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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; Plötner, 2005). This reproductive mode, which is termed hybridogenesis (Schultz, 1969), and the cytogenetics of which are not well understood, varies geographically.

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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

occurs before meiosis. The remaining R genome undergoes a premeiotic or occasionally a prediplotene meiotic endoreduplication (Tunner & Heppich-Tunner, 1991). This is followed by two apparently normal meiotic divisions without genetic consequences 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 & Plötner, 1988; Vinogradov *et al*., 1991; Plötner, 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 differences, have not been carried out. We made crosses to try to determine whether the modified gametogenesis 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 supported 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 genome 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 gametogenesis.

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*, intermediate 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 experiments, using artificial fertilization after hormone treatment of the female parent, followed standard procedures (Berger *et al*., 1994*a*). In cross designations, 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 *Stu*I, a clone of the centromeric satellite DNA family RrS1, was hybridized *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 *Stu*I-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 preparations, the centromeres of six chromosomes (1–5 and 8) are marked in *Rana ridibunda*, but not in *Rana lessonae* (Fig. 1*a*). The presence of a *ridibunda* genome was also confirmed by Southern blots of *Stu*I-digested genomic DNA hybridized with an RrS1 clone. Such blots give a distinctive autoradiographic pattern when a *Rana ridibunda* genome is present.

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

	Parents				Progeny			
	Female		Male					
Cross No.		Eggs		Sperm	N	Soma	Spermatogonia	Spermatocytes I
02.10	*R. lessonae* L-E system	L	*R. esculenta* L-E system	R	1	R L	2N = 26 R	
02.08	*R. lessonae* L-E system	L	*R. ridibunda* R-E system	R	5	R L	2N = 26 R; N = 13 R	N = 13 R bivalents
02.14	*R. esculenta* L-E system	R	*R. esculenta* R-E system	L	1	R L	2N = 26 R; N = 13 R	N = 13 R bivalents
02.41	*R. lessonae* L-E system	L	*R. esculenta* R-E system	R	4	R L	2N = 26 R; N = 13 R	N = 13 R bivalents
02.43	*R. lessonae* L-E system	L	*R. ridibunda* R-E system	R	1	R L	2N = 26 R; N = 13 R; 2N = 26L + R	N = 13 R bivalents
					1	R L	~2N = 26 L + R; ~3N = 39 2L + 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.

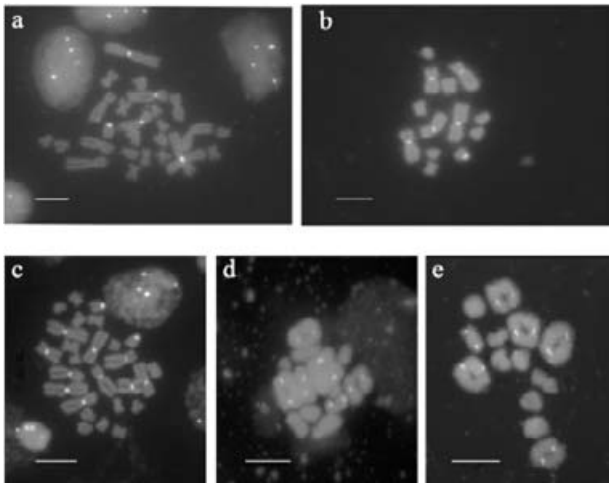

Fig. 1. Chromosome preparations of somatic and germinal tissues hybridized *in situ* with DIG-labelled insert pRr 300 *Stu*I. (*a*) Somatic metaphase of male 21 490 (cross 02.41): a heavy fluorescence is visible at the centromere of only one partner of chromosome pairs 1–5 and 8, revealing the frog's 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.

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. 1 *b–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. 2 *a*, *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.

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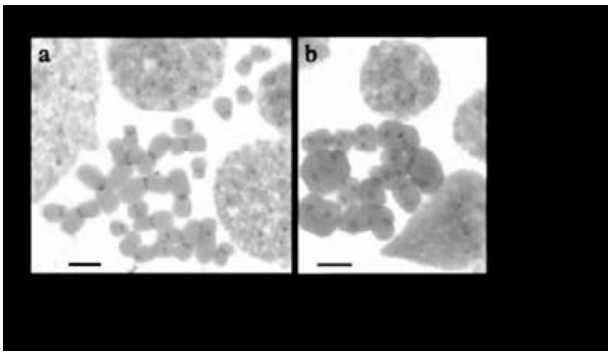

Fig. 2. Testis preparations of male 21 221 (cross 02.08) subjected to C-banding, which marks only the centromeres of *ridibunda* chromosomes. (*a*) Spermatogonial mitotic chromosomes. (*b*) Spermatocyte I at metaphase. Scale bars represent 20 μm.

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. 1*d*). 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 evidenced spermatogonial metaphases with 2N = 26 R chromosomes (Fig. 2*a*), and metaphases I with N = 13 R bivalents (Fig. 2*b*).

(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. 1*a*). 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. 1*c*). In three offspring, there were also spermatogonial metaphases with N = 13 R chromosomes (Fig. 1*b*). 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. 1*e*). 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 ridibunda* 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

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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*., 1994*b*; 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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