Evolutionary History of the Hybridogenetic Hybrid Frog *Rana esculenta* as Deduced from mtDNA Analyses[1]

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mtDNA of the hybridogenetic hybrid frog *Rana esculenta* from Switzerland, Austria, and Poland was compared to mtDNA of the parental species *R. ridibunda* and *R. lessonae* using electrophoretic analysis of restriction enzyme fragments. Two mtDNA phenotypes, with 3.4% sequence divergence, are present in *R. lessonae:* type C is found in Poland, and type D is found in Switzerland. *Rana ridibunda* from Poland has either of two mtDNA phenotypes: type A is the typical ridibunda mtDNA, and type B is a lessonae mitochondrial genome, introgressed into *R. ridibunda,* that differs from type C mtDNA of *R. lessonae* by only 0.3%. Each of the three lessonae genomes differs from A, the typical ridibunda mtDNA, by ~8%. All four types of mtDNA (A and B of *R. ridibunda,* C and D of *R. lessonae*) are found in *R. esculenta.* Of 62 *R. esculenta* from Poland, 58 had type C, three had type A, and one had type B mtDNA. All nine *R. esculenta* from Switzerland had type D mtDNA. All three *R. esculenta* from Austria, from a population in which males of *R. esculenta* are rare, had ridibunda mtDNA, two having type B and one having type A. Both field observations and studies of mating preference indicate that the primary hybridizations that produce *R. esculenta* are between *R. ridibunda* females and *R. lessonae* males; thereafter, *R. esculenta* lineages are usually maintained by matings of *R. esculenta* females with *R. lessonae* males. The presence of ridibunda mtDNA in the three *R. esculenta* sampled from Austria, its occasional presence in *R. esculenta* populations in Poland, and its absence from *R. esculenta* in Switzerland support both the direction of the original hybridization and the rarity of formation of new *R. esculenta* lineages. The preponderance of *R. esculenta* individuals with lessonae mtDNA in our samples from central Europe suggests that most lineages have gone through at least one mating between an *R. lessonae* female and an *R. esculenta* male. This reveals a greater reproductive role for *R. esculenta* males than their partial sterility and infrequent matings would suggest.

Introduction

Among the 50-odd clonally reproducing species of fishes, amphibians, and reptiles, *Rana esculenta* Linnaeus 1758, the common edible frog of Europe, is unique in that, in most populations, males as well as females occur in large numbers. Even so, the reduced fertility of males (Berger 1970, 1971; Günther 1973), the relative inviability of progeny fathered by them (Berger and Uzzell 1977), and their tendency to engage in combative rather than reproductive behavior in breeding congregations (Blankenhorn 1974, 1977) suggest that they have relatively little to do with maintaining populations of *R. esculenta.*

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Rana esculenta arose and, in areas of sympatry, arises by hybridization between the two Mendelian species *R. lessonae* Camerano 1882 and *R. ridibunda* Pallas 1771 (Berger 1967, 1968). *Rana ridibunda* is a large frog, distinguishable from *R. lessonae* on the basis of body size, call, and numerous morphological and biochemical features. *Rana lessonae* is a much smaller, more terrestrial, frog; the hybrid *R. esculenta* is intermediate in size and other morphological features between the two parental species. Of particular interest are the size differences between the taxa. The size range for sexually mature *R. ridibunda* (74–94 mm) does not overlap that for *R. lessonae* (42–71 mm); *Rana esculenta* spans the range (54–89 mm) between the two parental species (Berger 1966, 1970).

Both behavioral studies and field observations indicate that the original hybridizations that produce *R. esculenta* are between female *R. ridibunda* and male *R. lessonae.* Such matings—but never the reverse—between these two species have been observed in the field on many separate occasions (Berger 1957, 1959, 1970; Borkin et al. 1979). This directionality of interspecific matings depends both on size preferences in mating shown by males and on the large size difference between *R. ridibunda* males and *R. lessonae* females. In laboratory studies (Blankenhorn 1974, 1977), male water frogs showed a marked preference for larger females. In various combinations of pairings of *R. esculenta* and *R. lessonae,* larger females were preferred over smaller ones. No sexual behavior was displayed in any combination in which the female was smaller than the male; in fact, males actively avoided females smaller than themselves. As in most species of frogs (Wright and Wright 1949, p. 20), male water frogs are smaller on the average than conspecific females (Berger 1966, Borkin et al. 1978). Since the smallest sexually mature males of *R. ridibunda* are almost invariably longer than the largest female *R. lessonae,* the size difference, coupled with the size preference in mating shown by water frog males, virtually precludes pairing of *R. ridibunda* males and *R. lessonae* females in nature (Tunner 1974; H. Hotz, personal communication).

Reproduction in both sexes of *R. esculenta* is hybridogenetic (Tunner 1973). In hybridogenesis (Schultz 1969), the genome of one parental species is eliminated from germ line cells before the completion of gametogenesis but normally is restored at fertilization because the hybridogenetic individuals mate with that parental species. In the common form of *R. esculenta* in central Europe, the lessonae chromosome set is absent from both sperm and ova (Tunner 1974; Graf and Müller 1979; Uzzell et al. 1980), which contain only a ridibunda chromosome set. Such *R. esculenta* live with and reproductively depend on *R. lessonae* (the L-E system; Uzzell and Berger 1975). In each L-E system lineage, the ridibunda nuclear genome is passed clonally from generation to generation, while a new lessonae genome enters at each fertilization only to be lost before the next fertilization (fig. 1). In the L-E system, *R. esculenta* lineages are maintained mostly by matings between *R. esculenta* females and *R. lessonae* males (Blankenhorn 1974, 1977; L. Berger, personal communication), but because of the overlap in sizes, the reverse mating pattern—that is, between a large *Rana lessonae* female and a small *Rana esculenta* male—does occur at low frequency, both in nature and in the lab (Blankenhorn 1974; L. Berger, personal communication).

As a key to exploring the population dynamics and the evolutionary history of these frogs, we have examined mitochondrial DNA from numerous *R. esculenta* of the L-E system as well as from the two parental species. The maternal inheritance of mtDNA in Metazoa (Dawid and Blackler 1972; Hutchinson et al. 1974; Kroon et al. 1978; Giles et al. 1980) makes this genome useful in tracing maternal genealogies (Avise et al. 1979*a,* 1979*b;* Brown and Wright 1979; Ferris et al. 1981*b;* Brown et al.

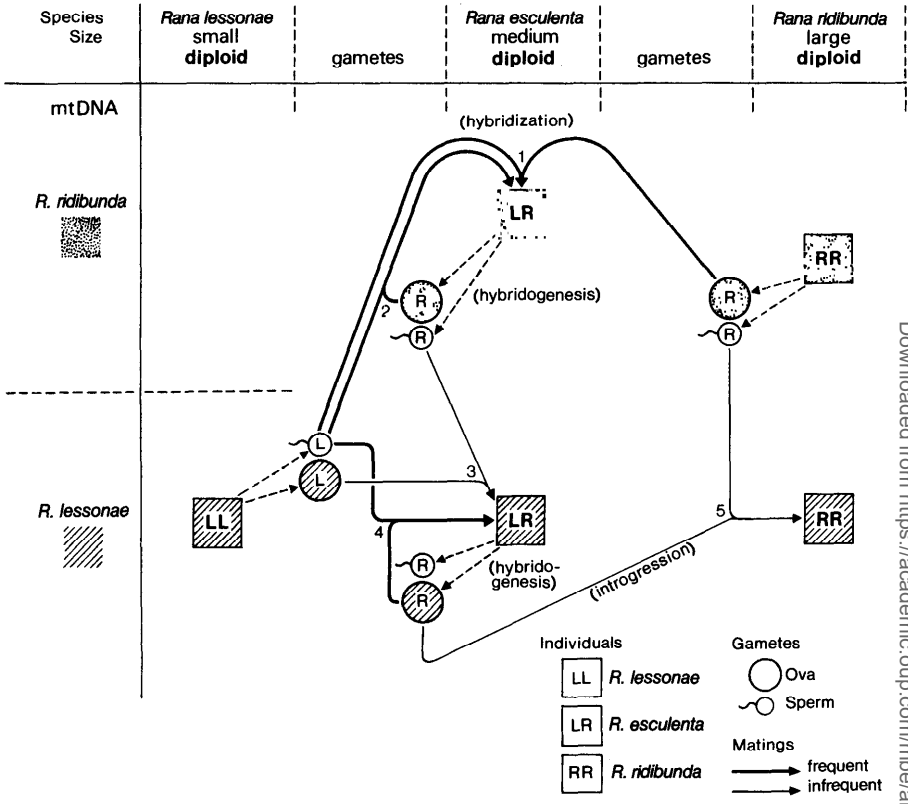

FIG. 1.—An overview of mating patterns within the *Rana esculenta* complex. Large boldface letters indicate the chromosomal complement of diploid individuals; single capital letters with dotted-line arrows to them indicate meiotic products. The mitochondrial types in diploid individuals and in ova are designated by shadings—stippled for ridibunda mtDNA and cross-hatched for lessonae mtDNA. Thin-line arrows represent pairings in which the male is to the right of the female and hence likely to be larger—and in which, therefore, the mating is less frequent than propinquity would allow. Thick-line arrows represent the reverse situation, in which the male is likely to be smaller than the female (the favorable case for mating). The LL female × RR male mating presumably does not occur. All other possibilities not shown are either intraspecific or are rare and produce *R. ridibunda.* Mating 1 produces *R. esculenta;* 2 maintains *R. esculenta* with ridibunda mitochondria; 3 introduces lessonae mitochondria into *R. esculenta;* 4 maintains *R. esculenta* with lessonae mitochondria; and 5 transfers lessonae mitochondria on to *R. ridibunda.*

1982; Hauswirth and Laipis 1982; Wright et al. 1983). In the present study, maternal genealogies traced by means of restriction fragment analysis of mtDNA from *R. esculenta* and from its parental species, *R. ridibunda* and *R. lessonae,* reveal a greater role in reproduction for male *R. esculenta* than previously has been suspected.

Material and Methods

Specimens

Frogs were identified as to species by morphology and electrophoretic phenotype. Ploidy was determined using red blood cell sizes (Berger and Uzzell 1975; Günther 1977); all *Rana esculenta* sampled were diploid. Most samples of frogs of all three species (132 individuals) were collected from six localities in western Poland, within

a 40-km radius of Poznan. At some of these localities only *R. lessonae* and *R. esculenta* occurred, but all three species occurred at others. These six localities were grouped in the analysis because no significant differences between localities in mtDNA ratios were found. In addition, nine *R. esculenta* were obtained from two localities near Zurich, Switzerland; eight *R. lessonae* came from Fehraltdorf, Canton Zürich, Switzerland; and three *R. esculenta* were collected at Neusiedlersee, Austria. All *R. ridibunda* came from Poland (table 1).

Preparation of mtDNA

mtDNA was isolated from individual frogs using methods described by Spolsky and Uzzell (1984). In some cases the mitochondrial fraction was enriched by banding in a sucrose step gradient (0.9 M and 1.8 M sucrose) prior to lysis. Purified mtDNAs were redissolved in 50–300 μl of 0.1 TE (1 mM tris, 0.1 mM ethylenediaminetetraacetate, pH 8.0) and stored at −70 C. The amount of mtDNA was estimated by minigel electrophoresis and ethidium bromide staining of a 5-μl aliquot of each preparation.

Restriction Endonuclease Analysis of mtDNA

Approximately 5–10 ng of each DNA were digested to completion with each restriction enzyme (table 2) under conditions recommended by the supplier (Boehringer Mannheim Biochemicals or Bethesda Research Labs). Resulting DNA fragments were end-labeled with a mixture of four (adenine, cytosine, guanine, and tyrosine) α-^{32}P-triphosphate deoxynucleosides and subjected to electrophoresis through 1% agarose gels and, for small fragments, 4% polyacrylamide gels (Brown 1980; Wright et al. 1983); separated fragments were detected by autoradiography. For each gel, fragment sizes were estimated from mobilities of DNA fragments of known size (*Hin*dIII-restricted lambda and PM2 DNAs, *Hin*cII-restricted ϕX174 DNA).

Estimation of Sequence Divergence and mtDNA Relatedness

The amount of sequence divergence was calculated from the fraction of restriction fragments shared by a pair of DNAs (Nei and Li 1979), using the formula derived by Upholt (1977). An unrooted Wagner network of mtDNA relationships was constructed from the matrix of divergence values.

Results

mtDNAs in the Parental Species

Four types of mtDNAs were found in *Rana ridibunda* and *R. lessonae*. For comparison between species and populations, fragment patterns were determined for one individual of each mtDNA type using 19 (types A, B, and C; Spolsky and Uzzell 1984) or 15 (type D; table 2) restriction enzymes. Paired comparisons of sequence differences were made for the four types of mtDNAs found in *R. ridibunda* and *R. lessonae* (table 3). Type A mtDNA of *R. ridibunda* is most divergent from the other three types; the sequence divergence of A from any of the other three types is approximately the same, 8.1%–8.5%. Types B and C are virtually identical, with a difference in sequence of 0.3%, so their mean divergence from A and their mean divergence from D were used for the analyses. The sequence difference between the D and C mtDNAs of *R. lessonae,* 3.4%, is within the range of intraspecific differences found in other vertebrates (Avise et al. 1979*b;* Ferris et al. 1981*a;* Wolstenholme et al. 1982; Lansman et al. 1983).

Table 1
Restriction Fragment Patterns in Type C and D mtDNAs[a]

Enzyme	mtDNA Type D	mtDNA Type C[a]
*Ava*I	8,800	7,000[b]
	7,000[b]	6,000
	2,950	5,100
	850	1,100
	130[b]	130[b]
		105
*Bam*HI	13,000	11,000
	6,300	8,000
	550	470
*Bcl*I	17,000	8,400
	2,150[b]	7,800
	900[b]	2,150[b]
		900[b]
*Bgl*II	5,300	5,700
	3,750[b]	3,750[b]
	3,000[b]	3,600
	1,650[b]	3,000[b]
	1,400	1,650[b]
	1,200	1,500
	1,100	760[b]
	760[b]	220
	590	
*Hinc*II	5,200	5,600
	3,200[b]	3,300
	2,900	3,200[b]
	2,150	1,900
	1,700[b]	1,700[b]
	1,300[b]	1,300[b]
	1,230	1,050
	1,150	830
	730	340[b]
	340[b]	110
	290	

Enzyme	mtDNA Type D	mtDNA Type C[a]
*Hind*III	5,700[b]	5,700[b]
	4,000[b]	4,000[b]
	4,000[b]	4,000[b]
	2,150[b]	2,150[b]
	1,500[b]	1,500[b]
	1,100[b]	1,100[b]
	600[b]	600[b]
	285[b]	285[b]
*Hpa*I	10,500[b]	10,500[b]
	6,000[b]	6,000[b]
	3,250[b]	3,250[b]
*Eco*RI	15,000[b]	15,000[b]
	4,400[b]	4,400[b]
*Eco*RV	19,500	16,000
		3,500
*Hae*II	8,500[b]	8,500[b]
	4,900[b]	4,900[b]
	4,200[b]	4,200[b]
	625[b]	625[b]
	500[b]	500[b]
*Pvu*II	14,200	15,000
	4,300	2,250
		600
		270
*Kpn*I[c]	7,300[b]	7,300[b]
	7,300[b]	7,300[b]
	3,300[b]	3,300[b]
	1,300[b]	1,500[b]
	620[b]	620[b]
*Pst*I	12,500[b]	12,500[b]
	3,600	7,100
	3,600	
*Sma*I	no cuts	19,500
*Xba*I	9,800	19,500
	9,800	

[a] Fragment patterns for type C mtDNA, as well as for types A and B mtDNA, have been previously published (Spolsky and Uzzell (1984).

[b] Shared fragments.

[c] The difference in the next-to-smallest *Kpn*I fragment represents size variation; we consider these fragments homologous in mtDNA types C and D.

These sequence divergence values were used to construct a Wagner network (fig. 2). Most of the divergence in the network, 6.6, is in the leg between A and the common node. The distances from D and from the B-C pair to the common node

Table 2
Distribution of mtDNA Types in the *Rana esculenta* Complex

Species and Region (*N*)	mtDNA Type (Frequency)			
	A	B	C	D
R. ridibunda:				
Poland (38)	.58	.42	...	...
R. lessonae:				
Poland (32)	...	...	1.00	...
Switzerland (8)	...	...	...	1.00
R. esculenta:				
Poland (62)	.05	.02	.94	...
Switzerland (9)	...	...	...	1.00
Austria (3)	.33	.67	...	...

are approximately the same, being 1.9 and 1.5, respectively. If the network is rooted at the midpoint of the longest inter-mtDNA distance, types B, C, and D cluster together to form a lessonae-like group.

Because *Bam*HI and *Sma*I are the only two restriction enzymes that distinguish between B and C mtDNAs and because they also distinguish among all four mtDNA types (fig. 3), these two enzymes were used to classify each mtDNA sample into one of the four types.

*Bam*HI cuts A mtDNA at a single position to produce a full-length linear molecule; it cleaves B mtDNA into three fragments approximately 8,000, 6,400, and 5,600 bp in length; and it cleaves C mtDNA into three fragments of 11,500, 8,000, and 470 bp. Type D mtDNA is cut at three sites to generate fragments 13,000, 6,300, and 550 bp in length (fig. 3).

*Sma*I digestion produced no common fragments between any of the four types. An mtDNA is cut by *Sma*I at three sites into fragments approximately 8,800, 8,800 and 1,800 bp long. B is cleaved into two fragments 14,000 and 5,500 bp long, and C is cut at a single site. There are no *Sma*I restriction sites on D mtDNA (fig. 3).

In addition to *Bam*HI and *Sma*I, between one and 10 other restriction enzymes were used on ~25% of the samples. Although more extensive sampling would probably have demonstrated some variation, in our limited sampling no gain or loss of restriction

Table 3
Matrix of mtDNA Comparisons

	A	B	C	D
A	...	.256	.258	.244
B	8.2 ± 1.0	...	.946	.556
C	8.1 ± 1.0	.3 ± .3	...	.560
D	8.5 ± 1.1	3.4 ± .8	3.4 ± .8	...

NOTE.—Values above the diagonal are the proportion of shared fragments; values below the diagonal are the percentages of sequence difference ± SD.

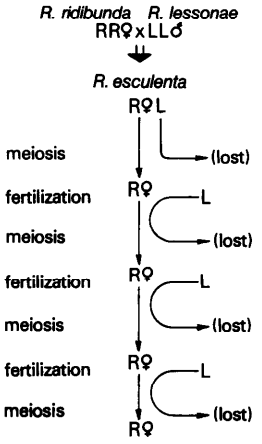

FIG. 2.—Inheritance patterns in hybridogenetic L-E system *Rana esculenta*. *Rana esculenta* hybrids originate from matings of *R. ridibunda* females with *R. lessonae* males. In the L-E system, diploid *R. esculenta* passes on only the ridibunda chromosome set through its gametes. *Rana esculenta* hybrid lineages are usually maintained by crosses of *R. esculenta* females with *R. lessonae* males, although the reverse cross—*R. lessonae* female × *R. esculenta* male—does rarely occur.

sites was observed within any of the four mtDNA types; variation in length of mtDNA within types was detected, however.

Individuals of *R. ridibunda* from Poland had one or the other of two very different types of mtDNA, i.e., A or B (Spolsky and Uzzell 1984; table 1). With the restriction enzymes used, mtDNA of *R. lessonae* was locally invariant but differed between Switzerland and Poland. *Rana lessonae* from Poland had type C mtDNA, whereas in Switzerland this species had type D mtDNA (table 1).

mtDNA in *R. esculenta*

In screening *R. esculenta* populations, each mtDNA sample was identified as to type by restriction with *Bam*HI and *Sma*I. Most *R. esculenta* from central Europe that were examined had *R. lessonae* mtDNA, rather than either of the mtDNA patterns found in *R. ridibunda* (table 1). All nine *R. esculenta* from Switzerland had the

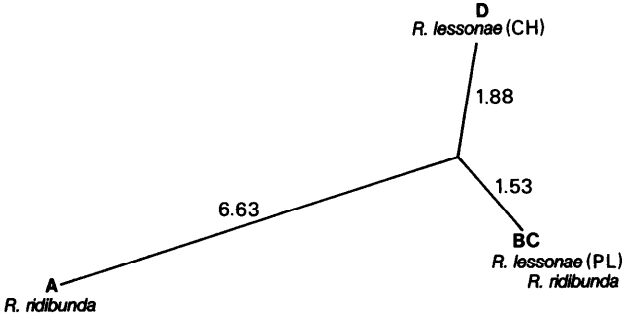

FIG. 3.—Rootless Wagner network of mtDNA relatedness. Distances are percentages of sequence divergences. CH = Switzerland; PL = Poland. The *Rana ridibunda* samples came from Poland. A, B, C, and D are the four mitochondrial types found.

D type of mtDNA found in *R. lessonae* in Switzerland. Of the 62 *R. esculenta* from Poland, 58 had the same mtDNA type, C, as did *R. lessonae* from this area. The remaining four *R. esculenta* from Poland had one of the mtDNA types seen in *R. ridibunda;* three, each from a different locality, had the A genome, and one had the B genome. A different pattern, however, was found in Austria: all three *R. esculenta* from there had ridibunda rather than lessonae mtDNA; two had B and one A mtDNA.

Discussion

Relationships within the *Rana esculenta* Complex

The distinction between *R. lessonae* and *R. ridibunda,* first clearly stated by Berger (1957), has been abundantly confirmed by subsequent morphological (Berger 1966), electrophoretic (Tunner 1970, 1972, 1973; Engelmann 1972, 1973; Uzzell and Berger 1975) and immunological (Uzzell 1979, 1982) studies. The mtDNA network (fig. 2), when rooted on the longest branch, suggests a phylogeny for *R. ridibunda* and *R. lessonae* that is inconsistent with these species limits, because in the rooted network the type B genome found in *R. ridibunda* clusters with the lessonae genomes. The mtDNA phylogeny can be reconciled with the species limits by postulating an introgression of *R. lessonae* mtDNA into *R. ridibunda* during the relatively recent past (Spolsky and Uzzell 1984).

Immunological comparisons of serum albumins place the divergence time of *R. ridibunda* and *R. lessonae* at ~12 Myr ago (Uzzell 1982). With a sequence divergence between the two species for A and C mtDNAs of 8.5% ± 1.1% (table 3), the rate of sequence change for mtDNA in this species pair is ~0.7%/Myr. If this rate of change were constant over this time period, which is far from certain, it would appear that the introgressed B mtDNA of *R. ridibunda* diverged from type C mtDNA of *R. lessonae* within the last 400,000 years and that mitochondrial genomes assayed from Swiss and Polish *R. lessonae* last shared a common ancestor 4–6 Myr ago. Since *R. lessonae* reinvaded the entire region between Switzerland and Poland after the Würm glaciation, probably within the past 15,000 years, the divergence time for the mtDNAs suggests that northern Europe was repopulated by already divergent stocks (cf. Avise et al. 1984).

Evolutionary Pathways in *R. esculenta*

Since the primary hybridizations that produce *R. esculenta* lineages involve at least primarily, if not exclusively, crosses of *R. ridibunda* females with *R. lessonae* males and since L-E system lineages are usually maintained by matings of *R. esculenta* females with *R. lessonae* males, we expected to find ridibunda mtDNA in our samples of *R. esculenta.* Instead, the majority of *R. esculenta* individuals had lessonae mtDNA rather than either of the mtDNAs found in *R. ridibunda.* Two alternative hypotheses could explain this observation. Either (1) the original hybridizations were, contrary to expectations, mainly between *R. lessonae* females and *R. ridibunda* males or (2) each *R. esculenta* lineage has gone through at least one mating between an *R. esculenta* male and an *R. lessonae* female.

Both field observations and laboratory studies, as reviewed in the Introduction, make the first hypothesis unlikely (the presence of both ridibunda and lessonae mtDNA in *R. esculenta* would in any case require a few original hybridizations involving *R. ridibunda* females). Furthermore, since lessonae mtDNA is very common in *R. es-*

culenta, despite the fact that the few observed matings between the parental species all involve *R. ridibunda* females, some additional factor must be invoked to account for this distribution if the first hypothesis is correct.

Support for the origin of *R. esculenta* from crosses of female *R. ridibunda* with male *R. lessonae* is provided by the small sample of *R. esculenta* from Neusiedlersee in eastern Austria. The *R. esculenta* population there, as throughout the Pannonian Basin, is exceptional among L-E populations in that it consists almost entirely of females (Tunner and Dobrowsky 1976; Berger et al. 1985). Since male *R. esculenta* are very rare here, matings of male *R. esculenta* with female *R. lessonae* virtually never occur. Although one might expect, a priori, to find the same proportion (94%–100%) of *R. esculenta* with lessonae mtDNA in Austria as in the other localities, all three individuals of *R. esculenta* from Neusiedlersee have ridibunda mtDNA. The probability of picking three individuals at random with ridibunda mtDNA, if 95% of the population had lessonae mtDNA, is vanishingly small (1.3×10^{-4}). Finding only ridibunda mtDNA in this population, where they can only very rarely be replaced with lessonae mtDNA, strongly suggests that the original hybridizations were between *R. ridibunda* females and *R. lessonae* males.

The alternative hypothesis that would account for the distribution of mtDNA in *R. esculenta*—that is, that males of *R. esculenta* have a significant role in maintaining populations of *R. esculenta* in the L-E system—is thus more plausible. It requires only one mating between a female *R. lessonae* and a male *R. esculenta* in each lineage. Once such a cross occurs in an *R. esculenta* lineage, that lineage becomes fixed for lessonae mtDNA, since subsequent crosses of the usual type (*R. esculenta* female × *R. lessonae* male) will maintain lessonae mtDNA in the lineage (fig. 4).

Given the small proportion of observed matings of esculenta males with lessonae females compared to the reverse cross, the widespread occurrence of lessonae mtDNA in *R. esculenta* is surprising. There are, however, a number of factors that could be responsible for the observed distribution: (1) Crosses between *R. lessonae* females and *R. esculenta* males may be more common than reported, although that alone would not account for the high proportion of *R. esculenta* with lessonae mtDNA. (2) There may be some selective advantage to *R. esculenta* in having lessonae mtDNA either in gametes or in the soma, although there is no direct evidence for this. (3) Sufficient time has elapsed for virtually all lineages to have gone through at least one mating of the rarer type; since a change to lessonae mtDNA is irreversible in an L-E system population (fig. 4), an increasing proportion of individuals with lessonae mtDNA will accumulate in a population as a result of additional matings of the rarer type in previously unchanged lineages. This is formally analogous to the increase in frequency of mutants in a bacterial system with a constant mutation rate (Kaplan 1947; Novick and Szilard 1950); it requires no assumption that either type of mtDNA in *R. esculenta* has a selective advantage over the other or that *R. esculenta* possessing either type of mtDNA reproduces more successfully. Crosses of the rarer type could thus account for most, if not all, of the distribution of lessonae mtDNA in *R. esculenta* in central Europe.

The increase with time of lessonae mtDNA in *R. esculenta* populations must be weighed against the increase of ridibunda mtDNA through the formation of new *R. esculenta* lineages. Since individuals of *R. esculenta* with ridibunda mtDNA may represent either old lineages that have not gone through a hybridogenetic mating of the unusual kind or newly arisen lineages, those few *R. esculenta* in Poland with ridibunda

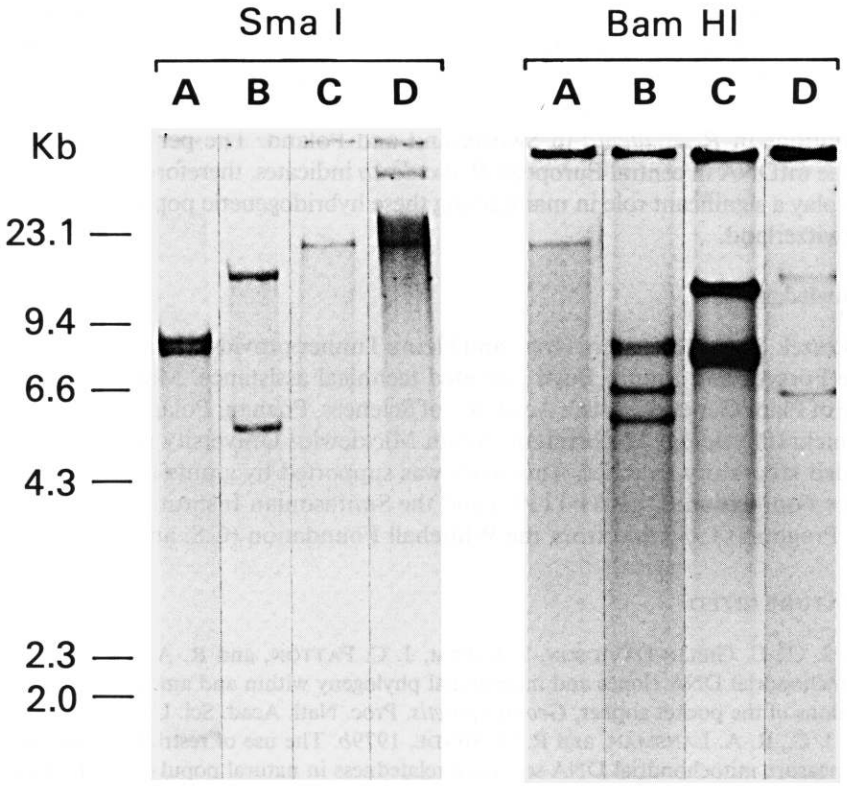

FIG. 4.—Autoradiogram of *Bam*HI and *Sma*I digestion patterns (two separate gels) for the four types of mtDNA found in the *Rana esculenta* complex. mtDNAs from individual animals were restricted with *Bam*HI or *Sma*I; the fragments thus generated were end-labeled with ^{32}P and separated according to size in 1% agarose gels. Fragment bands were visualized by autoradiography. Letters at the tops of lanes refer to the phenotype of mtDNA in that lane. Additional lanes with duplicated patterns were excised from the photographs of each gel. The band at ~19.5 kb in lane D for *Sma*I contains randomly linearized mtDNA; this band is always present in control mtDNA samples. Uncut mtDNAs can be distinguished from mtDNAs linearized by restriction enzymes with a single site *Sma*I, by the presence, in the uncut or control mtDNAs, of a slowly migrating band consisting of relaxed or nicked circles (*Sma*I, lane *C*; BamHI, lane *A*).

mtDNA also reflect the relative rarity of formation of new hybrid lineages. In Switzerland, where there are no autochthonous *R. ridibunda,* no primary hybridizations can occur; the lack of ridibunda mtDNA in *R. esculenta* in Switzerland suggests that all extant *R. esculenta* lineages there have gone through the rarer type of hybridogenetic mating.

To summarize, in areas where no primary hybridization can presently occur (e.g., most of Switzerland), *R. esculenta* do not have ridibunda mtDNA; where primary hybridizations can occur (e.g., Poland, Austria), at least some *R. esculenta* have ridibunda mtDNA; in Austria, where matings of male *R. esculenta* and female *R. lessonae* are extremely rare, *R. esculenta* have preponderantly, if not exclusively, ridibunda mtDNA. These data, taken together with behavioral data, fit the hypothesis that primary hybridizations are predominantly, if not exclusively, between female *R. ridibunda* and male *R. lessonae;* lessonae mtDNA gets into *R. esculenta* lineages through rare matings

between female *R. lessonae* and male *R. esculenta.* The alternative hypothesis—that is, that most primary hybridizations are between female *R. lessonae* and male *R. ridibunda*—besides being contrary to behavioral data and field observations, cannot hold in Austria and does not easily explain the differences between mtDNA type distributions in *R. esculenta* in Switzerland and Poland. The pervasive presence of lessonae mtDNA in central European *R. esculenta* indicates, therefore, that *R. esculenta* males play a significant role in maintaining these hybridogenetic populations in Poland and Switzerland.

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