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FROM CLONAL TO SEXUAL HYBRIDS: GENETIC RECOMBINATION VIA TRIPLOIDS IN ALL-HYBRID POPULATIONS OF WATER FROGS

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Speciation via interspecific hybrids is very rare in animals, as compared to plants. Whereas most plants overcome the problem of meiosis between different chromosome sets by tetraploidization, animal hybrids often escape hybrid sterility by clonal reproduction. This comes at the expense of genetic diversity and the ability to purge deleterious mutations. However, here we show that all-hybrid populations of diploid (LR) and triploid (LLR and LRR) water frogs (*Pelophylax esculentus*) have secondarily acquired sexual reproduction. First, in a crossing experiment analyzed with microsatellite markers, triploid hybrids of both sexes and genotypes (LLR and LRR) recombined their homospecific genomes. Second, the great majority of natural populations investigated had low multilocus linkage disequilibrium, indicating a high recombination rate. As predicted from mating system models, the L genome had constant, low levels of linkage disequilibrium, whereas linkage disequilibrium in the R genome showed a significant reduction with increasing proportion of recombining triploids. This direct evidence of sexual reproduction in *P. esculentus* calls for a change of the conventional view of hybridogens as clonally reproducing diploids. Rather, hybridogens can be independent sexually reproducing units with an evolutionary potential.

KEY WORDS: **Hybridogenesis, microsatellites, *Pelophylax esculentus*, polyploidy, *Rana esculenta*.**

Hybridization instantly creates individuals with a new genetic composition and is therefore a potentially powerful force in evolution (Wissemann 2007; Jiggins et al. 2008). Whether hybridization leads to speciation depends on the hybrids' ability to survive and reproduce (Arnold and Hodges 1995; Barton 2001; Chapman and Burke 2007). Two reproductive challenges need to be overcome for establishment of new hybrid taxa: First, the hybrids must be fertile, in spite of having two dissimilar chromosome sets that might interrupt meiosis (Arnold and Hodges 1995; Chapman and Burke 2007). Second, the hybrids must be spatially or reproductively isolated from the parental species (Wang et al. 2001; James and Abbott 2005; Chapman and Burke 2007).

Normal meiosis, as well as reproductive isolation, can instantly be restored by tetraploidization. Via this process, hybridization has had a large impact on plant evolution (Arnold 1997; Hegarty and Hiscock 2005; Wissemann 2007), whereas in animals, remarkably few examples of tetraploid speciation are known (Orr 1990; Otto and Whitton 2000). In animal hybrids, fertility and reproductive isolation are, however, often established by different kinds of clonal reproduction that may or may not be accompanied by polyploidy. Among clonal vertebrates, reptiles are parthenogenetic, whereas fish and amphibians depend on sperm from a sexual species for initiating embryogenesis (Vrijenhoek et al. 1989): In gynogenetic taxa, the sperm activates, but usually does not fertilize the eggs. In hybridogenetic taxa, fertilization takes place; yet, there is normally no recombination between the parental genomes. This is because the paternal genome is usually excluded from the germ line prior to meiosis while the remaining

maternal genome is transmitted clonally (Dawley 1989). Hybridity is restored in each generation by matings with the paternal species. The hybrids' soma is thus made up by both the sexual paternal and the clonal maternal genome, whereas the hybrids' germ line contains only the latter.

For hybrid speciation to be of evolutionary importance, a third factor is crucial: genetic recombination. Genetic recombination via sexual reproduction enhances genetic diversity and is generally agreed to convey three important benefits: One, high genetic diversity is required for defense against fast-evolving parasites (Red Queen hypothesis, Hamilton 1980). Two, the combination of beneficial mutations from different individuals enhances the efficiency of selection (Fisher 1930; e.g., Colegrave 2002; Cooper 2007). Three and most importantly, the combination of deleterious mutations allows their purging from the population (Muller 1932; e.g., Vrijenhoek 1994). Without recombination, clonal lines are predicted to accumulate deleterious mutations via Muller's ratchet, which will eventually lead to their extinction.

As a consequence of their clonal reproduction modes, parthenogenetic, gynogenetic, and hybridogenetic hybrid animal taxa lack the above-mentioned advantages of genetic diversity and the ability to purge mutations. Hence, they are generally considered to be "evolutionary dead ends," at least as far as individual lineages are concerned (e.g., Vrijenhoek et al. 1989; Maynard Smith 1992). In agreement with this, strictly clonal taxa are, with very few exceptions (Butlin 2002), distributed as short-lived tips on the tree of life mainly comprised of sexual taxa (Simon et al. 2003).

However, at least genetic diversity seems to be higher in clonally reproducing taxa than previously assumed, and various mechanisms have been described how this can be achieved. First, clonal hybrids often arise recurrently from different progenitors. Hence, they have a high genetic diversity possibly enabling them to fit different ecological niches (frozen niche variation hypothesis, Vrijenhoek 1984). Recurrent origin of clonal and polyploid sexual lineages is known from several plants (Soltis and Soltis 1999) and also from animals, including ostracods (Little and Hebert 1997), fish (Janko et al. 2003; Pala and Coelho 2005), reptiles (Moritz et al. 1989), and some anurans (Ptacek et al. 1994; Stöck et al. 2005). Second, some allegedly asexual organisms are not strictly clonal but occasionally incorporate new nuclear material from a sexual host (Hedges et al. 1992; Spolsky et al. 1992; Schartl et al. 1995). The most recent discovery of such a mechanism is "kleptogenesis" in unisexual salamanders of the genus *Ambystoma* (Bogart et al. 2007): all-female lines can incorporate (parts of) nuclear genomes from sperm from sympatric sexual species and presumably later discard other parts of the genome. Third, in bisexual hybridogenetic species, like the edible frog, *Pelophylax esculentus* (called *Rana esculenta* until Frost et al. 2006), and the Iberian minnow, *Squalius alburnoides*,

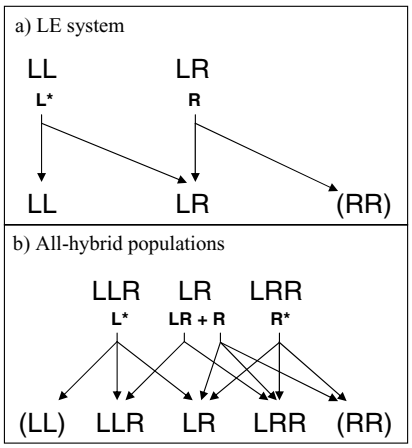

Figure 1. Adults, gametes and offspring of (A) the LE system with *P. lessonae* and *P. esculentus* and (B) all-hybrid populations of *P. esculentus*. *denotes gametes that could be recombined. Nonhybrid offspring from intraspecific *P. esculentus* matings are in parenthesis because they typically die before reproductive maturity. Note that in the LE system, the R genome is never recombined and the L genome is provided anew in every generation by *P. lessonae*. In the all-hybrid populations, both L and R genomes are supplied by hybrids and would regularly undergo recombination, if triploids have meiotic hybridogenesis.

hybrid × hybrid matings lead to offspring with parental genotype (cf. Fig. 1B). Although rarely viable, these offspring could recombine the otherwise clonal genomes if they succeed in reproducing (Hotz et al. 1992; Alves et al. 1998; Vorburger 2001c). Although the existence of these three mechanisms cannot be denied, their potential for lifting the doom of "evolutionary dead end" from the relevant hybrid taxa is subject to discussion.

Here we investigate the potential for systematic and frequent sexual reproduction in hybridogens through a mechanism called meiotic hybridogenesis. The term refers to the possibility that in polyploid hybridogens of the general type AAB, the homospecific chromosome sets from one parental species, A, recombine in a normal meiosis, whereas the set from the other parental species, B, is discarded (Alves et al. 1998). Preferential pairing of homologous chromosomes and elimination of the unmatched chromosomes has been shown for a number of triploid fish and frog hybrids (see Morishima et al. 2008 and references therein) but, so far, clear evidence for recombination through meiotic hybridogenesis comes from one species only: the Iberian minnow, *S. alburnoides* (Crespo-Lopez et al. 2006).

It might be argued that meiotic hybridogenesis is a rare and special phenomenon without much general relevance for the role of hybrids in animal evolution. However, meiotic hybridogenesis is interesting as a newly discovered possibility for hybridogenetic hybrids to obtain recombination in a regular, nonaccidental way. Besides, the list of taxa with meiotic hybridogenesis will surely

grow: First, with the increasing application of molecular tools to organisms from different populations, the list of known hybridogens has grown recently and is likely to grow further. Second, because hybridogenesis was originally discovered in the diploid topminnow, *Poeciliopsis monacha-lucida* (Schulz 1969), polyploidy in hybridogens increasingly appears to be the rule, rather than the exception. At present, polyploidy is known from four of the six genera with hybridogenesis. Water frogs (*Pelophylax*, Berger 1967), Iberian minnows (*Squalius*, Carmona et al. 1997), spined loaches (*Cobitis*, Saitoh et al. 2004), and Oriental weatherloaches (*Misgurnus*, Morishima et al. 2008) exhibit polyploidy whereas only hybridogenetic topminnows (*Poeciliopsis*) and stick insects (*Bacillus*, Bullini and Nascetti 1990) are purely diploid. Polyploidy is also known from a hybridogenesis-related mode of reproduction in the Batura toad (*Bufo viridis* complex, Stöck et al. 2002). We are thus just at the beginning of discovering the diversity and implications of hybrid reproduction modes.

Hence, investigating the extent of recombination during meiotic hybridogenesis and thus the long-term evolutionary potential for intraspecific hybrids seems timely and potentially relevant for more species than presently assumed. The edible frog, *P. esculentus*, provides a particularly interesting system for such an investigation, because it is the only hybrid yet known also to form self-sustaining, hybridogenetic, all-hybrid populations. In the absence of the parental species, meiotic hybridogenesis is the sole potential source of frequent recombination and could thus be of crucial evolutionary importance for these populations. Moreover, *P. esculentus* comes in various mating systems and, hence, offers an opportunity to study successive stages of incipient hybrid speciation.

The Pelophylax esculentus Systems

Pelophylax esculentus (*R. esculenta*) originated, and still originates, from interspecific matings between the two sexual water frog species, *P. lessonae* (the pool frog, genotype LL) and *P. ridibundus* (the marsh frog, genotype RR). The parental species, as well as the diploid *P. esculentus* hybrid with the genomic composition LR, have wide distributions in Europe. In the western part of this distribution area, LR excludes the L genome from the germ line prior to meiosis and transmits the R genome to the gametes clonally. As a result, matings between hybrids yield RR offspring, but these typically die due to homozygosity for deleterious mutations in the clonal R genome (Vorburger 2001a and references therein; Guex et al. 2002). To form a new generation of hybrid LR, *P. esculentus* is dependent on L gametes obtained from mating with *P. lessonae* (LE system, Fig. 1). In parts of Eastern Europe, the pattern is reversed: hybrid LR excludes the R genome, produces L gametes, and, therefore, lives in sympatry and mates with *P. ridibundus* (RE system). In both of these diploid systems, (reviewed by Graf and Polls Pelaz 1989) *P. esculentus* face disadvantages with respect to both of its genomes: the one in the hybrid's germ line is clonal, whereas the other, sexual, genome, must for every generation, be obtained by mating with the parental species. Various LE, RE systems, and *lessonae-esculentus-ridibundus* populations with both diploid and triploid *P. esculentus* also exist (Günther 1991; Tunner and Heppich-Tunner 1992; Rybacki and Berger 2001), but unfortunately hardly anything is known about how these diverse and complicated populations function.

The present study focuses on all-hybrid populations of *P. esculentus* (EE system) that, by definition, live and reproduce without any of the parental species. Thus, the propagation of both parental genomes, as well as any recombination within them, must be undertaken by hybrids alone. All-hybrid populations are found in large areas of Denmark, southern Sweden northeastern Germany, and patchily in northern Poland and probably a few localities in southeastern Europe (Mikulícek and Kotlík 2001; Rybacki and Berger 2001; Christiansen et al. 2005; Arioli 2007 ch. 5); (reviewed by Plötner 2005). These populations consist of diploid (LR) and one or two types of triploid hybrids (LLR and LRR). LLR frogs of both sexes provide L gametes whereas LRR make R gametes. Within the diploid LR, all males and some females produce R gametes, whereas all females and a few males make unreduced LR gametes yielding new triploids upon fusion with haploid gametes (Graf and Polls Pelaz 1989; Christiansen et al. 2005; Arioli 2007 ch. 1; Jakob 2007 ch. 5). Sex determination is an xx–xy system with a dominant male-determining y factor. The y factor is supposed to be present in the L genome only (Graf and Polls Pelaz 1989; Berger and Günther 1991–1992), which means that L genomes are either L_x or L_y whereas all R genomes are R_x. As a consequence, LLR and LR come in both sexes, while the great majority of LRR are females (Jakob 2007 ch. 2 and the present study). In this way, the mix of di- and triploid hybrid frogs forms self-sustaining populations producing all gametes needed for a new generation of similar composition (Fig. 1). Nonhybrid LL and RR offspring are also formed, but die off in natural ponds during the tadpole stage (Arioli 2007 ch. 3).

In all three systems, clonally propagated *P. esculentus* genomes face the risk of mutation accumulation. In the LE and RE systems, some accumulation can be tolerated, as the clonal genome is constantly paired with a healthy parental genome in the hemiclonal hybrids (confirmed in LE by Vorburger 2001b). Nevertheless, the life span of the clonal genomes in diploid systems appears limited, as old clones are likely to become inviable or replaced by new genomes that were more recently derived from primary hybridization between the parental species. In the all-hybrid populations, the situation was, so far, unknown. It was often assumed that the LLR recombine their two L chromosome sets after exclusion of the R, and that, likewise, LRR recombine

their two R sets after exclusion of the L genome (Günther et al. 1979; Graf and Polls Pelaz 1989; Som and Reyer 2006a). Under this assumption, the all-hybrid populations might be functionally sexual with a higher evolutionary potential than diploid LE and RE system populations. However, experimental evidence for recombination in triploids is scarce and controversial, due to low availability of polymorphic genetic markers. Based on allozyme and sex data, Günther et al. (1979) probably found recombination in one Polish LRR male (table 5, cross 25/26). Furthermore, Arioli (2007 ch. 1), using microsatellite analysis on Swedish frogs, detected recombination in an LRR female, but not in an LLR male. Although these data demonstrate the capability of triploids to recombine, it remains unclear whether recombination happens as a rule or as an exception and whether there are sex- and/or genotype-(LLR vs. LRR) specific differences in the recombination rate.

Here we present the first crossing experiment with a sufficient number of frogs (30) and polymorphic genetic markers (18) to conclude that intragenomic recombination takes place in triploids of both sexes and genotypes (LLR and LRR). We also provide previously unpublished microsatellite primers and new multiplex PCR protocols.

Confirming recombination in triploids does, however, not suffice to conclude that all-hybrid populations are functionally sexual. Therefore, assessment of the impact of triploid-mediated recombination on the genetic structure of the L and R genomes in wild populations was needed. One might expect populations with many triploids to be highly recombined and thus have low multilocus linkage disequilibrium. This, however, should be true only for the R genome; not for the L genome. The reason for the difference is that R gametes can originate from both recombining LRR and nonrecombining LR frogs whereas L gametes come from recombining LLR frogs alone (see Fig. 1). The monopoly on L gamete production guarantees LLR frogs a large and constant reproductive contribution to the next generation and, hence, should result in high recombination rates of L genomes, irrespective of the LLR/(LLR + LR) ratio. This prediction was previously confirmed by a mathematical model (Som and Reyer 2006a), but empirical data are lacking. In contrast, recombination rates of R genomes should, on average, be lower but increase with LRR/(LRR + LR) ratios. For this prediction, neither theoretical nor empirical studies were available.

Here we show that linkage disequilibrium was low in a large sample of natural populations from across the Danish and Swedish range, indicating that natural recombination rates are sufficiently high for these all-hybrid populations to be functionally sexual. We also provide evidence for the expected correlations between linkage disequilibrium and population structure. Finally, we confirm that pond-specific influences and method-specific biases were without importance for these results. In conclusion, the all-hybrid populations are an example of a hybridogen that, in a unique way, has become an independent evolutionary unit with sexual reproduction and thus a long-term evolutionary potential.

Methods

OVERVIEW

The study was carried out on Swedish and Danish all-hybrid populations, because these are geographically isolated from populations with parental species (Christiansen et al. 2005; Jakob 2007 ch. 2).

For direct evidence of whether triploids recombine, adult frogs were sampled, genotyped, and crossed and the offspring were reared and genotyped. Then, segregation and linkage analyses were performed on the inheritance pattern of the microsatellite alleles analyzed. Absence of linkage between the majority of loci, when compared pairwise, would indicate recombination.

For investigating the level of recombination in natural populations, frogs were sampled in ponds with different proportions of clone-propagating diploid (LR) and recombining triploid frogs (LLR and LRR). All individuals were genotyped, and the multilocus linkage disequilibria in the L and R genomes were calculated as $\bar{r}_d$ for each pond separately. Low $\bar{r}_d$ values would indicate high levels of recombination. The effects of genome, population structure, pond-specific effects, and method-specific biases on $\bar{r}_d$ were investigated to test the predictions outlined in the introduction and to test suspicions of artifacts. Finally, F statistics were calculated, because nonrandom mating, resulting in high F_{IS} values, would also affect $\bar{r}_d$.

CROSSES

Genetic variation in Swedish and Danish *P. esculentus* is very low (Christiansen et al. 2005; Arioli 2007 ch. 4). To obtain genetic data at multiple heterozygous loci for linkage analysis, it was therefore necessary to: (1) Screen both published and unpublished microsatellites for polymorphism in Scandinavia and design multiplex PCRs with the final selection of 18 primer pairs. (2) Select the most heterozygous triploids of a large sample of frogs for crossing. (3) Raise the larvae to an age where offspring genotypes could be inferred reliably when the heterozygous parents shared an allele. When parents share one allele, alleles, or even whole chromosomes missing in the offspring can lead to misinterpretation of the parental contributions. This was of a real concern, because many young larvae are aneuploid, i.e., they have mixed, uninterpretable genotypes with extra or missing alleles (Christiansen et al. 2005 and unpublished data from the present study). Raising the larvae to metamorphosis ensured that most aneuploid offspring died off and did not enter the analyses.

Crossing and rearing took place at Stensoffa Field Station, Scania, Sweden. Between May 12 and 22, 2006, i.e., after their emergence from hibernation and before breeding, 269 frogs

were caught at night using flashlight and dip net in one of the Danish (Alsønderup in Christiansen et al. 2005) and 10 of the Swedish ponds included in the investigation of natural populations described below. The frogs were marked individually with a transponder (Trovan ID101, Euro I.D., DE), toe-clipped for DNA analysis, and kept at approximately 7°C while the DNA samples were sent to the University of Zürich and analyzed for genome composition (LLR, LR, LRR) and heterozygosity (at LL in LLR and RR in LRR). The triploids with most heterozygous loci were preferred for the crossings, because recombination can only be assessed from combinations of heterozygous loci. This preference made a balanced design of source ponds impossible. Because males were more common than females among LLR frogs and females were predominant among LRR frogs, LLR males and LRR females were picked from a larger sample and were therefore more heterozygous than LLR females and LRR males. Most L genome data were therefore derived from males and most R genome data from females.

Six crossing tables were designed, each having three to four females and five to six males including at least one LLR, LR, and LRR female and at least one LLR, two LR, and one LRR male. Substitute frogs were added if the sperm or egg quality looked suboptimal. All females were crossed with all males within the same crossing table, so that all frogs were crossed to all genotypes (half-sibling design).

Offspring were produced on May 30, 2006 by artificial fertilization as described by Berger et al. (1994). Sperm solutions from the testes of hormone-injected males were distributed into three to five petri dishes per male. Eggs were then gently squeezed out of the hormone-treated females and dropped directly into the individual sperm solutions of the five to six different males, in small portions and in a random order. The following day, the egg clumps were transferred to 1 L tubs with 1–2 cm of water and subdivided for better oxygen supply.

The water was changed every 2–4 days and the egg jelly was removed after hatching. On June 12, when most tadpoles had just reached the feeding stage, 15 healthy-looking tadpoles (or fewer, if 15 were not available) from each sibship were randomly selected for rearing in 40 L outdoors tubs covered with mesh lids allowing air and sunlight through, but keeping predators out. Algae growing inside the tubs, supplemented with rodent pellets, ensured food ad libitum. Filamentous algae were regularly removed, fowling water exchanged, and *Daphnia sp*. added for good water quality. The tadpoles metamorphosed from July 18 onwards. Slow-growing tadpoles were eventually moved indoors into smaller tubs, where the last ones metamorphosed in mid-October. Offspring that died early during rearing disappeared, whereas offspring that died as metamorphs or nearly metamorphosing tadpoles were attempted DNA-analyzed although they were sometimes rotten. In total, 1628 tadpoles were selected for rearing, DNA samples were obtained from 1487 offspring (91%), and 1463 offspring (90%) were successfully genotyped.

NATURAL POPULATIONS

Population structure was investigated in 54 Danish and 12 Swedish ponds from mid May to mid August 2005. The Danish ponds were chosen as pairs of ecologically distinct ponds, maximally 5 km apart, from across the area of distribution. At each location, approximately 30 frogs (predominately adults) were caught at night with flashlight and dip net, were measured and had a toe-tip cut for DNA analysis before being returned to their pond.

The Swedish ponds constituted 11 ecologically variable ponds in the center of the small distribution area in Scania, Southern Sweden, and one from a satellite population near Malmö, 18 km west of the others ("core ponds" in Jakob 2007 ch. 2). The Swedish ponds were sampled as described above, but in both May and August, and the frogs were additionally marked with a transponder for individual identification. The Swedish samples are thus the sum of different individuals from the two catching rounds.

In total, 2296 Danish and Swedish frogs were caught and genotyped.

LABORATORY PROTOCOLS

DNA from the ethanol-stored toe-tips was extracted with Qiagen BioSprint 96 DNA Blood Kit (Qiagen, Valencia, CA) following Qiagen's protocol for tissue extraction. All samples were subjected to two PCRs with nine primer pairs each. The reactions were of 5 μl and contained 0.8 μl DNA extraction, 2.5 μl Qiagen Multiplex PCR Master mix, and 1.7 μl primer mix. PCR 1 contained primers Res16, Res20 (Zeisset et al. 2000), RlCA5, RlCA1b5 (Garner et al. 2000), Ca1b6, Ga1a19, Re2CAGA3 (Arioli 2007 ch. 4), RlCA2a34, and Rrid064A (Table 1). PCR 2 contained Res22 (Zeisset et al. 2000), RlCA18 (Garner et al. 2000), Rrid013A (Hotz et al. 2001), Rrid059A redesigned (Hotz et al. 2001 and Table 1: forward primer redesigned to extend the fragment amplified by 177 base pairs), Re1CAGA10 (Arioli 2007 ch. 4), RlCA1a27, ReGA1a23, Rrid169A, and Rrid135A (Table 1). Both forward and reverse primers appeared in 0.1 μM (or rarer 0.2 μM) in the PCR. Of the forward primers, 8–40% were color labeled with FAM, VIC, NED, or PET. PCR 1 was given 15 min of initial denaturation at 95°C, 30 cycles of 30 s at 94°C, 90 s at 57°C, and 60 s at 72°C, and a final extension of 30 min at 60°C. PCR 2 was run similarly, but with 31 cycles with 60°C instead of 57°C. PCR products (0.7 μl) were run on an ABI 3730 Avant capillary sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) with internal size standard (GeneScan-500 LIZ) and the alleles were scored with the Genemapper software (Applied Biosystems 2004).

Table 1. Primer sequences not previously published.

Locus	Sequence 5′–3′	Repeat	Genome specificity	GenBank ass. no.	Sequenced by
ReGA1a23	F: ATT GCT TTG GCA GTG AAG G R: TGA CAT CAC AGT GGG AGG AG	GA_n	L	EU445523	Garner et al., Arioli and Jakob
RlCA1a27	F: CAA ATG GGT CAT CCA CAC C R: GTT CAA GGG GGT CGA AAT AC	CA_n	L	EU445522	Garner et al.
RlCA2a34	F: GCT CCA TGC CAA AAG TCT TC R: TTG GGT ATG ATA CTA CAA GCT ATG C	GT_n	L+R[1]	EU445521	Garner et al.
Rrid059A redesigned	F: TTG GAG ACA GAC TTC CGT AGG	CA_n	L[1]+R	FJ024048	Hotz et al.
Rrid064A	F: TGT ACG GGC CTT TAG ACT GG R: AAC TTT TTG AAG GCC CCT TG	GT_n… …TA_nGT_n	R	EU445524	Hotz et al.
Rrid135A	F: TCT TTT GTT TTA GCG CAC CT R: CTG CCC GTC TAA GCA AGT GT	CA_nTA_n	R	EU445526	Hotz et al.
Rrid169A	F: CGG AAC TCC GCT TTA ATC AC R: CCC ATG TTG TCG TTG AGC TA	TA_n…CA_n	R	EU445525	Hotz et al.

[1]Monomorphic in this genome.

GENOTYPING

All samples were analyzed with 18 primer pairs amplifying loci in either the L genome, the R genome, or both. The 18 primers were scored at a total of 13 loci in each genome. With some primers, genome specificity changed slightly with PCR conditions, i.e., typically monomorphic L-specific alleles could arise or disappear beside the R allele(s) according to annealing temperature or primer concentrations. However, monomorphic loci conveyed no information of importance for the present study, so the choice of scoring or leaving out particular loci for technical reasons would not bias the data on homozygosity/heterozygosity, which was the focus of this study.

All alleles scored were specific to either the L or the R genome. Allele specificity was confirmed in *P. lessonae*, *P. esculentus*, and *P. ridibundus* from Estonia, Latvia, and Lithuania (D. G. Christiansen, unpubl. data), in nonhybrid LL and RR offspring from the crossings and through the distribution of L- and R-specific alleles on LLR, LR, and LRR frogs. Preliminary data from German and Swiss samples indicated, however, that in these more southern populations with higher genetic polymorphism, certain alleles were not genome-specific.

Four of the primer pairs (Res16, RlCA1b5, Ca1b6, and Ga1a19) amplifying both L- and R-specific alleles were used to distinguish LLR, LR, and LRR frogs by dosage effect, i.e., by the relative intensities (peak heights) of the L and R alleles amplified (see Christiansen 2005). L:R peak heights were evaluated separately per 96-well PCR, both per locus and per allele combination within that locus. The great majority of the L:R peak height ratios clustered into discrete groups corresponding to the LLR, LR, and LRR genotypes. Samples producing intermediate or extreme L:R ratios were subjected to repeated PCR analyses until each of the four dosage effect loci clearly signaled LLR, LR, or LRR. Assignment to LLR, LR, or LRR was thus determined independently at four loci. In nonhybrid offspring (LLL, LL, RR, RRR) the peak height ratios of heterozygous L or R loci were used to determine ploidy in the same way as just described. Not all loci and allele combinations proved diagnostic, but most did.

Samples that repeatedly gave conflicting results on genotype, i.e., had extra or missing alleles at particular loci, were classified as mixed genotypes. Mixed genotypes constituted 3.6% of the crossing experiment offspring and 2.1% (2.7% inclusive null alleles, see below) of the natural pond samples and were excluded from datasets in which the relevant loci could not be scored unambiguously.

Null alleles, i.e., alleles missing according to the overall ploidy of the individual, can be a nuisance in population genetics, because in high frequencies they bias estimates of allele frequencies and heterozygosity. However, in this study, they were generally not a problem, as they were often directly detectable and occurred in low frequencies. The adults used for crossings carried no problematic null alleles, as the analyses were made on the loci in which they were heterozygous for real alleles. Spontaneously missing alleles in mutant crossing experiment offspring, as well as null alleles in the frogs from the natural populations, were all directly detectable at the four dosage effect loci, and on average half of them were unmasked and detectable in a hemizygous state at the remaining loci. For example, a null allele at an L locus without dosage effect would be masked in LLR frogs but unmasked in LR and LRR frogs. Individuals with detected null alleles were handled as mixed genotypes described above. Only in two ponds

was the same locus found missing in more than two frogs (i.e., six and eight frogs, respectively), indicating that undetected null alleles could occur at potentially problematic frequencies in these ponds. In one of the two ponds, the entire locus was therefore recoded as missing data. In the second pond, all individuals were hemizygous at that locus, so that the null allele could always be detected. It was therefore coded as a real allele.

For determining LLR and LRR proportions in the ponds, mixed genotypes were assigned to the most similar euploid genotype.

STATISTICS: CROSSINGS

The crossings yielded data from 30 triploid frogs for segregation and linkage analyses. For males, the analyses were based on 19–58 (mean 41) offspring and for females on 30–86 (mean 66) offspring, as females were on average mated to more partners than males.

Nonrandom segregation would indicate selection during the experiment or unexpected genetic mechanisms. To check for random segregation at the heterozygous loci in the parents, offspring allele frequencies were tested with χ^2 tests for homogeneity with Yate's correction for continuity (Fowler and Cohen 1992). To correct for multiple tests ($n = 55$ L and 57 R loci), sequential Bonferroni correction of the P values was calculated according to Holm (1979) in the program MacBonferroni (Watkins 2002).

Linkage analysis involves analysis of the inheritance pattern at two loci that are heterozygous in a parent (e.g., Aa + Bb). Without recombination, all pairs of loci should show complete linkage, i.e., only two of the parent's allele combinations should be observed in the offspring (e.g., A + B and a + b). In contrast, with recombination all four possible combinations should be found in the offspring (A + B, a + b, A + b, and a + B) in approximately equal proportions of 0.25. Intermediate results, where the recombinant allele combinations (A + b and a + B) are significantly less frequent than the parental ones (A + B and a + b), would indicate reduced recombination and would be hard to explain if deriving from the majority of the locus pairs. However, a few locus pairs must, by chance, be expected to have reduced or no recombination, due to physical linkage. Linkage was investigated with 2×2 χ^2 tests with Yate's correction for continuity (Fowler and Cohen 1992) for every pairwise combination of loci that were heterozygous in the parent.

STATISTICS: NATURAL POPULATIONS

The rate of recombination is not easily measured directly. Instead, linkage disequilibrium between multiple genetic markers was used for an indirect measure, as recombination and linkage disequilibrium should be negatively related (see the Discussion). Pairwise and multilocus linkage disequilibria in natural populations were calculated as $\bar{r}_d$, as recommended by Halkett et al. (2005). $\bar{r}_d$ is an index of association adjusted for unequal sample size, calculated by the program Multilocus (Agapow and Burt 2001). First, L and R loci were divided into separate datasets. Then, the two homospecific allele sets in triploids were split up into haploid data by recoding all but one randomly chosen heterozygous locus into missing data. Recoding heterozygous loci into missing data is also how MULTILOCUS handles diploid data, according to the documentation file. Calculations were based on 20–71 (mean 37) haplotypes in Danish ponds and 56–110 (mean 78) in Swedish ponds. One pond was excluded from the L and another from the R dataset because less than our predefined minimum of 20 haploid genotypes had been sampled. Two further ponds were excluded from the L data and eight from the R data because no or only one locus was polymorphic. After that, the genomes had 2–11 variable loci (mean 3.8 for the L and 5.2 for the R), i.e., loci with at least five undeleted copies of an alternative allele.

Pairwise $\bar{r}_d$ was calculated in order check for locus pairs producing $\bar{r}_d$ values differing significantly from the mean $\bar{r}_d$ of the remaining pairs, when tested pairwise (locus pair in question vs. mean of remaining locus pairs) over all ponds. This pairwise within-pond approach was necessary because overall linkage was expected to differ between ponds. Significantly elevated linkage disequilibria could suggest physical linkage between the loci in question, whereas linkage disequilibria lower than the mean would be difficult to explain.

Multilocus $\bar{r}_d$ were calculated for each genome in each pond to test the predicted correlations between recombination and population structure outlined in the Introduction. All linear regressions, correlations, and t-tests were performed in SPSS (2004). The L and R slopes from the linear regressions were subjected to a test for difference between two regression lines (Fowler and Cohen 1992).

The expected relationships between linkage disequilibrium and population structure could be obscured by strong between-pond variation in the forces responsible for linkage disequilibrium, i.e., founder effect, drift, migration, and ecological selection on linked loci. If these forces affect the L and R genomes to a similar extent, the magnitude of this problem might be revealed by the degree of correlation between linkage disequilibrium in the L and R genomes in the ponds. To test for such pond-specific effects, we correlated $\bar{r}_d$ values for the L and R genome.

Genetic diversity varied between ponds and was generally lower in the L-specific than the R-specific markers. To investigate whether the estimates of multilocus linkage disequilibrium were affected by this variation in genetic diversity, we tested for correlation between $\bar{r}_d$ and genetic diversity measured as expected heterozygosity summed over all loci per genome. Expected

heterozygosity was for each locus calculated as $H_E = 1 - (al_1^2 + al_2^2 + al_3^2 \ldots)$ from allele frequencies (al_1, al_2, al_3, etc) computed by the software, SPAGeDi (see below).

As mentioned above, all but one of the heterozygous loci in triploid frogs had to be excluded for the constructing haplotypes for calculating $\bar{r}_d$. This affected the R genome the most, as its higher genetic diversity resulted in many R-heterozygous LRR frogs. Ponds rich in LRR frogs could thus theoretically have lower $\bar{r}_d$ values as a result of the lower resolution after the exclusion of the many heterozygous loci. To investigate whether $\bar{r}_d$ was affected by the resolution, it was tested whether the $\bar{r}_d$ values for the R genome were in correlation with the number of hemizygotes (LLR and LR which had no loci excluded) in the sample they were calculated from.

To investigate inbreeding and population structuring, F_{IS}, $F_{ST,}$ and F_{IT} were calculated in the program SPAGeDi (Hardy and Vekemans 2002), which accepts a mixture of different ploidy levels. These F statistics were calculated for each genome separately so that with respect to the L genome, LLR provided diploid data whereas LR and LRR provided haploid data. Similarly, LLR and LR gave haploid R data whereas LRR gave diploid R data. Excluding all haploid data from the analyses had very little effect on the results, though.

Results

CROSSES

Recombination data were obtained from seven LLR females, 10 LLR males, seven LRR females, and six LRR males. Due to multiple heterozygosity, most individuals provided data for several pairwise locus combinations. The LLR frogs provided recombination data for a total of 18 of 21 possible pairwise combinations of seven polymorphic L loci, and the LRR frogs for 47 of 66 possible combinations of 12 polymorphic R loci. All heterozygous loci in these triploids demonstrated random segregation, i.e., none of the allele proportions differed significantly from 0.5 at the 0.05 significance level after sequential Bonferroni correction performed within each genome separately. All triploids produced three or four gamete types per locus pair, corresponding to the two parental types and one or both recombinant types. All triploids thus recombined all their loci, and only for one locus pair were not all four gamete types present.

The uncorrected P values for the χ^2-tested frequency distributions of the four possible gamete types per locus pair are shown in Figure 2. As parental and recombinant gametes were indistinguishable because the genotypes of the parents of the frogs crossed were unknown, insignificant P-value deviations from zero do not necessarily imply reduced recombination. Insignificant P value would also have resulted from randomly derived excess of recombinant gametes and from uneven allele frequencies within the expected numbers of parental and recombinant gametes. When considered individually, the $-\log(P)$ values exceeding 1.30 were significant at the 0.05 level. After within-genome sequential Bonferroni correction for the 65 tests in the L genome and the 91 tests in the R genome, however, only four P values were significant. This indicates that the great majority of locus pairs were unlinked and freely recombined.

The four locus pairs showing significant linkage occurred in four different frogs (represented by four filled symbol types in Fig. 2) that all produced equilibrium offspring frequencies at their remaining locus pairs. The linkage was therefore rather a property of the loci than of the frogs involved. Unfortunately, replicate data were not obtained for the three locus pairs giving the most significant P values in this study, but the pair with strongest linkage, Re1CAGA10 + RlCA18 (L genome), was the same pair for which Arioli (2007 ch. 1) found no recombination. From the 40, 0, 0, 38 gamete frequency distribution in that and the 20, 0, 3, 23 gamete frequency distribution in the present study, it can be inferred that Re1CAGA10 and RlCA18 are linked, i.e., situated closely together on the same chromosome. Ca1b6 + Ga1a19 (R genome) had the offspring type distribution 10, 33, 18, 8 and Rrid169 + Rrid059A (R genome) had 36, 16, 7, 27. These locus pairs thus appear weakly linked, but replicate crossings would be needed to confirm linkage. Re2GAGA3 + Rrid135A appeared significantly linked in one female with gamete frequency distribution 12, 13, 20, 17, but unlinked in three other females. Overall, therefore, these two loci appear unlinked. Actually, a mutation happened in the germ line of this female so that some of her offspring had a new allele at locus Re2CAGA3. A rare allele at another locus confirmed that these offspring were indeed hers. The offspring with the new allele were excluded from the analyses involving Re2CAGA3, but when included by pooling the new and the lowest-frequency maternal allele, from which it most probably mutated, all four P values for locus pairs including Re2CAGA3 dropped substantially and the significant value became clearly nonsignificant.

Nearly significant P values appeared for several other locus pairs, but here also replicates raised the average for these loci to well above the 0.05 level, rendering no overall indication of linkage. Males and females did not have significantly different mean P values (male mean = 0.356, female mean = 0.285, t-test, $t_{154} = 1.606$, $P = 0.110$). Many species, probably including *P. esculentus* (Burt et al. 1991), have lower crossing-over rates in males than in females, but the present dataset can neither confirm or disprove this for *P. esculentus*.

NATURAL POPULATIONS

Triploids were found in all 55 ponds investigated, and both kinds (LLR and LRR) were found in 82% of the ponds. The proportion of LLR varied from 0% to 100% whereas that of LRR varied

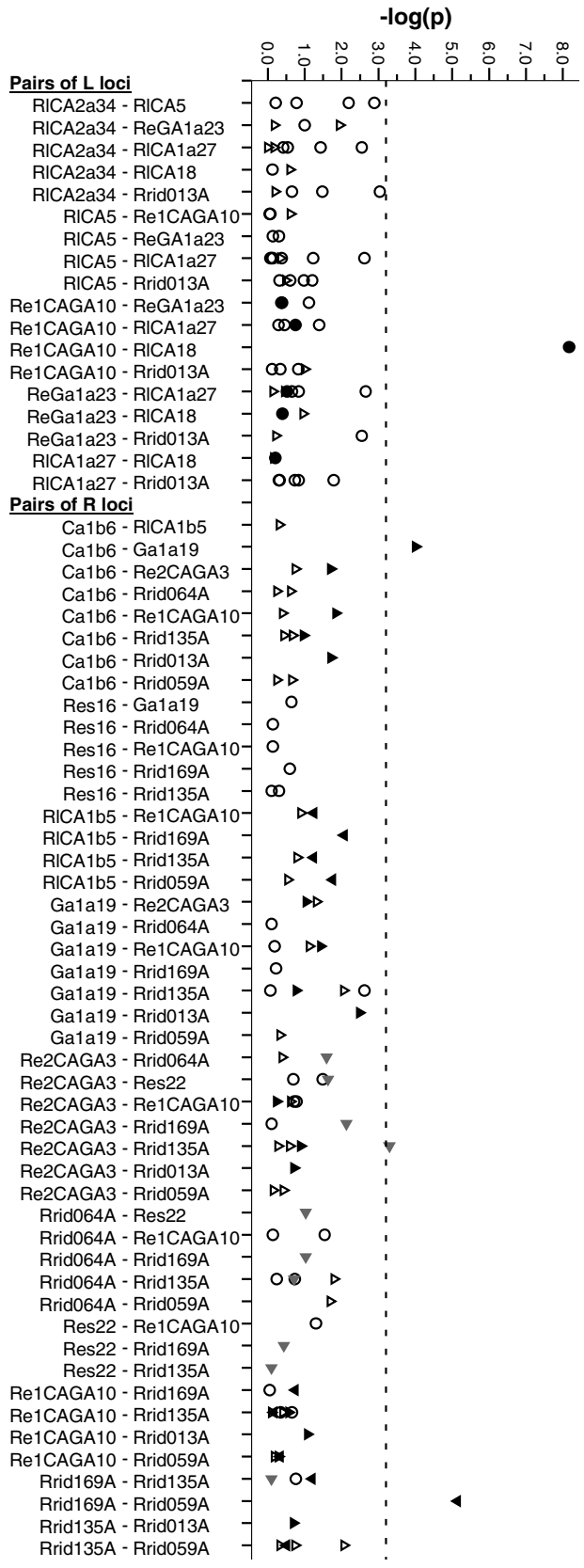

Figure 2. Linkage analysis of various locus combinations in crossing experiment with triploid *P. esculentus*. The symbols represent -$\log_{10}$ *P* values from χ^2 tests of the frequency distributions of the four potential (two parental and two recombinant) gamete types produced. Circles, males; triangles, females. Most individuals were heterozygous at several loci and therefore contributed data for several locus pairs. Each point left of the dashed line indicates a freely recombined locus pair in a frog. Points right of the dashed line indicate significant linkage at the 0.05 level after sequential Bonferroni correction within each genome separately. Filled symbols (circles and triangles pointing right, left and down) identify all *P* values derived from the four individuals that each gave a significant *P* value. The female identified by gray triangles pointing down had a mutation at Re2GACA3 in her germ line that she passed on to some of her offspring.

from 0% to 86% in the pond samples (Fig. 3). Of the 2296 frogs genotyped, only 0.2% were nonhybrid. These were five LL from two Swedish ponds. Multilocus linkage disequilibrium, measured as $\bar{r}_d$ on a scale from zero to one, averaged 0.01 in the L genome and 0.11 in the R genome, indicating that both genomes were well recombined in the majority of the natural populations. Mean $\bar{r}_d$ in the R genome was, however, significantly higher than in the L genome (*t*-test, $t_{111} = -3.819$, $P < 0.001$).

Multilocus disequilibrium in the L genome showed no relation with the proportion of LLR individuals (linear regression: $F_{1,61} = 2.269$, $P = 0.137$, $r^2 = 0.036$). In contrast, multilocus disequilibrium in the R genome was negatively associated with the proportion of recombining LRR frogs among the R gamete-producing LR and LRR frogs (linear regression: $F_{1,54} = 9.034$, $P = 0.004$, $r^2 = 0.143$, slope $= -0.214$). These results were thus fully in accordance with the expectations. The slopes of the L and the R regressions were, however, not significantly different ($t_{115} = 1.440$, $P = 0.153$).

The multilocus linkage disequilibria ($\bar{r}_d$) in the L and the R genomes were not positively correlated within ponds (Fig. 4). In fact, they were significantly negatively correlated (Pearson correlation: $r_{55} = -0.374$, $P = 0.005$); even excluding the L outliers far left and far right in Figure 4. This indicates an absence of strong pond-specific effects affecting $\bar{r}_d$ in the L and R genome simultaneously.

There was no correlation between $\bar{r}_d$ and genetic diversity, measured as the expected heterozygosity summed over all loci (Pearson correlation for L and R data pooled: $r_{119} = 0.013$, $P = 0.889$). The significant difference in mean multilocus disequilibrium between the L and the R genome can therefore not be explained by lower polymorphism in the L-specific microsatellite loci, but only by differences in recombination rates. The $\bar{r}_d$ values for the R genome also showed no correlation with the number of hemizygotes in the sample they were calculated from (Pearson correlation: $r_{55} = 0.016$, $P = 0.904$). The significant relation between LRR/(LR + LRR) and $\bar{r}_d$ in the R genome in Figure 3B can

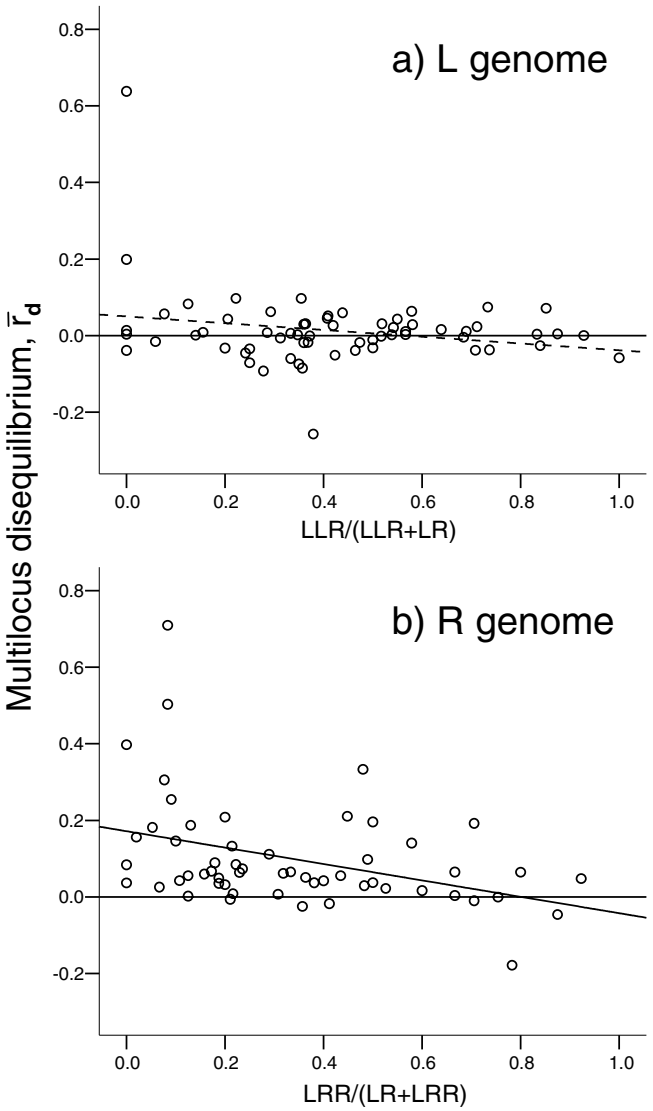

Figure 3. Multilocus linkage disequilibrium, $\bar{r}_d$, as a function of the proportion of frogs producing recombined gametes in 66 *P. esculentus* populations from Denmark and Sweden. (A) $\bar{r}_d$ in the L genome versus recombining LLR frogs of the total number of frogs propagating L genomes (LLR + LR). Linear regression line is dashed because it is nonsignificant. (B) $\bar{r}_d$ in the R genome versus recombining LRR/total R-propagating frogs; regression significant. $\bar{r}_d$ is an index of association adjusted for unequal sample size.

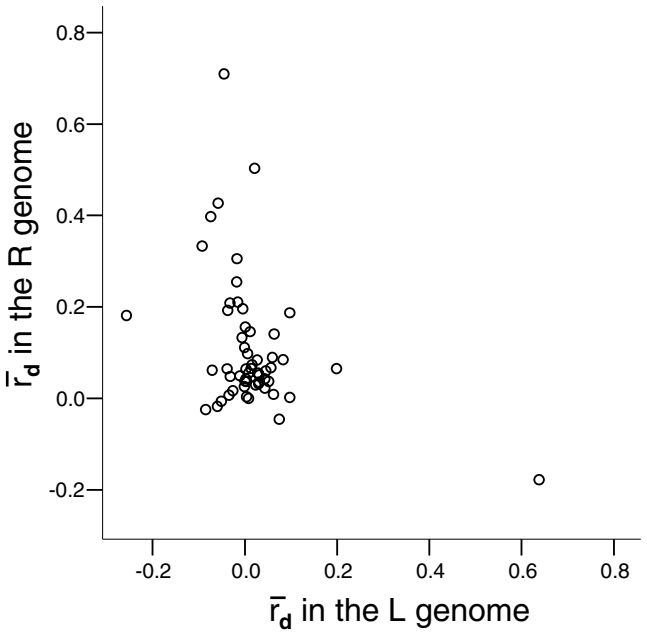

Figure 4. Multilocus linkage disequilibrium ($\bar{r}_d$) in the L versus the R genome in 56 ponds.

therefore not be explained by exclusion of heterozygous loci in LRR frogs, but must be attributed to differences in recombination rates.

An analysis of pairwise $\bar{r}_d$ values showed that only two locus pair had $\bar{r}_d$ values differing significantly from the mean pairwise $\bar{r}_d$ of the remaining locus pairs in the same ponds (28 L and 63 R, paired *t*-tests with sequential Bonferroni correction within each genome separately). These two locus pairs (L loci Res20 + Re1CAGA10 and the R loci RlCA1b5 + Rrid064A) both had significantly lower $\bar{r}_d$ than the remaining loci. Thus, most locus pairs gave similar results within ponds and none gave elevated values suggesting linkage. In spite of the tight linkage in the crossing experiment, pairwise $\bar{r}_d$ for Re1CAGA10 + RlCA18 was not significantly different from the mean, even without Bonferroni correction (paired *t*-test: $t_9 = 1.311$, $P = 0.222$). The same applies to the two potentially linked locus pairs (Ca1b6 + Ga1a19: $t_{10} = -0.654$, $P = 0.528$; Rrid169 + Rrid059A: $t_{22} = 1.261$, $P = 0.221$). Therefore, these three (potentially) linked locus pairs were not excluded from the analyses of natural populations.

Global F_{IS} was very low in both the L and R genome, i.e., −0.007 and −0.008, respectively, indicating random mating within ponds. Global F_{ST} values were rather high, i.e., 0.4561 and 0.6156 in the L and R genome, respectively, indicating much genetic structure among ponds, which is in accordance with the expectations for a low-mobility animal. As a consequence of the low F_{IS}, F_{IT} was very similar to F_{ST} for both genomes.

Discussion

Recombination was demonstrated in all 30 frogs tested in the crossing experiment including both males and females of both LLR and LRR. As a consequence of such triploid-mediated recombination, natural populations were found to have low multilocus linkage disequilibria. In agreement with predictions from the asymmetrical propagation of L and R genomes in the all-hybrid populations, L genomes were generally fully recombined whereas R genomes were recombined according to the proportion of LRR triploids. The unique all-hybrid populations of *P. esculentus* are thus functionally sexual; actually, they represent an obligate

symbiosis of two independent, functionally sexual genomes: the L and the R genome. Below, we will first describe the genetic mechanisms underlying these results and then outline the evolutionary, conceptual, and conservation-political implications for all-hybrid populations and hybridogenetic taxa.

RECOMBINATION IN ALL-HYBRID POPULATIONS

In normal meiosis, the combined effects of random segregation of chromosomes and chromosomal crossing-over assure equal proportions of parental and recombinant gametes for most locus pairs. Reduced recombination rates due to physical linkage are, however, observed between loci situated so closely together on the same chromosome that there is small probability of crossing-over between them. A random sample of genetic markers for any kind of organism might thus include a small proportion of linked loci. *P. esculentus* has 13 chromosomes per L or R set (e.g., Koref-Santibanez and Günther 1980). The physical locations of our microsatellite loci on these chromosomes are unknown, but the results from the crossing experiment suggested linkage between three of the 65 locus pairs investigated. Loci Re1CAGA10 and RlCA18 showed strong linkage in a male crossed by us as well as in one crossed by Arioli (2007 ch. 1); thus it can be inferred that these two loci are situated close together. The apparent linkage of the two remaining locus pairs in this study was weaker and assessed in only one frog each, so that linkage should not be concluded without further verification. This discovery of one to three linked loci does not suggest variation in recombination rates among individuals, as the three frogs with apparently linked loci had full recombination at their remaining locus pairs investigated.

Selection took place in the crossing experiment, as dead and sick-looking tadpoles were not reared, and 10% of the offspring chosen for rearing eluded genotyping—mainly by dying. Only selection on the interaction of nonneutral loci linked to our markers could, however, have affected the recombination results. Any such interaction effects were reduced by crossing every parent to several mates of different genotypes. As no significant bias in the segregation at any single locus was detected, bias of locus combinations by selection is unlikely. Furthermore, selection on the interaction of linked nonneutral loci would most likely bias the results toward less recombination, so it would not undermine the conclusion of recombination.

Unlike linkage, linkage disequilibrium can arise between loci without physical associations. Linkage disequilibrium, measured as $\bar{r}_d$ in the natural populations, is the net result of generating and deteriorating forces. Linkage disequilibrium-generating forces include founder effect, migration, drift, inbreeding, and selection on linked genes, called hitchhiking (Hedrick 2005). In clonal organisms, the entire genome hitchhikes with positively selected genes. The deteriorating force is recombination. Linkage disequilibrium is a negative linear function of recombination rate per generation, with half of the disequilibrium disappearing per generation at 100% recombination (Hedrick 2005). Provided that $\bar{r}_d$ is a good measure of linkage disequilibrium, that LRR/(LR + LRR) was a fair substitute for recombination frequency in the ponds, and that disequilibrium-generating forces did not depend on population structure (e.g., on LRR/(LR+LRR)), linear relationships were therefore expected in Figure 3.

With low $\bar{r}_d$ irrespective of population structure in the L genome (Fig. 3A) and a negative relationship between $\bar{r}_d$ and recombining triploids (LRR/LR + LRR) in the R genome (Fig. 3B), the expectations outlined in the introduction were met. According to the model by Som and Reyer (2006a), L genomes spend two-thirds of their generations in LLR frogs and one-third in LR frogs, which means that they are recombined two of three generations. The empirical data from the present study show that this recombination rate of two-thirds, whatever the type and strength of linkage disequilibrium-generating forces in the natural populations, is sufficient to reduce $\bar{r}_d$ values to around zero (mean $\bar{r}_d = 0.01$ on the scale from zero to one). For the R genome, no theoretical model is available. Before a reliable model can be made, more empirical data on the ratio of R and LR gametes produced by LR females and LR males are needed, as this ratio is important for population dynamics and has been shown to vary strongly between individuals and locations (Tunner and Heppich-Tunner 1991; Polls Pelaz 1994; Mikulícek and Kotlík 2001; Rybacki and Berger 2001; Christiansen et al. 2005; Arioli 2007 ch. 1; Jakob 2007 ch. 5). Central to such a model is also the question of why populations vary in structure. Although it is commonly accepted that population structure of *P. esculentus*, *P. lessonae*, and/or *P. ridibundus* depend on ecological components (Pagano et al. 2001; Holenweg Peter et al. 2002; Plötner 2005), attempts to identify the ecological components determining population structure in Swedish all-hybrid populations were so far rather inconclusive (Jakob 2007 ch. 3). In the absence of theoretical models, it was not known what level of linkage disequilibrium to expect in the R genome of natural populations, but the present empirical data show that it is generally low (mean $\bar{r}_d = 0.11$), although the genetic signature of clonal reproduction was visible in certain populations with few LRR frogs. In clonal populations of other organisms, $\bar{r}_d$ values have been found to be considerably higher than in the present study (e.g., Goyeau et al. 2007; Grundmann et al. 2008). Unfortunately, no thorough studies on multilocus disequilibrium in the *R. esculenta* LE or RE system have been conducted yet.

The variation not explained by the linear relations in Figure 3 is expected to derive from three main sources. (1) Error on the estimate of $\bar{r}_d$ from a random sample of 17–86 (mean 35) individuals. (2) Error on the estimate of population structure, e.g., LRR/(LR + LRR), from the same random sample and between-pond variation in the ratio of R gametes from LR frogs. If the proportion of R

gametes made by LR frogs varies between ponds, this will add further noise. (3) Between-pond variation in the strength of the various disequilibrium-generating forces listed above. The combined effects of these three sources explain the rather large variation for the R genome in Figure 3B. For the L genome, population structure (source 2) should have no relevance, however. Furthermore, if the recombination rate is so high that it always overpowers the local disequilibrium-generating forces (source 3), as seems to be the case in the L genome, variation comes only from the error on the estimate of $\bar{r}_d$ (source 1). This explains the relative low variation in Figure 3A. Unfortunately, disequilibrium-generating forces are difficult to measure. The only disequilibrium-generating force, we could measure in this study was inbreeding. The low F_{IS} values obtained indicated random mating, so that inbreeding would have little effect on $\bar{r}_d$. We did, however, test for those pond-specific effects that affect the L and R genome similarly. The lack of a positive correlation between $\bar{r}_d$ in the L and R genome across ponds (Fig. 4) indicates that such forces were absent. In conclusion, the forces generating multilocus linkage disequilibrium in the natural populations could not be indentified, but between-pond variation in their strength and composition did not pose a problem in this study. On the contrary: the good match of observed with expected relations in Figure 3A, B shows that $\bar{r}_d$ can be a useful tool in studies of recombination.

The extreme positive outlier in Figure 3A calls for a different explanation than those given for residual variation. This explanation has to apply to the L genome only, as the high L $\bar{r}_d$ value was not matched by a high R value (Fig. 4). Notably, in this pond, a null allele was scored as a real L allele, because it did not pose a technical problem. As pairwise $\bar{r}_d$ values were elevated for all locus pairs in this pond, the null allele cannot account for its outlier status, however. Exclusion of the locus with the null allele reduced $\bar{r}_d$ to 0.29, i.e., the point remained an outlier although less extreme. A better explanation for the high $\bar{r}_d$ value can be derived from the pond's extreme left position in the figure. Although necessary for reproduction, LLR frogs were absent from our sample of 23 adults. Also notable, although not exceptional for this pond, was that the population appeared small with few males, which are more often LLR than females. We could therefore speculate that the L genomes in the sampled frogs were derived from very few LLR ancestors. A linkage disequilibrium in the L genome caused by such a bottleneck in LLR frogs would persist for several generations with recombination.

EVOLUTIONARY CONSEQUENCES

Triploids are not restricted to all-hybrid populations, but have been found in various population types in Germany (Günther 1975), Poland (Rybacki and Berger 2001), and France (Regnier and Neveu 1986). The ability to make diploid eggs giving rise to triploid individuals provides all these *P. esculentus* populations with genetic recombination and potential reproductive independence—two important steps in the direction of speciation. Where hybrids live sympatrically with parental species, they do not reproduce independently, however, but interbreed with the parental species. Here, recombination by triploids might be of little genetic importance to the hybrids, because they can be supplied with recombined genomes from the parental species. In contrast, the all-hybrid populations of Denmark and southern Sweden must rely on recombination in triploids only, as they are isolated from the nearest parental populations by sea or large stretches of uninhabited land, and nonhybrid LL and RR offspring only very rarely survive to sexual maturity (Christiansen et al. 2005; Jakob 2007 ch. 2 and the present study). Here, *P. esculentus* has truly accomplished the transition from a clonal, gamete-dependent hybrid to an independent, sexually reproducing evolutionary unit.

Although the all-hybrid populations have a combination of clonal and sexual reproduction, the low multilocus linkage disequilibrium values indicate that the loci of natural populations were well mixed. Selection should thus have the whole range of genetic combinations to work on, enabling beneficial, as well as harmful, mutations to be combined for fast adaptation to changing environments (Fisher 1930) and for purging of deleterious mutations (Muller 1932). This hybridogenetic reproduction mode also ensures continuous genetic variation as a defense against fast-evolving parasites (Red Queen hypothesis, Hamilton 1980), because the combination of recombined and clonal gametes result in unique individuals. The all-hybrid populations thus seem to have all the advantages of sexual reproduction, including a long-term evolutionary potential. The ability of fast adaptation to changing environments might, however, be of more importance for the survival of *P. esculentus*, given that habitat loss and climate change increasingly threaten amphibians worldwide (Stuart et al. 2004).

It remains to be analyzed to what extent all-hybrid *P. esculentus* populations can also benefit from the clonal reproduction of diploids. In general, potential benefits of clonal reproduction include the possibility to save the costs of producing males and the ability to propagate favorable gene combinations (Otto and Gerstein 2006). In all-hybrid *P. esculentus* populations, the theoretical offspring sex ratio is only slightly female biased, which is in agreement with the mean observed adult sex ratio in large surveys (Som and Reyer 2006b; Jakob 2007 ch. 2 and the present study). Thus, only a few percent of the cost of males might be saved. Recombination takes place after maximum one generation in the L genome (Som and Reyer 2006a) and after one to a few generations in the R genome, suggesting that favorable gene combinations are not preserved for long, unless physically linked. Therefore, the benefit that all-hybrid populations of *P. esculentus* can potentially derive from the clonal component in their reproduction appears small—in contrast to cyclical parthenogens, such as aphids,

rotifers, water fleas that have successfully combined the advantages of sexual and clonal reproduction (Innes and Singleton 2000).

With sexual reproduction, the death of newly formed nonhybrid LL and RR in the all-hybrid populations is intriguing, because it cannot be attributed to clonal propagation of the genomes, as in the LE system. In the LE system, RR die because recessive deleterious mutations have become fixed in the clonally propagated R genome of the diploid LR hybrids (Vorburger 2001a; Guex et al. 2002). These deleterious mutations were either acquired through Muller's ratchet or were already present at hemiclone formation (Vorburger 2001a). In all-hybrid EE populations, both genomes are regularly recombined in triploid individuals, the L when in LLR and the R when in LRR. Hence, fixation of deleterious mutations by Muller's ratchet is unlikely, yet fixation may still have occurred by other mechanisms, for example founder effect. Fixation and low genetic diversity is certainly observed at microsatellite loci (Christiansen et al. 2005; Arioli 2007 ch. 4 and the present study). Explanations for how genetic diversity became and remained this low in spite of the presence of parental species just south of the German and north of the Swedish all-hybrid populations are, however, lacking.

Pelophylax esculentus most closely resembles the Iberian minnow, *S. alburnoides* (also called *Leuciscus*, *Rutilus*, and *Tropidophoxinellus*, reviewed by Alves et al. 2001) of other hybridogenetic taxa known: both hybrids often form mixed populations of di- and polyploid hybrids and one or both parental genotypes. All-hybrid di- and triploid populations are, however, not known from *S. alburnoides*. Instead, tetraploids occur in many *S. alburnoides* populations and, in special habitats, tetraploids can constitute 73% of the mixed populations. These tetraploids have an even sex ratio, have normal meiosis, produce tetraploid offspring when mating with each other, and appear to be reproductively isolated from other ploidy levels (Cunha et al. 2008). The discovery of these mainly tetraploid populations strongly suggests that meiotic hybridogenesis can act as a stepping stone to tetraploidization and ultimately to speciation. In *P. esculentus*, tetraploidy has so far only been found in very low frequencies in Swedish populations (Jakob 2007 ch. 2).

Given that recombination appears to be the rule in polyploid hybridogens and that polyploidy in hybridogenetic taxa appears to be more common than previously assumed, the prevailing view of hybridogens as clonally reproducing diploids may have to be changed. Should the discoveries of hybridogenetic breeding systems continue to increase, which is likely as more and more supposedly normal species are being genetically analyzed, this will also affect our perception of the importance of hybridization for speciation in animals.

Studies on hybrids are also relevant from a conservation point of view. Modern management concepts stress the importance of conserving "evolutionary significant units" (ESUs), i.e., populations representing significant adaptive variation; but how these units are to be identified, is strongly debated (reviewed by Crandall et al. 2000). Hybrids, for instance, are exempt from protection, because they do not seem to constitute independent evolutionary lineages (Kraus 1995). Although this may be true for F_1 progeny from many interspecific matings, it is not true for parthenogenetic, gynogenetic, and hybridogenetic taxa of hybrid origin, which are capable of self propagation (Ranker and Arft 1994; Kraus 1995). This, plus the finding that hybridogens like *P. esculentus* and *S. alburnoides* can form independent and sexually reproducing populations, makes these organisms evolutionary significant units and worthy of protection.

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