Experimental gynogenesis provides evidence of hybridogenetic reproduction in the *Rana esculenta* complex[1]

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Summary. The gynogenetic offspring of the hybrid frog *Rana esculenta (R. ridibunda* × *R. lessonae)* are exclusively of the *ridibunda* type. This is due to the premeiotic exclusion of the *lessonae* genome from the hybrid's germ cells.

The term 'hybridogenesis' has been applied by Schultz[2] to the particular mode of reproduction of some hybrid fishes of the genus *Poeciliopsis.* When a hybrid (AB) reproduces by backcrossing with one of the parental species (A), progeny are exclusively of the AB type. This implies that the hybrid AB produces only 1 kind of gamete (B type). Cytological evidence strongly suggests that the elimination of the A chromosome set occurs premeiotically[3]. A similar mechanism has been proposed to explain the unusual pattern of inheritance within the *Rana esculenta* complex[4] (Amphibia, Anura). The European green frog, *Rana esculenta,* is the natural hybrid of *R. lessonae* and *R. ridibunda*[5]. In most populations, *R. esculenta* can reproduce only with *R. lessonae;* the progeny of this backcross are exclusively of the *esculenta* type. The hybrid must therefore transmit only the *ridibunda* genome. Electrophoretical analysis of enzyme phenotypes supports this hypothesis: using oocytes I from *esculenta* females, only the typical *ridibunda* allozymes could be detected[6]. This suggests a premeiotic exclusion or an inactivation of the *lessonae* genome. In the present paper, we give conclusive evidence of the absence of the *lessonae* genome in oocytes of *R. esculenta.*

Adult frogs were collected from 2 ponds in the vicinity of Lausanne (Switzerland). 2 *esculenta* females were used for the study of lampbrush chromosomes and enzyme phenotypes in oocytes. 2 additional *esculenta* females and 1 *lessonae* male served for breeding experiments (table 1). Gynogenetic diploid progeny were obtained by exposure of eggs to hydrostatic pressure[7] following fertilization with UV-irradiated sperm[8]. Electrophoretic phenotypes of lactate dehydrogenase (LDH), aspartate aminotransferase (AAT) and glucosephosphate isomerase (GPI) were examined in parents and offspring. Results are given in table 2. Of the 4 enzyme loci examined in this study, 3 (Ldh-B, Aat-1, Aat-2) permit an unequivocal discrimination between *R. lessonae* and *R. ridibunda*[6]. *R. ridibunda* is polymorphic at the 4th locus (Gpi-1) and shares 1 allele with *R. lessonae.* At each of these 4 loci, all individuals of *R. esculenta* were found to be heterozygous for the typical alleles of both *R. lessonae* and *R. ridibunda.*

Table 1. Results of breeding experiments (♀ *R. esculenta* × ♂ *R. lessonae*)

Breeding method	Expected ploidy	Expected genotype[a]	Number of fertilized eggs	Blastulae (%)	Hatching tadpoles (%)	Ploidy[b] in a sample of embryos 1N	2N	3N	4N
Natural	2N	RL	♀ E1 880	100	93	–	100	–	–
Artificial fecundation	2N	RL	♀ E1 81	23.4	19.8	1	14	–	1
			♀ E2 150	11.3	11.3	2	15	–	–
Artificial fecundation + sperm irradiation	1N	R	♀ E1 139	14.4	7.2	5	5	–	–
			♀ E2 210	31.9	25.2	53	–	–	–
Artificial fecundation + pressure (450 at)	3N	RRL	♀ E1 42	76.2	21.4	2	2	6	–
			♀ E2 88	47.8	8.0	6	4	10	–
Artificial fecundation + sperm irradiation + pressure (450 at)	2N gynogenetic	RR	♀ E1 194	16.5	4.1	2	9	–	–
			♀ E2 374	68.2	13.6	11	67	–	1

[a] R = *Rana ridibunda* genome; L = *Rana lessonae* genome.
[b] Values obtained by squashing embryos and counting the number of nucleoli in the cell nuclei[7]. All tadpoles developed normally, except those being haploid or gynogenetic diploid. Haploid tadpoles died 2–3 days after hatching, with the typical haploid syndrome. Diploid gynogenetic survived 15–20 days after eclosion, and died before they began feeding.

Table 2. Electrophoretic phenotypes of parents and offspring

Parents and offspring	Nr.	Ldh-B 100	86	75	Aat-1 100	26	Aat-2 100	−21	Gpi-1 −100	−44
R. lessonae ♂ L	1			□		□	□		□	
R. esculenta ♀ E2	1	△		△	△	△	△	△	△	△
Triploid offspring E2 × L[a]	6	▲		▲	▲	▲	▲	▲	▲	▲
Gynogenetic diploid offspring ♀ E2	44	■			■			■		■
R. esculenta ♀ E1	1		△	△	△	△	△	△	△	△
Normal diploid offspring E1 × L	42		▲	▲	▲	▲	▲	▲	▲	▲
Gynogenetic diploid offspring ♀ E1	6		■		■			■		■
(*R. ridibunda* major alleles)		○	○		○			○	○	○

[a] The electrophoretic pattern of LDH from triploid larvae showed a gene dosage effect which is consistent with their expected genotype (RRL, see table 1).
Homozygosity at a locus is indicated by a square, heterozygosity by two triangles. Circles indicate the alleles that were detected in Swiss populations of *R. ridibunda.* The methods used for the analysis of adult frogs have been described elsewhere[6]. Tadpoles were killed 15–20 days after hatching and homogenized in an equal volume of 0.1 M Tris-HCl (pH 8.0).

The gynogenetic diploid progeny of the 2 *esculenta* females are homozygous at the 4 loci, displaying invariably the typical *ridibunda* allele (figure 1 and table 2). This demonstrates that the *lessonae* genome has been discarded from the germinal cells of the hybrid females, and that there has been no recombination between the 2 genomes. The diploid and triploid offspring of the same females, obtained through fertilization with non-irradiated sperm from *R. lessonae* (followed by application of hydrostatic pressure for the triploid offspring), exhibit invariably a typical *esculenta* phenotype. This result is perfectly consistent with our former conclusion that the *esculenta* females transmit only the *ridibunda* genome to their offspring.

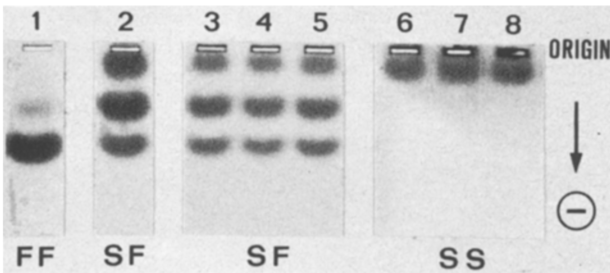

Fig. 1. Gel stained for GPI activity. Slot 1: *R. lessonae* parent (♂ L). Slot 2: *R. esculenta* parent (♀ El). Slots 3–5: normal diploid offspring of the cross El × L. Slots 6–8: gynogenetic diploid offspring of ♀ El. Genotypes: FF = homozygous for the 'fast' allele. SS = homozygous for the 'slow' allele. SF = heterozygous. Extracts of adult muscle tissue and whole tadpoles were run on a Tris-citrate system (Electrode buffer: 0.135 M Tris - 0.045 M citric acid; gel buffer: 1:15 dilution of electrode buffer) with a potential of 10 V/cm for 5 h.

In order to gain some insight as to the time when the elimination of *lessonae* chromosomes occurs, oocytes from 2 *esculenta* females were investigated for the morphology of their lampbrush chromosomes. These chromosomes (figure 2) form 13 bivalents, which correspond to the haploid number of both *R. lessonae* and *R. ridibunda*. Complete pairing is observed to occur, which is normal for homologous chromosomes of the same species. In amphibian species' hybrids, bivalents are rarely formed[9]. Therefore, the chromosomes of the *esculenta* oocyte are supposed to belong to 1 single species. Genetical evidence conclusively supports this hypothesis:

1. Breeding experiments clearly show that no recombination occurs between the 2 parental genomes in *R. esculenta*.

Fig. 2. Lampbrush chromosomes of *Rana esculenta*. Lampbrush chromosomes were prepared from half grown oocytes of about 0.7 mm diameter by the method developed for *Xenopus*[9]. Bar = 50 μm.

Nevertheless, the diplotene chromosomes show many chiasmata, which would necessarily lead to an introgression if the chromosome sets of the 2 parental species were involved.

2. In the *esculenta* oocytes, only the *ridibunda* allozymes could be detected, whereas transcriptional activity taking place on all chromosomes is indicated by the presence of well developed loops. If the 2 genomes were present, the allozymes of both species should also be present. This demonstrates that the *lessonae* genome has been eliminated before meiosis. The *ridibunda* genome must have undergone a supplementary duplication, which restored the diploid number of chromosomes. Bivalent chromosome partners are thus not only homologous, but sister-strand derived identical chromosomes. The ensuing meiotic division segregates then only identical chromosomes. Consequently, all gynogenetic progeny of 1 female form a 'clone'. In fact, the electrophoretic analysis reveals no difference between tadpoles derived from 1 single female. Differences exist however between the 2 females and their respective progeny.

In contrast to the gynogenetic offspring of other amphibians[7,10,11], the viability of the *esculenta* gynogenetics is dramatically reduced. No tadpole was able to survive longer than 20 days or enter into the feeding stage. This might be due to an accumulation of deleterious mutations in the hybrid's *ridibunda* genome, which is clonally reproduced in the mixed *lessonae-esculenta* populations. In *R. esculenta,* these deleterious mutations would be hidden by the *lessonae* genes, whereas in the homozygous gynogenetic progeny they are unrestricted and become lethal. This hypothesis, already suggested by Berger[12], could also explain the frequent occurrence of larval mortality in the offspring of the cross *esculenta* × *esculenta*.

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Selective enrichment technique for isolation of methanol-utilizing yeasts

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Summary. A simple, selective enrichment technique was developed for isolation of methanol-utilizing seasts by supplementing gentamicin or tetracycline in the medium.

In recent years there has been considerable interest in studies concerned with the utilization of methanol as an important raw material for the production of single-cell protein (SCP). The ability of bacteria to assimilate methanol has been known for many years[3], but the first yeast that utilized methanol was isolated as late as 1969[4]. Since yeasts as SCP surpass bacteria in some of their properties, e.g. lower nucleic-acid content and higher density, interest has been focused on the isolation of methanol-utilizing yeasts. This report describes simple, selective enrichment techniques for the isolation of methanol-utilizing yeasts.

Experiments and results. A 1 g soil sample (local) was suspended in 10 ml of sterile distilled water and 1 ml of this suspension was inoculated into 50 ml of half-strength Sabouraud's broth (Difco), pH 5.0, containing 1% (v/v) methanol and various (100–300 µg/ml) concentrations of gentamicin sulfate or tetracycline hydrochloride. These flasks were incubated on a rotary shaker at 25 °C for 5 days. A 1 ml portion of the turbid suspension was then transferred to a mineral-salts-methanol medium[5] supplemented with either gentamicin or tetracycline (100–300 µg/ml) and incubated on a rotary shaker for 1 week. This process was repeated 4 times with fresh medium, and, following the final incubation, a loopful of the suspension was streaked from each flask onto a solid mineral-salts-methanol medium[5]. These plates were incubated for 1 week at 25 °C, followed by microscopic examination of each colony from the plate. The results are summarized in the table. Inspection of the above table indicates that gentamicin and tetracycline were highly effective antibiotics for isolation of methanol-utilizing yeasts.

In conclusion, methanol-utilizing bacteria are generally gram-negative rods and susceptible to broad spectrum antibiotics. In contrast, yeasts are insensitive to these antibiotics, hence they will survive and multiply in the presence of such antibiotics if they can utilize the carbon source provided.

Enrichment for methanol-utilizing yeasts

Antibiotic	Concentration (µg/ml)	No. of yeast colonies	No. of bacteria colonies
None (control)	None	1	35
Gentamicin	100	30	3
	200	20	2
	300	28	0
Tetracycline	100	15	5
	500	25	3

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