavní chromosomy. Rozhodli jsme se tedy porovnat akumulaci celé škály repetitivních sekvencí včetně GATA u druhu *Coleonyx elegans* (Eublepharidae), kde sice nacházíme heteromorfní, nicméně euchromatický pohlavní chromosom Y a u druhu *Eremias velox* (Lacertidae), kde pohlavní chromosom W je v zásadě homomorfní, nicméně však značně heterochromatinizovaný. Použili jsme fluorescenčně značené sondy pro všechny dostupné mono-, di- a tri-nukleotidové sekvence mikrosatelitů a také pro GATA sekvenci a hybridizovali je s metafázemi uvedených dvou druhů šupinatých plazů. Výsledkem bylo zjištění, že chromosomy – a to ani pohlavní – u druhu *Coleonyx elegans* nevykazují žádnou specifickou akumulaci mikrosatelitových sekvencí. Podobná situace byla pozorována u fylogeneticky bazálně postavených druhů hadů (Singht et al. 1976, O'Meally 2010). Přestože jsou pohlavní chromosomy heteromorfní, jsou pravděpodobně evolučně poměrně mladé, čemuž odpovídá i značná podobnost v obsahu DNA mezi pohlavním chromosomem Y a chromosomy X_1 a X_2 popsaná ve třetí kapitole této práce.

U druhu *Eremias velox* jsme pozorovali značnou specifitu distribuce jednotlivých repetitivních sekvencí na pohlavním chromosomu W. Pozorovali jsme, že některé mikrosatelity jsou výrazně akumulované na části nebo po celé délce pohlavního chromosomu W, zatímco jiné, a to včetně GATA sekvence, na pohlavním chromosomu chybí, přestože na autozomech jsou rovnoměrně rozmístěny. Z našich výsledků vyplývá, že akumulace mikrosatelitových sekvencí není vždy asociována se stupněm heteromorfismu pohlavních chromosomů. Druhy začleněné do této studie leží fylogeneticky mezi hady a dalšími obratlovci, u kterých byla detekována akumulace GATA sekvencí na pohlavních chromosomech. Jelikož v naší studii nejsou sekvence GATA na pohlavních chromosomech přítomny, můžeme konstatovat, že jejich akumulace u různých druhů obratlovců je pravděpodobně homoplasická spíše než homologická. Podobně homoplasickou akumulaci jiných mikrosatelitových sekvencí můžeme pozorovat mezi pohlavními chromosomy druhu *E. velox* a pohlavními

chromosomy dalších obratlovců. Obecně lze tedy z našeho srovnání vyvodit závěr, že charakter mikrosatelitové akumulace na pohlavních chromosomech odráží spíše historickou náhodu než vlastnosti jednotlivých mikrosatelitů. Přestože tedy nemůžeme pozorovat žádnou závislost akumulace jednotlivých konkrétních mikrosatelitových sekvencí na pohlavních chromosomech, můžeme vyvodit, že u pohlavních chromosomů, u kterých předpokládáme, že vznikly v evoluci poměrně nedávno, nedochází zatím k akumulaci mikrosatelitových lokusů. Na druhé straně jejich distribuce u druhu s evolučně staršími pohlavními chromosomy je velice specifická. Tato pozorování nás myšlenkově vrací zpět k modelu vzniku a evoluce pohlavních chromosomů popsaném výše.

Při řešení disertační práce, která je rozšířeným tématem, kterým jsem se zabývala i během celého magisterského studia, jsem se seznámila s celou řadou metodických postupů klasické i molekulární cytogenetiky a vyzkoušela si úskalí i radost související s aplikováním těchto postupů na šupinaté palzy. Výsledky takto získané jsme se vždy snažili interpretovat v obecnějším evolučním kontextu a věřím, že jejich začlenění do obecného rámce současného poznání daného tématu přispěje k dalšímu rozvíjení diskuse o vzniku a diverzitě pohlavních chromosomů u obratlovců.

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Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap?

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Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap?

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Squamate reptiles possess two general modes of sex determination: (1) genotypic sex determination (GSD), where the sex of an individual is determined by sex chromosomes, i.e. by sex-specific differences in genotype; and (2) temperature-dependent sex determination (TSD), where sex chromosomes are absent and sex is determined by nongenetic factors. After gathering information about sex-determining mechanisms for more than 400 species, we employed comparative phylogenetic analyses to reconstruct the evolution of sex determination in Squamata. Our results suggest relative uniformity in sex-determining mechanisms in the majority of the squamate lineages. Well-documented variability is found only in dragon lizards (Agamidae) and geckos (Gekkota). Polarity of the sex-determining mechanisms in outgroups identified TSD as the ancestral mode for Squamata. After extensive review of the literature, we concluded that to date there is no known well-documented transition from GSD to TSD in reptiles, although transitions in the opposite direction are plentiful and well corroborated by cytogenetic evidence. We postulate that the evolution of sex-determining mechanisms in Squamata was probably restricted to the transitions from ancestral TSD to GSD. In other words, transitions were from the absence of sex chromosomes to the emergence of sex chromosomes, which have never disappeared and constitute an evolutionary trap. This evolutionary trap hypothesis could change the understanding of phylogenetic conservatism of sex-determining systems in many large clades such as butterflies, snakes, birds, and mammals. *Zoological Journal of the Linnean Society*, 2009, **156**, 168–183.

ADDITIONAL KEYWORDS: environmental sex determination – lizards – sex ratio – temperature.

INTRODUCTION

Explanation of the variation in sex-determining mechanisms across taxa represents an important goal of current evolutionary biology. The particular mode of sex determination is important not only at the individual organismal level (i.e. establishment of the sex during ontogeny), but it can also influence population sex ratio and thus total evolvability and susceptibility to extinction of the lineage in question (e.g. le Galliard *et al.*, 2005; Burt & Trivers, 2006). Tetrapods possess two general modes of sex determination: genotypic (also referred to as 'genetic') sex determination (GSD) and environmental sex determination (ESD). The most common ESD mechanism in vertebrates is the thermal effect on gonadal determination of developing embryos (temperature-dependent sex determination, TSD) (Janzen & Paukstis, 1991). In reptiles, GSD includes male or female heterogamety, which can be uncovered by cytogenetic examination of sex chromosomes; however, sex chromosomes may be morphologically undifferentiated (homomorphic) and may potentially differ only in the presence of a single sex-determining gene (Matsuda *et al.*, 2007). Recently, even microchromosomes were shown to be sex chromosomes in an agamid lizard species and in a turtle species (Ezaz *et al.*, 2005; Ezaz *et al.*, 2006). Thus, detection of sex chromosomes sometimes requires advanced molecular cytogenetic techniques such as comparative genomic hybridization.

ESD and GSD have been viewed as two ends of a continuum of sex-determining mechanisms (Shine,

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Elphick & Donnellan, 2002; Sarre, Georges & Quinn, 2004). For example, XX/XY sex chromosomes were described in a scincid lizard (*Bassiana duperreyi*) that presumably possessed TSD mechanisms (Shine *et al.*, 2002). The mechanism for long-term maintenance of these sex chromosomes in a population with ESD is not clear (Bull, 2008). The continuum between ESD and GSD can be explained by the existence of GSD with environmental effects (Valenzuela, Adams & Janzen, 2003), where environmental conditions can influence the observed sex ratio of hatchlings, but sex of an individual is still determined by its genotype (e.g. by differential fertilization of gametes producing particular sex or sex-specific mortality of embryos), or by the thermal induction of sex revertants (i.e. production of individuals with the wrong gonad type for their genotypes). Sex revertants induced by extreme developmental temperatures are well known in many GSD vertebrate lineages (e.g. Witschi, 1929) and were recently documented in two species of lizards as well (Quinn *et al.*, 2007 in the ZZ/ZW agamid *Pogona vitticeps*, and Radder *et al.*, 2008 in the XX/XY skink *Bassiana duperreyi*). In both cases, the existence of thermally induced sex reverted individuals was interpreted by the authors as evidence for the co-occurrence of GSD and TSD or for a transitional state between TSD and GSD. Nevertheless, gonadally and phenotypically reverted individuals still possess sex chromosomes corresponding to their genotypic sex, and when crossed with nonreverted mates, they produce progeny with a skewed sex ratio. We understand GSD and TSD as two dichotomous sex-determining systems that do not differ in thermal dependency of sex ratios, but basically in the presence or absence of sex chromosomes. Therefore, we treat both species as GSD species with environmental effects.

Often-debated questions concern the evolution and phylogeny of individual sex-determining mechanisms and the possibilities of reciprocal shifts between them. The historical approach deemed that the ancestral state was ESD and that GSD was always evolutionarily derived (Ohno, 1967). This scenario represents the shift from environmental (epigenetic) to genetic control of sex determination, and thus corresponds to the process of genetic assimilation proposed by Waddington (1953). Ohno (1967) predicted the evolution of sex chromosomes from a pair of autosomes, which was later repeatedly confirmed by comparative cytogenetics. For example, Matsuda *et al.* (2005) demonstrated that avian, turtle, and snake sex chromosomes were derived from different autosomes of their common ancestor. On the one hand, from the adaptive point of view, GSD may have emerged from ancestral ESD under selective pressure to ensure the maintenance of an equal sex ratio (Charnov, 1982) or for earlier and deeper differentiation of genitals and other sexual characteristics (Valenzuela, 2004). On the other hand, the possibility exists that the transition between alternative sex-determining systems was in the opposite direction from GSD to ESD. According to these hypotheses, ESD may be an adaptation to a heterogeneous environment, where certain 'patches' of environmental conditions confer greater fitness to individuals of a particular sex (Charnov & Bull, 1977). These 'patches' could also be temporal, and the offspring of a particular sex may be favoured at different times of hatching (Conover, 1984; Warner & Shine, 2005; reviewed by Shine, 1999). ESD may have evolved from GSD as a consequence of a pre-existing environmental sensitivity of sex differentiation (Bull, 1983). Nevertheless, current hypotheses on the origin of ESD from GSD do not account for the fate of sex chromosomes during the change of sex-determining systems. The transition from GSD to ESD requires the disappearance of sex chromosomes. This disappearance may have resulted from the complete elimination of the pair of these chromosomes from the genome. To our knowledge, the only example of the complete elimination of sex chromosomes in animals as a result of a change in sex-determining systems was reported in the fish order Aulopiformes following a switch from gonochorism to hermaphroditism (Ota *et al.*, 2000). Such a complete elimination leads to the total loss of genes present at sex chromosomes from the genome. The disappearance of sex chromosomes represented by their transition back to autosomes seems thus more likely. In order to facilitate a disappearance of sex chromosomes, the organism would have had to overcome potential constraints imposed by the existence of differentiated sex chromosomes including gene dosage compensation (Charlesworth, 2002). Moreover, shortly after their establishment, sex chromosomes start to accumulate sexually antagonistic alleles (Rice, 1996). For example, alleles beneficial to males, but harmful to females, should have a tendency to be linked to the Y chromosome. It is thus probable that transition from male heterogamety to ESD would be connected with a fitness deficit of ESD males in comparison to XY males (we can imagine an analogous situation concerning the W chromosome and female heterogamety).

In summary, although the transitions from ESD to GSD are well documented and understood, the transitions from GSD to ESD are somewhat obscure. The transitions between sex-determining systems and the direction of the transition can be readily assessed by phylogenetic comparative analysis within a group that exhibits diversity in the mechanisms of sex determination. Reptiles are an especially suitable group for such analysis as the sex-determining

mechanisms, in squamate reptiles in particular, are variable. Using phylogenetic analysis, Janzen & Krenz (2004); Janzen & Phillips (2006) determined that GSD may, in fact, be the ancestral state for all vertebrates and that ESD emerged from GSD among tetrapods repeatedly and frequently. The situation has not been completely resolved as they incorporated some unreliable data regarding the sex-determining mechanism (see Discussion for details). Moreover, only one of currently relevant hypotheses of squamate phylogeny was examined.

The first relevant phylogeny is the classical tree (Estes, de Queiroz & Gauthier, 1988), which is based primarily on morphological data and, for a long time, was accepted as a well-corroborated hypothesis for the relationships among squamate families. This phylogenetic hypothesis has sufficient statistical support and is based on many characters (Lee, 2005), and thus, this classical model should remain a viable scenario of squamate phylogeny. The molecular phylogenetic tree used by Janzen & Krenz (2004) and Janzen & Phillips (2006) was basically originally proposed by Townsend *et al.* (2004) and subsequently elaborated by Vidal & Hedges (2005). The molecular trees differ from the morphology-based tree in the position and monophyly of even basic groups. Although these new systems have sufficient molecular data support, the contradiction between the morphological and molecular data indicates that accepting the molecular trees as a definitive resolution of squamate phylogeny is still premature. Until now, no independent morphological or physiological evidence has been published in support of the new hypotheses. Expression of snake toxins found in dragon lizards and monitors was suggested to be a common apomorphy of Iguania ((agamids + chamaeleonids) + iguanids *s.l.*), varanids, helodermatids, anguids, and snakes and thus as a support for monophyly of the clade encompassing these groups (Fry *et al.*, 2006); however, this interpretation could not be accepted as members of the families outside the suggested monophylum were not studied. In contrast, several traits (most importantly tongue morphology) that were key supporting information for the traditional hypothesis directly contradict the molecular trees.

Here, we present a critical revision of the knowledge of sex-determining mechanisms and their phylogeny in the clade Squamata. We compiled a dataset of sex-determining mechanisms in as many squamate species as possible and conducted phylogenetic analyses based on both the traditional and molecular hypotheses of squamate phylogeny. Our aim was to reveal the sensitivity of the reconstruction of the ancestral state as well as the number and direction of transitions between GSD and ESD. Specifically, we examined the relationships between the number and the direction of transitions between GSD and TSD and the different scenarios for the emergence or loss of sex chromosomes.

METHODS

For the present phylogenetic analysis, we collected data from the literature on sex-determining mechanisms for 423 squamate taxa (species or subspecies). The data and the citation list are summarized in Supporting Information Table S1. Taxa with detectable sex chromosomes (regardless of whether heteromorphic or homomorphic) were considered GSD species according to the protocol described by Valenzuela *et al.* (2003). Each species was assigned as a TSD species when unequal sex ratios were detected at different temperatures in an adequately large sample size and the observed sex ratio pattern could not be explained by differential mortality. Some 'TSD' species thus could be in fact GSD species with environmental effects (Valenzuela *et al.*, 2003). For reasons elucidated in the Discussion, we omitted some problematic data concerning a varanid, a lacertid, an anguid, and four scincids from the phylogenetic reconstructions.

For the phylogenetic analyses, we defined four character states: (1) 'XY' for male heterogamety; (2) 'ZW' for female heterogamety; (3) 'GSD' for cases where equal sex ratios were observed at several constant incubation temperatures, but sex chromosomes have not yet been identified by cytogenetic methods (Janzen & Krenz, 2004 and Janzen & Phillips, 2006 incorrectly indicated that such species have homomorphic sex chromosomes); and (4) 'TSD'. X1X2Y systems occurring in some polychrotids and *Lialis burtonis* (Gekkota) (Gorman & Gress, 1970) were incorporated into male heterogamety. Analogously, Z1Z2W (lacertids, elapid snakes), Z1Z2W1W2 (lacertids, elapid snakes), and ZW1W2 (elapid snakes) systems were considered to have female heterogamety (Odierna *et al.*, 2001; Olmo, 2004). These peculiar sex chromosomal systems are unequivocally derived and are well nested within a clade originally possessing ordinary XY and ZW systems. Traditionally, three different categories of TSD (TSD Ia, TSD Ib, TSD II) defined by patterns of the sex ratios produced as a function of constant incubation temperature have been recognized (Valenzuela, 2004). Under TSD Ia, males are produced at lower and females at higher viable temperatures, whereas the pattern is opposite in TSD Ib. Females are produced at both low and high incubation temperatures, but predominantly males hatch at intermediate incubation temperatures in reptiles assigned as TSD II species. However, the classification of particular species into each TSD category is rather problematic

(reviewed by Mitchell *et al.*, 2006). For example, in the leopard gecko (*Eublepharis macularius*), which used to be classified as TSD II species, high temperatures during the whole period of development are lethal for embryos. In the laboratory, 'hot' females can be obtained only by decreasing the temperature after the period critical for sex determination (Viets *et al.*, 1993). Therefore, from our point of view, the categories of TSD reflect the intersection between thermal dependence of mortality and sex ratio, and the observation of the three different TSD patterns does not imply that particular types of TSD are nonhomologous. From these reasons, we decided to treat 'TSD' as a single character state. However, Janzen & Krenz (2004: 123) advocated in detail against grouping all GSD systems into a single category for phylogenetic analyses of the evolution of sex-determining systems. Following their recommendations, we decided not to combine our three GSD categories ('XY', 'ZW', 'GSD'), because it might considerably increase homoplasy in our phylogenetic reconstructions (see Discussion).

Subsequently, character states were mapped on three alternative phylogenies for squamate reptiles. The morphological phylogenetic hypothesis (further referred to as 'morphological tree') was basically originally proposed by Estes *et al.* (1988). These authors treated snakes, dibamids, and amphisbaenians as Scleroglossa *incertae sedis*. Nevertheless, they pointed out that snakes are most probably members of Autarchoglossa (= 'the most recent ancestor of Scincomorpha and Anguimorpha, and all of its descendants'; Estes *et al.* (1988)), and that the position of snakes within or close to anguimorphs (including varanoids) is 'better supported than any of the other possibilities.' Subsequent morphological studies further supported nested position of snakes within Anguimorpha as the sister taxon to varanids or their sister position to anguimorphs (Lee, 1998, 2000; Reynoso, 1998; Lee & Caldwell, 2000). Therefore, in our morphological tree, we placed snakes as sister to varanids (no other members of Anguimorpha are present in our dataset). No such consistency can be found in the literature concerning the position of the dibamids, therefore, we excluded *Dibamus novaeguineae* from our morphological tree. Estes *et al.* (1988) suggested that Amphisbaenia are nested within Scincomorpha, probably close to Lacertiformes (the monophylum of Lacertidae, Gymnophtalmidae and Teiidae; Estes *et al.*, 1988), therefore, we placed them as sister to this monophylum (see also Cooper & Vitt, 2002). However, their position anywhere within Scincomorpha does not affect our analysis of the transitions between TSD and GSD.

In addition, we used the molecular phylogenies proposed by Townsend *et al.* (2004: fig. 8) and Vidal & Hedges (2005: their fig. 1). The latter phylogeny differs from the tree suggested by Townsend *et al.* (2004) in the position of the Dibamidae and the resolution of the position of Lacertiformes. The varanids are traditionally thought to be a sister clade to snakes (see above), but may alternatively be a sister clade to snakes + Iguania (Vidal & Hedges, 2005). The latter topology, however, is poorly supported by bootstrap analysis. Therefore, in our third alternative phylogeny, we treated the mutual relationship of varanids, snakes and Iguania as unresolved. In all alternative phylogenies, we assigned Rhynchocephalia (tuataras), which possesses TSD (Cree, Thompson & Daugherty, 1995), as the outgroup to the Squamata (but see Hedges & Poling, 1999).

Where appropriate, we supplemented the trees with topology of taxa within individual major clades from other sources. We followed the relationships within agamids according to Hugall *et al.* (2008), and within Chamaeleonidae according to Raxworthy, Forstner & Nussbaum (2002). *Bradypodion ventrale* was missing in the chamaeleonid phylogeny, therefore, we estimated its position from the topology of *B. pumilum* (*B. ventrale* was originally described as the subspecies of *B. pumilum*; see e.g. Uetz, 2006). Within Iguanidae *s.l.*, we followed the topologies by Schulte, Valladares & Larson (2003). We are aware that the phylogenetic relationships within Iguanidae *s.l.* are not well understood; however, the alternative placement of clades within this group does not change our conclusions as all possess GSD. Unfortunately, there are currently not available any phylogenetic hypotheses covering all taxa of geckos. We followed the basic relationships within Gekkota according to Donnellan, Hutchinson & Saint (1999), within the genus *Phelsuma* (Gekkonidae) according to Austin, Arnold & Jones (2004) and Rocha *et al.* (2006), and within the eye-lid geckos (family Eublepharidae) according to Grismer (1988). We placed the family Eublepharidae (in our dataset, the genera *Eublepharis*, *Coleonyx*, and *Hemitheconyx*) traditionally as the basal gecko group (e.g. Grismer, 1988). Recently, they were considered as sister to the Gekkonidae, and the Diplodactylidae + Carphodactylidae + Pygopodidae clade was considered to be basal (e.g. Gamble *et al.*, 2008). The alternative placement of the eye-lid geckos does not change any conclusion concerning the evolution of sex-determining modes. Although there are some recent papers concerning phylogenetic relationships within Scincidae (e.g. Smith *et al.*, 2007), none covers all taxa of skinks from our dataset. Within Teiidae, we placed *Cnemidophorus uniparens* as sister to *Cnemidophorus inornatus*. *Cnemidophorus uniparens* is the parthenogenetic triploid species, which evolved by hybridization of the sexual ancestors, and *C. inornatus* is believed to be one of its ancestors (e.g. Lowe & Wright, 1966). We utilized as

much phylogenetic information as possible in clades with transitions in sex-determining modes, but for our purposes, it was not necessary to resolve phylogenetic position within clades where all members shared the same sex-determining mechanism (Gymnophtalmidae, Lacertidae, Serpentes, Varanidae).

For phylogenetic analysis of sex-determining modes, we employed the unordered maximum parsimony method implemented in the MESQUITE program 2.01 (Maddison & Maddison, 2008). All polytomies were treated as soft. For reconstruction of the phylogeny of sex-determining modes within squamate reptiles, we preferred maximum parsimony over other methods, such as maximum likelihood and Bayesian reconstruction of character states, as maximum parsimony does not require knowledge of branch lengths and can operate with numerous character states and polytomies as observed in our phylogenetic trees. Relatively small numbers of transitions between sex-determining modes further justifies the use of maximum parsimony. However, we supplemented our analyses by maximum likelihood reconstruction and confidence estimation of ancestral states as implemented in MESQUITE 2.01 (Maddison & Maddison, 2008), assuming a single rate for all transitions between character states. To test the statistical support of reconstructed ancestral state at each node across the trees, we determined its significance using the likelihood-ratio test after random resolution of polytomies (1000 simulated trees generated, branch length set to 1 except for the randomly resolved nodes, where it was set to 0.0001). Specifically, if the log-likelihoods of a reconstruction assuming a specific character state at a node differed by 2.0 or more than alternative reconstructions across all trees, then the state was considered to be the significantly best estimate for that node (following Pagel, 1999). Otherwise, the reconstruction for that node was considered as poorly supported.

Table 1. Taxonomic distribution of particular sex-determining modes across squamate families

Family	*N*	GSD	TSD	XY	ZW
Agamidae	25	11	13		1
Chamaeleonidae	4	1	2		1
Polychrotidae	33			33	
Corytophanidae	1	1			
Iguanidae	1	1			
Crotaphytidae	1	1			
Tropiduridae	6			6	
Phrynosomatidae	43			43	
Eublepharidae	6	4	2		
Diplodactylidae	5		5		
Pygopodidae	2			2	
Gekkonidae	32	5	15	3	9
Dibamidae	1			1	
Teiidae	3	2		1	
Gymnophthalmidae	7			7	
Lacertidae	45		1*		44
Amphisbaenidae	1				1
Scincidae	19	3	4*	12	
Anguidae	1		1*		
Varanidae	5		1*		4
Boidae	6				6
Colubridae	63				63
Elapidae	18				18
Hydrophiidae	58				58
Viperidae	37				37

Asterisks denote the questionable data (discussed in detail in the Discussion) excluded from the phylogenetic analyses. For the references and detailed list of taxa see Supporting Information Table S1. GSD, genotypic sex determination; TSD, temperature-dependent sex determination.

RESULTS

The data showed uniformity of the sex-determining system in most of the main squamate clades (Table 1). All snakes, monitor lizards, and lacertids possess female heterogamety. All iguanids *s.l.* possess male heterogamety with the only exceptions being *Basiliscus*, *Dipsosaurus*, and *Crotaphytus*, where sex chromosomes have not yet been identified. After karyotypic examination by more sensitive cytogenetic methods, these reptiles may, in fact, possess male heterogamety as was inferred from the phylogenetic patterns. Skinks, gymnophtalmids, and teiids possess either male heterogamety or GSD without identification of sex chromosomes, which most parsimoniously is also male heterogamety. We found well-supported variability in sex-determining systems only in agamids and chamaeleonids and in gekkotan lizards.

The resultant phylogenetic analyses of the sex-determining systems in squamate reptiles and the significance of the reconstructions of ancestral states in each node are depicted in Figure 1 (the morphological phylogeny), Figure 2 (the molecular phylogeny according to Townsend *et al.*, 2004) and Figure 3 (the molecular phylogeny following Vidal & Hedges, 2005). TSD emerged as the ancestral state for squamate reptiles after mapping of sex-determining mechanisms onto the morphological phylogeny and the molecular phylogeny according to Townsend *et al.* (2004). The ancestral state for Lepidosauria (= Rhynchocephalia + Squamata) as well as for Squamata alone is not resolved in the third alternative tree. In this case, the ancestral state was either TSD or male heterogamety; however, the outgroups of Lepidosauria (turtles and archosaurs) resolved this

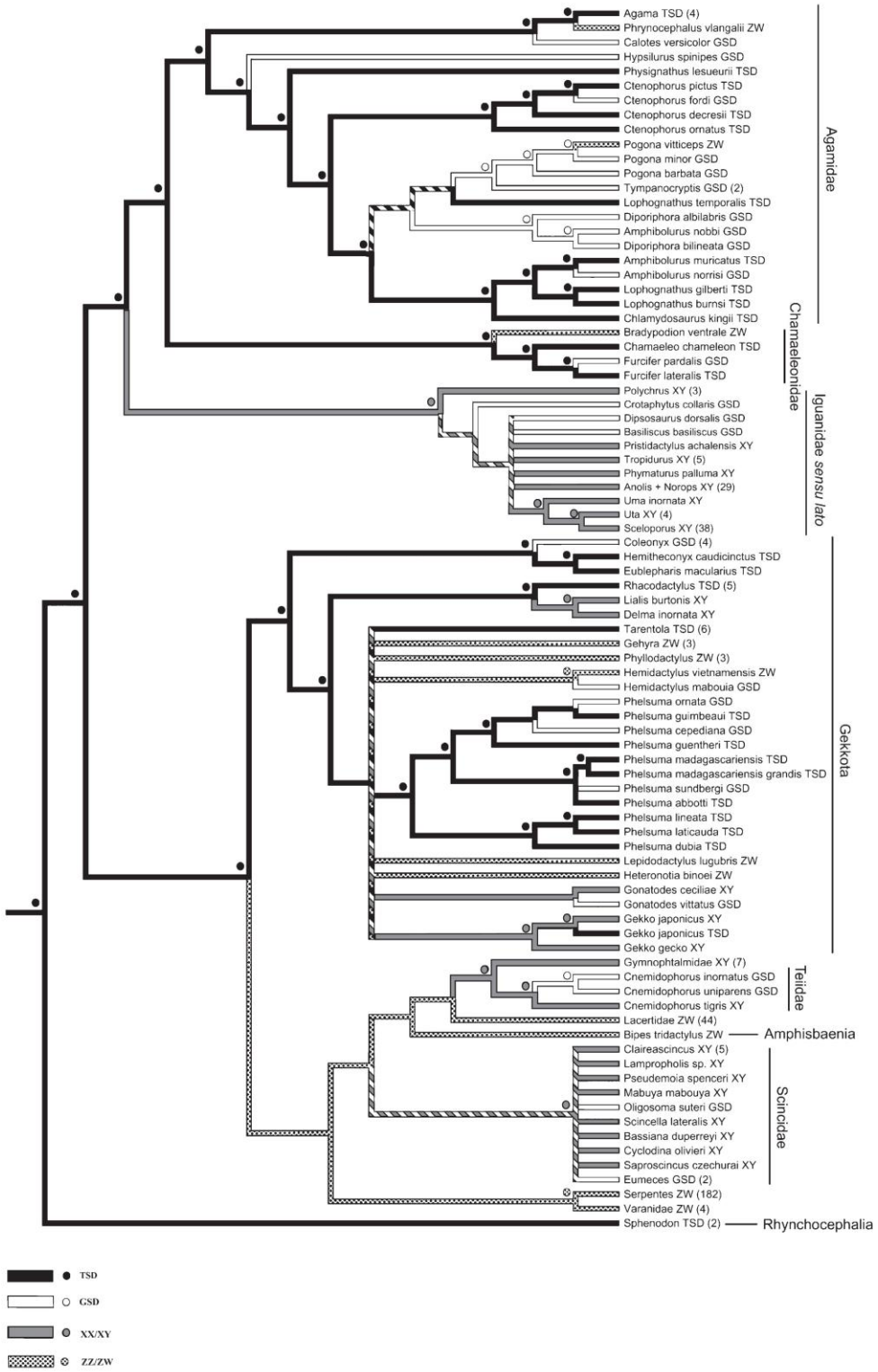

Figure 1. Parsimony analysis of sex-determining mechanisms in squamate reptiles based on the 'morphological' tree. Circles indicate maximum-likelihood reconstructions of ancestral states, only nodes with significant reconstruction are shown (tested by likelihood-ratio test). Numbers in parentheses indicate the counts of species within a genus that share a given mechanism of sex determination.

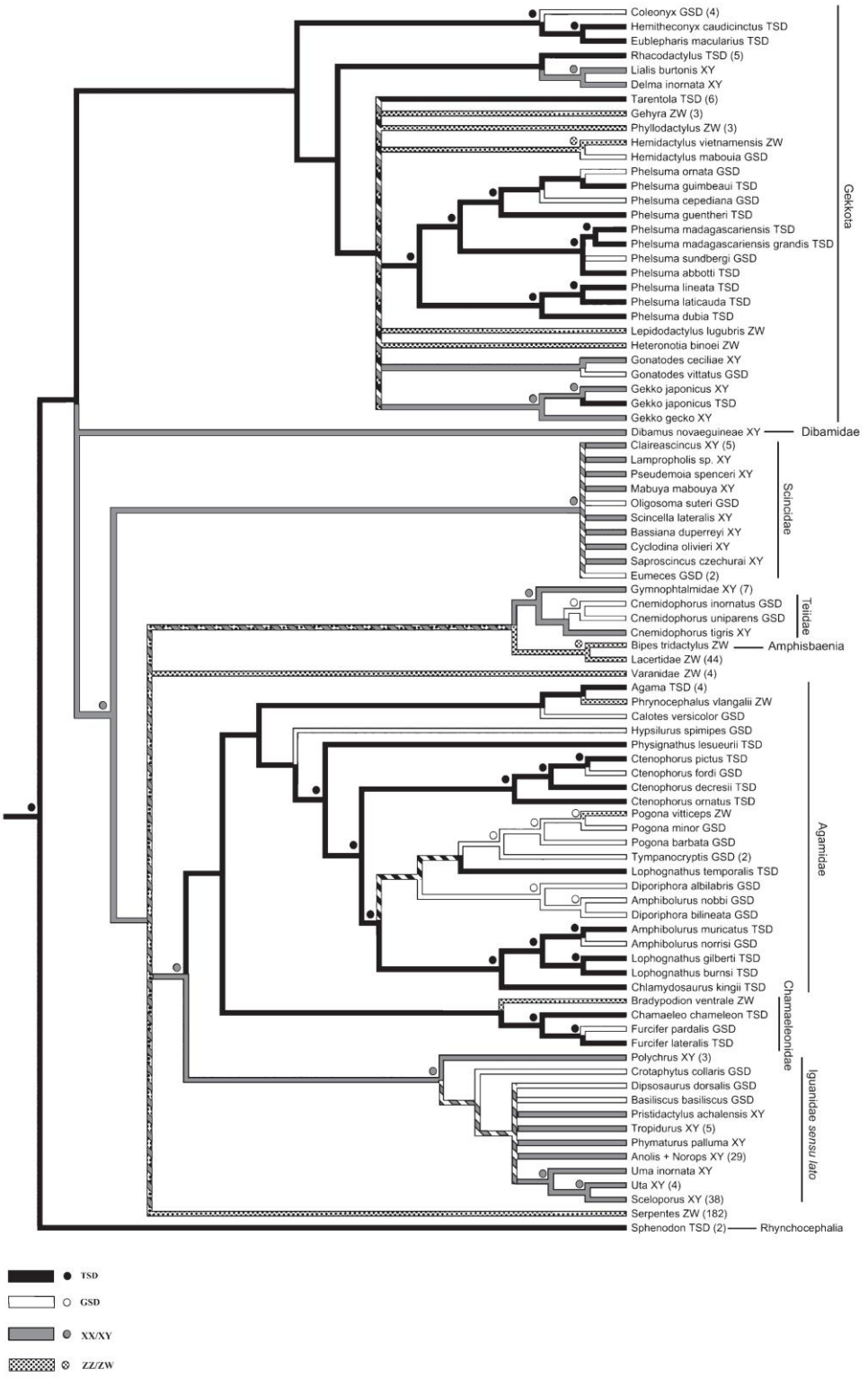

Figure 2. Phylogenetic reconstruction of sex-determining mechanisms in squamate reptiles based on the molecular tree according to Townsend *et al.* (2004). For details see legend to Figure 1.

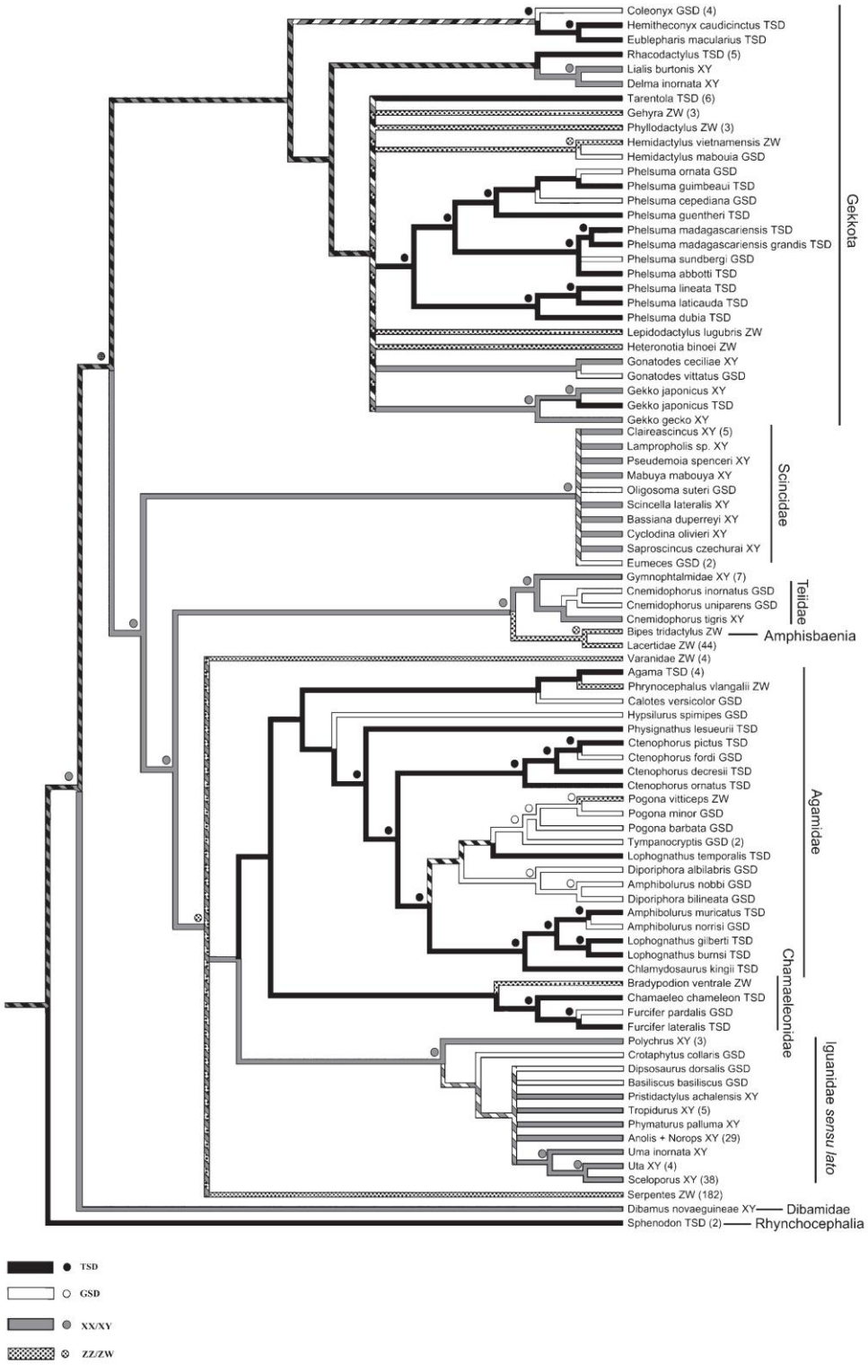

Figure 3. Phylogenetic reconstruction of sex-determining mechanisms in squamate reptiles based on the molecular tree according to Vidal & Hedges (2005). For details see legend to Figure 1.

situation in favour of the ancestral state of TSD. TSD is the ancestral state for turtles, and archosaurs possess either TSD (crocodiles) or female heterogamety (birds) (Janzen & Krenz, 2004). Thus, we conclude that regardless of the underlying phylogeny, TSD and the lack of sex chromosomes were probably the ancestral mechanism in squamate reptiles.

Using the morphological tree, we have found:

1. Five independent well-supported appearances of GSD from the ancestral TSD within the Agamidae (in the ancestors of *Calotes versicolor, Phrynocephalus vlangalii, Hypsilurus spinipes, Ctenophorus fordi*, and *Amphibolurus norrisi*). The ancestral character state is unresolved in the clade encompassing *Pogona, Tympanocryptis, Diporiphora, Lophognathus temporalis*, and *Amphibolurus nobbi*: there are either the sixth and the seventh independent origins of GSD from TSD within Agamidae, or the sixth transition from GSD to TSD and one transition in the opposite direction (in the ancestor of *Lophognathus temporalis*).
2. It seems that there were two independent transitions from the ancestral TSD to GSD within the family Chamaeleonidae.
3. All Iguania *s.l.* possess independently evolved GSD.
4. Independent origin of GSD from TSD in the ancestor of the genus *Coleonyx* (Eublepharidae).
5. Independent origin of GSD in the ancestor of pygopodids (*Delma* + *Lialis*).
6. Unresolved phylogeny does not allow us to reconstruct the situation within Gekkonidae. TSD, male, and female heterogamety are present within this family suggesting high evolutionary plasticity. The reconstruction of the phylogeny of sex-determining modes within gekkonids will be possible only after resolution of their phylogeny. However, three independent origins of GSD from the ancestral TSD – although unsupported by the likelihood-ratio tests – emerged within the genus *Phelsuma*. The phylogenetic reconstruction suggests evolution of TSD from male heterogamety in a population of *Gekko japonicus*.
7. Independent origin of GSD from ancestral TSD in the common ancestor of Gymnophtalmidae + Teiidae + Lacertidae + Amphisbaenia + Scincidae + Varanidae + snakes.

The main differences between mapping on the morphological tree and the molecular phylogeny according to Townsend *et al.* (2004) concerning the evolution of sex-determining modes are the origin of sex chromosomes in Dibamidae, and the possible independent origin of GSD in the common ancestor of Scincidae + Gymnophtalmidae + Teiidae + Lacertidae + Amphisbaenia + (Agamidae + Chamaeleonidae) + Iguanidae *s.l.* + Varanidae + snakes. According to this tree, TSD evolved from GSD within this clade in the common ancestor of Agamidae + Chamaeleonidae.

In comparison to the first molecular tree, the altered position of the *Dibamus* (XY) in the molecular phylogeny following Vidal & Hedges (2005) changes the reconstruction of the ancestral state in the common ancestor of Gekkota to unresolved. It could be either TSD (and the reconstruction of evolution of sex-determining modes within this group is then the same as in the previous analyses), or male heterogamety (and TSD would then evolved from GSD in geckos several times).

DISCUSSION

Omission of data on sex determination in several species

We assume that the detection of sex chromosomes is sufficient proof of GSD. Therefore, species in which male or female heterogamety has been verified by cytogenetic methods possess these sex-determining systems. Assignment as GSD to species exhibiting equal sex ratios at several constant incubation temperatures but no sex chromosomes should be treated with more caution. In many cases (e.g. *Oligosoma*, *Cnemidophorus*, and *Basiliscus*), however, such species are nested within clades with identified sex chromosomes in relatives, and therefore, these species are likely to share the same sex-determining system.

The most problematic assignment is in species possessing TSD. In many cases, experiments confirming TSD have been repeated many times with large sample sizes, and the validity of TSD in these species, e.g. in *Sphenodon punctatus* (Cree *et al.*, 1995), *Eublepharis macularius* (Viets *et al.*, 1993), *Agama impalearis* (el Mouden, Znari & Pieau, 2001), or *Physignathus lesueurii* (Harlow, 2004), is not questioned. In others, although temperature-biased sex ratios were reported, the legitimacy of TSD warrants further testing.

The literature on sex ratios is considered to suffer from high 'publication bias' and sensitivity to small sample size (Ewen, Cassey & Møller, 2004; Silk, Willoughby & Brown, 2005). Many temperature-dependent sex ratios may mirror the small sample size. For example, the published temperature-dependent sex ratio in a brush turkey (*Alectura lathami*), which differed significantly from 1 : 1 (77% of males at 31 °C; 29% of males at 36 °C), was based on only 13 eggs at 31 °C and 17 eggs at 36 °C. As a result, only 3.5 eggs at each temperature deviated in their sex from the null expectation suggesting equal sex ratios. Moreover, an unbiased sex ratio was found

at the temperature of 34 °C when the sample size was almost twice as large ($N = 32$; Göth & Booth, 2004). Thus, statistically significant results observed with small sample sizes may easily represent random variation. Similar effects of small sample sizes may be invoked to explain temperature-sensitive sex ratios in many 'TSD' species of reptiles as well. In conclusion, we expect the highest potential bias in our dataset in TSD species.

We have not included several species of lizards (a varanid, a lacertid, an anguid, and four scincids) in our phylogenetic analyses because of questionable evidence for their sex-determining modes. As criticized by Harlow (2004), the evidence that *Varanus salvator* (Varanidae), *Podarcis pityusensis* (Lacertidae), and *Gerrhonotus multicarinatus* (Anguidae) possess TSD is extremely poor. The conclusions are based on a single description of the production of one male to '10–15 females' at 29 °C in *Podarcis pityusensis* and on two contradictory reports of biased sex ratios in *Varanus salvator* at similar temperatures. Evidence of TSD in *Gerrhonotus multicarinatus* (Anguidae) was described by Langerwerf (1984) *ex* Harlow (2004), who found that this species 'produces more males at 27–28 °C, but it is not very obvious'.

Temperature-dependent sex ratios were also reported in three viviparous species of Australian skinks from the genera *Niveoscincus* and *Eulamprus* (Robert & Thompson, 2001; Wapstra *et al.*, 2004; Langkilde & Shine, 2005) and the Asian skink *Sphenomorphus indicus* (Ji *et al.*, 2006). The reported TSD in *Sphenomorphus indicus* may be attributed to the small sample size. Ji *et al.* (2006) reported that 'Females gave birth to predominantly female offspring (85.7% of the 14 sexed offspring were females) at 24 °C and to predominantly male offspring (76.5% of the 17 sexed offspring were males) at 28 °C. Females with the opportunity to regulate body temperature produced a mix of sexes that did not differ from equality'. In addition, offspring of both sexes were produced over a wide range of incubation regimes in *Sphenomorphus indicus*, which is not the typical situation for most TSD species (Viets *et al.*, 1994; Harlow, 2004). Early reports of temperature-dependent sex ratios in *Eulamprus heatwolei* (D. Allsop, unpubl. data cited by Langkilde & Shine, 2005), were not confirmed by a subsequent study (Langkilde & Shine, 2005). In all skinks with reported TSD, we cannot unequivocally reject the possibility of GSD with environmental effects (as suggested by Valenzuela *et al.*, 2003) or that the sex was not determined properly. In some of these studies (Wapstra *et al.*, 2004), the sex was not determined histologically but by the eversion of hemipenises in juveniles. The reported sex ratios may, then, actually reflect thermally induced phenotypic plasticity of hemipenial structure during development. Other possibilities such as differential fertilization or mortality also cannot be excluded (Harlow, 2004). Several species closely related to *Niveoscincus* possess heteromorphic sex chromosomes and thus possess GSD (Donnellan, 1985) *ex* (Olsson & Shine, 2001). The karyotypes of 'TSD' scincid species are not known. Although the consensus is not clear, these scincids are candidates for the transition from GSD to TSD and require further studies including cytogenetic analysis.

Of course, the mechanism of TSD for some species of agamids and geckos included in our dataset was also inferred from relatively small sample sizes. Nevertheless, as these species are members of clades in which TSD is well-documented in other species, it is reasonable to suggest that they possess TSD as well.

Taxon sampling and variability of sex-determining modes within clades

After withdrawal of the questionable data discussed above, our dataset comprised 416 taxa of squamate reptiles, which represents more than 5% of all currently recognized living species (Uetz, 2006). Although the maximum amount of data was accumulated, taxon sampling is one of the weak points of our study. Nonetheless, the phylogenetic analysis of the sex-determining mechanisms within Squamata is worthwhile. The results indicated that sex-determining mechanisms in Squamata are not as variable as commonly thought. In addition, these findings showed that particular large taxonomic groups usually share the mode of sex determination. For example, GSD of the same type characterizes all snakes and lacertids (both female heterogamety), and probably also all iguanids *s.l.* (male heterogamety). Such phylogenetic conservatism among relatively well-sampled clades suggests that the sex-determining mode of even small numbers of members of the other clades may indicate the mode of many of their relatives. Excluding the problematic cases of 'TSD' skinks, relatively well-supported variability in the mode of sex determination was found in the geckos and the common clade of agamids and chamaeleonids.

Are sex chromosomes an evolutionary trap?

Besides taxon sampling, the phylogenetic analyses of sex-determining modes in Squamata suffer from poorly known phylogenies (not even the relationships of the major groups are yet reliably resolved) and the lack of knowledge of the type of GSD in many species included in our analyses. We use the category 'GSD' in species, where GSD was inferred from the equal sex ratios at several constant incubation temperatures,

but where differences in genotypes between sexes – the only direct proof of GSD – have not been found yet (either because karyotypes are not known or sex chromosomes were not identifiable in karyotypes by cytogenetic methods used). We expect that after closer cytogenetic inspections most (if not all) cases of 'GSD' will be transferred to the XY or ZW category, as polygenic sex determination in vertebrates seems to be very rare (see Vandeputte *et al.*, 2007). In lineages where male, eventually female heterogamety is known in other members, we can assume that the same type of sex chromosomes will be uncovered in 'GSD' species (i.e. XY in 'GSD' skinks, iguanids and teiids, or ZW in *Pogona minor, Pogona barbata* and two species of the genus *Tympanocryptis*). From the view of transitions between TSD and GSD, the 'GSD' category is also nonproblematic in species where all closely related species possess TSD (*Phelsuma sundbergi, Amphibolurus norrisi, Ctenophorus fordii*). However, we have to note that the category 'GSD' in some other taxa could affect our results and interpretations. For example, 'GSD' in *Calotes versicolor* could be homologous with ZW of *Phrynocephalus vlangalii*, 'GSD' in *Furcifer pardalis* to ZW of *Bradypodion ventrale*, or 'GSD' in *Coleonyx* to XY in the Pygopodidae. Such findings would influence the reconstruction of the phylogeny of sex-determining systems not only in these specific lineages, but potentially also the whole reconstruction of the ancestral states in Squamata. The sex-determining mechanisms in the mentioned 'GSD' taxa clearly require further cytogenetic examinations.

Excluding the unresolved situation in the Gekkonidae, mapping on the morphological tree revealed 11–12 transitions in the direction from TSD to GSD. Based on this analysis, GSD and sex chromosomes evolved repeatedly and independently from the ancestral TSD. The origin of TSD from GSD within Agamidae is a product of the unresolved character state in the ancestor of the ((*Pogona* + *Tympanocryptis*) + *Lophognathus temporalis*) + (*Diporiphora albilabris* + (*Diporiphora bilineata* + *Amphibolurus nobbi*)) clade. The second putative origin of TSD from GSD was found within the genus *Gekko*, where male heterogamety is reported in one population of *Gekko japonicus* and *Gekko gecko*, whereas TSD was documented in another population of *G. japonicus*. Nevertheless, the reconstructed ancestral state can reflect poor taxon sampling and the need for further investigation of sex determination in other members of the genus *Gekko* and in related genera is evident. Based on the morphological tree, we conclude that TSD seems to be homologous in all reptiles that possess this sex-determining system; however, sex chromosomes are synapomorphies of particular groups with GSD and are not homologous among such groups. Sex chromosomes that evolved in parallel may have emerged from different pairs of autosomes. This hypothesis can be tested in the future by comparative cytogenetic analysis. For example, our phylogenetic analysis detected at least five independent appearances of sex chromosomes within agamids. An independent origin of agamid sex chromosomes is supported by the discovery of sex chromosomes in *Pogona vitticeps* (Ezaz *et al.*, 2005). In this species, the sex chromosomes are microchromosomes, and females are heterogametic. This system clearly evolved independently of the sex chromosomes in iguanids (sister group to agamids + chamaeleonids), which display large sex chromosomes and heterogametic males (Olmo, 2004).

The phylogenetic analysis based on the morphological tree matches the expectation of Ohno's (1967) hypothesis on the repeated origin of sex chromosomes from autosomes well. Correspondingly, we suggest that sex chromosomes in general and especially well-differentiated sex chromosomes serve as an evolutionary trap. In other words, once the sex chromosomes have evolved, they can never be lost again.

In the reconstruction based on the molecular tree according to Townsend *et al.* (2004), we can find an additional transition from GSD to TSD: the common ancestor of agamids and chamaeleonids evolved TSD from GSD secondarily (Fig. 2). This putative conversion of GSD to TSD deserves further study, and this notion could be supported or rejected by examination of karyotypes of agamids and chamaeleons and their sister groups. If this TSD mechanism indeed evolved from GSD, the XY sex chromosomes of the iguanids *s.l.* and the gymnophtalmids + teiids and the scincids (and maybe also the dibamids) should be homologous. This assumption is contradicted by the pattern described in the classical tree, where XY sex chromosomes in the iguanids *s.l.* evolved independently from those of the skinks and gymnophtalmids + teiids (cf. Figs 1, 2). Moreover, the fate of sex chromosomes in the ancestor of chamaeleonids and agamids after the shift to TSD should be thoroughly examined. Fortunately, comparative analyses of chromosomal morphology and structure or gene content could potentially give extensive information on the homology of the pairs of chromosomes and their evolutionary transitions (Dobigny *et al.*, 2004; Carvalho & Clark, 2005; Matsuda *et al.*, 2005). The accumulation of new cytogenetic data is, therefore, needed not only to test alternative hypotheses on the evolution of sex-determining systems in squamate reptiles but also to resolve the inconsistencies among the competing views on squamate phylogeny.

The phylogenetic topology following Vidal & Hedges (2005) does not provide resolved ancestral states at the base of the tree, which is caused by the altered

topology of *Dibamus*. The ancestral state for the Gekkota can be either TSD or male heterogamety. In the first case, the transitions between sex-determining mechanisms would be the same as in the previously discussed trees. If male heterogamety was ancestral in the Gekkota, TSD would emerge repeatedly within this clade. Remarkably, based on morphology, Rieppel (1984) concluded that the dibamids are nested within the family Scincidae, which concurs with their male heterogamety. Further information on the phylogenetic position of the Dibamidae, as well as on their sex chromosomes, is highly needed.

Taken together, the phylogenetic analyses demonstrate the rarity and/or uncertainty of GSD to TSD transitions in the phylogeny of squamate reptiles. All potential transitions from GSD to TSD in Squamata are either rather questionable (e.g. the cases of 'TSD' in skinks, or emergence of TSD within the genus *Gekko*), or reflect inconsistencies in underlying phylogenetic hypotheses (e.g. the putative transition of GSD to TSD in the common ancestor of agamids + chamaeleonids) or ambiguous reconstruction of character states. In all these cases, the cytogenetic information supporting the disappearance of sex chromosomes is lacking. Janzen & Krenz (2004) found six transitions from TSD to GSD and not even a single transition in the opposite direction in turtles, in which TSD is the ancestral state as well. GSD in the common ancestor of birds was probably also derived from TSD, as the closest living relatives (crocodiles) possess TSD. Thus, the transitions from TSD to GSD, as originally hypothesized by Ohno (1967), are well supported by phylogenetic analyses and cytogenetic information in reptiles, whereas the transitions in the opposite direction are scarce and largely unsupported.

We should note that combining all three GSD character states ('XY', 'ZW', 'GSD') into a single category and repeating the analyses with just two character states (GSD and TSD) changes the results of the analyses: (1) the ancestral state in Squamata is either unresolved or GSD; and (2) TSD emerges from GSD repeatedly and rather frequently (results not shown). This pattern emerges mainly as a result of the ubiquity of GSD among squamate reptiles. Just from the comparative analyses alone, we cannot unequivocally demonstrate that these conclusions are false; nevertheless, we can test this scenario – the opposite of the 'evolutionary trap hypothesis' – in the future by additional data from comparative cytogenetic studies. However, even recent evidence (although rather limited) suggests that taking all GSD as a single category is misleading. Because of the general rarity of TSD in squamate reptiles (we advocate that well-supported TSD can be found only in geckos and dragon lizards), mixing all types of GSD into a single category would imply that GSD in the majority of squamate species is homologous. If, for example (and we think this very likely), the comparative cytogenetics demonstrates that sex chromosomes (and sex-determining genes) in *Phrynocephalus vlangalii* (ZW) are not homologous to those of *Polychrus* (XY) or snakes (ZW), mixing these nonhomologous characters into a single category would bias the analyses by the introduction of homoplasy. Next, assuming homology of GSD in nearly all squamates requires frequent changes between male and female heterogamety. Under the 'evolutionary trap hypothesis', we are inclined to view female and male heterogamety as independently derived from the ancestral TSD.

A recent phylogenetic analysis of sex-determining mechanisms in teleost fishes (Mank, Promislow & Avise, 2006) contradicts the 'evolutionary trap hypothesis' to some extent. The authors revealed substantial evolutionary lability in sex-determining modes and numerous transitions between alternative modes in both directions. Nevertheless, the problem of taxon sampling in fishes is even worse than in the case of squamate reptiles, and the phylogenetic trees for the fishes are not very stable. Moreover, considerably more GSD than ESD data exist for fishes (based on the presence of sex chromosomes). This bias could potentially lead to the overestimation of GSD as the ancestral state as discussed above. Also, we must consider the potential bias in phylogenetic analyses concerning traits with highly unequal probabilities of transitions in a given direction. For example, if the ancestral situation was TSD and transitions from TSD to GSD were frequent and always unidirectional, GSD would occur in the majority of the terminal branches of the phylogenetic tree. Reconstruction of the ancestral trait, then, incorrectly indicates GSD to be ancestral. Stireman (2005) described an analogous situation in the reconstruction of host spectra in herbivorous insects. If specialists emerged frequently from generalists, all phylogenetic reconstructions, regardless of whether based on maximum parsimony or maximum likelihood principles, would indicate the specialist way of life as the ancestral state. The solution in the case of specialists vs. generalists is rather problematic as past host spectra are extremely difficult to reconstruct; however, cytogenetic data may provide independent evidence for particular evolutionary scenarios in the reconstruction of ancestral sex-determining systems. In addition, fishes may prove to be different from reptiles as they have less often differentiated sex chromosomes and higher developmental plasticity of gonadal tissues in comparison to other vertebrates. In fact, the differentiation of gonads proceeds in relatively later stages of ontogenetic development in fishes, and the gonads are generally more labile (Mank *et al.*, 2006). In addition,

it is not clear whether most fish species with reported ESD really lack sexual differences in genotypes, or possess just GSD with environmental effects.

Transitions between male and female heterogamety

Our phylogenetic analyses detected independent putative transitions between XY and ZW sex-determining systems. The morphological tree suggests two independent transitions from female to male heterogamety: the first one in the common ancestor of skinks, and the second one in the common ancestor of the gymnophthalmids + teiids (Fig. 1). Transitions between male and female heterogamety are apparent (and even more numerous) under the molecular trees as well, but the direction in some of them depends on the resolution of polytomies (Figs 2, 3). The first explanation for the occurrence of such 'transitions' is an independent origin of sex chromosomes from autosomes of the now extinct or unsampled ancestor with TSD. The second possibility is the direct change of male heterogamety to female heterogamety. Recently, both possibilities were suggested to explain the synteny of avian sex chromosomes with parts of sex chromosomes in the platypus *Ornithorhynchus anatinus* (Veyrunes *et al.*, 2008). Under the first explanation, sex chromosomes in birds and monotremes evolved independently from the same ancestral pair of autosomes, whereas the second possibility (less plausible to us) would suggest homology of GSD between these two groups and thus independent evolution of TSD in the ancestors of turtles, crocodilians, and tuataras (+ Squamata?) and a direct conversion from female to male heterogamety. Such conversions have been well documented (e.g. Kozielska *et al.*, 2006; Miura, 2008), but this possibility seems to be restricted to newly emerged, not yet fully differentiated sex chromosomes, in which YY or WW individuals are still viable and fertile (Marín & Baker, 1998). As inferred from the extensive phylogenetic conservatism of sex-determining systems in many highly radiated animal clades (birds, mammals, snakes, iguanids, butterflies and their sister taxon sedge flies), highly differentiated sex chromosomes prevent the switch between male and female heterogamety (Traut & Marec, 1996; Scherer & Schmid, 2001). In squamate reptiles, the direct conversion from female to male heterogamety assumes the homology of ZW sex-determining systems in the Lacertidae + Amphisbaenia and Varanidae + snakes under the morphological tree (Fig. 1). Under the molecular trees (Figs 2, 3), the transitions between female and male heterogamety can be supported by the homology of ZW sex chromosomes in Varanidae and snakes (and possibly also in Lacertidae + Amphisbaenia in the tree following the topology of Townsend *et al.* (2004), or of XY sex chromosomes in the gymnophtalmids + teiids, iguanids *s.l.* and the Scincidae (and possibly also the dibamids).

CONCLUSIONS

In summary, our analyses have demonstrated that the mechanisms of sex determination in squamate reptiles do not seem to be phylogenetically as labile as usually viewed and that the variability in sex determination is likely to occur only in the gekkotan and agamid + chamaeleonid clades. Moreover, TSD appears to be the ancestral state in squamates, and transitions between sex-determining modes were probably unidirectional from TSD to GSD (or at least all transitions in the opposite directions are not well supported by the recent evidence). Thus, the ancestral absence of sex chromosomes was probably replaced by the repeated emergence of sex chromosomes. Evolved sex chromosomes appear to be permanent fixtures in the evolution of the species. Thus, we propose that the presence of sex chromosomes (GSD) constitutes an evolutionary trap that blocks the backward transition to TSD. This evolutionary trap hypothesis shines new light on phylogenetic conservatism of sex-determining systems in many large clades such as butterflies, snakes, birds, and mammals. However, our analyses have established a clear need for further studies to examine the evolutionary trap hypothesis. In general, additional information on sex-determining mechanisms in some included and many additional species, on the homology of sex chromosomes among animal clades by comparative cytogenetic analysis, as well as improved knowledge of the tree of life, are needed to verify this hypothesis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. List of squamate taxa included in phylogenetic analyses with their sex-determining modes and references.

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Kapitola II.

Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 My divergence.

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Chromosoma, v tisku (DOI 10.1007/s004 12-011-0322-0)

Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 My divergence

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Abstract

The divergence of lineages leading to extant squamate reptiles (lizards, snakes and amphisbaenians) and birds occurred about 275 million years ago. Birds, unlike squamates, have karyotypes that are typified by the presence of a number of very small chromosomes. Hence, a number of chromosome rearrangements might be expected between bird and squamate genomes. We used chromosome-specific DNA from flow sorted chicken (*Gallus gallus*) Z sex chromosomes as a probe in cross-species hybridization to metaphase spreads of 28 species from 17 families representing most main squamate lineages and single species of crocodiles and turtles. In all but one case, the Z chromosome was conserved intact despite very ancient divergence of sauropsid lineages. Furthermore, the probe painted an autosomal region in seven species from our sample with characterized sex chromosomes, and this provides evidence against an ancestral avian-like system of sex determination in Squamata. The avian Z chromosome synteny is, therefore, conserved albeit it is not a sex chromosome in these squamate species.

Keywords chicken Z - comparative genomics - cross-species chromosome painting - FISH - karyotype evolution - reptilian chromosomes

Introduction

With the exception of mammals, all extant amniotes are members of the clade Sauropsida. The group includes archosaurs (birds and crocodiles), turtles, and lepidosaurs (tuataras and squamate reptiles). The phylogenetic relationship of archosaurs, turtles and lepidosaurs is not resolved unequivocally, but a recent view prefers the sister position of turtles and archosaurs (e.g. Shedlock and Edwards 2009). Sauropsids consist of around 18000 species. The lineages with the largest diversity are birds with more than 9000 species and squamates (snakes, lizards and amphisbaenians) with 8200 described species. In contrast, there are only 300 turtle and 23 crocodile species. The divergence of the lineages leading to extant birds and squamates occurred about 275 million years ago (Shedlock and Edwards 2009). Species richness of squamates is associated with great karyotype variability, with levels of karyotypic changes similar to those of some mammals, such as carnivores and bats (reviewed in Olmo 2008). On the contrary, turtles and crocodiles show a low variability in number, morphology and G-banding pattern of chromosomes (reviewed in Olmo 2008). Conserved karyotypes are observed also in birds, despite a number of species with low diploid numbers comparable to squamates. Birds show a slow rate of interchromosomal rearrangements, with most species showing diploid numbers between 76 and 80 (reviewed in Ellegren 2010). Most bird karyotypes are typified by a number of very small chromosomes resulting from fissions in the lineage leading to birds (e.g. Nakatani et al. 2007). This series of fissions would explain the difference in diploid numbers between birds and squamates, where 2n is usually between 24 and 46 (reviewed in Olmo 1986). Hence, many chromosome rearrangements might be expected that would reduce whole chromosome homology between bird and squamate chromosomes. Despite the karyotype variability of squamates, emerging data on the whole genome sequence in the iguanian lizard *Anolis carolinensis* suggest relatively low levels of interchromosomal rearrangements between these two clades (*cf.* AnoCar2.0; http://www.ensembl.org/Anolis_carolinensis). Similarly, Giovannotti et al. (2009) recently showed highly conserved karyotypes within a single family (Scincidae) by cross-species chromosome painting. At a different taxonomic level, comparative gene mapping has revealed highly conserved linkage homology between an agamid lizard (*Leiolepis reevesii*) and a snake (*Elaphe quadrivirgata*) (Srikulnath et al. 2009). However, this conservation may be explained by the fact that snakes and the family Agamidae seem to be members of the same phylogenetic lineage (Toxicofera; Vidal and

Hedges 2005). Homology of most chromosomes across other phylogenetic lineages within Squamata has not been studied using comparative mapping.

Particular attention has been paid to the investigation of avian Z chromosome synteny among various lineages. Avian Z chromosome has a relatively stable genomic content and was subjected only to intrachromosomal rearrangements among birds (Itoh et al. 2006). In chicken, the Z chromosome encompasses about 1000 genes (Bellott et al. 2010). Preliminary painting results have been reported between chicken (*Gallus gallus*) Z (GGAZ) and acrocentric chromosome 6 of both *Crocodylus niloticus* and *Trachemys scripta* (Kasai et al. 2003). GGAZ genes mapped to both arms of a metacentric chromosome in a crocodile (*Crocodylus siamensis*; Kawai et al. 2007) and to a single acrocentric chromosome pair in a turtle (*Pelodiscus sinensis*; Matsuda et al. 2005). Orthologues of GGAZ genes were also localised on an arm of a metacentric chromosome pair in two species of agamid lizards (*Leiolepis reevesii* and *Pogona vitticeps*), in an iguanian lizard (*Anolis carolinensis*) and in snakes (Matsubara et al. 2006; Srikulnath et al. 2009; Ezaz et al. 2009a; http://www.ensembl.org/Anolis_carolinensis), and on a pair of acrocentric sex chromosomes in a gecko (*Gekko hokouensis*; Kawai et al. 2009). Conserved synteny of part of the genome homologous to GGAZ has been indicated in amphibians, the sister group to amniotes, e.g. in salamanders of the genus *Ambystoma* (Smith and Voss 2007) and in a frog *Xenopus tropicalis* (O'Meally et al. 2010).

Conservation of the avian Z chromosome is lost in mammals, the sister lineage to sauropsids, and GGAZ orthologues can be found on several different chromosomes. In humans, most of the GGAZ genes can be found on four chromosomal pairs (Nanda 2002; Bellott et al. 2010). These chromosomes were already separate in the eutherian ancestor (Yang et al. 2003). With the exception of a small region, most of GGAZ was probably still syntenic in the marsupial ancestor. Further loss of synteny occurred during subsequent marsupial radiation (Rens et al. 2001, 2003). The loss of synteny also occurred in the mammalian monotreme lineage; GGAZ genes are found on six autosomal pairs and X_1, Y_1, X_2, Y_2, X_3 and X_5 sex chromosomes in platypus (Rens et al. 2007). The situation in mammals may be a consequence of chromosome instability factors such as mobilization of retrotransposons or increase activity of interspersed repetitive elements in the mammalian lineage (Janes et al. 2010).

Bird Z chromosome evolution was studied particularly because of the general interest in sex determination. Different sex determining systems can be found across

amniotes. Birds have a ZZ/ZW sex determining system. Viviparous mammals (Theria) ancestrally possess XX/XY sex determining system with sex chromosomes homologous to autosomes in birds (Fridolfsson et al. 1998; Nanda et al. 2002). Monotremes, the sister group of Theria, have male heterogamety but with multiple sex chromosomes. Their sex chromosomes are not homologous to sex chromosomes in Theria, and are only partly syntenic with sex chromosomes of birds (Rens et al. 2007; Veyrunes et al. 2008). This partial synteny, together with the fact that sex chromosomes in a species of gecko (*Gekko hokouensis*; Kawai et al. 2009) are syntenic with bird sex chromosomes, led to the hypothesis of an ancestral bird-like ZZ/ZW system (Graves 2009; O'Meally et al. 2010). According to this scenario, this system of sex determination was ancestral for all amniotes and the ancestor of sauropsids including the ancestor of their inner group Squamata. However, several other reports are not in favour of this model. The bird Z chromosome is syntenic with one arm in a pair of autosomes in elapid, viperid and colubrid snakes, documenting that although both birds and advanced snakes possess ZZ/ZW sex determining systems, their sex chromosomes were derived from different ancestral chromosomal pairs (Matsubara et al. 2006; O'Meally et al. 2010). Molecular cytogenetic studies also demonstrate that sex chromosomes evolved at least twice independently in the agamid lizards (Ezaz et al. 2009b). However, the limited sampling of other phylogenetic lineages of squamate reptiles with potentially independently evolved sex chromosomes (Pokorná and Kratochvíl 2009; Ezaz et al. 2010) has not permitted further tests of this hypothesis.

The aim of the present paper is to extend genomic studies of chromosomes syntenic with the bird Z chromosome in representative species of the main lineages of squamates, and in crocodiles and turtles using cross-species chromosome painting and comparative mapping. We specifically focussed on sampling squamate phylogenetic lineages to test the conservation status of the Z chromosome. We tested for synteny of avian sex chromosomes with the known sex chromosomes of several squamate species to investigate the ancestral bird-like ZZ/ZW hypothesis.

Material & Methods

Species studied

We selected 28 species of squamate reptiles from 17 families representing the main lineages in the taxonomic nomenclature and phylogeny suggested by Vidal and Hedges (2005). We included members of the clades Gekkota (families Pygopodidae,

Diplodactylidae, Eublepharidae and Gekkonidae), Scinciformata (families Scincidae, Cordylidae and Gerrhosauridae), Teiformata (Teiidae), Lacertibaenia composed of Lacertiformata (Lacertidae) and Amphisbaenia (Trogonophidae), and Toxicofera (Iguania: families Agamidae, Chamaeleonidae, Iguanidae and Polychrotidae; Anguimorpha: families Varanidae, Anguidae; Serpentes: Colubridae). For the list of the species and sex of the individuals examined (where known) see Table 1. Only the dibamids, probably the most basal squamates (Vidal and Hedges 2005), are missing due to difficulties in obtaining material from this secretively living tropical lineage.

All squamate individuals were captive bred animals maintained in the laboratory breeding rooms at the Faculty of Science, Charles University in Prague, Czech Republic (accreditation No. 24773/2008-10001) and in the Dipartimento di Biochimica, Biologia e Genetica, Università Politecnica delle Marche, Ancona, Italy. Blood sample of *Anguis fragilis* was kindly provided to MG and VC by Dr Raffaele Gattelli (Aquae Mundi - Ravenna, Italy). We also included a turtle (Testudines: Emydidae: *Trachemys scripta elegans*) and a crocodile (Crocodylia: Crocodylidae: *Crocodylus niloticus*) thanks to La Ferme aux Crocodiles (Pierrelatte, France).

Metaphase chromosome preparation

Metaphase chromosome spreads were prepared from cultures of whole blood or from fibroblast cell cultures obtained from embryonic epithelium following protocols described in Ezaz et al. (2005) and Rens et al. (2006) with slight modifications. For the list of tissues collected in each species see Table 1.

Probe preparation

The paints from chicken Z chromosome and chromosomes of *C. niloticus* and *S. scincus* were prepared from chromosomes sorted with a dual laser cell sorter (Mo-Flo, Dako) at the Cambridge Resource Centre for Comparative Genomics, Department of Veterinary Medicine, University of Cambridge, Cambridge, UK, as previously described (Yang et al. 1995). Sorted chromosomes were used as templates for DNA amplification by DOP-PCR (Telenius et al. 1992). Primary DOP-PCR product was used as a template in a secondary DOP-PCR to incorporate biotin-16-dUTP (Roche).

Fluorescent in situ hybridization and signal detection

FISH was performed using the protocol described in Yang et al. (1995) and Rens et al. (1999; 2006) with several modifications. Briefly, slides were dehydrated through ethanol series; aged for 1 h at 65 °C; denatured in 70% formamide/0.6x saline-sodium citrate (ssc) at 70 °C for 1 up to 4 min (time depending on species and metaphase

preparation) and dehydrated again. Eight microliters of biotinylated probe were precipitated in ethanol and resuspended in 11 µl of hybridization buffer [40% deionized formamide (v/v), 10% dextran sulfate, 2x ssc, 0.05 M phosphate buffer, pH 7.3]. This mixture was denatured for 10 min at 75 °C, preannealed at 37 °C for 30 min and applied to each slide. Hybridization was carried out at 37 °C for three nights. Posthybridization washes were performed in 40 % formamide/1.8x ssc twice for 5 min each, followed by 2x ssc twice for 5 min each and 4x ssc with 0.05% Tween-20 (4xT) once for 4 min. Washes were carried out at 42 °C. Probe detection was carried out using 200 µl of diluted (1:500) Cy3-Streptavidin antibody (Amersham) per slide at 37 °C for 30 min. After detection, slides were washed in 4xT three times for 3 min each at 42 °C and mounted in Vectashield Mounting Medium with DAPI (VECTOR Laboratories).

Microscopy and data analyses

Images were captured using the Leica DMRXA microscope equipped with CCD camera (Photometrics Sensys). Leica CW4000 FISH software (Leica Microsystems) was used to capture grey-scale images and to superimpose the source images into colours to visualize the results of the FISH. For the reciprocal FISH, where the probe from *Scincus scincus* (SSC) chromosome was hybridized to GGA metaphases, a new image enhancement procedure was written in Perl software code to visualise the signal. Due to the necessary low stringency wash, the signal is sandwiched between two types of background. The first conventional background is caused by non-specific binding of SSC DNA. The second type of background, a by-product of low stringency washes, consists of relative bright spots randomly distributed over the microscope slide. The Perl code removes the first type of background and differentiates the second type of background from the signal by presenting it in a different green pseudo-colour. The signal is presented in a yellow pseudo-colour. Chromosome analyses were made from images that were processed with a 9 x 9 high-pass spatial filter, displayed in contrast-adjusted reversed greyscale images and classified using Leica CW4000 Karyo software (Leica Microsystems). The final composition of the images was performed in CorelDraw X5 software (Corel Corporation).

Gene mapping

Chromosome-specific DNA from flow-sorted chromosomes of *A. carolinensis, G. japonicus, C. niloticus, T. scripta* (details to be published elsewhere) and *S. scincus* (Giovannotti et al. 2009) was used as template for PCR mapping of chicken Z-linked genes (*ATP5A1, CHD1, DMRT1, GHR, MUSK, PRLR, PRR16*) using conserved gene-

specific primers for confirmation of chromosome homologies. Primers designed by Brunner et al. (2001) were used to amplify the conserved B region of *DMRT1* gene. Primers for amplification of the other five genes were newly designed according to *Anolis carolinensis* DNA sequence available as Ano.Car1.0 assembly (Broad Institute, available at https://www.broadinstitute.org/ftp/pub/assemblies/reptiles/lizard/AnoCar1.0). Genomic regions of *A. carolinensis* encompassing *ATP5A1, CHD1, GHR, MUSK, PRLR* genes were aligned in CLUSTALW (Larkin et al. 2007) with the orthologous regions of *G. gallus*, *X. tropicalis* (http://www.ensembl.org) and *G. hokouensis* (GeneBank accession number AB326214). For primer sequences see Table 2. The gene was considered localised to a particular chromosome if the PCR product had the expected size and was obtained only from that particular chromosome template.

Results

Karyotyping

We present the karyotypes of three species (*Chamaeleo calyptratus, Oedura monilis*, and *Rhacodactylus ciliatus*) which, to our knowledge, are the only ones in our series that have not been described previously (Fig. 1). The karyotype in both sexes of *Chamaeleo calyptratus* (*2n* = 24) consists of 12 pairs of chromosomes, the six largest pairs are metacentric, the chromosome pairs 7 and 8 are submetacentric and the chromosome pairs 9-12 are dot-like (Fig. 1a). No heteromorphic sex chromosomes were observed in either sex of this species with genotypic sex determination (Andrews 2005). The karyotype of *Oedura monilis* (*2n* = 38) is composed of 19 pairs of chromosomes, from which four pairs are metacentric, eight pairs submetacentric/subtelocentric and seven pairs acrocentric (Fig. 1b). We did not observe any differences between the male and female karyotypes. The karyotype of *Rhacodactylus ciliatus* (*2n* = 38) consists of 19 mostly acrocentric elements of gradually decreasing size although the small chromosomes 16 and 18 may be subtelocentric (Fig. 1c). No heteromorphic sex chromosomes were observed in either sex, which is in agreement with the putative temperature-dependent sex determination reported in this species (Seipp and Henkel 2000).

Cross-species painting with GGAZ probe and reciprocal painting

The GGAZ probe hybridized to a region on a single arm of the second largest biarmed chromosome pair in 15 species studied: *Varanus acanthurus* (Fig. 2a), *Hierophis viridiflavus* (Fig. 2c), *Chamaeleo calyptratus* (Fig. 2d), *Pogona vitticeps* (Fig. 2e),

Anolis carolinensis (Fig. 2f), *Iguana iguana* (Fig. 2g), *Trogonophis wiegmanni* (Fig. 2h), *Cordylus tropidosternum* (Fig. 2k), *Gerrhosaurus flavigularis* (Fig. 2l), *Chalcides ocellatus* (Fig. 2m), *Eumeces schneideri* (Fig. 2n), *Lepidothyris fernandi* (Fig. 2o), *Trachylepis quinquetaeniata* (Fig. 2p), *Scincus scincus* (Fig. 2q) and *Tiliqua scincoides* (Fig. 2r). In some species (e.g. Fig. 2f,m) the paint did not cover the whole arm which may be due to the presence of a heterochromatic block (co-localization of heterochromatic blocks and the unpainted region was confirmed in *C. ocellatus* and *A. carolinensis* by C-banding; data not shown).

The probe hybridized to a pair of acrocentric chromosomes in 12 species studied: *Anguis fragilis* (Fig. 2b), *Eremias velox* (Fig. 2i), *Cnemidophorus deppei* (Fig. 2j), *Rhacodactylus ciliatus* (Fig. 2t), *Lialis burtonis* (Fig. 2u), *Eublepharis macularius* (Fig. 2v), *Coleonyx elegans* (Fig. 2w), *Coleonyx variegatus* (Fig. 2x), *Goniurosaurus luii* (Fig. 2y), *Gekko japonicus* (Fig. 2z), *Gekko ulikovskii* (Fig. 2α) and *Gekko vittatus* (Fig. 2β). In *Oedura monilis*, the probe hybridized to a pair of subtelocentric chromosomes (Fig. 2s). In the case of *E. velox* an extra small region of hybridization was observed at the end of the long arm of a small acrocentic pair (Fig 2i). This was the only species that showed a rearrangement of the chicken Z.

In *Trachemys scripta elegans* and *Crocodylus niloticus* the probe assigned a pair of acrocentric chromosomes (chromosome 6 in both organisms; Fig. 3a,b). The probe from chromosome 6 of *C. niloticus* painted the p-arm of chromosome 2 in *Iguana iguana* (Fig. 3c), the same region painted by the GGAZ probe (Fig. 2g). The GGAZ probe hybridized to a region on a single arm of the second largest biarmed chromosome pair in *S. scincus* (SSC2). Therefore, we used the probe from SSC2 for the reciprocal FISH between a lizard species and the bird. The probe painted the Z chromosome and a part of the W chromosome of *Gallus gallus*, however, no clear signal was detected on any chicken autosomes (Fig. 3d).

Synteny of GGAZ with squamate sex chromosomes

The homology of sex chromosomes with GGAZ was assessed in members of lineages of squamate reptiles with identified sex chromosomes. We recognised the sex chromosomes according to their size and morphology. In *C. elegans* ($X_1X_1X_2X_2/X_1X_2Y$), the Y chromosome is the only metacentric chromosome in the male karyotype and it has a similar DNA content to X_1 and X_2 together, a result of a putative Robertsonian fusion between the proto-Y chromosome with an autosome. The probe from the Y chromosome of *C. elegans* hybridized to all three (X_1, X_2, Y) sex

chromosomes (Pokorná et al., 2010). On the other hand, the GGAZ probe has a different painting pattern; it paints just a pair of acrocentric chromosomes in this species (Fig. 2w). In *L. burtonis* ($X_1X_1X_2$ X_2/X_1X_2Y), the X_1, X_2 and Y sex chromosomes are distinctly smaller (Gorman and Gress 1970) than the chromosome pair painted by the GGAZ probe (Fig. 2u). In *P. vitticeps* (ZZ/ZW), the sex chromosomes are microchromosomes (Ezaz et al. 2005), and the GGAZ labelled only the p-arm of the chromosome 2 (Fig. 2e). Sex chromosomes of *V. acanthurus* (ZZ/ZW) are small acrocentrics (King et al. 1982), but the GGAZ painted an arm of the second largest metacentric chromosome pair (Fig. 2a). Sex chromosomes are acrocentric in *E. velox* (ZZ/ZW) (Ivanov et al. 1973), but much smaller than the acrocentric pair painted by the GGAZ probe (Fig. 2i). The chromosome pair with the additional signal is not the *E. velox* sex chromosome pair either; the *E. velox* W chromosome can be easily identified as a DAPI positive chromosome and was not painted by the GGAZ probe (Fig. 2i). In *H. viridiflavus* (ZZ/ZW), we identified the W chromosome as a small metacentric chromosome using FISH with a probe derived from the flow-sorted W chromosomes of the same species (results not shown). The GGAZ probe painted a chromosome different from the W and Z chromosomes, it hybridized to the p-arm of the second largest chromosome pair in this species (Fig. 2c). Sex-specific polymorphism in the regions bearing nucleolar organisers was found in *S. scincus* (XX/XY; Caputo et al. 1994). This polymorphism is linked to a medium-sized subtelocentric pair, while GGAZ painted the p-arm of the chromosome 2 (Fig. 2q), as in the other species of skinks analysed. Thus, in all these cases GGAZ was not syntenic with the sex chromosomes of these squamate species.

Gene mapping

PCR-based mapping with seven genes linked to both arms of the avian Z chromosome confirmed the results of chromosome painting in the respective squamates. Five genes (*ATP5A1, DMRT1, GHR, MUSK, PRLR*) were mapped in *A. carolinensis*, four (*CHD1, DMRT1, MUSK, PRR16*) in *S. scincus*, and three (*CHD1, DMRT1, GHR*) in *G. japonicus*. In all three species, the genes mapped onto the chromosomes labelled by the GGAZ probe. Moreover, PCR gene mapping localised *DMRT1* on chromosome 6 of both *T. scripta* and *C. niloticus* (results not shown) and thus confirmed the homologies found by GGAZ chromosome painting. For an example of PCR gene mapping see Fig. 4.

Discussion

In the present study, we demonstrate that comparative painting with a whole-chromosome chicken Z probe can be used effectively in distant lineages of sauropsids. The estimated divergence time between crocodile and bird lineages is about 220 MYA, between turtle and bird lineages about 230 MYA, and the deepest divergence between squamate and bird lineages occurred about 275 MYA (Shedlock and Edwards 2009). So far, chromosome painting was applied successfully mostly among members of phylogenetic lineages with more recent divergence, for example among mammalian infraclasses with a maximum of 166 My divergence (Glas et al. 1999; Ferguson-Smith and Trifonov 2007). The maximum divergence bridged by chromosome painting was previously reported between a human (HSA4) and chicken (GGA4) chromosome (Chowdhary and Raudsepp 2000). Chromosome painting has also been performed in bird orders (e.g. Shetty et al. 1999), among turtle families (Mühlmann-Díaz et al. 2001) and between chicken and turtle (Graves and Shetty 2001), or among members of a single family in bony fish (Ráb et al. 2008) and lizards (Giovannotti et al. 2009). In contrast, the technique usually fails in angiosperms, which have typically high levels of interchromosomal rearrangements and higher turnover of non-coding sequences (Kejnovský et al. 2009).

The applicability of chromosome painting across highly distant lineages of sauropsids that we report here suggests conservatism of both coding and non-coding sequences of at least that part of the genome which is homologous to GGAZ. The sequences within avian micro-chromosomes have a relatively higher mutation rate (Ellegren 2010) and cross-species painting with probes from these chromosomes is expected to be more troublesome. Our painting results are supported by the reciprocal hybridization of SSC2 on chicken metaphases, where SSC2 painted the Z and a part of the W chromosome (presumably the pseudo-autosomal region) in chicken (Fig. 3d). However, because the SSC2 chromosome is metacentric and only part of the p-arm is syntenic with GGAZ, we expected, but did not observe, a signal on those chicken autosomes that are homologous to the q-arm of SSC2. These autosomes are presumably microchromosomes as similar homology was demonstrated in another lizard and snake species (e.g. Srikulnath et al. 2009; http://www.ensembl.org/Anolis_carolinensis). The lack of signal on bird autosomes in reciprocal painting with the SSC2 probe could be attributed to small size or lower conservation of chicken chromosomes syntenic with the q-arm of SSC2. Our conclusions on conserved synteny of GGAZ in reptiles are further

supported by the gene mapping results and the hybridization of the GGAZ chromosome paint and the paint of the homologous chromosome in *C. niloticus* (CNI6) to the same chromosome region in a lizard (*Iguana iguana*; Figs. 2g, 3c). Moreover, the probe from the SSC2 chromosome painted the chromosomes painted by the GGAZ probe in other skink species included into our dataset (*Chalcides ocellatus*, *Eumeces schneideri*, *Lepidothyris fernandi*, *Trachylepis quinquetaeniata*; cf. to Giovannotti et al. 2009; *Tiliqua scincoides* – M. Giovannotti, unpublished results). Nevertheless, the reliability of our results is best supported by the genetic content of the p-arm of the chromosome 2, painted by the GGAZ probe in our experiments (Fig. 2f), in *Anolis carolinensis.* The ENSEMBL database demonstrates strong synteny of this part with GGAZ in the annotated genome of this species (http://www.ensembl.org/Anolis_carolinensis) as well.

The GGAZ paint identified homologous chromosomes in reptiles, although the bird chromosome Z is known to be subjected to substantive changes characteristic for sex chromosomes, such as accumulation of non-coding sequences and duplications of male-specific genes (Bellott et al. 2010; Li et al. 2010). One may speculate that these genomic changes that took place after the chromosome started to function as a sex chromosome in birds could affect the efficiency of the probe from GGAZ in cross-species chromosome painting in reptiles. However, the results described here demonstrate that the genomic changes due to the sex-chromosome evolution of GGAZ did not interfere with its hybridization to homologous regions in phylogenetically distant lineages.

Previous results on karyotypic evolution and genome dynamics in reptiles have been obtained mainly by physical mapping of protein-coding genes (e.g. Matsuda et al. 2005; Kawai et al. 2009; Srikulnath et al. 2009; Ezaz et al. 2009a; but see Mühlmann-Díaz et al. 2001 and Giovannotti et al. 2009 for chromosome painting). The advantage of gene mapping is its usefulness for detailed studies of intrachromosomal rearrangements, but at the same time, its applicability is limited mainly to coding sequences. On the other hand, chromosome painting points also to the dynamics of non-coding sequences in detecting conserved synteny. Although the techniques differ in several important aspects, our results obtained by chromosome painting confirm and extend the previously published results obtained by physical gene mapping (Fig. 5). Our results largely confirm conserved synteny of part of the genome homologous with GGAZ in crocodiles (Kasai et al. 2003; Kawai et al. 2007 in *C. siamensis*), turtles (Matsuda et al. 2005 in *Pelodiscus sinensis*) and many lineages of squamates

(previously reported in several species of snakes, the gecko *Gekko hokouensis*, the dragon lizards *Leiolepis reevesii* and *Pogona vitticeps*, and the iguanian lizard *Anolis carolinensis*; reviewed by Ezaz et al. 2009a; Srikulnath et al. 2009, O'Meally et al. 2010, http://www.ensembl.org/Anolis_carolinensis). GGAZ-linked genes localize on the opposite sides of the centromere in *C. siamensis* (Kawai et al. 2007). This probably reflects a centromere repositioning in an ancestor of this species. Ezaz et al. (2009a) mapped five genes linked to GGAZ to the chromosome 2 of *Pogona vitticeps* (PVI2), four of them to the p-arm, while a single one (*APTX*) mapped to the q-arm of this chromosome. Our chromosome painting correctly identified the p-arm of the PVI2 as syntenic with GGAZ, but there was no homology with the long arm of PVI2.

The hybridisation patterns of the chromosome paint of the metacentric GGAZ are similar across various reptile lineages and showed a clear phylogenetic signal, e.g. the signal covers only acrocentric chromosomes in all gekkotan lizards, and a region on the p-arm of a metacentric chromosome pair in all members of Iguania and Scinciformata (Fig. 5). Despite the selection of a small number of species as representatives of their particular lineages, the phylogenetic distribution of the two character states (GGAZ homologous to a pair of acrocentric/part of metacentric chromosomes) suggests that the chromosome homologous to the avian Z was ancestrally a single acrocentric chromosome in sauropsids (Fig. 5). It is known that the q-arm in the metacentric chromosome is syntenic with GGA12,13,16,18 in an agamid lizard (*Leiolepis reevesii*). Similarly, in the snake *Elaphe quadrivirgata* and the iguanian lizard *Anolis carolinensis*, the q-arm of the chromosome 2 contains homologs of genes linked to the GGA12,13,18 chromosomes, while the p-arm is syntenic with GGAZ (Srikulnath et al. 2009; http://www.ensembl.org/Anolis_carolinensis), which suggests that this state might be homologous in these groups. Future research should test whether the biarmed chromosome containing the part homologous with avian Z in other squamate groups is also syntenic with the same chicken chromosomes.

According to our results, GGAZ is not syntenic with sex chromosomes in *Lialis burtonis* (Pygopodidae), *Coleonyx elegans* (Eublepharidae), *Eremias velox* (Lacertidae), *Varanus acanthurus* (Varanidae), *Scincus scincus* (Scincidae) and *Hierophis viridiflavus* (Colubridae). Non-homology of sex chromosomes with avian sex chromosomes among squamate reptiles was previously reported in *Pogona vitticeps* (Agamidae; Ezaz et al. 2009a) and several species of advanced snakes (*Notechis scutatus*: Elapidae; *Elaphe quadrivirgata*, *Stegonotus cucullatus*: Colubridae;

Protobothrops flavoviridis: Viperidae; Matsubara et al. 2006; O'Meally et al. 2010). The only species of squamates known to possess sex chromosomes syntenic with avian sex chromosomes is the gecko *Gekko hokouensis* (Kawai et al. 2009). Thanks to better phylogenetic coverage, we were able to give arguments that do not support the ancestral bird-like ZZ/ZW hypothesis for squamates. Phylogenetic distribution of two character states (sex chromosomes syntenic/not syntenic with GGAZ; Fig. 6) shows that none of the species studied in the non-gekkotan squamate lineages (Scincidae, Lacertidae, Varanidae, Agamidae, Serpentes) have sex chromosomes syntenic to chicken Z. Furthermore, *G. hokouensis* is a member of the family Gekkonidae, which is a nested crown group in the gekkotan lineage (Gamble et al. 2008). More basal gekkotans (here represented by the species *Lialis burtonis* and *Coleonyx elegans*) do not possess sex chromosomes syntenic with avian sex chromosomes. In addition, closely related species congeneric with *G. hokouensis* have either homomorphic sex chromosomes or even XX/XY sex chromosomes (Solleder and Schmid 1984; Shibaike et al. 2009). Most likely, the situation in *G. hokouensis* is a derived condition. The sex chromosomes seem to be an evolutionary novelty of this species and evolved most likely via independent co-option of the same chromosomal pair for sex determination in the avian and the gecko ancestors. In summary, the ancestral bird-like ZZ/ZW hypothesis for squamates receives little support from molecular-cytogenetic analyses of synteny of sex chromosomes. Recent phylogenetic analyses argue for temperature-dependent sex determination (Pokorná and Kratochvíl 2009) and temperature-dependent sex determination or XY sex-determining system (Organ and Janes 2008) as ancestral for Squamata. Nevertheless, more data are needed for better understanding of the evolution of sex determination in this group.

In conclusion, the results show that the part of genome homologous with the avian Z chromosome exhibits conserved synteny across reptiles, despite the very ancient times of their divergence. We specifically demonstrated this conservative synteny across Squamata, the group with extensive radiation and phenotypic and karyotypic diversification. In addition, the results lead to phylogenetic arguments against the hypothesis of avian-like sex determination in the squamate and reptilian ancestor. Our study demonstrates that chromosome painting may be used for testing chromosome homology across widely phylogenetically distributed sauropsids despite the very ancient times of their divergence. Future studies should be aimed at testing

whether other sauropsid chromosomes share equally conservative syntenies to gain further insights into reptilian genome dynamics.

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Table 1 The list of species, sex of the individuals and origin of material used in the study.

Species	**Family**	**Sex (where known)**	**Tissue collected**
Varanus acanthurus	Varanidae	-	blood
Anguis fragilis	Anguidae	female	blood
Hierophis viridiflavus	Colubridae	female	blood
Chamaeleo calyptratus	Chamaeleonidae	male	blood
Pogona vitticeps	Agamidae	female	embryonic epithelium
Anolis carolinensis	Polychrotidae	male	embryonic epithelium
Iguana iguana	Iguanidae	male	blood
Trogonophis wiegmanni	Trogonophidae	female	blood
Eremias velox	Lacertidae	female	blood
Cnemidophorus deppei	Teiidae	male	blood
Cordylus tropidosternum	Cordylidae	female	blood
Gerrhosaurus flavigularis	Gerrhosauridae	male	blood
Chalcides ocellatus	Scincidae	male	blood
Eumeces schneideri	Scincidae	-	blood
Lepidothyris fernandi	Scincidae	-	blood
Trachylepis quinquetaeniata	Scincidae	female	blood
Scincus scincus	Scincidae	male	blood
Tiliqua scincoides	Scincidae	male	blood
Oedura monilis	Diplodactylidae	female	blood
Rhacodactylus ciliatus	Diplodactylidae	female	blood
Lialis burtonis	Pygopodidae	male	blood
Eublepharis macularius	Eublepharidae	-	blood
Coleonyx elegans	Eublepharidae	male	blood
Coleonyx variegatus	Eublepharidae	-	blood
Goniurosaurus luii	Eublepharidae	-	embryonic epithelium

Gekko japonicus	Gekkonidae	-	embryonic epithelium
Gekko ulikovskii	Gekkonidae	male	blood
Gekko vittatus	Gekkonidae	female	blood
Trachemys scripta	Emydidae	-	embryonic epithelium
Crocodylus niloticus	Crocodylidae	male	embryonic epithelium
Gallus gallus	Phasianidae	female	embryonic epithelium

Table 2 Primers for GGAZ-linked genes mapped to sorted chromosomes by PCR. Exon numbers correspond to *Anolis carolinensis* genome.

B = G + T + C; D = G + A + T; K = G + T; R = A + G; S = G + C; W = A + T; Y = C+T; V = G + A + C

Gene	Exon	PCR product size (base pairs)	Forward primer (5′→3′)	Reverse primer (5′→3′)
ATP5A1	8	217	TACCGYCAGATGTCYCTGCT	CCTGYCCATCDGTGATRGAG
CHD1	36	281	CATAGAAARYTAGATGACCACAGGA	TCTGWTGARTGTTCAAATGGAGA
GHR	7	772	GAGAGRACYGRAGGSTCRGATACTG	TGTGGAGAYTGTACYACRTGAATGG
MUSK	14	580	GYCTKCTGTTTGARTACATGG	TCAGMWGGYAKCTTGCTCCAA
PRLR	17	112	AAWCTGAAGAAYTGYTGAGTGC	AGYTGCTGRTCYTCGCTGTC
PRR16	1	205	CAGGGAGGTBCACTTRCACA	VGTCTTCTGKGGTTTTGGTG

Legends:

Fig. 1 Karyotypes of a) *Chamaeleo calyptratus* male, b) *Oedura monilis* female and c) *Rhacodactylus ciliatus* female arranged from DAPI stained metaphases. Bars equal 10 μm.

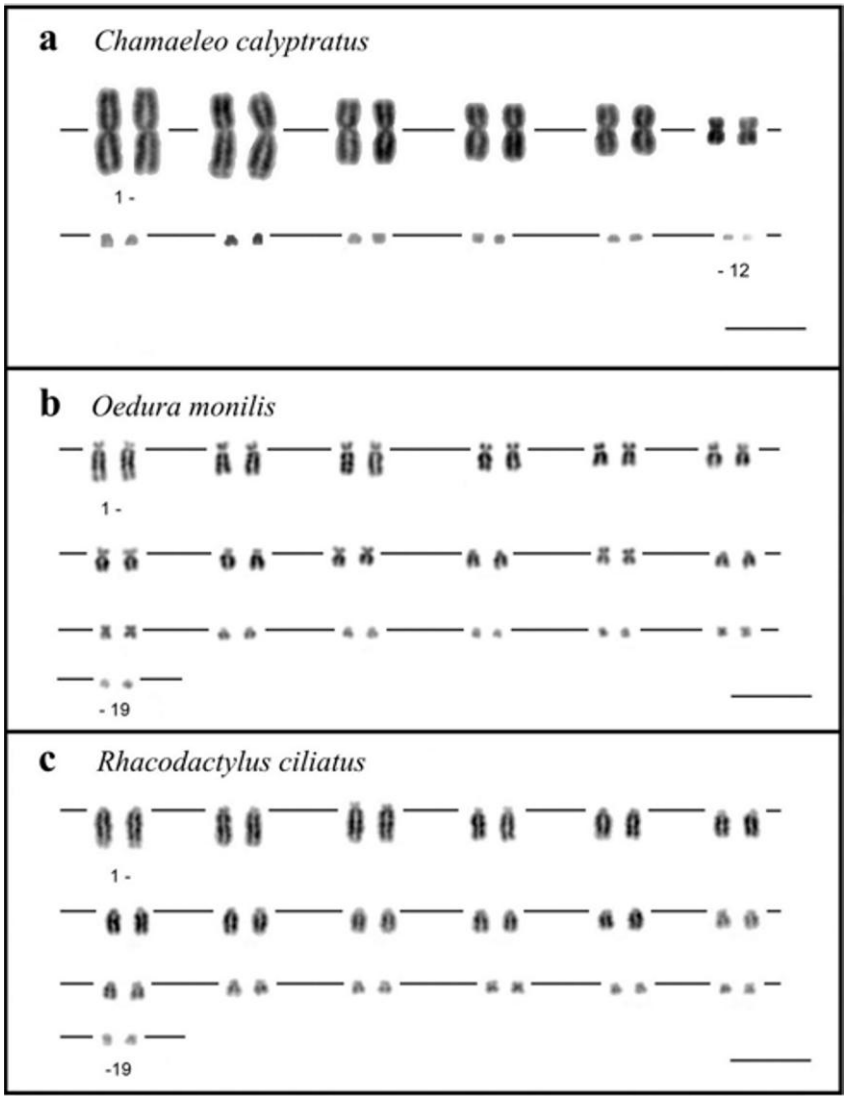
Fig. 2 Cross-species FISH with GGAZ probe on metaphases of a) VAC- *Varanus acanthurus*; b) AFR- *Anguis fragilis*; c) HVI- *Hierophis viridiflavus*; d) CCA- *Chamaeleo calyptratus*; e) PVI- *Pogona vitticep*s; f) ACA- *Anolis carolinensis*; g) IIG- *Iguana iguana*; h) TWI- *Trogonophis wiegmanni*; i) EVE- *Eremias velox*; j) CDE- *Cnemidophorus deppei*; k) CTR- *Cordylus tropidosternum*; l) GFL- *Gerrhosaurus flavigularis*; m) COC- *Chalcides ocellatus;* n) ESC- *Eumeces schneideri;* o) LFE- *Lepidothyris fernandi;* p) TQU- *Trachylepis quinquetaeniata*; q) SSC- *Scincus scincus*; r), TSC- *Tiliqua scincoides*; s) OMO- *Oedura monilis*; t) RCI- *Rhacodactylus ciliatus*; u) LBU- *Lialis burtonis*; v) EMA- *Eublepharis macularius*; w) CEL- *Coleonyx elegans*; x) CVA- *Coleonyx variegatus*; y) GLU- *Goniurosaurus luii*; z) GJA- *Gekko japonicus*; α) GUL- *Gekko ulikovskii*; β) GVI- *Gekko vittatus*. Arrows and asterisks indicate FISH signals.

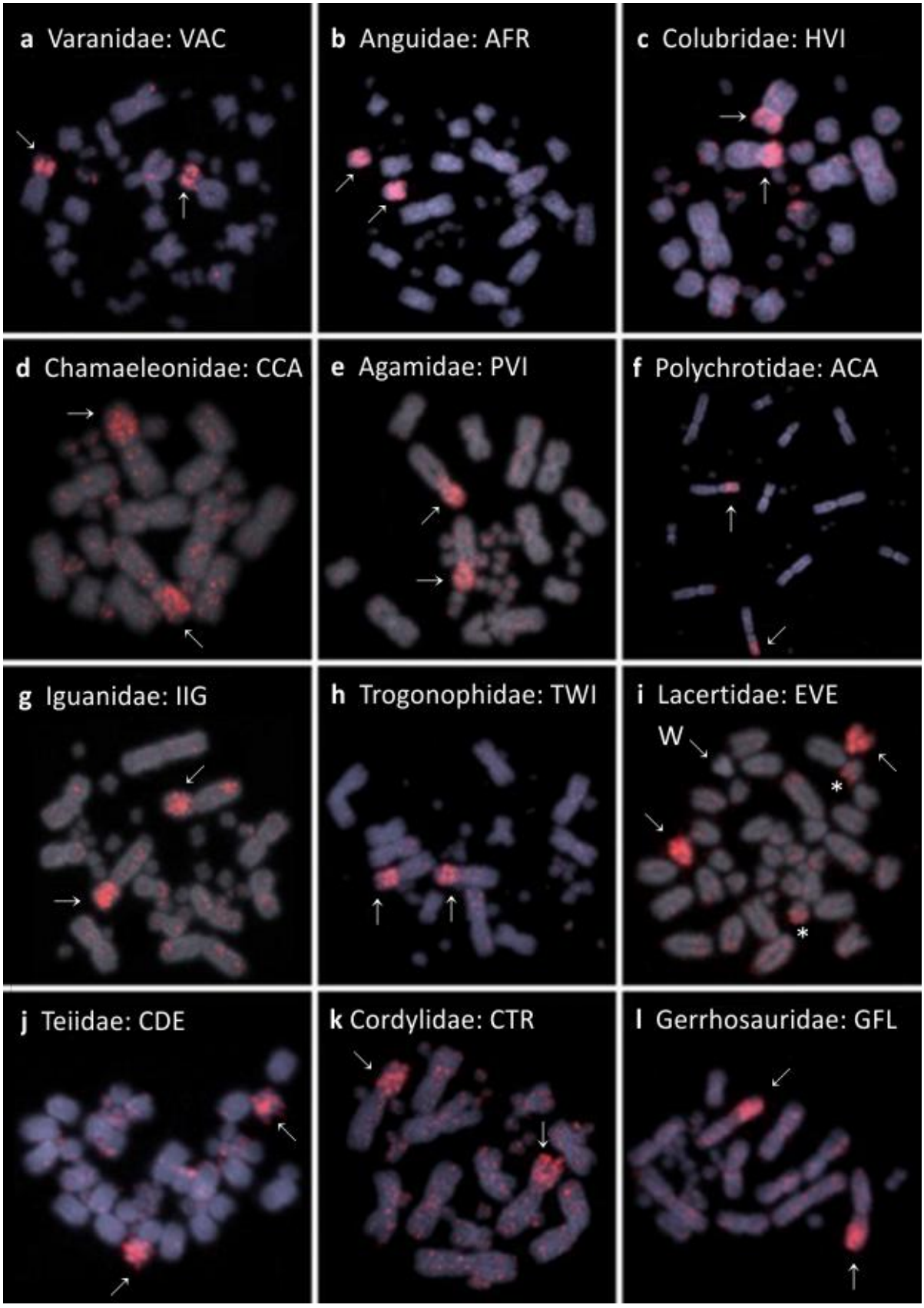
Fig. 3 Cross-species FISH with GGAZ probe on metaphases of a) TSCR- *Trachemys scripta* and b) CNI- *Crocodylus niloticus*. c) Cross-species FISH with probe from CNI 6 chromosome on metaphase of IIG- *Iguana iguana*. d) Cross-species FISH with probe from SSC2 chromosome on metaphase of GGA- *Gallus gallus* (the signal is yellow).

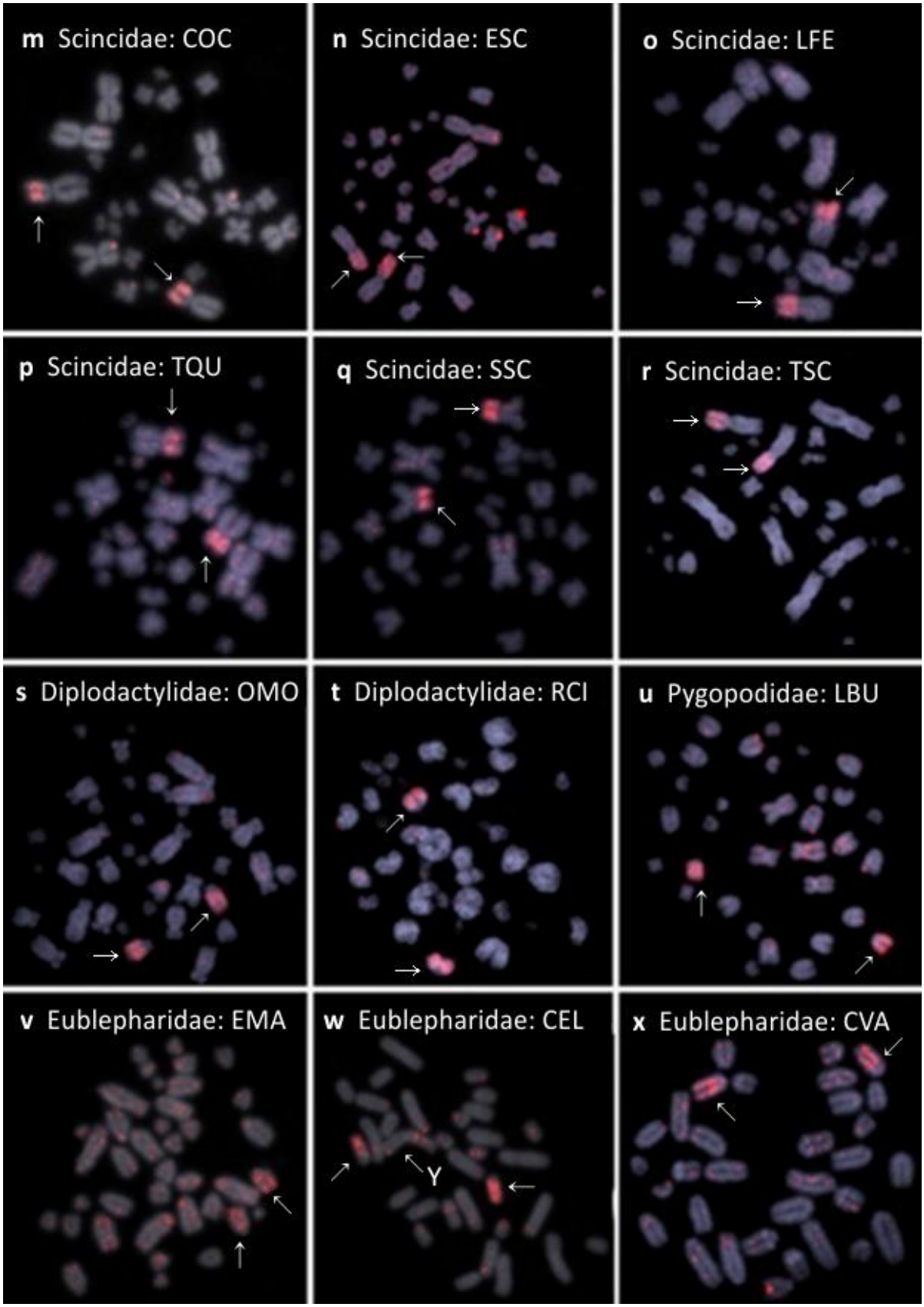
Fig. 4 An example of PCR-gene mapping. Numerals refer to chromosome pairs in *Scincus scincus* karyotype according to Giovannotti et al. (2009). *MUSK* exon 14 mapped to *S. scincus* chromosome 2. L = DNA ladder; BL = blank control; SSC = genomic DNA of *S. scincus* used as a positive control.

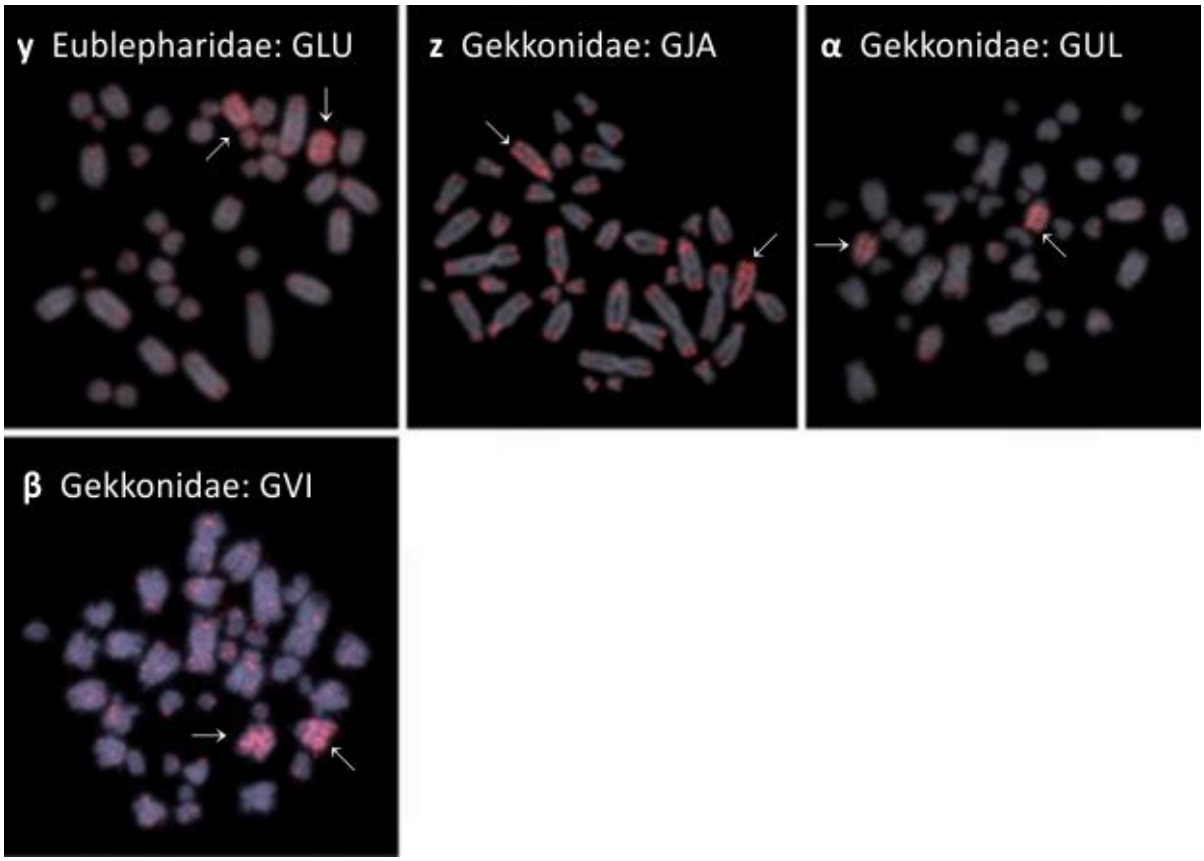
Fig. 5 Phylogenetic distribution of homology between GGAZ and either a single arm of a metacentric chromosome pair (black line) or acrocentric chromosomes (white line) in reptiles. In *C. siamensis*, GGAZ is syntenic with the p and part of q arm of metacentric chromosome (grey line). Diagram combines our results with data from Srikulnath et al. (2009). Only ancestral situation (acrocentric Z chromosome; Nishida-Umehara et al. 2007) is for simplicity depicted in birds and turtles.

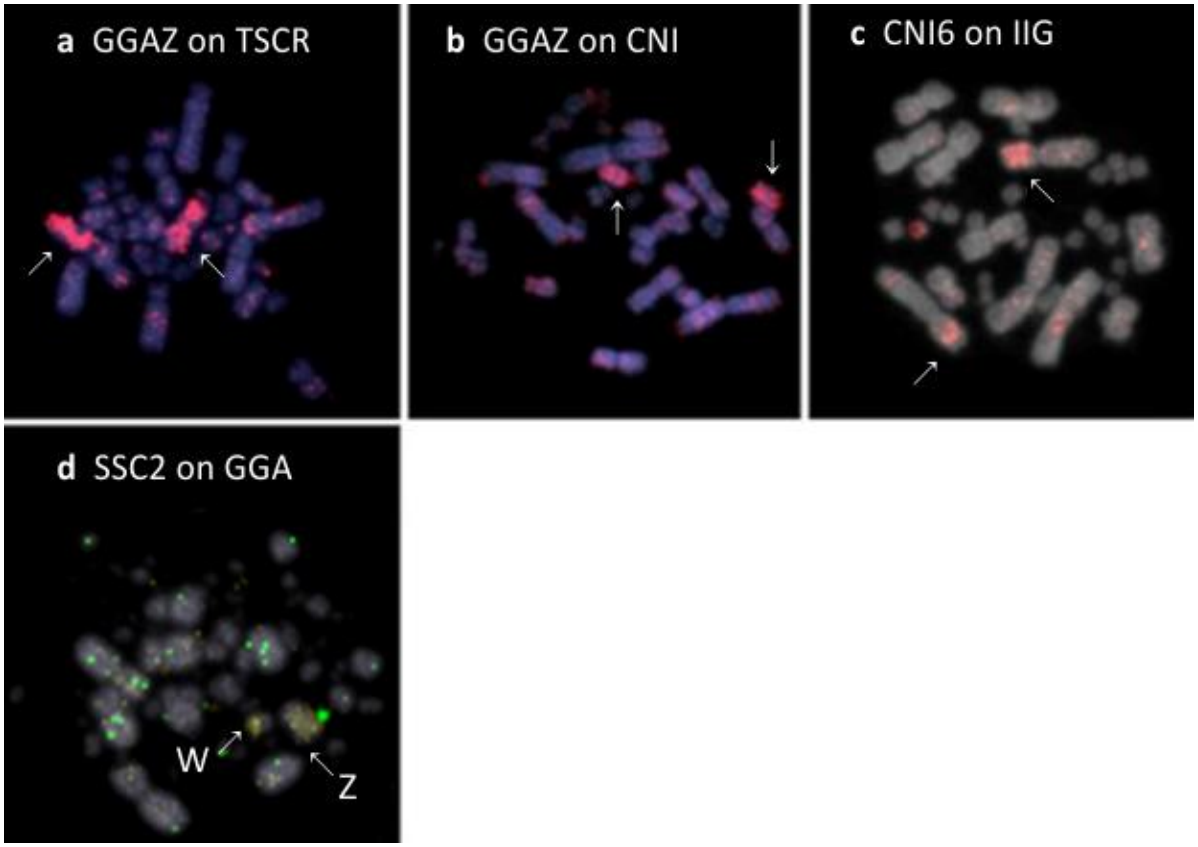
Fig. 6 Phylogenetic distribution of synteny (white line) or non-synteny (black line) of sex chromosomes of members of several squamate lineages with GGAZ. XY stands for male heterogamety, ZW for female heterogamety and X_1X_2Y for male hetogamety with multiple sex chromosomes. Note that five species are members of the single clade (Serpentes) and share the homologous sex chromosomes. Diagram combines our results with the data from other papers (*Elaphe quadrivirgata* and *Protobothrops flaviviridis*: Matsubara et al. 2006; *Gekko hokouensis*: Kawai et al. 2009; *Stegonotus cucullatus* and *Notechis scutatus*: O'Meally et al. 2010).

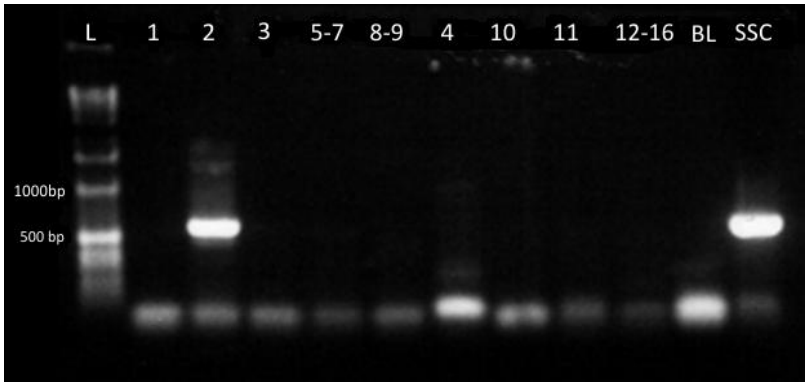

a Chamaeleo calyptratus
1 -
- 12
b Oedura monilis
1 -
- 19
c Rhacodactylus ciliatus
1 -
-19

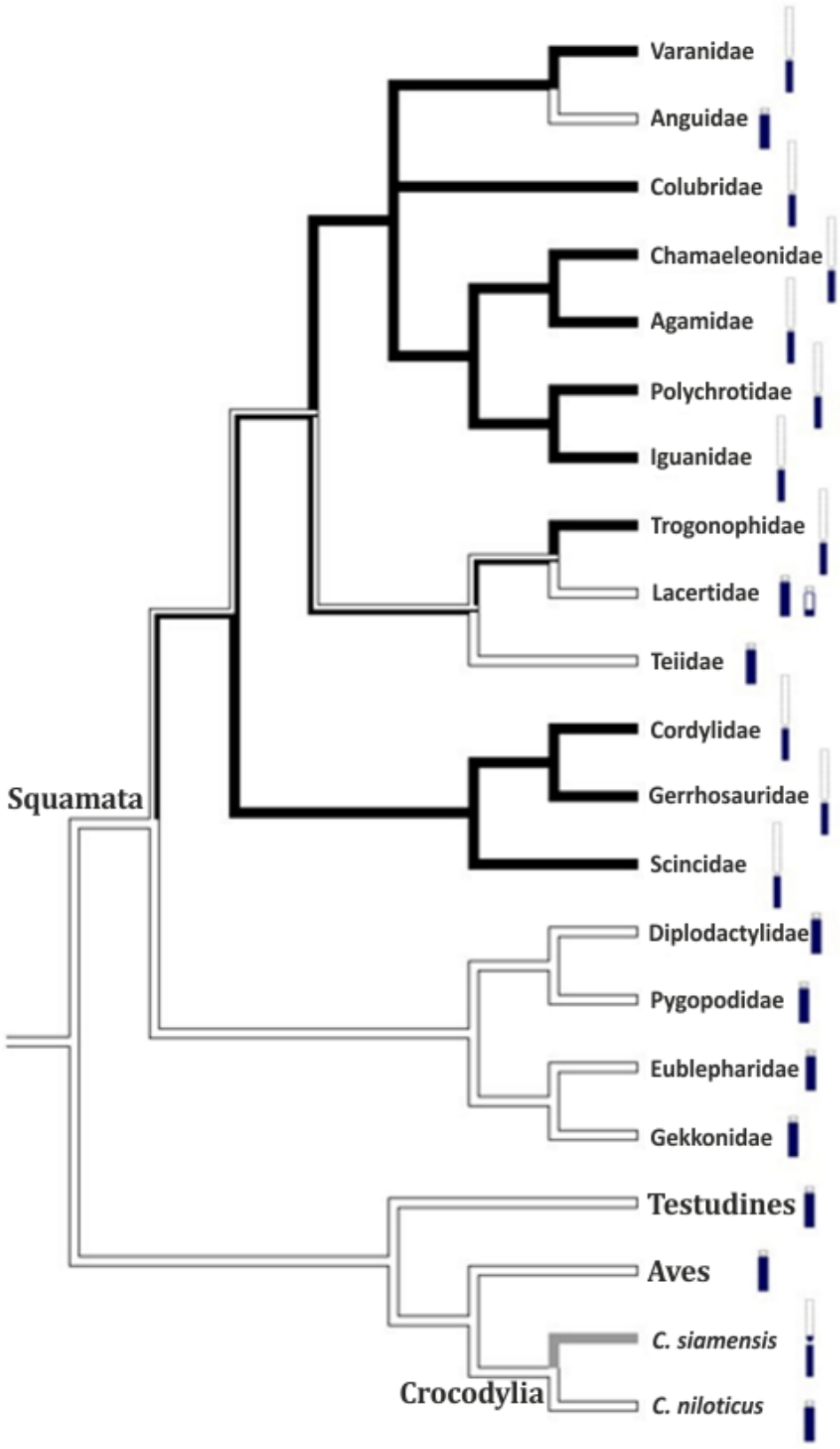

a Varanidae: VAC
b Anguidae: AFR
c Colubridae: HVI
d Chamaeleonidae: CCA
e Agamidae: PVI
f Polychrotidae: ACA
g Iguanidae: IIG
h Trogonophidae: TWI
i Lacertidae: EVE
W
j Teiidae: CDE
k Cordylidae: CTR
l Gerrhosauridae: GFL

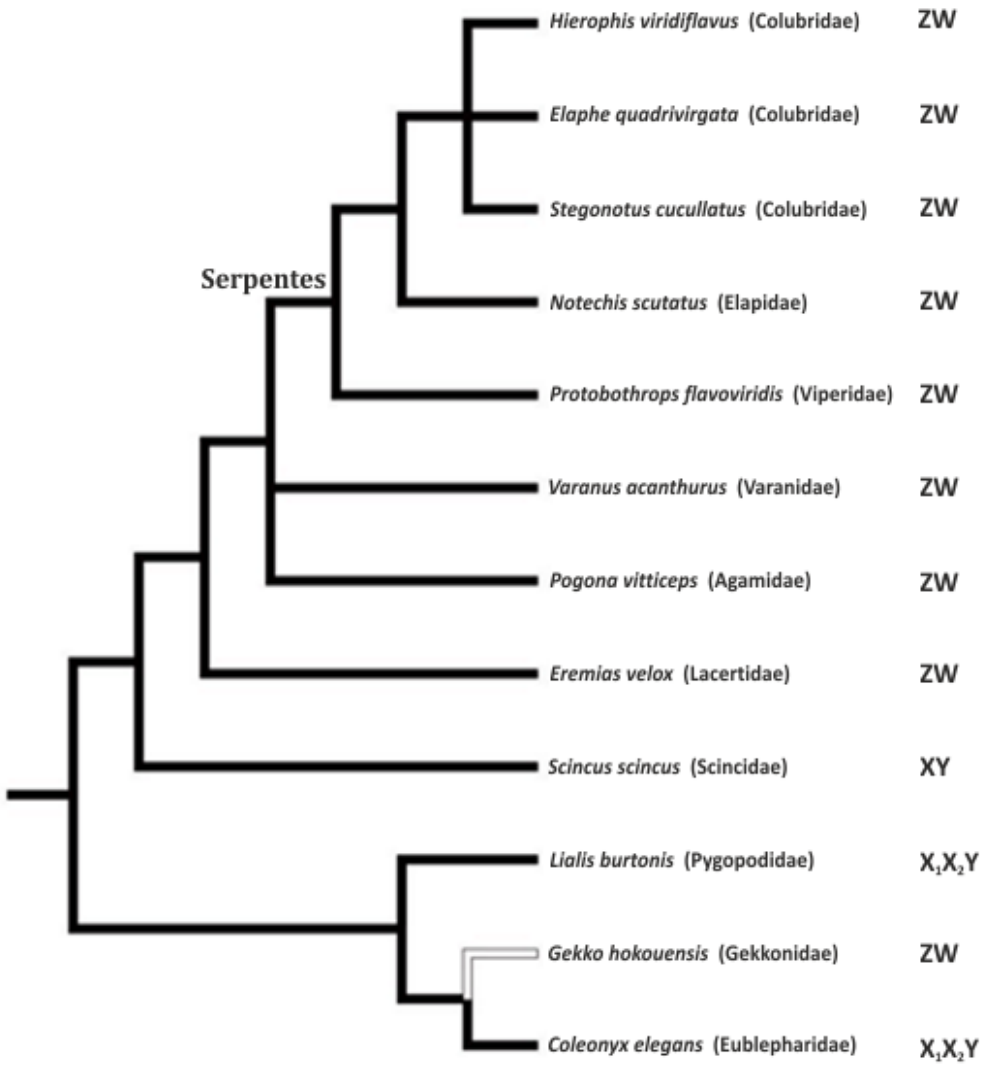

m Scincidae: COC
n Scincidae: ESC
o Scincidae: LFE
p Scincidae: TQU
q Scincidae: SSC
r Scincidae: TSC
s Diplodactylidae: OMO
t Diplodactylidae: RCI
u Pygopodidae: LBU
v Eublepharidae: EMA
w Eublepharidae: CEL
Y
x Eublepharidae: CVA


y Eublepharidae: GLU
z Gekkonidae: GJA
α Gekkonidae: GUL
β Gekkonidae: GVI


a GGAZ on TSCR
b GGAZ on CNI
c CNI6 on IIG
d SSC2 on GGA
W
Z


L
1
2
3
5-7
8-9
4
10
11
12-16
BL
SSC
1000bp
500 bp


Varanidae
Anguidae
Colubridae
Chamaeleonidae
Agamidae
Polychrotidae
Iguanidae
Trogonophidae
Lacertidae
Teiidae
Cordylidae
Gerrhosauridae
Scincidae
Squamata
Diplodactylidae
Pygopodidae
Eublepharidae
Gekkonidae
Testudines
Aves
C. siamensis
Crocodylia
C. niloticus


Serpentes
Hierophis viridiflavus (Colubridae) ZW
Elaphe quadrivirgata (Colubridae) ZW
Stegonotus cucullatus (Colubridae) ZW
Notechis scutatus (Elapidae) ZW
Protobothrops flavoviridis (Viperidae) ZW
Varanus acanthurus (Varanidae) ZW
Pogona vitticeps (Agamidae) ZW
Eremias velox (Lacertidae) ZW
Scincus scincus (Scincidae) XY
Lialis burtonis (Pygopodidae) X1X2Y
Gekko hokouensis (Gekkonidae) ZW
Coleonyx elegans (Eublepharidae) X1X2Y

Kapitola III.

Can we predict emergence of a key evolutionary novelty? Reconstruction of ancestral sex-determining mechanism in Amniote vertebrates questions predictions for extinct marine reptiles.

Martina Pokorná, Lukáš Kratochvíl, Willem Rens

Rukopis

Can we predict emergence of a key evolutionary novelty? Reconstruction of ancestral sex-determining mechanism in Amniote vertebrates questions predictions for extinct marine reptiles

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Amniote vertebrates (mammals and reptiles including birds) possess various sex-determining mechanisms. Based on data in living species, Organ *et al.*[1] described a coevolutionary relationship between sex-determining mechanism and egg laying versus bearing live young. The authors used this relationship to reconstruct the evolution of genotypic sex determination before acquiring live birth in three extinct marine lineages and concluded that genotypic sex determination was a key trait enabling adaptive radiations of these lineages in marine environment. Here we alert that their conclusions are questioned by reconstruction of ancestral sex-determining mechanism in amniote vertebrates. The prediction of genotypic sex determination in the ancestors of extinct marine lineages is based only on the expectation of analogous evolution of sex-determining mechanisms in extant and extinct lineages.

Two alternative sex-determining mechanisms can be found in amniotes: environmental sex determination, frequently realized as temperature-dependent sex determination (TSD), and genotypic sex determination (GSD). Sex-determining mechanisms can influence evolutionary potential and its knowledge would help us to understand causes of evolutionary success of particular lineages. Unfortunately, it is impossible to uncover sex-determining mechanisms directly from fossil records. Based on the analysis of the distribution of sex-determining mechanisms (TSD versus GSD) and reproductive modes

(oviparity: egg laying versus viviparity: bearing live young) among living amniotes, Organ *et al.*[1] concluded that GSD was an important precondition for the evolution of viviparity. Subsequently, they predicted that the *Propalaeotherium*, an extinct horse, and the ancestors of three extinct amniote lineages (mosasaurs, sauropterygians and ichthyosaurs) possessed GSD. The authors proposed that GSD was the key trait enabling the three marine reptile lineages to evolve viviparity and pelagic lifestyle.

The presence of GSD in the *Propalaeotherium* is highly probable. As all living viviparous mammals (Theria) share the same pair of sex chromosomes[2] we can assume that they inherited GSD from their common ancestor. The *Propalaeotherium*, deeply nested within living members of Theria, simply possessed GSD homologous with GSD of recent therians.

However, the evidence that the three extinct reptile lineages inherited GSD from their respective ancestors as well is poor. TSD is ancestral for turtles, crocodiles, tuataras, and probably also for Squamata, the clade encompassing lizards and snakes[3-6]. The ancestors of birds and mammals probably evolved GSD independently. Bird sex chromosomes and parts of the multiple pairs of sex chromosomes in monotremes share gene content[7], nevertheless, this cannot be taken as a support for the common origin of GSD in these lineages. More likely, the same chromosomal pair independently happened to switch into sex chromosomes in these taxa. In amniotes, thus, the ancestral sex-determining mechanism seems to be TSD, not GSD (Fig. 1). The alternative scenario, ancestral GSD, would require more evolutionary changes and four transitions from GSD to TSD, i.e. four losses of sex chromosomes. As no transition from presence to absence of sex chromosomes among amniotes has been supported by cytogenetic evidence[6] the feasibility of this transition is speculative. This view is further supported by the low rates of transitions in this direction estimated by Organ *et al.*. The phylogenetic position of ichthyosaurs and sauropterygians, not nested within lineages with ancestral GSD, suggests that GSD would be an evolutionary novelty of these lineages. Mosasauroids are nested within squamates, where GSD is common, but even here we lack any evidence that GSD in their putative sister lineages (snakes, varanids, iguanas) was inherited from their common ancestor.

Evolution is a historical accidental process and we cannot predict past or future singular events, such as emergence of a key evolutionary novelties, based only on their

statistical probabilities[8,9]. Unlike in the extinct horse, the predictions of GSD for at least two extinct marine lineages are not based on ancestral coherency, but on an estimation of the evolutionary transition rates between sex-determining mechanisms and reproductive modes. Moreover, statistical predictions by Organ *et al.´*s[1] are biased by the non-uniform distribution of transitions among states in living lineages. Viviparity evolved from oviparity more than 100 times within amniotes, and only one of these transitions occurred outside Squamata, i.e. within mammals[10]. Consequently, the association between GSD and viviparity is based largely on the frequent parallel transitions to viviparity and ubiquity of GSD in squamates, where well-supported TSD is restricted to dragon lizards and geckos [6,11]. It is highly questionable to expect that evolution in any extinct lineage proceeded in a direct analogy to patterns found among members of a single lineage of living organisms. Importantly, also the evolutionary advantage of the transition to GSD in mesozoic marine amniots is questionable. Transition from TSD to GSD is often hypothesised to occur in environments instable in temperature[12]. The stable ocean temperature can be expected as a convenient environment for adjustment of TSD to produce equal sex ratio at this particular temperature, giving the same result as GSD. However, as even other possibilities cannot be excluded, e.g. selection of proper thermal environments by gravid mothers or social sex determination, the sex-determining mechanisms in these extinct lineages cannot be easily predicted.

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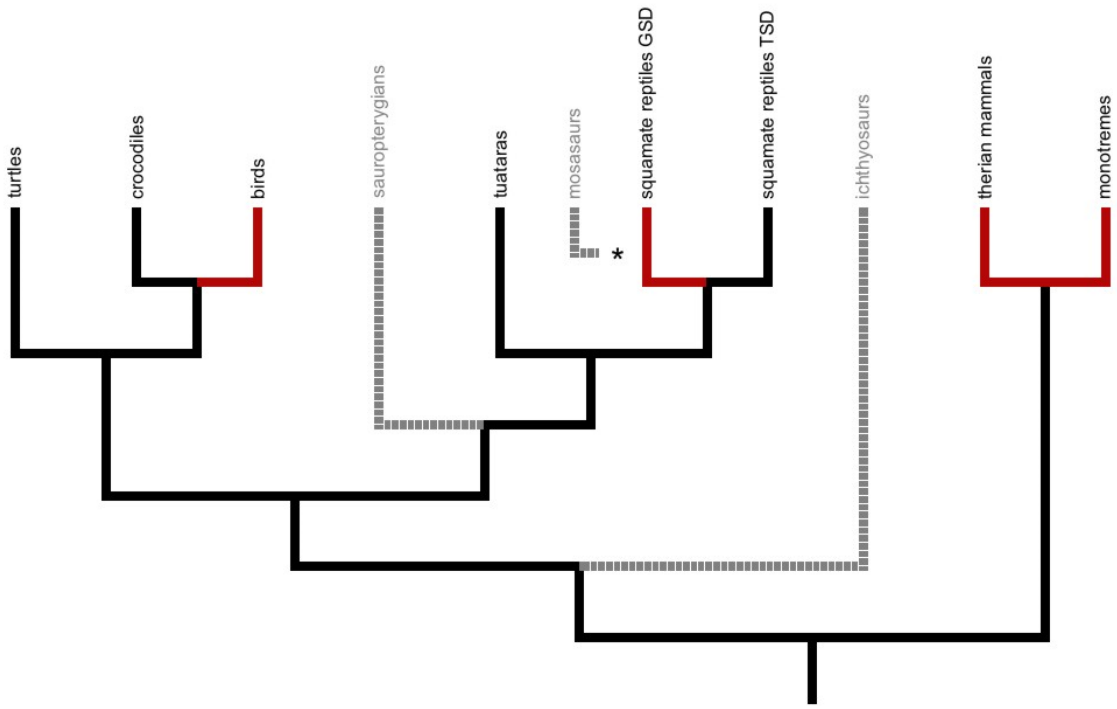
Figure 1. Phylogenetic reconstruction of the evolution of sex-determining mechanisms in amniotes. The topology follows Organ *et al.*[1]. For each lineage, only the ancestral states are depicted for simplicity: black – TSD, red – GSD. Note the phylogenetic position of the extinct marine reptile lineages (mosasaurs, sauropterygians, and ichthyosaurs). * The phylogenetic position of mosasaurs within squamates is ambiguous as well as reconstruction of phylogeny and evolution of sex- determining mechanisms within squamates.



Kapitola IV.

Differentiation of sex chromosomes and karyotypic evolution in the eye-lid geckos (Squamata: Gekkota: Eublepharidae), a group with different modes of sex determination.

Martina Pokorná, Marie Rábová, Petr Ráb, Malcolm A. Ferguson-Smith, Willem Rens, Lukáš Kratochvíl

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Differentiation of sex chromosomes and karyotypic evolution in the eye-lid geckos (Squamata: Gekkota: Eublepharidae), a group with different modes of sex determination

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Abstract The eyelid geckos (family Eublepharidae) include both species with temperature-dependent sex determination and species where genotypic sex determination (GSD) was suggested based on the observation of equal sex ratios at several incubation temperatures. In this study, we present data on karyotypes and chromosomal characteristics in 12 species (*Aeluroscalabotes felinus*, *Coleonyx brevis, Coleonyx elegans, Coleonyx variegatus, Eublepharis angramainyu, Eublepharis macularius, Goniurosaurus araneus, Goniurosaurus lichtenfelderi, Goniurosaurus luii*, *Goniurosaurus splendens, Hemitheconyx caudicinctus,* and *Holodactylus africanus*) covering all genera of the family, and search for the presence of heteromorphic sex chromosomes. Phylogenetic mapping of chromosomal changes showed a long evolutionary stasis of karyotypes with all acrocentric chromosomes followed by numerous chromosomal rearrangements in the ancestors of two lineages. We have found heteromorphic sex chromosomes in only one species, which suggests that sex chromosomes in most GSD species of the eyelid geckos are not morphologically differentiated. The sexual difference in karyotype was detected only in *C. elegans* which has a multiple sex chromosome system (X_1X_2Y). The metacentric Y chromosome evolved most likely via centric fusion of two acrocentric chromosomes involving loss of interstitial telomeric sequences. We conclude that the eyelid geckos exhibit diversity in sex determination ranging from the absence of any sexual differences to heteromorphic sex chromosomes, which makes them an interesting system for exploring the evolutionary origin of sexually dimorphic genomes.

Key words reptile cytogenetics · FISH · neo-sex chromosomes · eublepharid lizards

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Abbreviations

Ag-NOR	Silver-stained nucleolar organizer region
DAPI	4,6-diamidino-2-phenolindole
FISH	Fluorescence in situ hybridization
GSD	Genotypic sex determination
MYA	Million years ago

NF	Fundamental number, the number of chromosome arms in a somatic cell of a particular species
PBD	Phosphate-buffered detergent
SSC	Standard sodium citrate
TSD	Temperature-dependent sex determination

Introduction

In reptiles, sex determination occurs basically in two different modes: the sex of an individual is set primarily either by its sex-specific genotype (genotypic sex determination, GSD), or the sexes do not differ in genotype and sex determination is triggered by environmental factors such as temperature during the sensitive period of incubation (temperature-dependent sex determination, TSD; Valenzuela et al. 2003; Pokorná and Kratochvíl 2009; for an alternative definition of sex-determining modes that are considered to be a continuum, see, e.g., Sarre et al. 2004 or Radder et al. 2008).

As far as is known, sex-determining mechanisms are phylogenetically conservative in many reptilian clades, e.g., in birds (all female heterogamety; Fridolfsson et al. 1998), crocodiles (all TSD, Deeming 2005), snakes (all female heterogamety, Matsubara et al. 2006), iguanids sensu lato (all GSD, reviewed in Pokorná and Kratochvíl 2009), while other clades possess evolutionary lability in sex-determining mechanisms (e.g., turtles: Janzen and Krenz 2004, Janzen and Phillips 2006; agamids: Harlow 2004; geckos: Viets et al. 1994, Gamble 2010; reviewed by Pokorná and Kratochvíl 2009). The latter groups provide an opportunity to study the origin of particular sex-determining systems and their evolutionary transitions within the phylogenetic comparative framework.

In the present study, we focus on the eyelid geckos (family Eublepharidae; Squamata: Gekkota), a monophyletic group of lizards with phylogeny supported by numerous morphological (Grismer 1988) and molecular data (Jonniaux and Kumazawa 2007). Traditionally, the eye-lid geckos were considered to be the basal gecko group, sister to all the remaining gekkotan species (e.g., Kluge 1987; Grismer 1988). However, recent molecular evidence suggests that this group is nested within Gekkota being sister to the Gekkonidae+Phyllodactylidae+Sphaerodactylidae, with the Diplodactylidae+Carphodactylidae+Pygopodidae being the basal gekkotan clade (Gamble et al. 2008 and references therein). The family Eublepharidae includes two species with TSD (*Eublepharis macularius*: Viets et al. 1993, Bragg et al. 2000, Rhen and Crews 2000, Kratochvíl et al. 2008; *Hemitheconyx caudicinctus:* Bragg et al. 2000). The leopard gecko, *E. macularius*, serves as the model TSD laboratory animal (e.g., Rhen et al. 2005). In contrast, in *Coleonyx brevis*, *Coleonyx variegatus* (Viets et al. 1994) and *Coleonyx mitratus* (Bragg et al. 2000), equal sex ratios were observed at several incubation temperatures, which suggests that these species may have GSD. We also observed equal sex ratios at three constant temperatures (26, 28, and 30°C) in *Coleonyx elegans* (L. Kratochvíl, L. Kubička, and E. Landová, unpublished data; Kratochvíl et al. 2008). Equal sex ratio at a single constant temperature was also reported in *Goniurosaurus luii* (Seufer et al. 2005) and observed in *Goniurosaurus lichtenfelderi* (L. Kratochvíl, unpublished data). If the species of eyelid geckos with equal sex ratios across incubation temperatures indeed have GSD, we predict that they should exhibit sexually divergent genotypes, which could be represented by sexually dimorphic karyotypes. In contrast, genotypes of TSD species should not differ between sexes. However, the lack of information on karyotypes in most species of the family prevented testing of these predictions. So far, karyotypes in only four out of approximately 28 described eyelid geckos' species have been described, namely in *E. macularius* ($2n$=38; Gorman 1973), *C. variegatus* ($2n$=32, only males were studied; Matthey 1933), *Coleonyx switaki* ($2n$=24, only males were studied; Murphy 1974) and *Goniurosaurus kuroiwae* ($2n$=24; Ota et al. 1987). Although limited, these data suggest extensive karyotype changes within the family. No sexual differences in karyotype or heteromorphic pairs of chromosomes were detected in any of these species.

Here, we report the cytogenetic screening of 12 species of eyelid geckos covering all currently described genera (*Aeluroscalabotes, Eublepharis, Holodactylus, Hemitheconyx, Goniurosaurus,* and *Coleonyx*). We specifically focused on searching for possible sexual differences in karyotypes, particularly in species suspected of possessing GSD.

Materials and methods

Animals

In this study, karyotypes were determined in 85 adult males and females of 12 species of the family Eublepharidae, namely *Aeluroscalabotes felinus*, *Eublepharis angramainyu, E. macularius, Holodactylus africanus, Hemitheconyx caudicinctus, Goniurosaurus lichtenfelderi, G. luii, G. araneus, G. splendens, Coleonyx brevis, C. variegatus,* and *C. elegans* (for the list of specimens see Table 1). All individuals were captive bred animals maintained in the laboratory breeding room at the Faculty of Science, Charles University in Prague, Czech Republic (accreditation No. 18847/2003-1020).

Metaphase chromosome preparation

Four different sources were used to harvest metaphase chromosomes (leukocyte cultivation, gut epithelium, bone marrow, and regenerating tail tips).

For leukocyte cultivation, peripheral blood was cultured at 30°C for a week in T 199 medium (Sigma-Aldrich), enriched with 10% fetal bovine serum (Baria), 0.5% antibiotic–antimycotic solution (Sigma-Aldrich), 1% canamycin (Sigma-Aldrich), 0.2% phytohaemaglutinin (Biomedica), and 1% lipopolysacharide (Sigma-Aldrich). Chromosome preparations were made following standard procedures that included a 45 min 0.1% colchicine treatment (1 μl/ml) and fixation in 3:1 methanol–acetic acid.

Metaphases were obtained from the intestinal epithelium in seven individuals of *C. elegans* following the technique basically described by King and Rofe (1976). Animals were colchicinized by an intraperitoneal injection of 10 μl of colchicine solution (0.1%) per gram of body weight 1 h before euthanasia. The longitudinally-cut small intestine was incubated for 15 min in 5 ml of KCl solution (0.056 g/ml), and then fixed in 3:1 methanol–acetic acid for 2 h. Subsequently, the tissue was transferred to 45% acetic acid for 24 h. The epithelial cells were scraped from the internal intestinal surface and suspended in the fixative before dropping. Correspondingly, femur and humerus marrow cells were treated with KCl solution for 10 min followed by standard fixation.

Chromosomes were also prepared from regenerating tail tips following the methods of Cattin and Ferreira (1989), as later modified by Völker (2006) with a few necessary modifications for reptiles. The tail tip was removed and subsequently left to regenerate for about 3 weeks. Approximately 2 mm^3 of the regenerated tissue was removed and incubated for 45 min in NaCl (0.107 g/100 ml), KCl (0.0026 g / 100 ml), $CaCl_2$ (0.0029 g/100 ml), $NaHCO_3$ (0.00023 g/100 ml) and colchicine (0.025 g/100 ml) solution at 30°C. After fixation, the tissue was gently minced in 50 μl of 50% acetic acid. The cell suspension was dropped on slides heated to 45°C.

Table 1 Number of analyzed specimens/metaphases of the eyelid geckos examined in this study

Species	Sex determination	Number of females	Number of female metaphases	Number of males	Number of male metaphases
Aeluroscalabotes felinus	?	2	6	1	4
Eublepharis angramainyu	?	4	17	2	2
Eublepharis macularius	TSD	5	11	5	6
Holodactylus africanus	?	1	2	1	2
Hemitheconyx caudicinctus	TSD	3	9	3	4
Goniurosaurus lichtenfelderi	GSD?	3	6	2	2
Goniurosaurus luii	GSD?	9	24	10	22
Goniurosaurus araneus	?	2	4	2	3
Goniurosaurus splendens	?	4	12	2	10
Coleonyx brevis	GSD	1	3	-	-
Coleonyx variegatus	GSD	1	2	1	4
Coleonyx elegans	GSD	8	21	13	56

Meiotic chromosome preparation

Testes from four males of *C. elegans* were used for meiotic chromosome preparation according to the protocol described in Schmid et al. (1982). Testes were gently minced and the suspension was treated by a standard procedure.

Chromosome staining

Slides were stained using the standard Giemsa solution and Chromomycin A3 (Serva). The nuclear organizer regions (NORs) were visualized by the silver staining method (Howell and Black 1980). C-banding in selected species was performed as described by Sumner (1972) with slight modifications. Slides were aged at 65°C for 1 h, soaked in 0.4% HCl for 20 min, treated with $Ba(OH)_2$ for 4 min at 45°C and finally for 1 h at 60°C in 2xSSC. Slides were rinsed in distilled water and stained with 3% Giemsa.

Fluorescent in situ hybridization (FISH) with (TTAGGG)n telomeric probe

Chromosome preparations of males of *C. elegans* were hybridized with $(TTAGGG)_n$ telomeric probe (Cambio) for direct observation following the manufacturer's instructions. Briefly, the slides were dehydrated through an ethanol series, air dried, and kept for 15 min at 65°C. The RNase solution (200 μl, concentration 100 μg/ml; TopBio) was placed on each slide. The slides were covered by cover slides and kept for 1 h at 37°C. Subsequently, they were washed at 2xSSC and denatured for 2 min at 70°C in 70% deionized formamide, dehydrated through an ethanol series, air dried, and kept at 37°C. Hybridization buffer (11.5 μl) was mixed with 1 μl of specific (TTAGGG)n telomeric probe. The hybridization mixture was denatured at 85°C for 10 min, chilled on ice, and placed on a slide. Hybridization took place at 37°C for 16 h in a humid chamber. After its termination, the slides were washed at 37°C in 2xSSC and 42°C in 0.5xSSC, respectively. The slides were air dried and mounted using the anti-fade medium Vectashield (Vector Laboratories) containing 1.5 μg/ml 4,6-diamidino-2-phenolindole (DAPI).

FISH with 28 S rDNA probe

Metaphase chromosomes of males of *C. elegans* were hybridized with 28 S rDNA probe derived from a teleost fish kindly provided by the lab of Castherine Ozouf-Costaz (Museum National d'Histoire Naturelle, Paris, France). Briefly, the slides were dehydrated through an ethanol series, air dried, and denaturated for 2 min at 72°C in 70% deionized formamide/2xSSC. 28 S rDNA probe in total concentration 50 ng/μl was denaturated at 80°C for 5 min. Hybridization took place at 37°C for 48 h in a humid chamber. After its termination, the slides were washed at 72°C in 2xSSC and in 1xPBD (phosphate-buffered detergent) at room temperature. The slides were incubated with FITC-Avidin for 5 min at 37°C to detect the signals, washed in 1xPBD at room temperature and mounted with the anti-fade medium Vectashield containing 1.5 μg/ml DAPI.

Flow chromosome sorting and chromosome painting

Flow sorting of chromosomes from cultivated cells was performed according to the protocols described previously (Yang et al. 1995; Rens et al. 1999, 2007) using a dual-laser cell sorter (MoFlo, DAKO). Chromosome paint was prepared from flow-sorted Y chromosomes of *C. elegans* that were used as templates for DNA amplification by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) (Telenius et al. 1992). Primary DOP-PCR product was used as templates in a secondary DOP-PCR to incorporate biotin-16-dUTP (Roche). The probe was hybridized to metaphase spreads of *C. elegans* males. FISH and probe detection were carried out exactly as described by Rens et al. (1999).

Microscopy and data analyses

Images were captured using the epifluorescence microscopes (Provis AX, Olympus; 70; DMRXA, Leica) equipped with CCD cameras (DP30BW, Olympus; Photometrics Sensys). The IKAROS, ISIS (Metasystems) and Leica CW4000 (Leica Microsystems FISH Software) imaging programs were used to capture gray-scale images and to superimpose the source images into colours to visualize the results of the FISH.

The karyotype data were mapped on the molecular phylogeny of the eyelid geckos reported by

Jonniaux and Kumazawa (2007). Their phylogeny is in concordance with that by Grismer (1988) based on morphological characters, but three species (*C. switaki, E. angramainyu,* and *G. splendens*) included in our study are lacking in their cladogram. We supplemented the position of *C. switaki* and *E. angramainyu* according to Grismer (1988) morphological tree. *G. splendens* is a member of the *kuroiwae* group, the Japanese lineage of the genus *Goniurosaurus* characterized by several morphological synapomorphies (e.g., Grismer et al. 1999). Therefore, we placed *G. splendens* as sister to *G. kuroiwae,* the only other species of the *kuroiwae* group in our phylogeny. Close relationship of these two species was also supported by Ota et al. (1999).

Results

The karyotype of *A. felinus* ($2n=34$) consisted exclusively of acrocentric chromosomes of gradually decreasing size (Fig. 1a). No heteromorphic sex chromosomes were observed in either sex.

The diploid chromosome number in *E. angramainyu* (Fig. 1b), *E. macularius* (Fig. 1c), *H. africanus* (Fig. 1d), *H. caudicinctus* (Fig. 1e), *G. lichtenfelderi* (Fig. 1f), *G. luii* (Fig. 1g), and *G. araneus* (Fig. 1h) was $2n=38$ and the karyotype consisted exclusively of acrocentric chromosomes of gradually decreasing size. No heteromorphic sex chromosomes were observed in either sex.

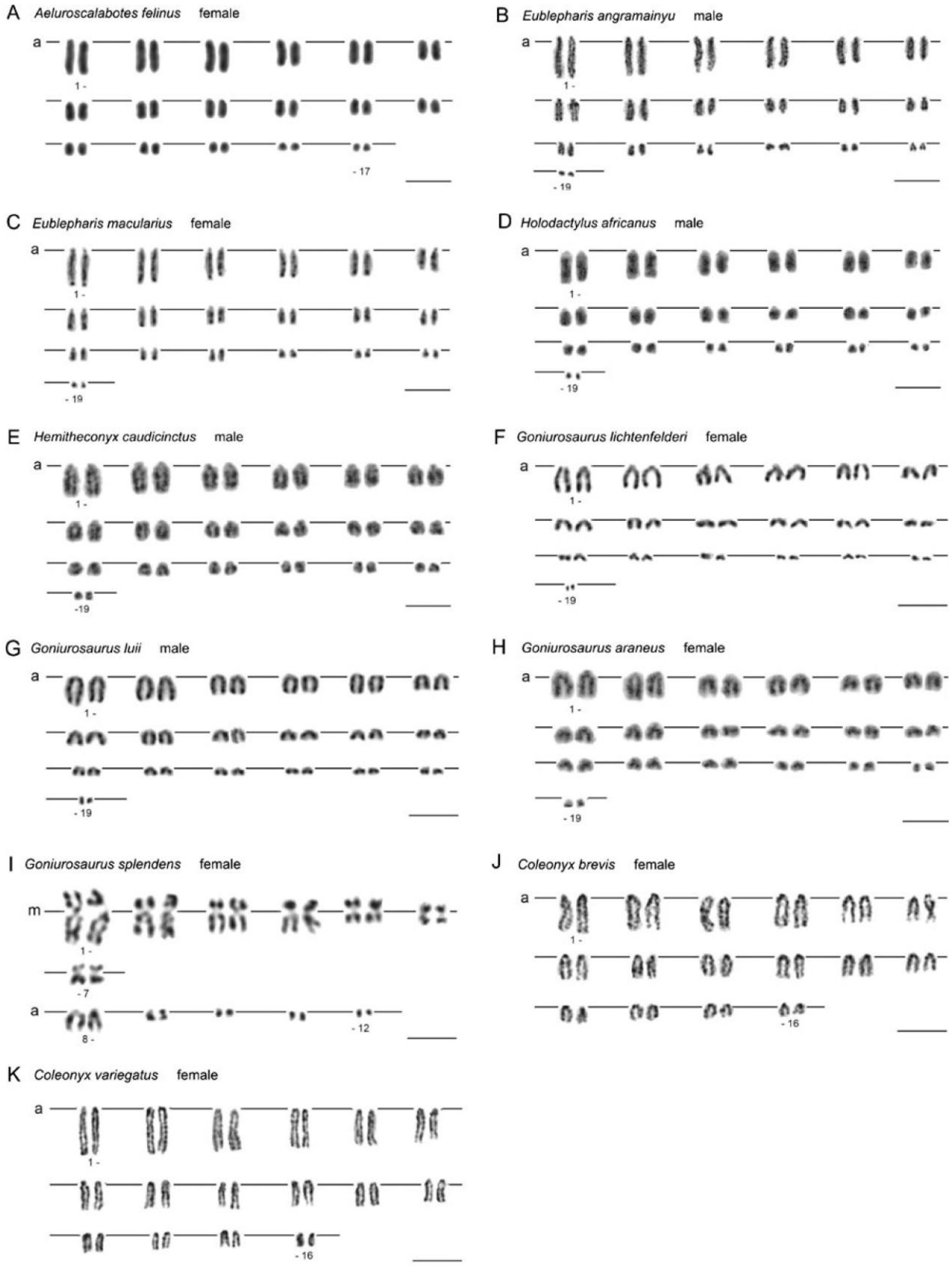
The karyotype of *G. splendens* ($2n=24$) comprised seven pairs of metacentric and five pairs of acrocentric chromosomes, from which four were microchromosomes (Fig. 1i). No heteromorphic chromosomes were observed in either sex.

The diploid chromosome number in *C. brevis* (Fig. 1j) and *C. variegatus* (Fig. 1k) was $2n=32$ and the karyotype was composed exclusively of acrocentric chromosomes of gradually decreasing size. No heteromorphic sex chromosomes were observed (only a female was examined in *C. brevis*).

In *C. elegans,* we found sex-linked variability in karyotypes. Diploid chromosome number of females was $2n=32$ and their karyotypes were composed exclusively of acrocentric chromosomes of gradually decreasing size (Fig. 2a). The diploid chromosome number of males was $2n=31$ and the karyotype contained 30 acrocentric chromosomes and one metacentric element (Fig. 2b). In the female karyotype, one middle-sized pair of chromosomes had a distinct achromatic constriction in the telomeric region after Giemsa staining (Fig. 2a). This region was negative after DAPI fluorescent staining but positive following impregnation with Ag-nitrate, suggesting the presence of NOR sites (Fig. 2c). In males, one of the NOR-bearing chromosomes was acrocentric while its homologue was part of the large metacentric element (Fig. 2b, d). The hybridization with the 28 S rDNA probe confirmed that the region contains genes for 28 S rRNA (Fig. 2i). In both sexes, chromomycin A3 did not stain any specific chromosomal region (Fig. 2e, f). Examination of male chromosomes using FISH with a telomeric probe did not reveal any hybridization signal in the pericentromeric region of the large metacentric chromosome (Fig. 2h). During meiosis, this chromosome—further assigned as sex chromosome Y—formed a trivalent with two middle-sized acrocentric chromosomes (assigned as X_1, X_2; Fig. 2g). In male metaphase spreads, the chromosome painting probe derived from the flow sorted Y-chromosomes hybridized not only with the metacentric Y chromosome, but also with two acrocentric chromosomes (Fig. 2j).

The differential staining (C-banding) carried out in three species did not reveal any substantial heterochromatic regions (Fig. 3). This selection includes a species from the genus with putative GSD (*G. lichtenfelderi*; Fig. 3a, b), a TSD species (*E. macularius*; Fig. 3c), and a GSD species with identified sex chromosomes (*C. elegans*; Fig. 3d).

Phylogenetic mapping (Fig. 4) suggests that the karyotype composed of 38 acrocentric chromosomes was ancestral for the *Eublepharis-Hemitheconyx-Holodactylus-Goniurosaurus* clade. Within this clade, rearrangement of chromosome numbers occurred only in the common ancestor of *G. splendens* and *G. kuroiwae*. The diploid chromosome number of $2n=32$ was probably ancestral for the genus *Coleonyx*, where the reduction of chromosomal number appeared in the ancestor of *C. switaki*. The phylogenetically most basal species of the family (*A. felinus*) may possess a diploid number of $2n=34$. The analysis cannot decide whether $2n=34$, $2n=32$ or $2n=38$ was ancestral for the whole family Eublepharidae.


A Aeluroscalabotes felinus female
a
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B Eublepharis angramainyu male
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- 19
C Eublepharis macularius female
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D Holodactylus africanus male
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E Hemitheconyx caudicinctus male
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F Goniurosaurus lichtenfelderi female
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H Goniurosaurus araneus female
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J Coleonyx brevis female
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K Coleonyx variegatus female
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- 16

Fig. 1 Karyotypes of **a** *A. felinus* female, **b** *E. angramainyu* male, **c** *E. macularius* female, **d** *H. africanus* male, **e** *H. caudicinctus* male, **f** *G. lichtenfelderi* female, **g** *G. luii* male, **h** *G. araneus* female, **i** *G. splendens* female, **j** *C. brevis* female, and **k** *C. variegatus* female arranged from Giemsa-stained chromosomes. *Bars* 10 μm

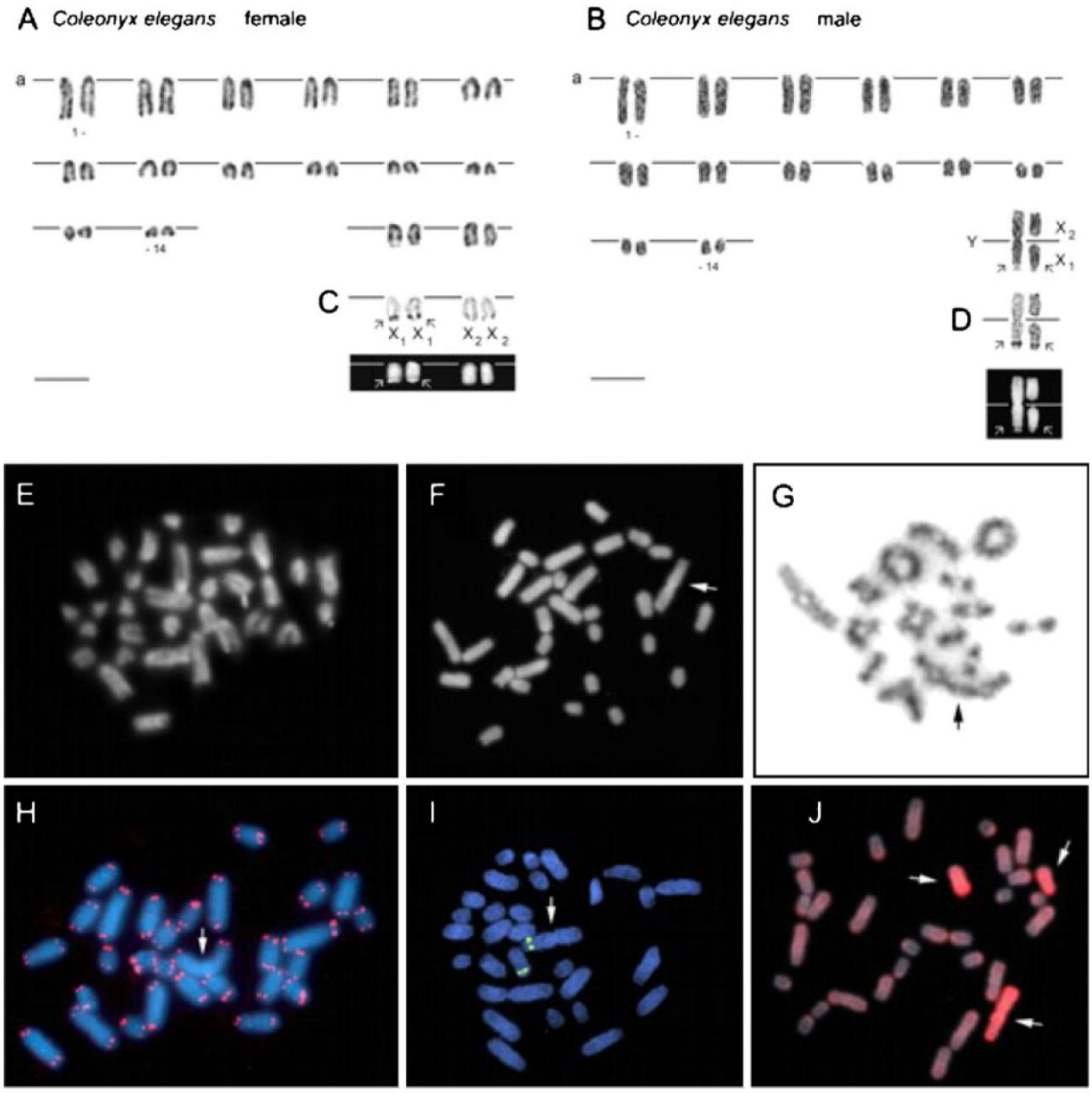

Fig. 2 Mitotic and meiotic chromosomes of *Coleonyx elegans*. **a** Karyotype of female and **b** male arranged from Giemsa-stained chromosomes; *bars* 10 μm. **c** Silver and DAPI stained sex chromosomes in females and **d** males; *arrows* NORs by silver staining and negative signals in DAPI on one pair of sex chromosomes in females and on the metacentric Y and acrocentric X_1 sex chromosomes in males. **e** Mitotic chromosomal spread stained by Chromomycin A3 in females and **f** males (*arrow* points to Y chromosome). **g** Male meiotic metaphase I. (*arrow* highlights X_1X_2Y trivalent). **h** Male mitotic chromosomes hybridized with vertebrate pantelomeric probe (*arrow* points to Y chromosome). **i** FISH with 28 S rDNA probe (*arrow* points to Y chromosome). **j** Chromosome painting of male metaphase with probe from flow sorted Y chromosome (*arrows* indicate painted Y and X_1, X_2 chromosomes)

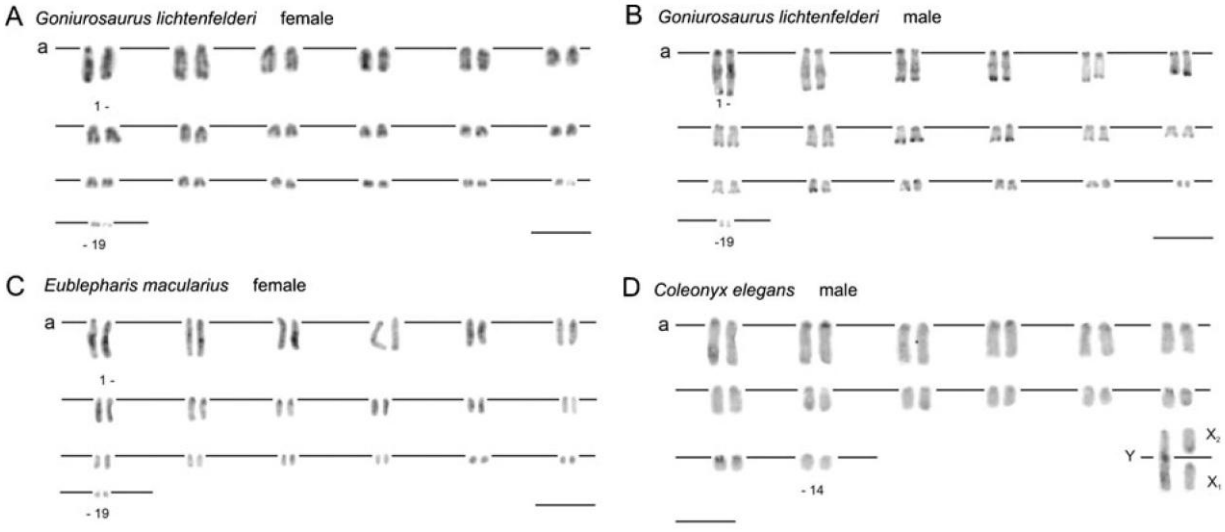

Fig. 3 C-banded karyotypes of **a** *G. lichtenfelderi* female, **b** male, **c** *E. macularius* female, and **d** *C. elegans* male

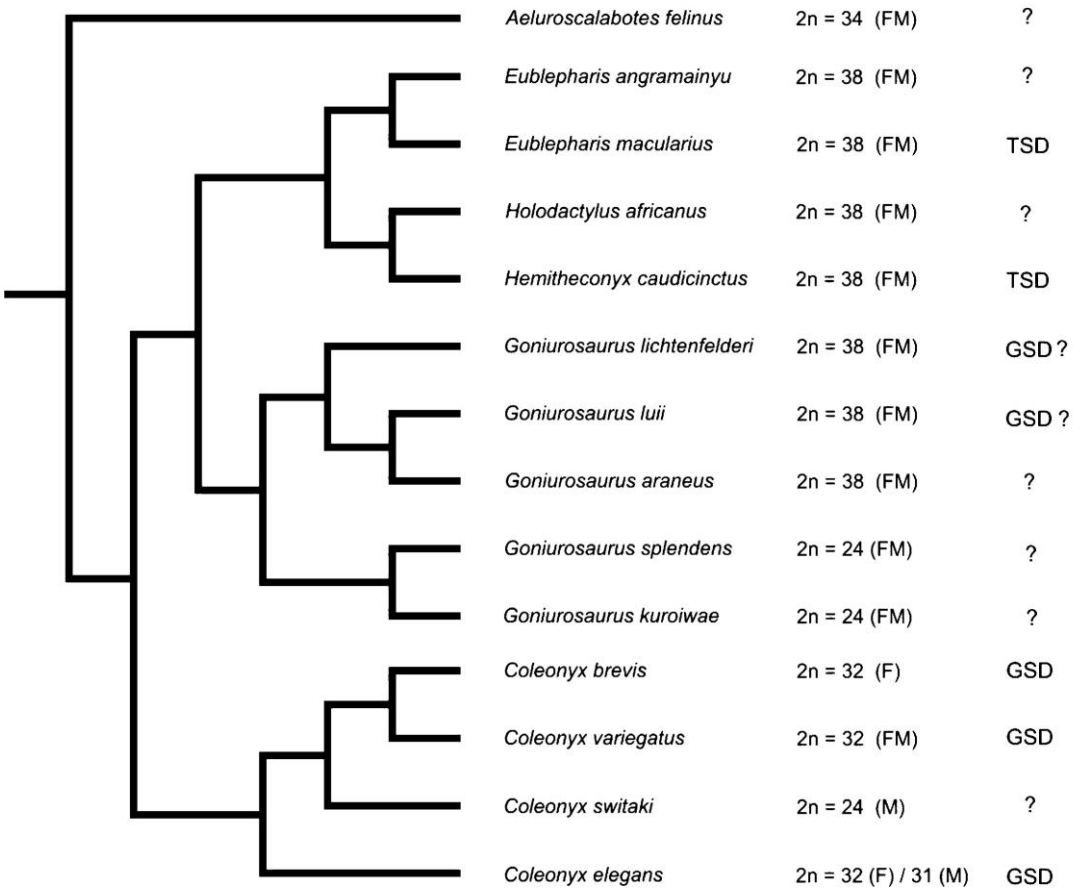

Fig. 4 Phylogenetic relationships among the eyelid geckos in this study with information on chromosomal numbers and mode of sex determination. Cytogenetic data for *G. kuroiwae* taken from Ota *et al.* (1987) and for *C. switaki* from Murphy (1974). *F* only female karyotype is known, *M* only male karyotype is known, *FM* both male and female karyotypes are known

Discussion

Karyotype differentiation and evolution

Our results confirm the previously published karyotypes in *E. macularius* (Gorman 1973) and *C. variegatus* (Matthey 1933). The karyotype of *G. splendens* described herein concurs with the description of the karyotype in the closely related *G. kuroiwae* (Ota et al. 1987). Altogether, we increased the number of eyelid gecko species with a known karyotype to 14.

It appears that 38 acrocentric chromosomes is the ancestral chromosomal number of the Afro-Asian clade encompassing genera *Goniurosaurus*, *Eublepharis, Hemitheconyx,* and *Holodactylus* (Fig. 4). The common ancestor of the American genus *Coleonyx* probably possessed 32 all-acrocentric chromosomes. The sister topology of these two clades and yet another chromosome number ($2n$=34) we found in the Malaysian cat gecko (*A. felinus*), the basal species of the whole family Eublepharidae (Grismer 1988; Jonniaux and Kumazawa 2007), does not permit a decision on the ancestral number of chromosomes for the whole family Eublepharidae. Gorman (1973) concluded that the typical gecko karyotype is represented by 32–46 acrocentric or subtelomeric chromosomes, and that metacentric chromosomes in geckos are rather exceptional (see also King 1990). Our findings on the eyelid geckos concur basically with these observations. As the ancestral karyotypes of the genus *Coleonyx* and the Afro-Asian clade *Goniurosaurus-Eublepharis-Hemitheconyx-Holodactylus* as well as the basal species of the family (*A. felinus*) involve only acrocentric chromosomes, the ancestral karyotype of the family Eublepharidae was probably composed of all acrocentric chromosomes.

Based on the calibrated molecular clocks (Jonniaux and Kumazawa 2007), the estimated age of the divergence between the genus *Coleonyx* and the Afro-Asian *Eublepharis-Hemitheconyx-Holodactylus-Goniurosaurus* clade is about 150 MYA. The divergence time between the genus *Goniurosaurus* and the *Eublepharis-Hemitheconyx-Holodactylus* clade was reconstructed to about 120 MYA. Even the time of the most recent split between the African genera of the eye-lid geckos (*Hemitheconyx-Holodactylus*) is estimated to be about 90 MYA. This means that the karyotype with 38 acrocentric chromosomes was conserved by several lineages of the Afro-Asian eyelid geckos for at least 120 MYA. This situation contrasts sharply with the karyotypes of the Japanese members of the genus *Goniurosaurus* (*G. kuroiwae* and *G. splendens*) that have a highly derived chromosomal number ($2n$=24) and the presence of metacentric chromosomes. As both ancestral and derived karyotypes possess the same fundamental number (NF=38), the latter karyotype probably originated through chromosomal rearrangements involving several Robertsonian fusions. The cause of this relative high number of chromosome rearrangements in this genus remains to be investigated.

A karyotype with 24 chromosomes was also reported for *C. switaki* (Murphy 1974). Unfortunately, no photograph of the karyotype of *C. switaki* is presented in the original paper. The karyotype is displayed there in the form of an idealized drawing. Murphy (1974) concluded that 22 chromosomes including microchromosomes are metacentric and that two chromosomes are acrocentric, which would mean a highly altered fundamental number in *C. switaki*.

Sexual differences in karyotypes

Sex of an individual in TSD species is set by environmental conditions and hence both sexes share the same genotype (see Valenzuela et al. 2003, Pokorná and Kratochvíl 2009). In accordance, we did not find any sexual differences in the karyotypes of the two TSD species of eyelid geckos, *E. macularius* and *H. caudicinctus* (Viets et al. 1993, Bragg et al. 2000, Rhen and Crews 2000, Kratochvíl et al. 2008; Bragg et al. 2000). Rigorous experiments on the incubation of eggs across a wide spectrum of constant temperatures have not been carried out in most species of eyelid geckos. We did not detect sexual differences in the genomes of *A. felinus, G. luii, G. lichtenfelderi* (not even after differential staining in this species, Fig. 3a–b), or in *G. araneus, H. africanus, E. angramainyu,* and *G. splendens*; similar findings were previously reported in *G. kuroiwae* (Ota et al. 1987) and *C. switaki* (only the male karyotype was studied; Murphy 1974). Thus, the mode of sex determination in these species is still unknown. The balanced sex ratio across several constant temperatures was reported in *C. elegans, C. variegatus,* and *C. brevis* (Viets et al. 1994; Kratochvíl

et al. 2008). We did not find any heteromorphic pair of chromosomes in the female of *C. brevis* (males were not available to us at the time of the study) and in either sex in *C. variegatus*. The sex chromosomes in *C. variegatus* are thus probably homomorphic and more sensitive cytogenetic methods will be necessary for their identification. Our discovery of sex chromosomes in the Mexican banded gecko (*C. elegans*) is the first demonstration of sexual differences in genotypes, the unequivocal proof of GSD (Valenzuela et al. 2003, Pokorná and Kratochvíl 2009), in the eye-lid geckos.

From our results, we conclude that the Mexican-banded gecko possesses X_1X_2/X_1X_2Y sex chromosome system. This sex chromosome system evolved independently within squamate reptiles several times. It occurs in three species of the gymnophtalmid genus *Calyptommatus*, several species of Iguanidae sensu lato and in the skink *Scincella lateralis*, where closely related species or populations possess the simple XY system (Bertolotto et al. 2001; Yonenaga-Yassuda et al. 2005; recently reviewed in Ezaz et al. 2009). It was also identified in the gekkotan lizard *Lialis burtonis* (Gorman and Gress 1970), where the related genus (*Delma*) from the same family (Pygopodidae) also posesses an XY system (King 1990). We propose that the metacentric Y chromosome in *C. elegans* evolved via a Robertsonian fusion of two acrocentric chromosomes coupled with the loss of the adjacent telomeric regions (Fig. 2h). The homologous chromosomes now serve as X_1 and X_2 sex chromosomes. One of the original chromosome pairs involved in the fusion leading to the Y sex chromosome had a NOR-site (Fig. 2d, i). The observation of sex chromosomes during male meiosis (Fig. 2g) confirmed that both arms of the Y chromosome pair and recombine with their particular X chromosomes. The probe from the flow sorted Y chromosome also distinctly paints its acrocentric counterparts (X_1, X_2), which demonstrates that these three chromosomes have a similar DNA content (Fig. 2j). This situation suggests a relatively early stage of differentiation of these sex chromosomes. Differential staining (C-banding) did not reveal any substantial heterochromatin accumulation in the sex chromosomes of *C. elegans* (Fig. 3d), which further supports an early stage of their differentiation.

Unlike in many other vertebrates with poorly differentiated sex chromosomes (e.g., ostriches, boids, and other basal snakes, medaka killifish and many teleost fish; Matsubara et al. 2006; Tsuda et al. 2007; Volff et al. 2007), in GSD eyelid geckos, we know of close relatives with environmental sex determination, i.e., without any sexual differences in genotype. Further examination of the sex chromosomes in *C. elegans* will establish their homology to autosomes of TSD species. The eyelid geckos could serve as an important group for the identification of the processes associated with the transition between sex determining modes and the evolution of sex chromosomes at the early stages of their differentiation.

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Kapitola V.

Microsatellite distribution on sex chromosomes at different stage of heteromorphism and heterochromatinization in lizards (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*).

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Rukopis

Microsatellite distribution on sex chromosomes at different stage of heteromorphism and heterochromatinization in lizards (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*)

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Abstract

Background: The accumulation of repetitive sequences such as microsatellites during the differentiation of sex chromosomes has been largely unstudied in squamate reptiles (lizards, amphisbaenians and snakes), a group which has a large diversity of sex determining systems. It is known that the *Bkm* repeats containing tandem arrays of GATA quadruplets are highly accumulated on the degenerated W chromosomes in advanced snakes. Similar, potentially homologous, repetitive sequences were found on sex chromosomes in other vertebrates. Using FISH with probes containing all possible mono-, di-, and tri-nucleotide sequences and GATA, we studied the genome distribution of microsatellite repeats on sex chromosomes in two lizard species (the gecko *Coleonyx elegans* and the lacertid *Eremias velox*) with independently evolved sex chromosomes. The gecko possesses heteromorphic euchromatic sex chromosomes, while sex chromosomes are homomorphic and the W chromosome is highly heterochromatic in the lacertid. Our aim was to test whether microsatellite distribution on sex chromosomes correspond to the stage of their heteromorphism or heterochromatinization. Moreover, because the lizards lie phylogenetically between snakes and other vertebrates with the *Bkm*-related repeats, the knowledge of their repetitive sequence is informative for the determination of the homology of repetitive sequences across vertebrate lineages.

Results: Heteromorphic sex chromosomes of *C. elegans* do not show any sign of microsatellite accumulation. On the other hand, in *E. velox*, certain microsatellite sequences are extensively accumulated over the whole length or parts of the W chromosome, while others, including GATA, are absent on this heterochromatinized sex chromosome.

Conclusion: The accumulation of microsatellite repeats is not connected with heteromorphism of sex chromosomes, but it does correspond to the stage of their heterochromatinization. The lack of GATA repeats on the sex chromosomes of both lizards suggests that the *Bkm*-related repeats on sex chromosomes in snakes and other vertebrates evolved convergently. The comparison of microsatellite sequences accumulated on sex chromosome in *E. velox* and in other eukaryotic organisms suggests that historical contingency, not characteristics of particular sequences, plays a major role in the determination of which microsatellite sequence is accumulated on the sex chromosomes in a particular lineage.

Introduction

The evolution of sex chromosomes from autosomes (Ohno 1967) has been documented many times in different organisms (recently reviewed e.g. in Charlesworth and Mank 2010; but see e.g. Carvalho 2002). During their evolution, sex chromosomes go progressively through several steps. Briefly, the first step is the acquisition of sex determining locus or loci. Subsequently, the genomic content of both members of the pair diverge. The specialization of sex chromosomes for their sex-specific roles (e.g. Rice 1984) selects for the reduction of the interchange of genetic material between sex chromosomes and thus for lower levels of recombination. However, lack of recombination leaves the unpaired sex chromosomes (Y and W) without the possibility to correct mutations in coding sequences, which leads to an unusually low content of functional genes linked to these chromosomes. Moreover, cessation of recombination opens doors for the accumulation of various repeats on sex chromosomes (e.g. microsatellites, transposons, rDNA sequences, Charlesworth 2002). Alternatively, the accumulation of repetitive sequences may not be a consequence of reduced recombination, but its cause. By generating asynchrony in the DNA replication pattern of X and Y, respectively Z and W chromosomes, it can reduce the frequency of crossing-overs between them (e.g. Singh et al. 1976). The accumulation of repeats on a heterogametic sex chromosome may be so massive that the chromosome is finally much larger than its homologous counterpart in the pair. The heterogametic sex chromosome may even become the largest chromosome in the genome such as the Y chromosome in the plant *Silene latifolia* (Kejnovsky et al. 2006). On the other hand, in some lineages, heterogametic sex chromosomes may progressively decrease in size (e.g. Matsubara et al. 2006) and such degeneration can result in their elimination from the genome (e.g. Fredga 1988). In yet another cases, sex chromosomes may stay homomorphic for a long evolutionary time (e.g. Olmo et al. 1987; Matsubara et al. 2006; Tsuda et al. 2007). In many organisms, the heterogametic sex chromosome has been found to be highly heterochromatinized (e.g. Olmo et al. 1987; Ezaz et al. 2005). The heterochromatinization may be a mechanism for the defence against the activity of transposable elements or other repetitive sequences to safeguard genome integrity (e.g. Grewal and Jia 2007).

Squamate reptiles, the lineage encompassing lizards, snakes and amphisbaenians, represent an interesting group for the exploration of the evolution of

sex chromosomes, as they possess substantial variability in sex determining mechanisms (Janzen and Phillips 2006; Organ and Janes 2008; Pokorná and Kratochvíl 2009). Squamate reptiles include species with environmental sex determination, i.e. without sex chromosomes; species with homomorphic sex chromosomes, and those with heteromorphic sex chromosomes. All three situations can be found even in a single family, for example in dragon lizards or eyelid geckos (Ezaz et al. 2009; Pokorná et al. 2010; Gamble 2010). Phylogenetic distribution (Pokorná and Kratochvíl 2009) as well as differences in size, shape and type (male or female heterogamety) of sex chromosomes (Ezaz et al. 2009) or molecular-cytogenetic tests of synteny of sex chromosomes (Matsubara et al. 2006; Ezaz et al. 2009; Pokorná et al., in press) indicate that sex chromosomes evolved within squamates independently several times and that they are at various stages of the general process of sex chromosome evolution in different species.

Up-to-date, to our knowledge, the accumulation of repeats during the degeneration of sex chromosomes has been studied only in a single lineage of squamate reptiles, in snakes (Singh et al. 1976; Jones and Singh 1985; O'Meally et al. 2010). Pythons, the rather basal snakes with homomorphic sex chromosomes, do not show any accumulation of repeats, while the degenerated W sex chromosomes in more advanced snakes such as colubrids or elapids, exhibit a massive accumulation of repeats (Jones and Singh 1985; O'Meally et al. 2010). For example, the W chromosome in an elapid snake *Notechis scutatus* is composed almost entirely of repetitive sequences, including 18S rDNA and the banded krait minorsatellite (*Bkm*) repeats (O'Meally et al. 2010). The *Bkm* repeats consist of tandem arrays of 26 and 12 copies, respectively, of two quadruplets, GATA and GACA (Epplen et al. 1982). *Bkm*-related repeats are also accumulated on the heterogametic sex chromosomes in many vertebrates, including humans, and also in plants (Jones and Singh 1981; Arnemann et al. 1986; Schäfer et al. 1986; Nanda et al. 1990, 1991; Parasnis et al. 1999). It was speculated that the *Bkm*-related repeats are functional, playing a role in the transcriptional activation of sex chromosome heterochromatin (Singh et al. 1976). A common origin of the *Bkm*-related repeats across different eukaryotic lineages was assumed (e.g., Epplen et al. 1983), however, a convergent evolution is also likely (Epplen 1988). Recently, based on the results of chicken W chromosome painting in snakes, O'Meally et al. (2010) concluded that heterogametic sex chromosomes in birds and derived snakes may share repetitive

sequences. They suggested that this observation could be explained by yet undetected synteny of parts of the sex chromosomes between these lineages. The homology of repetitive sequences accumulated on sex chromosomes could be tested by the evaluation of the identity of repeats on sex chromosomes in other lineages of squamates phylogenetically nested between snakes, birds and vertebrates with the *Bkm*-related repeats.

The aim of the present study is to compare the distribution of microsatellite sequences on differently differentiated sex chromosomes in two lizard species with independently evolved sex chromosomes and to determine whether the distribution of microsatellite repeats on sex chromosomes corresponds to the stage of their heteromorphism or heterochromatinization. Multiple sex chromosomes ($X_1X_1X_2X_2/X_1X_2Y$) are heteromorphic and fully euchromatic in the first studied species, the gecko *Coleonyx elegans* from the family Eublepharidae (Pokorná et al. 2010). On the other hand, the ZZ/ZW sex chromosomes in the second species, *Eremias velox* from the family Lacertidae, are homomorphic and the W chromosome is highly heterochromatic (Ivanov et al. 1973). Moreover, the gekkotan lizards represent one of the basal groups of squamate reptiles, while lacertids are much more closely related to snakes (Vidal and Hedges 2005). The knowledge of repetitive sequences on the sex chromosomes in the two selected species should therefore be informative for the determination of the homology of the *Bkm*-related and other repeats across vertebrate lineages.

Material and Methods

The lizard individuals involved in the study were captive bred animals maintained in the breeding room at the Faculty of Science, Charles University in Prague, Czech Republic (accreditation No. 24773/2008-10001). Metaphase chromosome spreads were prepared from cultures of whole blood following the protocols described in Ezaz et al. (2005) with slight modifications.

Oligonucleotides containing microsatellite sequences were directly labelled with Cy3 at the 5´ end during synthesis by VBC-Biotech (Wien, Austria). All possible mono- ($d(A)_{30}$, $d(C)_{30}$), di- ($d(CA)_{15}$, $d(GA)_{15}$, $d(GC)_{15}$, $d(TA)_{15}$), and tri-nucleotides ($d(CAA)_{10}$, $d(CAG)_{10}$, $d(CGG)_{10}$, $d(GAA)_{10}$, $d(CAC)_{10}$, $d(CAT)_{10}$, $d(GAC)_{10}$, $d(GAG)_{10}$, $d(TAA)_{10}$, $d(TAC)_{10}$) and $d(GATA)_8$ were used. The tetranucleotide was

included to test for the presence of the *Bkm*-related repeats. Slide denaturation was performed in 7:3 (v/v) formamide: 2xSSC for two minutes at 72 °C, then the slides were dehydrated using 50%, 70% and 100% ethanol (-20 °C) serie and air-dried. The probe was denatured at 70 °C for 10 minutes in a mix containing 50% formamide (v/v), 2xSSC and 10% dextransulfate (w/v) and subsequently applied to the slides, covered with plastic coverslips, and hybridized for 18 hours at 37 °C. The slides were washed at room temperature twice for 5 minutes in 2xSSC and twice for 5 minutes in 1xSSC. The slides were analysed using an Olympus Provis microscope and the image analysis was performed using ISIS software (Metasystem).

The heterogametic sex chromosomes in both studied species of lizards are easily recognizable. The Y chromosome is the largest and the only metacentric chromosome in karyotype of *C. elegans* (Pokorná et al. 2010). The W chromosome of *E. velox* is an acrocentric chromosome of similar size to the Z chromosome, but only the W is conspicuously DAPI-negative (Pokorná et al., in press).

Results

There was no accumulation of mono-, di-, tri-nucleotides or GATA repeats detected on sex chromosomes in *C. elegans*. All tested sequences showed relatively uniform distribution throughout the genome of this species (Fig. 1).

Strong accumulations of several microsatellites were detected on the W chromosome or some autosomes in *E. velox* (Fig. 2). The W chromosome showed an interspersed presence of microsatellite sequences typical for the Z chromosome and autosomes only in three cases, i.e. in the probes $d(CAA)_{10}$, $d(GAC)_{10}$ and $d(C)_{30}$. The $d(CGG)_{10}$ and $d(CAC)_{10}$ sequences exhibited notable accumulations just on small autosomes. Only in two cases ($d(A)_{30}$, $d(TA)_{15}$) was there a concurrently notable accumulation of microsatellites on the whole W chromosome and on two different pairs of autosomes. In all other cases, the W chromosome showed a more highly distinct pattern than the Z chromosome and all autosomal pairs. Some microsatellites with tri-nucleotide motifs ($d(CAG)_{10}$, $d(CAT)_{10}$, $d(GAG)_{10}$, $d(TAC)_{10}$, $d(TAA)_{10}$) were extensively accumulated over the whole length of the W chromosome, while three di-nucleotide repeats ($d(CA)_{15}$, $d(GA)_{15}$, $d(GC)_{15}$) were accumulated just in the centromeric parts of the W chromosome. Three microsatellite sequences ($d(GA)_{15}$,

d$(GAA)_{10}$, d$(GATA)_8$) were conspicuously lacking on the W chromosome, although the signal was otherwise uniformly distributed across the rest of the genome.

Discussion

The FISH patterns using the probes bearing microsatellite repeats in the two lizard species contrasted greatly. The FISH experiments did not reveal a substantial accumulation of microsatellites on X_1, X_2 and Y sex chromosomes nor on autosomes in the gecko *C. elegans* (Fig. 1). This finding is rather surprising, because the Y chromosome is the largest chromosome in the karyotype and certain parts of the Y and the X_1 chromosome consist largely of repetitive elements. Specifically, both these chromosomes carry nucleolus organizer regions (NORs) with accumulated 28S rDNA repeats (Figs. 2d,i in Pokorná et al. 2010). The massive amplification of rDNA-related repeats was documented over the whole W chromosome of the Chinese soft shell turtle, *Pelodiscus sinensis*. The Z chromosome in the turtle is much smaller than the W and contains only an accumulation of the rDNA-related repeats typical for functional NORs (Kawai et al. 2007). The distribution of rDNA-related repeats on the X_1 and the Y sex chromosomes in *C. elegans* is restricted just to their specific parts and corresponds to NORs. Previously, through chromosome painting, we documented that the Y and X_1 and X_2 chromosomes in *C. elegans* share similar DNA content (Pokorná et al. 2010) and concluded that sex chromosomes of this species are only poorly differentiated. The results of the painting with the microsatellite probes further support this conclusion.

The W sex chromosome and some autosomes show strong microsatellite accumulation in the lacertid lizard *E. velox* (Fig. 2). The distribution of microsatellite sequences on the W chromosome differed greatly in various microsatellites. We found enrichment of some microsatellite sequences over the whole W chromosome, while the accumulation of some sequences (CA, GA, and GC repeats) was restricted to a part of the W chromosome near the centromere. This unequal distribution could reflect constitutional characteristics of individual microsatellites, e.g. their convenience for centromere formation; however, no accumulation of CA, GA, and GC repetitive sequences was detected in the centromeres of the Z chromosome and the autosomes (Fig. 2). Alternatively, the unequal distribution could reflect an instantaneous stage of competition of individual microsatellite sequences over a limited number of positions on the W chromosome. Some sequences are present on the Z chromosome and

autosomes, but they are notably lacking on the W chromosome (Fig. 2). It seems likely that they were ancestrally present on the W chromosome as well, but that they were outcompeted by more successful invaders. The alternative explanations on unequal distribution of particular repeats can be tested by the reconstruction of evolutionary dynamics of microsatellite distribution on sex chromosomes across lacertids in future comparative analyses.

The microsatellite distribution in *C. elegans* and *E. velox* corresponds to the general scenario of sex chromosome evolution. Sex chromosomes in *C. elegans*, although heteromorphic, probably represent an early stage of sex chromosome differentiation. The lack of microsatellite accumulation on sex chromosomes in this species is comparable to the situation on homomorphic sex chromosomes in relatively basal snakes (Singh et al. 1976; O'Meally et al. 2010). The present study documents that the DNA content strongly differs between the euchromatic Z and the heterochromatic W chromosomes in *E. velox* (Fig. 2), although the sex chromosomes are homomorphic in this species and in many other lacertids (Olmo et al. 1987). It seems that the accumulation of microsatellites precedes the evolution of heteromorphy of sex chromosomes. Heteromorphic sex chromosomes with accumulated repeats, e.g. in the advanced snakes from the families Elapidae and Colubridae (O'Meally et al. 2010), may represent only the later stage of the evolution of sex chromosomes.

Members of many genera in the family Lacertidae from both its subfamilies (Gallotiinae and Lacertinae) have the ZZ/ZW sex-chromosome system with sex chromosomes at various stage of differentiation (Olmo et al. 1987; Arnold et al. 2007; Olmo and Signorino 2011). Phylogenetic distribution of species with known sex chromosomes suggests that female heterogamety is ancestral for the family (e.g. Pokorná and Kratochvíl 2009; *cf.* to phylogenetic relationships within the family in Arnold et al. 2007 or Mayer and Pavlicev 2007). A molecular clock based on mitochondrial DNA sequences indicates that the separation of the Gallotiinae and Lacertinae occurred around 20 My ago (Arnold et al. 2007). The mechanism keeping homomorphy of sex chromosomes in the lineage leading to *E. velox* and in other lacertids for such a long time in the face of the highly divergent DNA content of sex chromosomes is not known. A candidate for this process could be a protection against female meiotic drive (Rutkowska and Badyaev 2008).

It remains to be investigated why only microsatellites used to be accumulated on Y and W sex chromosomes after or during recombination cessation. It is possible that the accumulation of microsatellite sequences merely reflects their high mutation rate in copy numbers. However, the accumulation of microsatellites can be adaptive. Microsatellite accumulation may guarantee the conservation of chromosome morphology and at the same time they may represent a relatively small threat to genome integrity in comparison to other repetitive sequences such as transposable elements (Grewal and Jia 2007). In this respect, it is notable that the high accumulation of most microsatellites in *E. velox* concerns exclusively the W chromosome and does not spread over autosomes and the Z chromosome (Fig. 2).

The *Bkm*-related repeats containing tandem copies of GATA sequence have been shown to be accumulated on the sex chromosomes of various eukaryotes including advanced snakes (O'Meally et al. 2010). The GATA repeats are uniformly distributed over the autosomes and sex chromosomes in *C. elegans* , and the W chromosome of *E. velox* even exhibits a conspicuous depletion of this sequence (Fig. 2), which supports the independent origins of the *Bkm*-related repetitions on sex chromosomes in snakes and other vertebrates. O'Meally et al. (2010) reasoned that as derived snake and bird sex chromosomes share common repetitive sequences, this may be due to cryptic homology of parts of the sex chromosomes between these lineages. However, no FISH signal on the W chromosome was observed after hybridization of the *Bkm* probe to chicken metaphase chromosomes in their study. Moreover, sex chromosomes in snakes and birds evolved from different autosomal pairs (Matsubara et al. 2006) and basal snake and avian lineages have homomorphic sex chromosomes without an accumulation of repetitive sequences (Jones and Singh 1985; Tsuda et al. 2007; O'Meally et al. 2010). The independent origins of repetitive sequences on degenerated W sex chromosomes in both these lineages therefore seem more likely.

Due to different biochemical characteristics, particular microsatellite sequences should differ in their potency to accumulate on sex chromosomes. We should then observe an accumulation of the same sequences on independently evolved sex chromosomes in different organisms. Nevertheless, the data accumulated so far does not support this prediction. For example, among all possible trinucleotide sequences, tandem copies of CAA, CAG, GAA and TAA showed the most notable accumulation on the Y chromosome in the plant *Silene latifolia* (Kubat et al. 2008). However, out of

these four sequences, only CAG and TAA tandem copies are accumulated on the W chromosome, while CAA repeats are uniformly distributed across all chromosomes and GAA repeats are even lacking on the W chromosome in *E. velox* (Fig. 2). Similarly, the CGG repeats are accumulated on the Y chromosome of the fish *Hoplias malabaricus* (Cioffi et al. 2011), but they are missing on the W chromosome of the lizard. In conclusion, various repetitive sequences follow very different trajectories on sex chromosomes in different organismal lineages. The identity of particular microsatellite sequences accumulated on sex chromosomes seems to largely reflect historical contingency.

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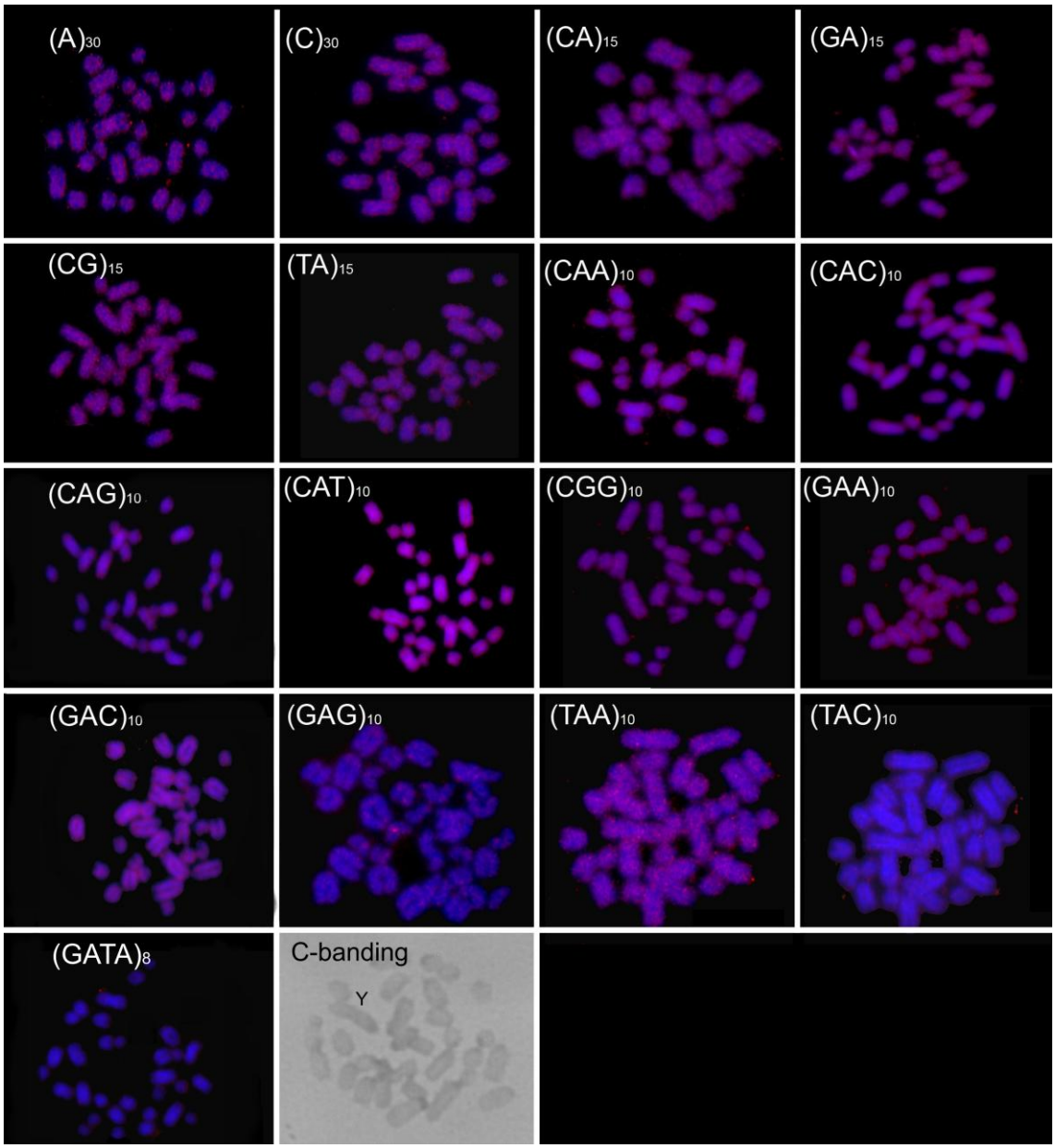
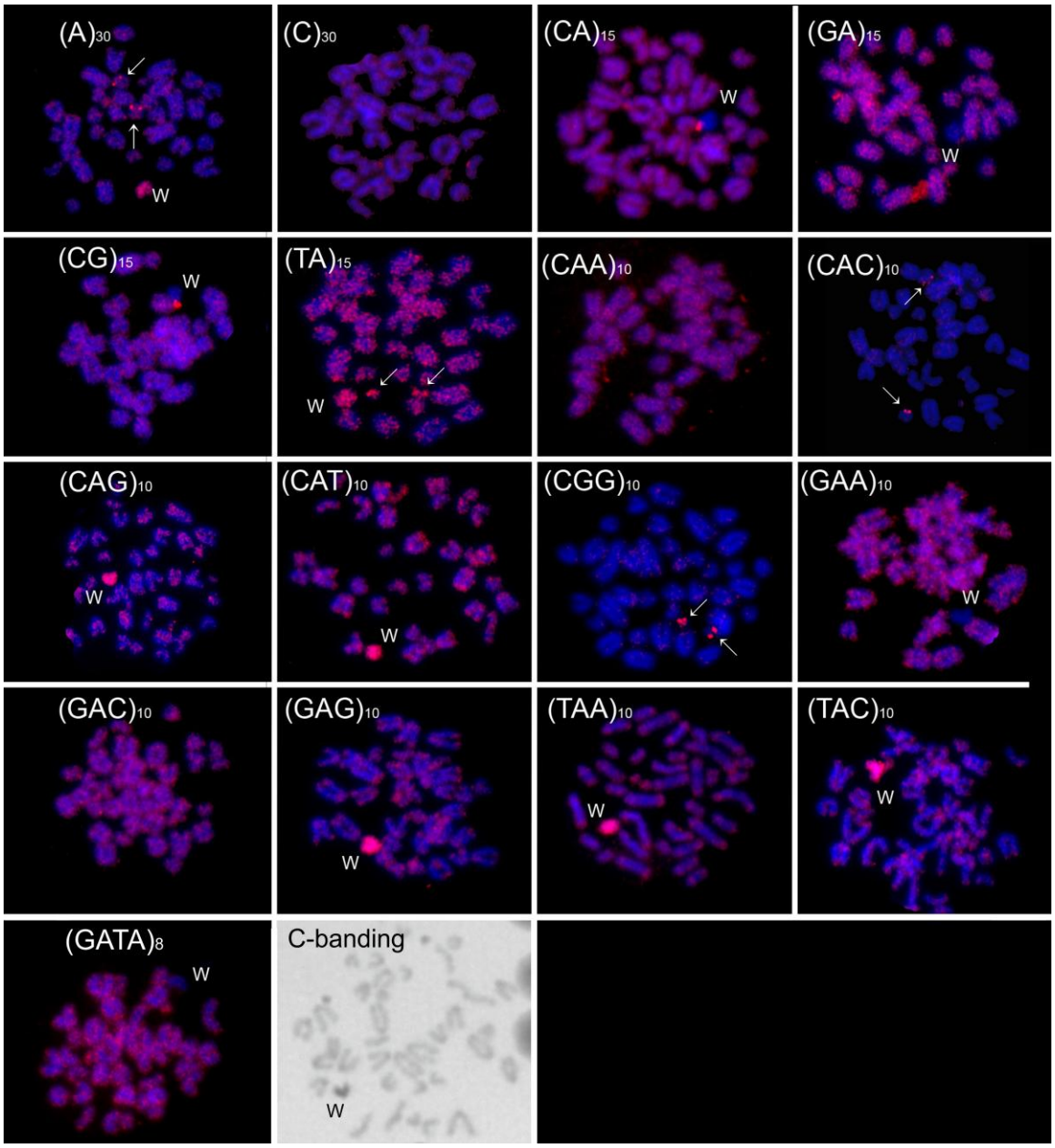
Figure 1 Mitotic metaphase chromosomes of *Coleonyx elegans* males hybridized with different microsatellite-containing oligonucleotides. Chromosomes were counterstained with DAPI (blue) and microsatellite probes were labelled with Cy3 (red signals). Last figure represents C-banded metaphase chromosomes; letter marks the Y sex chromosome.

Figure 2 Mitotic metaphase chromosomes of *Eremias velox* females hybridized with different microsatellite-containing oligonucleotides. Chromosomes were counterstained with DAPI (blue) and microsatellite probes were labelled with Cy3 (red signals). Last figure represents C-banded metaphase chromosomes. Letters mark the W sex chromosomes, arrows assign autosomal signals.


(A)30
(C)30
(CA)15
(GA)15
(CG)15
(TA)15
(CAA)10
(CAC)10
(CAG)10
(CAT)10
(CGG)10
(GAA)10
(GAC)10
(GAG)10
(TAA)10
(TAC)10
(GATA)8
C-banding
Y


(A)30
W
(C)30
(CA)15
W
(GA)15
W
(CG)15
W
(TA)15
W
(CAA)10
(CAC)10
(CAG)10
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(CAT)10
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(CGG)10
(GAA)10
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(GAC)10
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(TAA)10
W
(TAC)10
W
(GATA)8
W
C-banding
W

Závěr

Z jednotlivých kapitol vyplývá, že šupinatí plazi představují ideální modelovou skupinu pro studium obecnějších principů souvisejících s evolucí pohlavně determinačních mechanismů, pohlavních chromosomů a genomů. Přestože jednotlivé práce přistupují ke studované problematice rozličnými způsoby a řeší jednotlivá témata na různých fylogenetických škálách, závěry je vždy možné do značné míry zobecnit a usuzovat podle nich na obecné evoluční mechanismy. Ústředním tématem celé práce je evoluce pohlavně determinačních mechanismů. To je ve vědecké literatuře nesmírně diskutované téma, o kterém vyšla celá řada převážně teoretických publikací. Největší zájem byl věnován teoretickým úvahám o evoluci pohlavních chromosomů a o možnostech evolučních přechodů mezi jednotlivými způsoby determinace pohlaví. Práce, které by tyto hypotézy experimentálně testovaly a přinášely dostatečně kvalitní výsledky, na základě kterých bychom se posunuli od úvah ke konkrétnějším závěrům, vznikají jen velmi pomalu. Je to dáno tím, že studovat tyto fenomény je experimentálně nesmírně náročné a současná věda dává jen omezené nástroje k tomu, abychom mohli studovat dostatečně podrobně dostatečně široké spektrum linií. Soustředíme-li se na obratlovce, musíme konstatovat, že z celého širokého spektra druhů a fylogenetických linií, máme informace o primárním faktoru hrajícím rozhodující roli v determinaci pohlaví jen u velmi malého zlomku. Do současné chvíle známe gen *SRY*, který je primárním spouštěčem k diferenciaci varlat a jeho přítomnost je pravděpodobně synapomorfií všech živorodých savců. Otázkou však nadále zůstává, jaká je situace u těch savců, kde došlo k úplné redukci pohlavního chromosomu Y, a kde gen *SRY* v genomech nebyl nalezen (Kobayashi et al. 2008). Další informace o primárním faktoru determinace pohlaví máme pro dva druhy ryb z rodu *Oryzias*, u kterých je za určení pohlaví zodpovědný paralog genu *DMRT1* (Matsuda et al. 2007). Sesterský druh z tohoto rodu má však pravděpodobně odlišný způsob determinace pohlaví a stejný gen u něj nebyl nalezen. Předpokládá se, že paralog genu *DMRT1* by mohl být zodpovědný za determinaci pohlaví u drápatky *Xenopus laevis* (Yoshimoto et al. 2008) a také u ptáků (Smith et al. 2009), nicméně na jednoznačné potvrzení těchto předpokladů si budeme muset ještě počkat. Z celé škály obratlovců jsou tedy tyto informace zatím tím jediným, co skutečně víme o primární podstatě molekulární determinace pohlaví. Pokud se nebudeme zaměřovat čistě na spouštěč kaskády diferenciace gonád, informací o

pohlavních chromosomech a způsobech determinace pohlaví je samozřejmě u obratlovců známo mnohem víc. Nicméně i toto spektrum, pokud uvážíme druhovou početnost obratlovců, není příliš široké. Největší nedostatek znalostí nacházíme u ryb a ve stejné míře i u plazů a to zejména u nejpočetnější skupiny, tedy u šupinatých plazů. Uvážíme- li, že z celkového odhadovaného počtu druhů šupinatých plazů máme informace o pohlavně determinačních mechanismech jen pro něco málo přes 400 druhů, což představuje asi 5% celkového počtu, vidíme, že naše současné poznání představuje pouze zlomek. Přitom právě šupinatí plazi představují ideální skupinu pro mnohá fylogenetická srovnání. Budoucnost studií týkajících se pohlavních chromosomů a evoluce karyotypu jako takového leží jednoznačně v celogenomovém sekvenování a porovnávání jednotlivých genů náležících do sítě genů zajišťujících diferenciaci gonád, anebo celých bloků genomů jednotlivých linií. Už i jen data, která jsou k dispozici po osekvenování genomu leguána *Anolis carolinensis* představují rozsáhlý zdroj informací. Bohužel ani u takto intenzivně studovaného druhu, který slouží jako plazí modelový organismus, dosud neznáme pohlavní chromosomy. Předpokládá se, že *A. carolinensis* pohlavní chromosomy má, protože poměr pohlaví mláďat z vajec inkubovaných v různých teplotách je vyrovnaný, ale jejich identita zatím nebyla odhalena. A zde narážíme patrně na největší úskalí studia pohlavních chromosomů a evoluce pohlavně determinačních mechanismů u šupinatých plazů a zároveň na důvod, proč stále nemáme dostatek informací pro podrobnější srovnání. U šupinatých plazů se setkáváme poměrně často s přítomností homomorfních pohlavních chromosomů. Je proto nesmírně obtížné takové chromosomy odhalit a proto mnoho druhů plazů, i když u nich předpokládáme, že mají pohlaví určeno genotypicky, nemá známy pohlavní chromosomy. Ideálně by v budoucnosti bylo možno posuzovat syntenii pohlavních chromosomů mezi jednotlivými druhy různých linií nejen šupinatých plazů na základě osekvenovaných genomů. Tato data by pak sloužila pro fylogenetické srovnání a zhodnocení homologie a to by nám přineslo mnoho odpovědí na otázky o evolučních zákonitostech vzniku a diferenciace pohlavních chromosomů a evoluci genomu a jeho uspořádání. Nicméně vstupní informací by mělo být, že víme, který pár z karyotypu tvoří pohlavní chromosomy, jsme schopni jej identifikovat a tedy i porovnat jeho syntenii s jinými rozpoznatelnými a jasně identifikovanými chromosomy. Zde stále zůstává veliké pole působnosti pro metody molekulární cytogenetiky a na dlouhou dobu budou tyto metody také významné pro posuzování syntenií a homologií jednotlivých chromosomů mezi fylogeneticky významnými liniemi obratlovců, protože i když se celogenomové

sekvenování stále zrychluje a zlevňuje, uspořádání sekvencí tak, abychom v nich byli schopni zachytit důležité informace je stále časově velice náročné, a zástupci mnoha fylogeneticky významných linií obratlovců stále nejsou na seznamu druhů čekajících na sekvenaci.

Metody molekulární cytogenetiky jsou v současnosti ideálním nástrojem pro studium evoluce uspořádání genomů u rozličných skupin organismů. Podobně jako v případě pohlavních chromosomů, sekvenční data v budoucnu přinesou obrovské množství informací, na základě kterých bude možné srovnávat genomy a jejich uspořádání a tak poodhalovat čím dál tím více z obecných zákonitostí podílejících se na formování karyotypů. V dnešní době si ale musíme vystačit s daty, která máme a nejvhodnějším přístupem je kombinace a srovnání údajů známých ze sekvenování s cytogenetickými daty. Příkladem takového postupu může být práce, kterou publikoval Ellegren (2010), v níž srovnává dynamiku karyotypů u savců a ptáků. Závěrem jeho srovnání je pozorování, že ptáci mají na rozdíl od savců velice konzervativní karyotyp. Savčí karyotyp se vyznačuje vysokou dynamičností s množstvím chromosomových přestaveb. Důvodem může být nízká aktivita retrotranspozonů u ptáků. Nakatani (2007) dokonce hovoří o karyotypové stázi u ptáků, přestože jejich karyotyp prošel v evoluci mnohačetnými chromosomovými štěpeními, což je považováno za evoluční novinku této skupiny. Jednotlivé bloky genomu jsou však značně konzervativní. Ellegren (2010) to ve své práci považuje za unikátní vlastnost ptačího genomu a ptáky na rozdíl od savců považuje v tomto ohledu za výjimečné. Na základě našich zjištění z výsledků pocházejících z experimentů s ptačím Z chromosomem jako sondou i z experimentů, v nichž jsme použili sondy pro FISH z ptačích autosomů, jasně vyplývá, že genom je konzervativní nejen u ptáků, ale i u šupinatých plazů a ostatních skupin sauropsidů. To, že se nám vůbec podařilo hybridizovat spolu části genomů linií vzdálených 275 milionů let, vypovídá o značné konzervativnosti. Naše data však také ukázala, že z nějakého důvodu jednotlivé segmenty genomů drží pohromadě a nepodléhají mnohačetným přestavbám, jako to známe u savců. Samozřejmě k významným přestavbám karyotypů v rámci skupiny Sauropsida docházelo a o jejich důvodech se můžeme stále jen dohadovat. Můžeme spekulovat o nějakém funkčním významu chromosomových přestaveb, anebo na ně můžeme nahlížet jako na výsledek náhodného působení např. transposonů. To ale nic nemění na tom, že v rámci sauropsidů se setkáváme se značně konzervativním uspořádáním genomů a můžeme tedy uzavřít, že ptáci nejsou výjimeční

ve svých konzervativních genomech, ale výjimeční jsou právě savci se svými dynamickými a mnohokrát přeuspořádanými genomy.

Na úplný závěr bych chtěla poznamenat, že přestože předkládaná disertační práce představuje výsledek několika let intenzivní práce na sevřeném tématu a jak doufám její výsledky přispívají k novým náhledům a úvahám o evoluci pohlavně determinačních mechanismů a uspořádání genomů, většina informací o pohlavně determinačních mechanismech u obratlovců zůstává neznámá. To přináší mnohé možnosti pro další směry výzkumu, nové úvahy a metodické přístupy, které přispějí k detailnějšímu porozumění evoluce tohoto fascinujícího tématu.

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