Gametogenesis of intergroup hybrids of hemiclonal frogs

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Summary

European water frog hybrids Rana esculenta (R. ridibunda × R. lessonae) reproduce hemiclonally, by hybridogenesis: in the germ line they exclude the genome of one parental species and produce haploid gametes with an unrecombined genome of the other parental species. In the widespread L-E population system, both sexes of hybrids (E) coexist with R. lessonae (L). They exclude the lessonae genome and produce ridibunda gametes. In the R-E system, hybrid males coexist with R. ridibunda (R); they exclude either their ridibunda or their lessonae genome and produce sperm with a lessonae or with a ridibunda genome or a mixture of both kinds of sperm. We examined 13 male offspring, 12 of which were from crosses between L-E system and R-E system frogs. All were somatically hybrid. With one exception, they excluded the lessonae genome in the germ line and subsequently endoreduplicated the ridibunda genome. Spermatogonial metaphases contained a haploid or a diploid number of ridibunda chromosomes, identified through in situ hybridization to a satellite DNA marker, and by spermatocyte I metaphases containing a haploid number of ridibunda bivalents. The exception, an F1 hybrid between L-E system R. lessonae and R-E system R. ridibunda, was not hybridogenetic, showed no genome exclusion, and evidenced a disturbed gametogenesis resulting from the combination of two heterospecific genomes. None of the hybridogenetic hybrids showed any cell lines excluding the ridibunda genome, the pattern most frequent in hybrids of the R-E system, unique to that system, and essential for its persistence. A particular combination of R-E system lessonae and R-E system ridibunda genomes seems necessary to induce the R-E system type of hemiclonal gametogenesis.

1. Introduction

European water frog hybrids of the Rana esculenta complex reproduce hemiclonally; in the germ line, they generally exclude the genome of one parental species and produce haploid gametes with a functional, intact, unrecombined genome of the other parental species (reviewed by Graf & Polls Pelaz, 1989; Ploëtner, 2005). This reproductive mode, which is termed hybridogenesis (Schultz, 1969), and the cytogenetics of which are not well understood, varies geographically.

In the most widespread population system, the L-E system (Uzzell & Berger, 1975), the hybrids (Rana esculenta, genomic composition RL) exclude the genome of their parental species Rana lessonae (LL) and produce haploid gametes, whether ova or sperm, that contain an intact genome of their parental species Rana ridibunda (RR). Such hybrids coexist with and reproductively depend on their sexual host, the parental species LL, matings with which restore somatic hybridity in each generation of such Rana esculenta lineages. Most of such matings are with Rana lessonae males, the heterogametic sex, so that L-E system populations normally contain both male and female Rana esculenta. Exclusion of the L genome

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occurs before meiosis. The remaining R genome undergoes a premeiotic or occasionally a prediplo-
tene meiotic endoreduplication (Tunner & Heppich-
Tunner, 1991). This is followed by two apparently
normal meiotic divisions without genetic conse-
quences of segregation or crossing over because the
two R genomes are identical, sister chromatid-derived
copies. It has been hypothesized that the R genome
induces the germ line exclusion of the L genome (e.g.
Hotz et al., 1985; Guerrini et al., 1997).

There are other hybridogenetic population systems,
geographically localized in limited areas, of which
the R-E system (Uzzell & Berger, 1975) of eastern
Germany and northwestern Poland is the best known.
It consists of diploid male Rana esculenta and both
sexes of Rana ridibunda (Uzzell et al., 1977; Günther
& Plötsner, 1988; Vinogradov et al., 1991; Plötsner,
2001). Such Rana esculenta males produce either L
or R sperm or a mixture of both; on average, about
two thirds of transmitted genomes are L genomes.
In contrast to the L-E system, in which gametogenesis
is usually very regular (e.g. Uzzell et al., 1980), there
is a low frequency (2–3%) of recombination between
the L and R genomes in hybrids of the R-E system
(Uzzell et al., 1977).

The L-E and R-E systems have been known and
studied for three decades, but the molecular-genetic
basis for the gametogenetic differences between them
is not known: basic crosses between frogs of the L-E
and the R-E systems, necessary to explore these dif-
ferences, have not been carried out. We made crosses
to try to determine whether the modified gameto-
genesis in R-E system Rana esculenta depends on (1) their
L genome, (2) their R genome, or (3) the combination
of the two. We here report cytogenetic data on
gametogenesis of male hybrids between frogs of the
two reproductive systems. Hypothesis (1) would be
supported if hybrid progeny with an L genome from
the R-E system and an R genome from the L-E system
had an R-E system-type gametogenesis (producing
mainly L gametes) but progeny with the reciprocal
genome origin did not; hypothesis (2) would be sup-
ported by the reverse; and hypothesis (3) would be
supported if neither progeny with an R-E system L
genome and an L-E system R genome nor those with
an R-E system R genome and an L-E system L gen-
nome had an R-E system-type gametogenesis, so that
a combination of L and R genomes from the R-E
system is necessary for the R-E system-type gameto-
genesis.

2. Materials and methods

(i) Animals and experimental crosses

Frogs of the L-E system were collected near
Rogaczewo, 40 km south of Poznań, central Poland.
Frogs of the R-E system were collected at the Alte
Oder near Lebus, about 100 km east of Berlin, eastern
Germany. Morphology permits clear distinction
among Rana ridibunda, Rana esculenta and Rana
lessonae; the best measures (e.g. Berger, 1966, 1977)
include the relative length and height of the callus
internus (shortest and lowest in Rana ridibunda, in-
termediate in Rana esculenta, longest and highest in
Rana lessonae), relative length of the first toe (longest
in Rana ridibunda, intermediate in Rana esculenta,
shortest in Rana lessonae), and relative length of the
tibia (longest in Rana ridibunda, intermediate in Rana
esculenta, shortest in Rana lessonae). Crossing ex-
periments, using artificial fertilization after hormone
treatment of the female parent, followed standard
procedures (Berger et al., 1994a). In cross designa-
tions, the female parent is reported first.

(ii) Cytological procedures and fluorescence in situ
hybridization (FISH)

Mitotic and meiotic chromosomes from intestinal
tissue and/or testis were prepared as previously
described (Bucci et al., 1990). Mitotic and meiotic
preparations were banded using Arrighi & Hsu’s
(1971) method for staining of heterochromatin. DIG-
labelled DNA insert from pRr300 StuI, a clone of the
centromeric satellite DNA family RrS1, was hy-
bridized in situ to mitotic and meiotic chromosome
preparations using standard procedures (Ragghianti
et al., 1995).

(iii) DNA extraction and Southern blot analysis

Genomic DNA was prepared from somatic tissues
using the DNeasy Tissue Kit (QIAGEN). For
Southern blot experiments, fragments of completely
StuI-digested genomic DNA of all offspring were
separated electrophoretically and then transferred
(Southern, 1975) to Hybond-N filters (Amersham).
Southern hybridizations were carried out as described
by Ragghianti et al. (1995).

3. Results

We examined a total of 13 male progeny from five
crosses (Table 1). Somatic genome constitution of the
offspring was examined by in situ hybridization of
metaphase plates of intestinal cells to a clone of the
centromeric satellite DNA RrS1. In such prepara-
tions, the centromeres of six chromosomes (1–5
and 8) are marked in Rana ridibunda, but not in Rana
lessonae (Fig. 1a). The presence of a ridibunda genome
was also confirmed by Southern blots of StuI-digested
genomic DNA hybridized with an RrS1 clone. Such
blots give a distinctive autoradiographic pattern when
a Rana ridibunda genome is present.
In chromosomal preparations of testis tissues we examined germ line cells by in situ hybridizations to the RrS1 clone, which for spermatogonial and meiotic metaphases show the same centromeric markings of ridibunda chromosomes as do somatic cells (Fig. 1b–e).

For some offspring, we subjected testis preparations to C-banding, which marks the centromeres of all ridibunda chromosomes, but not lessonae chromosomes, as Giemsa-positive granules (Fig. 2a, b).

(i) Cross 02.10: Rana lessonae L-E system × Rana esculenta L-E system (control)

As a control of the L-E system type of gametogenesis, we examined one offspring from this cross between two frogs of the L-E system (14 spermatogonia and 10 spermatocytes I). This offspring, a diploid male, obviously received a lessonae genome from its mother. It was somatically hybrid, as shown by intestine metaphases; Southern blots confirmed the presence of a ridibunda genome. Spermatogonial plates with $2N = 26$ chromosomes indicated the presence of two R genomes characteristic of germ line cells of hybrids of the L-E system.

(ii) Cross 02.08: Rana lessonae L-E system × Rana ridibunda R-E system

We examined five offspring from this cross, and a total of 64 spermatogonia and 25 spermatocytes I.

One female parent was used in crosses 02.10 and 02.08; a second female parent was used in crosses 02.41 and 02.43; all other parents were used only in single crosses.

Genome composition: L, Rana lessonae genome; R, Rana ridibunda genome.

Table 1. Gametogenesis of male progeny from crosses between frogs from the L-E system and frogs from the R-E system

| Cross No. | Parents            | Progeny          | N   | Soma  | Spermatogonia | Spermatocytes I |
|-----------------|-------------------|------------------|-----|-------|---------------|-----------------|
| 02.10           | R. lessonae L-E   | R. esculenta L-E | 1   | R L   | 2N = 26 R     |                 |
| 02.08           | R. lessonae L-E   | R. ridibunda R-E | 5   | R L   | 2N = 26 R; N = 13 R |                 |
|                 | R. esculenta L-E  | R. esculenta R-E | 1   | R L   | 2N = 26 R; N = 13 R |                 |
| 02.41           | R. lessonae L-E   | R. esculenta R-E | 4   | R L   | 2N = 26 R; N = 13 R |                 |
| 02.43           | R. lessonae L-E   | R. ridibunda R-E | 1   | R L   | 2N = 26 R; N = 13 R; 2N = 26L + R |                 |

One female parent was used in crosses 02.10 and 02.08; a second female parent was used in crosses 02.41 and 02.43; all other parents were used only in single crosses.

Genome composition: L, Rana lessonae genome; R, Rana ridibunda genome.

In chromosomal preparations of testis tissues we examined germ line cells by in situ hybridizations to the RrS1 clone, which for spermatogonial and meiotic metaphases show the same centromeric markings of ridibunda chromosomes as do somatic cells (Fig. 1b–e). For some offspring, we subjected testis preparations to C-banding, which marks the centromeres of all ridibunda chromosomes, but not lessonae chromosomes, as Giemsa-positive granules (Fig. 2a, b).

Fig. 1. Chromosome preparations of somatic and germinal tissues hybridized in situ with DIG-labelled insert pRr 300 Stul. (a) Somatic metaphase of male 21 490 (cross 02.41): a heavy fluorescence is visible at the centromere of only one partner of chromosome pairs 1–5 and 8, revealing the frog’s hybrid genome composition. Spermatogonial metaphases of the same male that contained 13 chromosomes, six of which have RrS1-marked centromeres (b) and 26 chromosomes, 12 of which have marked centromeres (c) are shown. Meiotic metaphase I from male 21 221 (cross 02.08) with 13 bivalents, six of which have marked centromeres (d) and from male 21 493 (cross 02.41) again with 13 bivalents, of which six have marked centromeres (e), indicating the hybridogenetic genome exclusion in both males. Scale bars represent 10 μm.
All offspring examined received a lessonae genome from their female parent and a ridibunda genome from their male parent. They were all diploid and had the expected hybrid somatic genome composition. For one offspring this was evidenced by in situ hybridizations of metaphase plates of intestinal cells, which indicated the presence of a single ridibunda genome. Southern hybridizations confirmed for all five the presence of a ridibunda genome.

In four offspring we observed spermatogonial metaphases that contained 2N = 26 chromosomes, with two R genomes. In addition, all four of these offspring had spermatogonial metaphases that contained N = 13 R chromosomes (for one offspring we found neither spermatogonial nor meiotic processes in testis preparations). In two of these offspring we examined spermatocyte I preparations. These meiotic metaphases I had 13 R bivalents (Fig. 1d). A single preparation from one offspring had a nearly tetraploid metaphase I that probably contained four R genomes. C-banded testis preparations of one offspring had spermatogonial metaphases that contained 2N = 26 R chromosomes (Fig. 2a), and metaphases I with N = 13 R bivalents (Fig. 2b).

(iii) Cross 02.14: Rana esculenta L-E system × Rana esculenta R-E system

We examined one offspring from this cross (12 spermatogonia and 12 spermatocytes I). It was diploid and somatically hybrid, as evidenced by somatic metaphases from intestinal cells and by Southern blots revealing the presence of an R genome. Assuming that this frog’s R genome stemmed, as expected, from its L-E system Rana esculenta mother, its somatic hybridity demonstrates that the R-E system Rana esculenta father contributed an L sperm. Newly metamorphosed progeny of this cross had two morphological phenotypes: green, with an esculenta-like callus internus (13%), and gray, with a ridibunda-like callus internus (87%). These must correspond to L sperm and R sperm, respectively, of their R-E system father. The frog examined had differently sized testes (one normal, one small). Spermatogonial metaphases from both testes had either 2N = 26 R chromosomes or N = 13 R chromosomes. Metaphases I had 13 R bivalents.

(iv) Cross 02.41: Rana lessonae L-E system × Rana esculenta R-E system

We examined four offspring from this cross (a total of 27 spermatogonia and 17 spermatocytes I), all of which had an L-E system lessonae genome from their mother. Somatic hybridity of one offspring was shown by somatic metaphases with 2N = 26 chromosomes, of which N = 13 were R chromosomes (Fig. 1a). Southern blots confirmed the presence of a ridibunda genome in all four offspring. The somatic hybridity of these frogs shows that, in each case, their Rana esculenta father from the R-E system produced an R sperm. In total, we obtained two females and six males from this cross, all of which, judged by their morphology, received an R genome from their R-E system hybrid father. Three of the four offspring examined had asymmetrical testes (differently sized in two frogs, one atrophied in one frog). Spermatogonial metaphases of all four offspring contained 2N = 26 R chromosomes (Fig. 1c). In three offspring, there were also spermatogonial metaphases with N = 13 R chromosomes (Fig. 1b). The larger and smaller testes of two offspring had the same reproductive mode. Meiotic preparations were examined for three offspring. Metaphases I had N = 13 R bivalents (Fig. 1e). In one offspring, we found metaphases I with 12 bivalents and two univalents; all of these spermatocytes I contained two R genomes.

(v) Cross 02.43: Rana lessonae L-E system × Rana lessonae R-E system

We examined two offspring from this cross (a total of 36 spermatogonia and 24 spermatocytes I). Both were diploid and received an L genome from their mother and an R genome from their father. Somatic hybridity was confirmed for one offspring by intestine metaphases with N = 13 L and N = 13 R chromosomes, and for both by Southern blots that revealed the presence of an R genome. For one offspring, which had a single testis, we observed spermatogonial metaphases with 2N = 26 R chromosomes and others with N = 13 R chromosomes. In addition, a few spermatogonial metaphases contained 2N = 26 chromosomes, of which half were R and half L chromosomes. Spermatocytes I had N = 13 R bivalents; a few had R bivalents and some had R univalents. For the second
offspring, we found spermatogonial metaphases with about 24 chromosomes, of which five had RrS1-marked centromeres; with about 21 chromosomes, of which four had marked centromeres; with about 26 chromosomes, of which five had marked centromeres; and with about 39 chromosomes, of which six had marked centromeres. This individual was thus not hybridogenetic because spermatogonial cells contained both parental genomes and there was no evidence of genome exclusion. There were disturbances in germ line mitotic processes, reflected, for example, in the metaphase figures with approximately 3N chromosomes that contained one R genome.

4. Discussion

The hybrids between frogs from the two population systems we examined included progeny having ridibunda genomes of the L-E system combined with lessonae genomes of the R-E system (cross 02.14) as well as progeny with lessonae genomes of the L-E system combined with ridibunda genomes of the R-E system (crosses 02.08, 02.41, 02.43). The two Rana esculenta males of the R-E system we used as parents differed in their reproductive mode: one male (cross 02.14) transmitted an L sperm to one offspring, the other (cross 02.41) transmitted an R sperm to each of four offspring. Somatic morphology of newly metamorphosed progeny of cross 02.14 shows that the R-E system father produced both L sperm and (more frequently) R sperm.

With one exception (an offspring of cross 02.43), all hybrids between L-E system and R-E system frogs examined resembled the control cross (02.10; between an L-E system Rana lessonae and an L-E system Rana esculenta) in having an L-E system type of gametogenesis, with germ line exclusion of the L genome and subsequent endoreduplication of the R genome. No examined cells of any individual showed exclusion of the R genome. Exclusion of the R genome is the usual pattern in hybrids of the R-E system, a reproductive pattern that is unique to that system and that is essential to the R-E system’s persistence. Several offspring had asymmetrically sized testes, but in each case the two different testes showed the same type of gametogenesis. The absence of R genome exclusion was independent of whether the lessonae or the ridibunda genome stemmed from either population system, whether the maternal or the paternal parent stemmed from either population system, or whether Rana esculenta or Rana ridibunda from the R-E system was used as parent.

The exceptional offspring without L-E system type gametogenesis, an F1 hybrid between an L-E system Rana lessonae female and an R-E system Rana ridibunda male (cross 02.43), was not hybridogenetic. Like other non-hybridogenetic hybrids between Rana ridibunda and Rana lessonae (Hotz et al., 1985; Bucci et al., 1990), it evidenced a disturbed gametogenesis caused by the combination of the two heterospecific genomes. The observed approximately triploid spermatogonial metaphases, for example, probably reflect endoreduplication of the L genome without prior elimination of the R genome. It is possible that a few cell lines of the otherwise hybridogenetic second offspring of cross 02.43 (those with allodiploid spermatogonia) also revealed such non-hybridogenetic spermatogenesis.

Some of our present data are unexpected with respect to sex determination. In the water frog group, genetic sex determination is via a male-heterogametic XX-XY mechanism (Berger et al., 1988 and literature cited therein). Because primary hybridizations between the two species are, for size-related behavioural reasons, regularly between females of Rana ridibunda and males of Rana lessonae rather than the reciprocal, the clonally transmitted ridibunda genome of Rana esculenta carries no male determinants. Sex of L-E system hybrids is therefore determined by their lessonae genome and, as a result, L-E system Rana esculenta males have all-female progeny (Berger et al., 1988). Two findings contradict these generalizations: (1) An offspring from an L-E system Rana esculenta male was male (cross 02.10). The causes of this exception are unknown; the Rana esculenta father stemmed from a persisting L-E system population without Rana ridibunda so that its origin from a Rana ridibunda male is ruled out. Such exceptional male offspring of L-E system Rana esculenta males have occasionally occurred in other crosses (G.-D. Guex & H. Hotz, unpublished data). (2) Four offspring that received an R genome from their R-E system hybrid father were male (cross 02.41). If the male-determining factors lie, as they do in the L-E system, on the L rather than the R genome (which is consistent with the all-maleness of R-E system hybrids), offspring of an R-E system Rana esculenta receiving from it an L genome are expected to be male, while offspring receiving an R genome are expected to be female. This is in fact usually observed in progeny of R-E system Rana esculenta (Uzzell et al., 1977). Whether there was frequent recombination in the Rana esculenta father of cross 02.41 involving the sex-determining region or locus (cf. Hotz et al., 1997), whether there was sex reversal, or whether the male-determining factors were exceptionally located on the R genome, is not known.

In a majority of the offspring discussed, spermatogonial metaphases were of two types: cells with a diploid number of 2N = 26 ridibunda chromosomes, and cells with a haploid number of N = 13 ridibunda chromosomes. The latter obviously reflect germ line cells after the elimination of the lessonae genome but prior to the endoreduplication of the remaining
ridibunda genome. This shows both the separateness and the temporal sequence of these two processes. Such haploid spermatogonia have been reported as an exception for one L-E system Rana esculenta (Heppich et al., 1982); oogonial metaphases with a haploid number of ridibunda chromosomes were observed in L-E system Rana esculenta (Tunner & Heppich, 1981; Tunner & Heppich-Tunner, 1991).

The effects of combining heterospecific genomes in biological systems are many and varied. They often lead to developmental failure or sterility. Particular heterospecific genome combinations account for the modified reproductive patterns that have produced the 80-odd clonally reproducing taxa of vertebrates (e.g. Dawley & Bogart, 1989; Alves et al., 2001). The diversity of patterns in western Palearctic water frogs is striking. Hybrids between Rana ridibunda and Rana lessonae, for example, are sterile when the R genome stems from southern parts of the Rana ridibunda range (Hotz et al., 1985; Berger et al., 1994b; unpublished data). Such non-hybridogenetic hybrids show incomplete pairings between the Rana ridibunda and Rana lessonae genomes in meiosis I, which probably lead to aneuploid gametes and thus render the individuals sterile (Hotz et al., 1985; Bucci et al., 1990). The non-hybridogenetic offspring of cross 02.43 is an example of such hybrids. In contrast, crosses between central European Rana ridibunda and Rana lessonae almost always show hybridogenetic reproduction (Hotz et al., 1985).

What is observed in frogs from natural populations is probably not representative of the kinds of gametogenesis that actually occur: gametogenetic modes that lead to continuing lineages are much more likely to be detected than those that do not. Even among the successful kinds of gametogenesis, however, diversity is great. Gametogenetic modes leading to similar continuing lineages can evolve independently and have entirely different mechanical bases. For example, all-male allotriploid LLR frogs that produce diploid LL sperm have been observed in a population in northern France (Graf & Polls Pelaz, 1989; Polls Pelaz, 1994) and in a population of the Danube basin in western Hungary (Tunner & Heppich-Tunner, 1992; Brychta & Tunner, 1994); in both cases, these males exclude their R genome before meiosis, but in France they reproduce ameiotically, omitting the reductional division, whereas in Hungary they endoreduplicate both L genomes and proceed with a quasi-normal meiosis.

In some cases the variation is apparently in the R genome, as in hybrids between Rana lessonae and Rana ridibunda, which are sterile when the R genome is derived from southern parts of the Rana ridibunda range but usually hybridogenetic when the R genome is derived from a central European Rana ridibunda. In the Pannonian Basin, RL hybrids are almost exclusively female, again, apparently as a result of variation in the R genome (Berger et al., 1988). The unique gametogenesis shown by male Rana esculenta from the R-E system, a frequent exclusion of the R genome rather than the exclusion of the L genome as in the L-E system, is a third specialized case.

It has been unknown before whether the different reproductive mode in hybrids of the R-E system is caused by their lessonae or their ridibunda genome or by their combination. The present results suggest that a particular combination of R-E system lessonae and R-E system ridibunda genomes is necessary to lead to the R-E system type of hybridogenetic gametogenesis.

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