MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2010) doi: 10.1111/j.1755-0998.2010.02861.x

PERMANENT GENETIC RESOURCES ARTICLE

Strong correlation between cross-amplification success and genetic distance across all members of 'True Salamanders' (Amphibia: Salamandridae) revealed by *Salamandra salamandra*-specific microsatellite loci

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Abstract

The unpredictable and low cross-amplification success of microsatellite loci tested for congeneric amphibian species has mainly been explained by the size and complexity of amphibian genomes, but also by taxonomy that is inconsistent with phylogenetic relationships among taxa. Here, we tested whether the cross-amplification success of nine new and 11 published microsatellite loci cloned for an amphibian source species, the fire salamander (*Salamandra salamandra*), correlated with the genetic distance across all members of True Salamanders (genera *Chioglossa*, *Lyciasalamandra*, *Mertensiella* and *Salamandra* that form a monophyletic clade within the family of Salamandridae) serving as target species. Cross-amplification success varied strongly among the species and showed a highly significant negative relationship with genetic distance and amplification success. Even though lineages of *S. salamandra* and *Lyciasalamndra* have separated more than 30 Ma, a within genus amplification success rate of 65% was achieved for species of *Lyciasalamandra* thus demonstrating that an efficient cross-species amplification of microsatellite loci in amphibians is feasible even across large evolutionary distances. A decrease in genome size, on the other hand, paralleled also a decrease in amplified loci and therefore contradicted previous results and expectations that amplification success should increase with a decrease in genome size. However, in line with other studies, our comprehensive dataset clearly shows that cross-amplification success of microsatellite loci is well explained by phylogenetic divergence between species. As taxonomic classifications on the species and genus level do not necessarily mirror phylogenetic divergence between species, the pure belonging of species to the same taxonomic units (i.e. species or genus) might be less useful to predict cross-amplification success of microsatellite loci between such species.

Keywords: cross-amplification, genome size, microsatellite loci, neutral genetic distance, *Salamandra salamandra*, Salamandridae, True Salamanders

Received 29 September 2009; revision received 24 January 2010; 11 March 2010; accepted 22 March 2010

Introduction

Microsatellite loci are the most important and frequently used population genetic markers in amphibian research, addressing questions related to general biological issues, such as landscape ecology (e.g. Palo *et al*. 2003; Funk *et al*. 2005), fine-scale population structure (e.g. Palo *et al*. 2004; Jehle *et al*. 2005; Steinfartz *et al*. 2007a), mating systems (e.g. Jones *et al*. 2002; Steinfartz *et al*. 2006; Jehle *et al*. 2007), amphibian decline-linked disease infections (e.g. Pearman & Garner 2005; Teacher *et al*. 2009) and

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conservation biology (reviewed by Jehle & Arntzen 2002; Beebee 2005). Although the development of microsatellite loci has been improved drastically by enrichment methods (Zane *et al.* 2002), the cloning of microsatellite loci for amphibian species remains, compared to other groups of organisms (e.g. fish and birds), a time-consuming and money-consuming endeavour (see Steinfartz *et al.* 2004 for comments). Being aware of this problem, several studies (Krupa *et al.* 2002; Primmer & Merilä 2002; Zhan & Fu 2008) tested the cross-amplification suitability of species-specific amphibian microsatellite loci in congeneric species that were thought to be closely related based on taxonomic criteria (e.g. Krupa *et al.* 2002; Garner *et al.* 2003). Similar approaches have been also applied to other vertebrate groups such as birds (e.g. Primmer *et al.* 1996) or pinniped species (e.g. Hoffman *et al.* 2007) quite successfully. However, in the case of amphibians, the results often showed unexpected low rates of amplification success among species of the same taxonomic genus. It was assumed that the reasons for this might be associated with the large genome sizes typical for amphibians (see http://www.genomesize.com; Garner 2002), and/or by a taxonomy that is inconsistent with phylogenetic relationships among taxa and therefore obscures the existing genetic divergence between taxa (see Primmer & Merilä 2002).

To our knowledge, there is no other study than the one of Garner (2002) that has specifically tested for the influence of genome size on cross-amplification success of microsatellite loci between species, but two studies that have tested for the impact of neutral genetic divergence between tested species on cross-amplification success. Primmer *et al.* (2005) could show that pairwise sequence divergence of the mitochondrial Cytochrom *b* gene provided relatively accurate estimations of the cross-species amplification success rate in birds (32 tested species), amphibians (five tested species) and cetaceans (three tested species). On the basis of the divergence of partial sequences of the mitochondrial 12S and 16S ribosomal genes, a significant negative correlation between genetic divergence and cross-species amplification success was found for eight fish species belonging to the family of Serranidae (Carreras-Carbonell *et al.* 2008). A similar result was found in a third study carried out on a specific group of reptiles. Glenn *et al.* (1996) observed a decrease in cross-species amplification success between phylogenetic divergent source–target species pairs when studying the allelic diversity in Alligator microsatellite loci compared to other crocodilian species. Although this study was mainly based on the taxonomic status of the species, the general phylogeny of the order Crocodylia has recently been confirmed also on molecular grounds (Roos *et al.* 2007; Willis 2009).

The family Salamandridae represents one of the best-studied amphibian families, and phylogenetic relationships among its species are well resolved by molecular markers (Weisrock *et al.* 2006; Steinfartz *et al.* 2007b; Zhang *et al.* 2008). Within the Salamandridae, the so-called True Salamanders (genera *Salamandra*, *Lyciasalamandra*, *Chioglossa* and *Mertensiella*) form a highly supported monophyletic clade (Weisrock *et al.* 2006; Steinfartz *et al.* 2007b; see Fig. 1). In addition, the taxonomy within this clade adequately reflects the phylogenetic relationships on the species level (e.g. Veith & Steinfartz 2004). Accordingly, species of True Salamanders provide an ideal system to test for the influence of genetic distance on cross-amplification success of microsatellite loci in a comprehensive setup. In detail, we determined whether cross-amplification success of eleven published (Steinfartz *et al.* 2004) and nine novel microsatellite loci of a True Salamander source species, the fire salamander (*Salamandra salamandra*), was correlated with genetic distance among all 17 species and four additional subspecies of True Salamanders serving as target species. Furthermore, we tested in which way genome size influenced cross-amplification success. Our results showed that cross-amplification success of species-specific microsatellite loci of *S. salamandra* across all species of True Salamanders was highly negatively correlated with an increasing genetic distance between the source–target species. Additionally, we observed the contrary effect of genome size on amplification success as previously suggested. We conclude that the reported low cross-amplification success rates in amphibians may have mainly been obscured by the congeneric taxonomic classification of species that are in fact genetically rather divergent.

Methods

Cloning and development of new microsatellite loci for Salamandra salamandra

Tissue samples of fire salamander larvae were collected from the Kottenforst (50°41′09 N, 7°07′03 E; 180 m a.s.l.) near Bonn in Germany with the permission of the 'Untere Landschaftsbehörde der Stadt Bonn' for the development of microsatellite loci that followed the published procedure of Hauswaldt *et al.* (2008).

A total of 48 positive clones of an appropriate size (500–1000 bp) were sequenced with T3 and T7 primers using BigDye version 3.1 chemistry (Applied Biosystems). Primer design was carried out for 18 microsatellite loci with at least six repeat units using the software PRIMER3 (Rozen & Skaletsky 2000), and primers were tested in 24 individuals from the Kottenforst in multiplex PCRs, performed in 10 μL volumes using the Qiagen Multiplex

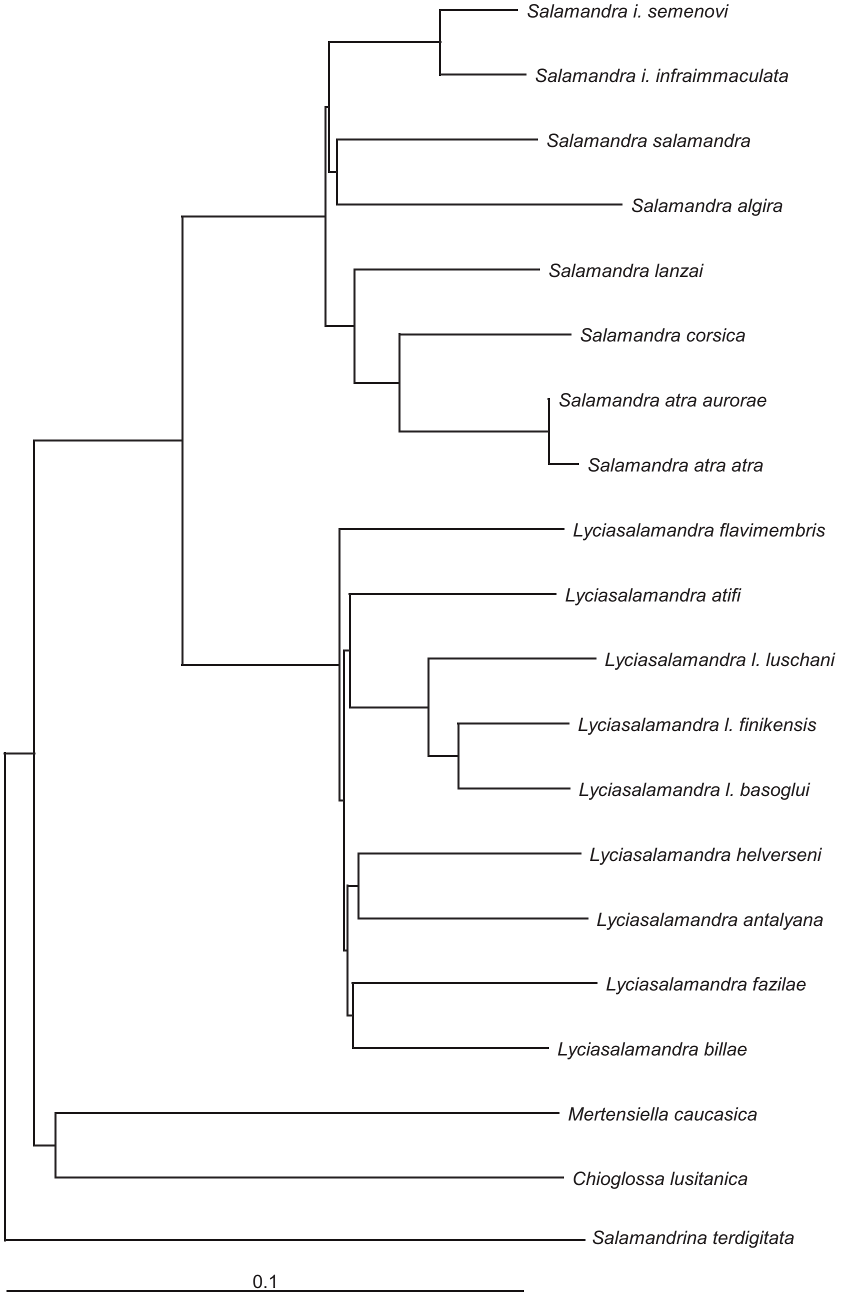

Fig. 1 Unrooted Neighbour-Joining (NJ) tree based on the uncorrected p-distance for all species of True Salamanders, modified from Weisrock *et al*. (2006).

kit. All reactions included 3 μL Multiplex PCR Master Mix, 0.4 μL of each primer (10 μM) and 1 μL DNA (10–20 ng/μL). PCR cycling was based on the following conditions: initial denaturation at 95 °C for 15 min followed by 45 cycles of 95 °C for 30 s, 60 °C for 90 s, 72 °C for 60 s and a final extension at 72 °C for 10 min. PCR products were genotyped on an ABI 3730 capillary sequencer (Applied Biosystems) with the Genescan LIZ 500 size standard (Applied Biosystems), and fragment sizes were scored using the GENEMARKER v.1.70 software (Softgenetics). Primer pairs of nine polymorphic loci consistently amplified scorable products from genomic DNA, whereas four of the 18 primer pairs amplified monomorphic loci and five primer pairs amplified no clear products.

A total of 79 individuals of *S. salamandra* from the Kottenforst were genotyped using the same PCR cycling conditions as described earlier with the minor modification of using a final extension time of 30 min at 72 °C. The program CERVUS 3.0 (Kalinowski *et al.* 2007) was used to calculate observed and expected heterozygosities, null allele frequencies and the number and size range of the alleles. Deviations from Hardy–Weinberg equilibrium and linkage disequilibrium were estimated in GENEPOP 4.0 (Rousset 2007).

Testing for cross-species amplification success across members of True Salamanders and correlation analyses

Genomic DNA of all tested samples was extracted using the SDS-proteinase K/Phenol-Chloroform extraction method as described in Steinfartz *et al*. (2000). The cross-species amplification success was tested for 11 established (Steinfartz *et al*. 2004) and nine newly developed (this study, see above) *S. salamandra*-specific microsatellite loci for 98 individuals representing all 17 species and four subspecies of True Salamanders using the PCR conditions and allele scoring methods as described in detail elsewhere (see Steinfartz *et al*. 2004). Obtained amplified products were analysed on an ABI 3730 capillary sequencer, and alleles were scored using the program GENEMARKER v.1.70 (Softgenetics). The amplification success of microsatellite loci was classified into four categories: (i) loci showing clear signals of amplification, (ii) loci with identifiable, but weak signals, (iii) loci without amplification success, and (iv) loci that showed multiple banding patterns (i.e., three or more alleles, which could not be scored unambiguously according to size and intensity). Amplified products that referred to categories (iii) and (iv) were considered as negative result of cross-amplification. Subsequently, the cross-species amplification success of *S. salamandra*-specific microsatellite loci (expressed as the mean number of amplified loci) was tested for a correlation with the corresponding genetic distance between *S. salamandra* and the tested species using the program PAST (Hammer *et al*. 2001). The uncorrected p-distances of the True Salamander species were calculated with the program PAUP 4.0b10 (Swofford 2002), using 2607 bp of the mitochondrial sequence alignment (*nd1*, *nd2*, *coI*, *cytb*, 12S, 16S, and the Leu, Ile, Gln, Trp, Met, Ala, Asn, Cys and Tyr tRNA genes) of Weisrock *et al.* (2006) deposited in TreeBase (http://www.treebase.org; TreeBase S1513). As *S. s. salamandra* and no other member of the so called C-clade according to Steinfartz *et al.* (2000) were included in the sequence alignment of Weisrock *et al.* (2006), we took *Salamandra s. longirostris* as the phylogenetically, most closely related taxon of *S. s. salamandra* (see Steinfartz *et al.* 2000) as the basis to infer genetic distances for the correlation analyses. Also, because similar sequence information was not available for *Salamandrina perspicillata*, it was not included in the correlation analysis.

To test for a possible bias of low sample sizes on amplification success rate of microsatellite loci, we performed the correlation analysis separately only with taxa for which at least three distinct individuals were tested (i.e. 13 taxa were included; see Table 1). Additionally, a similar correlation analysis was performed with a 1457-bp fragment of the nuclear-coded Rag-1 gene for a subset of the complete set of taxa, for which this sequence was available. Accordingly, we analysed *S. salamandra* (AY650135), *Lyciasalamandra l. luschani* (AY323753), *Lyciasalamandra atifi* (AY456261), *Chioglossa lusitanica* (AY583347) and *Salamandrina perspicillata*.

Genome sizes (expressed as *C*-values) were available for six taxa of True Salamanders from the animal genome size database (see http://www.genomesize.com). The following values (in cases of multiple values for a specific taxon, e.g. *S. salamandra*, the mean was taken) were used for correlating genome size with amplification success (respective *C*-value is provided in brackets): *S. s. salamandra* (35.20), *Salamandra infraimmaculata* (39.30), *S. s. longirostris* (35.02), *Salamandra atra* (32.55), *C. lusitanica* (29.22) and *Salamandrina terdigitata* (20.06).

Results

Locus characteristics of new Salamandra salamandra-specific microsatellite loci

The standard characteristics of the newly developed microsatellite loci for the fire salamander are described in Appendix 1. Null allele frequencies for the different loci were as follow: SST-A6-I: −0.045, SST-A6-II: +0.064, SST-B11: +0.082, SST-C2: +0.022, SST-C3: +0.031, SST-E11: +0.015, SST-F10: +0.4593, SST-G6: +0.011 and SST-G9: +0.324. The tests for linkage disequilibrium detected three significant *P*-values ($P < 0.05$) for SST-A6-II, SST-B11 and SST-C2, but were nonsignificant after having been adjusted by sequential Bonferroni correction (Rice 1998). Two loci (SST-F10, SST-G9) deviated significantly from Hardy–Weinberg equilibrium, likely because of the presence of null alleles.

Cross-species amplification success and correlation analyses

Out of 1960 individual locus amplifications (98 individuals × 20 loci), 1332 (68%) positive amplicons were obtained, of which 1278 (96%) were heterozygous. The number of nonamplified loci differed across the genera of True Salamanders, with $n = 4$ for *Salamandra* ssp., $n = 7$ for *Lyciasalamandra* ssp., $n = 8$ for *Chioglossa lusitanica* and $n = 10$ for *Salamandrina* ssp. as well as for *Mertensiella caucasica*. Loci Sal 29 and Sal E5 resulted in almost no amplifications across the tested target species. The total number of polymorphic loci (Fig. 2) per taxon ranged from 12 to 16 in *Salamandra* ssp., 9 to 15 in *Lyciasalamandra* ssp., 10 to 13 in *C. lusitanica*, 6 to 10 in *M. caucasica* and *Salamandrina* ssp.

The number of amplified microsatellite loci cloned from *S. salamandra* decreased significantly with an increase in the mitochondrial-based genetic distance

Table 1 Cross-species amplification success of 20 *Salamandra salamandra*-specific microsatellite loci for all 17 closely related species of True Salamanders and four additional subspecies as represented by 98 individuals. Microsatellite loci lacking amplification success for a certain species are indicated by (−), and those of a successful amplification are marked with (+). The markers that weakly amplified alleles are indicated by (w), whereas those that amplified multiple bands are labelled with (mb)

		Locus																			
Species	No.	SST-A6I	SST-A6II	SST-B11	SST-C2	SST-C3	SST-E11	SST-F10	SST-G6	SST-G9	Sal 3	Sal E2	Sal E14	Sal 29	Sal E11	Sal E5	Sal E6	Sal E8	Sal E12	Sal 23	Sal E7
Salamandra algira	(*n* = 7)	−	+	w	w	+	+	w	+	+	+	+	+	−	−	−	+	+	+	+	+
Salamandra atra	(*n* = 7)	−	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
Salamandra atra aurorae	(*n* = 2)	−	−	+	−	+	+	w	+	+	+	−	+	−	−	−	+	+	+	+	+
Salamandra corsica	(*n* = 3)	−	+	+	−	+	+	+	+	+	+	−	+	−	−	−	+	+	+	+	+
Salamandra i. infraimmaculata	(*n* = 4)	−	+	w	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
Salamandra i. semenovi	(*n* = 1)	−	+	+	+	+	+	+	+	+		+	+	−	−	−	+	+	+	+	+
Salamandra lanzai	(*n* = 11)	−	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
Salamandra s. longirostris	(*n* = 1)	−	+	+	+	+	+	−	+	+	+	w	+	−	−	−	+	+	+	+	+
Mertensiella caucasica	(*n* = 2)	w	w	+	−	+	−	−	w	+	−	−	−	+	w	−	−	+	w	−	−
Chioglossa lusitanica	(*n* = 4)	w	−	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	w	−	−
Lyciasalamandra antalyana	(*n* = 1)	+	−	−	+	+	+	−	−	−	+	+	+	−	+	−	+	+	+	−	+
Lyciasalamandra atifi	(*n* = 3)	+	−	−	+	+	+	−	w	−	+	+	+	−	+	−	+	−	+	−	+
Lyciasalamandra billae	(*n* = 5)	+	−	−	+	+	+	−	+	−	+	+	mb	−	+	−	+	+	+	−	w
Lyciasalamandra fazilae	(*n* = 13)	mb	+	−	+	+	+	−	+	−	+	+	mb	−	w	−	+	+	+	−	+
Lyciasalamandra flavimembris	(*n* = 1)	+	−	−	+	+	+	−	+	−	+	+	mb	−	+	−	+	+	+	−	+
Lyciasalamandra helverseni	(*n* = 1)	+	+	−	+	+	+	−	−	−	+	+	mb	−	+	−	+	+	+	−	+
Lyciasalamandra l. luschani	(*n* = 10)	+	−	−	w	+	+	−	w	−	+	+	mb	−	+	−	+	+	+	−	+
Lyciasalamandra l. basoglui	(*n* = 10)	+	−	−	+	+	+	−	+	−	+	+	mb	−	+	−	−	+	+	−	+
Lyciasalamandra l. finikensis	(*n* = 4)	+	−	−	+	+	+	−	+	−	+	+	mb	−	+	−	+	+	+	−	+
Salamandrina perspicillata	(*n* = 4)	−	+	+	−	−	w	w	−	−	−	−	w	−	+	+	−	−	w	−	−
Salamandrina terdigitata	(*n* = 4)	−	+	+	−	−	−	w	w	−	−	−	−	−	+	+	−	+	+	−	−

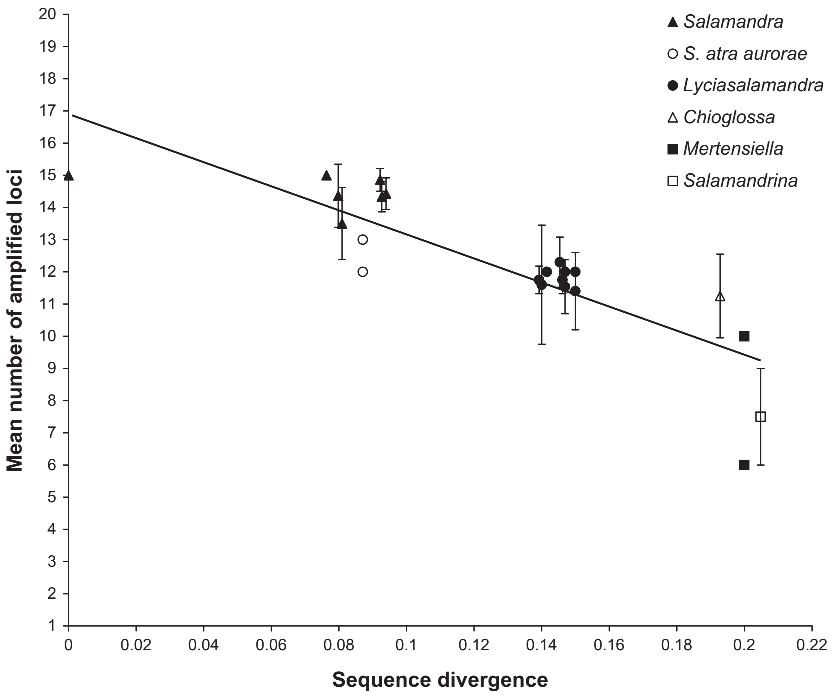

Fig. 2 Relationship between mean number of amplified loci and uncorrected pairwise sequence divergence of 2607 bp of mitochondrial DNA sequences for each target species (all taxa but *Salamandrina perspicillata* as listed in table 2) and *Salamandra s. longirostris* ($r = -0.84$, $r^2 = 0.71$, $P < 0.001$). Target species associated bars show standard deviation of amplification success of microsatellite loci that have been calculated if at least three individuals per taxon were tested. Amplification success of taxa tested by less than three individuals (e.g. *Salamandra atra aurorae*) are shown by single symbols.

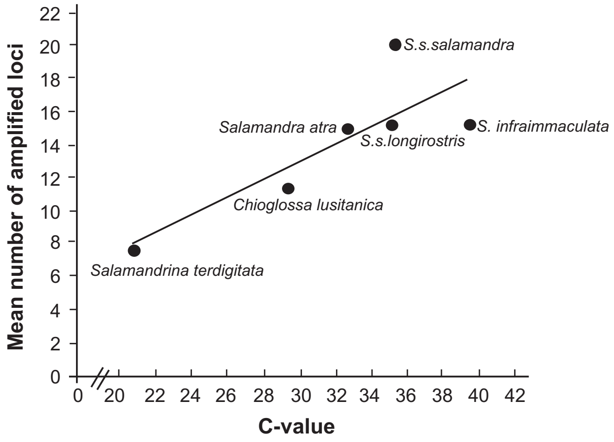

Fig. 3 Relationship between mean numbers of amplified loci and corresponding genome size expressed as *C*-value for six taxa of True Salamanders. A positive correlation between mean number of amplified loci and an increase in genome size has been found ($r = 0.818$; $r^2 = 0.67$; $P = 0.046$).

across the species of True Salamanders ($r = -0.84$, $r^2 = 0.71$, $P < 0.001$; see Fig. 2). An additional analysis without loci SST-F10 and SST-G6 that harboured high null allele frequencies resulted in a slightly weakened correlation coefficient ($r = -0.746$, $r^2 = 0.556$), but the correlation was still highly significant ($P < 0.001$). Also, if correlation analysis was restricted to taxa for which at least three distinct individuals were tested, similar results were obtained ($r = -0.91$; $r^2 = 0.84$; $P < 0.001$) thus showing that low sample sizes of some taxa have not biased the results based on the complete dataset. The analysis of the Rag-1 gene for a limited number of taxa showed a trend for a negative correlation ($r = -0.49$ $r^2 = 0.239$, $P = 0.40$).

The analysis of genome size – amplification success revealed contrary results as expected from the previous study of Garner (2002). As shown by Fig. 3, the mean number of amplified loci was positively correlated with an increase in genome size ($r = 0.818$; $r^2 = 0.67$; $P = 0.046$).

Discussion

The most important results of our study are (i) cross-species amplification success of microsatellite loci is negatively correlated with underlying increasing neutral genetic distances of the mitochondrial genome in salamanders and (ii) a considerable portion of loci cross-amplified over large evolutionary distances. In the following, we will discuss these points and their general implications more deeply.

So far, the unpredictable and low cross-amplification success of microsatellite loci tested for congeneric newts (genus *Triturus*; Krupa *et al.* 2002; Garner *et al.* 2003) and frog species, e.g. with only a 21% within genus amplification success rate in the genus *Rana* (Primmer & Merilä 2002), has mainly been explained by the size and complexity of amphibian genomes (Garner 2002), but also by taxonomy that is inconsistent with phylogenetic relationships among taxa (Primmer & Merilä 2002). In a study that tried primarily to identify potential factors affecting cross-species amplification success of microsatellite loci in birds Primmer *et al.* (2005) also analysed a small set of amphibian species in parallel. Accordingly, five species of *Rana* displayed a negative correlation between neutral genetic distance (based on the Cytochrom *b* gene) of source–target species pairs and cross-amplification success of microsatellite loci. Our systematic analysis of cross-species amplification success of 20 *Salamandra salamandra*-specific-microsatellite loci across all of the species of True Salamanders clearly adds important support to the findings of Primmer *et al.* (2005) by showing the same negative correlation and allows us to extend this preliminary trend to apply to amphibians in general. The most reasonable and straightforward explanation for obtained observations accounts for mutations that will occur over evolutionary time scales in the primer site of microsatellite loci: Base substitutions in the flanking region of the microsatellite loci will decrease the amplification success of microsatellite loci because of evolving mismatches between the primer region of the source and target species, if genetic distance between both increases. Given that we found the same trend, although nonsignificant because only five species could be used for this analysis (see Methods), also for the nuclear coding Rag-1 gene, we suppose that the observed negative correlation is restricted not only to neutral mitochondrial divergence, but also to genetic divergence of the nuclear genome.

Another factor that has been discussed in the context of low cross-amplification success rate of microsatellite loci in amphibian species is their large genome. Garner (2002) tested the amplification success of microsatellite loci in several individuals of the respective source species across nine different metazoan taxa including amphibians as represented by species of the genera *Rana* and *Triturus*. He found that amplification success decreased significantly with an increase in genome size and concluded that genome size may affect amplification success negatively. For our dataset, we were able to test the influence of genome size on cross-species amplification success in six distinct taxa of True Salamanders. In contrast, as suggested by Garner (2002), we found the opposite effect: the amplification success of microsatellite loci established for *S. salamandra* as a source species decreased significantly with a decrease in genome size (see Fig. 3). Although one might argue that this result is expected because the microsatellite loci were cloned in a source species (i.e. *S. salamandra*) with a comparable large genome size with respect to Salamandrina, we do not think that this might have had an effect on our results for the following reason. Although it might be more difficult to clone microsatellite loci from large genomes as those from salamanders than from fish (see Steinfartz *et al.* 2004), once loci have been established in a source species, their cross-amplification success should be independent of the genome size of the source species. Indeed, our results are not surprising as the decrease in genome size from *S. salamandra* to *Salamandrina terdigitata* is paralleled by an increase in neutral genetic distance (see Fig. 2). Given that both *Triturus* (see discussion below) and *Rana* are polyphyletic species assemblages (see Frost *et al.* 2006), their choice to test for the impact of genome size on amplification success was unlucky. In our view, two aspects will be important to test for the influence of genome size on cross-amplification success in amphibians in the future: (i) tested microsatellite loci should have been shown to work properly for a certain number of individuals of the source species (e.g. for a specific population as in our case) and (ii) comparisons should also control for the effect of genetic distance between source–target species.

Because an accurate dating of the phylogenetic divergence of major clades and many lineages is available for the family of Salamandridae, we can infer over which evolutionary time scales a considerable amount of microsatellite loci will amplify in target species. Although lineages of *Salamandra* and *Lyciasalamandra* diverged more than 30 Ma (Zhang *et al.* 2008), still a cross-amplification success rate of 65% (i.e. 13 of 20 *S. salamandra*-specific microsatellite loci amplified in species of *Lyciasalamandra*) was observed. On the other hand, the low cross-amplification success documented for *Triturus* species, as experienced by Krupa *et al.* (2002) and Garner *et al.* (2003), is not surprising given that *Triturus* is no longer considered monophyletic and the divergence of *Triturus* species dates back more than 60 Ma (Steinfartz *et al.* 2007b).

Considering our results and the observations of correlation of cross-species amplification success of microsatellite loci and genetic distance between source–target species in birds (Primmer *et al.* 2005), fish (Carreras-Carbonell *et al.* 2008) and crocodile species (Glenn *et al.* 1996), we feel that this is a general trend applicable also to amphibians.

Acknowledgements

We thank Prof. Dr. Diethard Tautz (Max-Planck-Institute for Evolutionary Biology, Plön, Germany) for logistic support, Dr. David Weisrock (University of Kentucky, Lexington, KY, USA) and Dr. David Vieites (Museo Nacional de Ciencias Naturales, Madrid, Spain) for providing the sequence data of the true salamander species. The numerous comments made by two anonymous reviewers and Dr. Travis Glenn helped to increase the quality of this manuscript substantially. This study was supported by grants (STE 1130/3-1, STE 1130/3-2) from the German Research Foundation (DFG), awarded to Sebastian Steinfartz, to study the mechanisms of adaptive speciation in *Salamandra*

salamandra and by a doctoral stipend of the VolkswagenStiftung (I/83 230) awarded to Ralf Hendrix.

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Appendix 1 Characteristics of nine newly developed polymorphic microsatellite loci for the fire salamander (*Salamandra salamandra*)

Locus	Primer sequence (5′ → 3′)	Label	Size range (bp)	Repeat motif	N_A	T_a(°C)	H_O	H_E	*P*-value	GenBank accession nos
SST-A6-I	F: TTCAGTGCTCTTGCAGGTTG	HEX	210–226	$(ATCT)_9ATCA(ATCT)_{10}$	5	64.2	0.633	0.593	0.887	FJ384990
	R: AGTCTGCAAGGATAGAAAGATCG									
SST-A6-II	F: ATTCTCTCTGACAAGGATTGTGG	FAM	191–223	$(TATC)_{11}$	8	63.3	0.671	0.744	0.224	FJ384992
	R: GGTAGACAGACATCAAGGCAGAC									
SST-B11	F: TCAAACGGTGCCAAAGTTATTAG		177–225	$(TATC)_{14}$	9	63.7	0.734	0.738	0.134	FJ384993
	R: TTAATTGGCAGTTTTCTTTCCAG	HEX								
SST-C2	F: CTTTGGGTCAGCCCTCTTC		203–235	$(TATC)_{16}$	8	63.8	0.759	0.738	0.173	FJ384989
	R: CAGAGCAACATTGGATGTATCAG	FAM								
SST-C3	F: CCGTTTGAGTCACTTCTTTCTTG		220–228	$(TAGA)_7TAGG(TAGA)_3$	3	63.8	0.278	0.287	0.367	FJ384996
	R: TTGCTTTACCAACCAGTTATTGTC	HEX								
SST-E11	F: AGACAAAAATGGGGACTAACCAC		231–311	$(TAGA)_{10}AAGATAGG(TAGA)_5$	10	63.9	0.772	0.805	0.602	FJ384988
	R: TGTCTACCTGTTTGTTTATCTACTGG	HEX								
SST-F10	F: GGCCAACGTCAGAGGTTTC		168–180	$(TAGA)_{13}$	4	64.7	0.127	0.508	0.000	FJ384994
	R: TCATATTCCTCTTATGTTCCTACTCC	HEX								
SST-G6	F: GAGGCCCATTTTCTTTACTTACC	FAM	220–236	$(TATC)_{12}$	5	62.6	0.532	0.526	0.277	FJ384995
	R: GTAAAGAGGGCCGCTTAGTTG									
SST-G9	F: CCTCGTCAGGGGTTGTAGG	FAM	237–257	$(ATCT)_{13}$	6	64.5	0.342	0.661	0.000	FJ384991
	R: CTTTCCAGGAAGAAACTGAGATG									

N_A, number of alleles; T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; *P*-value, *P*-value from exact tests for deviation from Hardy–Weinberg equilibrium.