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MORPHOMETRIC AND GENETIC VARIABILITY OF *RODENTOLEPIS ASYMMETRICA* (HYMENOLEPIDIDAE) FROM THE PYRENEAN MOUNTAINS

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ABSTRACT: *Rodentolepis asymmetrica* (Janicki, 1904), is a common hymenolepidid cestode recorded in several vole species (rodents) in the Palearctic. Here, we report a detailed analysis of this species, which includes metrical features and multilocus enzyme electrophoresis. Worms isolated from 4 species of arvicolid hosts in 3 localities in Spain and France from 1994 to 1997 were studied. All the worms used in the morphological study ranged between 1 and 5 individuals per host. Furthermore, all individuals were analyzed electrophoretically. Statistical analysis of metrical features in scolex, sexual segments, and eggs was carried out, and significant differences were detected only in sexual structures of mature segments. These differences were found in worms from each host species in different localities and in the same host species in 2 localities. Multivariate statistical analysis shows correct classification of worms in all cases. Surprisingly, we observed a lack of genetic variability at the 11 enzymatic loci analyzed, which could be explained by 2 nonexclusive hypotheses: (1) a preferential selfing mode of reproduction for these parasites, and (2) a weak effective size of parasite populations.

Current classifications of cestodes are mainly based on morphological traits in adult parasitic phenotypes (Khalil et al., 1994). The taxonomy in hymenolepidid cestodes relies largely on morphologic and morphometric features (Czaplinski and Vaucher, 1994). Difficulties may arise in specific differentiation when using data of variability. Moreover, coherent specific determination is hindered by incomplete descriptions and confusing faunistic data. Several species of armed hymenolepidids are described for *Rodentolepis* Spasski, 1954. The morphological characters most commonly used in specific differentiation are scolex, shape and size of rostellar hooks, gonad arrangement, gravid segments, and eggs. A set of morphometric characters believed to be of primary importance for the taxonomy of Hymenolipididae Ariola, 1899, was proposed by Mas-Coma and Galán-Puchades (1991). A review of the literature on hymenolepidids in rodents shows that these species often exhibit low host specificity. This observation has been made for rodents, humans, and birds (Schmidt, 1986).

Rodentolepis asymmetrica (Janicki, 1904) is currently considered a typical parasite of Palearctic arvicolid rodents and exhibits great morphological variability. Its identification is mainly based on rostellar and embryonic hooks (Tenora and Murai, 1972). Characteristics of rostellar hooks are widely used in *Rodentolepis* spp. systematics. But in some cases (*Rodentolepis straminea* and *Rodentolepis microstoma*), rostellar hooks are not confirmed as discriminant specific criteria (Casanova et al., 2001).

Isozyme differentiation of cestodes has been carried out by several research groups and has helped resolve some specific problems in similar parasitic phenotypes (Renaud et al., 1983; Baverstock et al., 1985; Renaud and Gabrion, 1988; Ba et al., 1993; De Chambrier and Vaucher, 1994; Andrews and Chilton, 1999; Casanova et al., 2001). In the Hymenolepididae these studies are very rare, however, and have focused mainly on species that are important for human health or laboratory research, e.g., *Hymenolepis diminuta* Rudolphi, 1819, *Rodentolepis nana* (von Siebold, 1852), and *R. microstoma* (Dujardin, 1845) (Arai, 1980; Casanova et al., 2001). Valuable genetic information on morphological variability in cestodes is scarce (Snabel et al., 1994).

Here, we investigated the genetic variability of *R. asymmetrica* through a genetical approach by multilocus enzyme electrophoresis (MEE). Morphological and morphometrical features were studied in specimens of this species from different arvicolid hosts and different localities. The study aims to examine the morphometric and enzymatic variability of this species.

MATERIALS AND METHODS

Hymenolepidids from 4 arvicolid hosts were studied morphologically and electrophoretically. Parasitized hosts were found in 3 localities in the Pyrenean Mountains (Spain and France) between 1994 and 1997: Vall d'Aran (Spain, 1994, 1997; 42°35′21″N, 1°19′40″E), Eugi (Spain, 1995, 1996; 42°58′49″N, 1°30′26″W), and Moulis (France, 1997; 42°57′35″N, 1°5′33″E). A total of 291 hosts were examined: 169 *Clethrionomys glareolus* (25 from Vall d'Aran, 66 from Eugi, and 78 from Moulis), 25 *Microtus agrestis* (17 from Eugi and 8 from Vall d'Aran), 82 *M. arvalis* (46 from Vall d'Aran and 36 from Moulis), and 15 *M. gerbei* (from Eugi) (Table I).

Adult worms were used for the morphological study (scolex, mature segments, and the last gravid segment with eggs) and for electrophoretic purposes (the rest of the worm). Voucher specimens are deposed in the Museum of Natural History, Genève, Switzerland (32861 INVE, 32862 INVE, 32863 INVE).

Morphological and morphometrical analysis

For the morphological study, live worms were initially placed in water. Mature segments were stored without pressure in 70% ethanol, stained in acetic ferric carmine (Georgiev et al., 1986), dehydrated in a series of ethanol, cleared in xylol, and mounted in Canada balsam. Scolices were cleared in lactophenol and examined without pressure. Eggs were obtained by disrupting the last gravid segments. All measurements are given in micrometers. In *M. gerbei*, no fully gravid individual was found. Metric characters were considered for the scolex, mature segments, and eggs (Table II).

Multilocus enzyme electrophoresis

A total of 49 worms were analyzed by MEE. Parts of the strobila that were not used in morphological studies were frozen in liquid nitrogen in the field, and these served as a source of enzymes for electrophoretic analyses. The electrophoretic procedure was performed using the method described in Pasteur et al. (1987). A total of 10 enzyme systems were studied: aspartate amino transferase (AAT, E.C. 2.6.1.1), adenylate kinase (AK, E.C. 2.7.4.3), glucose phosphate isomerase (GPI,

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TABLE I. Samples of host species parasitized by *R. asymmetrica* and individual worm intensities.*

Site (year)	*C. glareolus* (n = 169)			*M. agrestis* (n = 25)			*M. arvalis* (n = 82)			*M. gerbei* (n = 15)			Total hosts	Total
	NS	NH (P)	NW	NS	NH (P)	NW	NS	NH (P)	NW	NS	NH (P)	NW	(n = 291)	worms
Vall d'Aran (1994, 1997)	25	1 (4.0)	1	8	1 (12.5)	1	46	12 (26.0)	5, 1, 1, 3, 1, 1, 1, 1, 1, 1, 1, 1		—	—	14 (17.7)	20
Eugi (1995–1996)	66	2 (3.0)	1, 1	17	5 (29.4)	1, 5, 3, 2, 3		—	—	15	2 (13.3)	1, 3	9 (9.1)	20
Moulis (1997)	78	1 (1.2)	1		—	—	36	5 (13.8)	2, 2, 2, 1, 1		—	—	6 (5.2)	9
Total hosts		4 (2.4)			6 (24.0)			17 (20.7)			2 (13.3)		29 (9.9)	
Total worms			4			15			26			4		49

* Abbreviations: NS, number of hosts per site; NH, number of parasitized hosts; P, prevalence in per cent; NW, number of individual worms per host.

E.C. 5.3.1.9), malate dehydrogenase (MDH, E.C. 1.1.1.37), malic-enzyme (ME, E.C. 1.1.1.40), mannose phosphate isomerase (MPI, E.C. 5.3.1.8), nucleoside phosphorylase (NP, E.C. 2.4.2.1), peptidases (PEPC, PEPD, E.C. 3.4.1.1, 3), phosphoglucomutase (PGM, E.C. 2.7.5.1). The NP system displayed 1 enzymatic pattern, which reflects the expression of 2 separated loci, NP1 and NP2.

Statistical methods

Metric data obtained from worms isolated from the 3 host species (*C. glareolus*, *M. agrestis*, and *M. arvalis*) were evaluated using Student's *t*-test. Significant levels were considered at $P < 0.05$ and $P < 0.001$. Discriminant multivariate analysis using all morphometric variables was performed to determine the *P* levels. Mahalanobis distances and percentage of correct classification of worms assigned to a single host, or geographical origin of host, or both, were calculated. Because of the low number of individuals found in *M. gerbei*, data for these worms were omitted from the statistical analysis. For all statistical studies the software SPSS 9.0 was used.

RESULTS

The worms isolated from the host species in the 3 localities were identified as *R. asymmetrica* following Baer and Tenora (1970) and Tenora and Murai (1972). The metric parameters studied in all individuals are given in Table II. In hosts infected by 1 or more individuals, intra- and interselfing were found in segments with fully developed ovaries and testes. Measurements of the scolices and eggs from distinct host species from the same and from different localities did not show significant differences in any parameter studied. In mature segments all features except the width of the external seminal vesicle showed significant differences ($P < 0.05$; $P < 0.001$), at least between individuals from 2 host species (Table III). The most dissimilar worms were those in *C. glareolus* and *M. arvalis*. In general, worm measurements from *M. arvalis* were larger in all the parameters measured. The size of testes was similar to the size of those from *M. agrestis*. The general minimal mean values for sexual structures were found in segments from *M. gerbei*. Multivariate discriminant analysis showed correct classification of all the worms with respect to host identity. All variables were selected in the model, except for width of cirrus sac, length of seminal reservoir, and diameter of testes. Maximal values of Mahalanobis distances were found between worms from *C. glareolus* and *Microtus* spp. (220.1 and 227.2). The most similar individuals were isolated from *M. agrestis* and *M. arvalis* (66.91). Significant statistical differences appeared mostly between individuals from Vall d'Aran (*M. agrestis*, *M. arvalis*) and the 2 other localities, i.e., Eugi (*C. glareolus*, *M. agrestis*) (97.4) and Moulis (*C. glareolus*, *M. agrestis*) (161.4). Worms from these 2 areas differed in only 4 features. Discriminate analysis indicated that classification was correct for all the worms with respect to the geographical origin of the host. The variables used as the best discriminant features, which were also used to differentiate between individuals isolated from different hosts, were length of cirrus sac and diameter of ovary. Statistical differences in 7 characters were found (in all cases at $P < 0.001$) in worms from *M. arvalis* in Moulis and Vall d'Aran. Discriminant analysis of host species and geographical origin showed that minimal Mahalanobis distances were between *M. agrestis* from Eugi and *M. arvalis* from Moulis and Vall d'Aran (118.4; 183.8).

Multilocus enzyme genotypes at 11 loci displayed a lack of genetic variability within and between parasite populations. In-

TABLE II. Metrical data (μm) of *R. asymmetrica* from *C. glareolus, M. gerbei, M. arvalis*, and *M. agrestis*.

	C. glareolus		*M. gerbei*		*M. arvalis*		*M. agrestis*	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Width of scolex	276.5 ± 10.9	268.8–284.2	268.8	—	254.1 ± 28.8	192–286.7	270.5 ± 15.4	256–286.7
Length of scolex	221.4 ± 41.6	192–250.9	199.7	—	205.6 ± 27.2	151.0–243.2	221.0 ± 5.9	217.6–227.8
Maximal sucker diameters	131.8 ± 5.4	128–135.7	140.8	—	132.8 ± 22.7	87.0–166.4	123.7 ± 19.5	102.4–140.8
Length of rostellum	64 ± 1.4	64	64	—	57.7 ± 9.1	38.4–64	64 ± 12.8	58.9–84.5
Width of rostellum	60.2 ± 1.8	58.9–61.4	56.3	—	46.4 ± 15.6	23.0–76.8	71.7 ± 12.8	51.2–76.8
Length of rostellar sac	166.4 ± 36.2	140.8–192	169.0	—	156.2 ± 27.0	97.3–186.9	204.8 ± 35.8	169.0–240.6
Width of rostellar sac	99.8 ± 32.6	76.8–122.9	102.4	—	87.8 ± 28.0	58.9–151.0	99.8 ± 16.8	81.9–115.2
Total number of hooks	22 ± 1.4	21–23	22	—	22.8 ± 1.4	21–25	21 ± 1.0	20–22
Total length of hooks	14.1 ± 1.8	12.8–15.4	15.4	—	16.8 ± 2.2	12.8–20.48	16.2 ± 1.4	15.4–17.9
Length of blade hooks	10.2 ± 0.1	10.2	10.2	—	9.4 ± 1.2	7.7–10.2	9.4 ± 1.4	7.7–10.2
Maximal diameter of testes	110.6 ± 7.3	103.2–123.7	76.5 ± 3.4	73.4–80.2	139.1 ± 19.6	102.4–166.4	131.8 ± 27.3	93–176.6
Minimal diameter of testes	81.6 ± 4.8	76.8–88	70.1 ± 1.4	68.3–70.8	99.0 ± 33.0	49.5–128	103.0 ± 18.6	80.2–126.3
Length of cirrus sac	186.5 ± 10.6	171.5–204.8	189.4 ± 4.4	184.3–192	220.4 ± 10.1	204.8–238.1	208.2 ± 12.0	204.8–225.3
Width of cirrus sac	35.8 ± 3.4	30.7–38.4	37.5 ± 2.9	35.8–41.0	48.1 ± 7.6	38.4–56.3	38.1 ± 3.5	30.7–43.5
Length of internal seminal vesicle	180.6 ± 10.1	163.8–197.1	149.3 ± 9.0	58.7–150.8	110.6 ± 35.7	87.0–115.2	171.0 ± 53.9	76.8–212.5
Width of internal seminal vesicle	31.8 ± 2.9	28.2–35.8	35.8 ± 2.6	33.3–38.4	46.9 ± 6.5	38.4–53.7	35.8 ± 2.6	30.7–38.4
Length of external seminal vesicle	76.5 ± 12.9	64–107.5	69.1 ± 8.9	64–79.4	117.8 ± 36.9	76.8–138.2	85.3 ± 24.1	64–120.3
Width of external seminal vesicle	64.6 ± 8.6	51.2–81.9	57.2 ± 6.4	51.2–64	61.4 ± 10.5	51.2–84.48	66.0 ± 7.9	58.9–81.9
Length of seminal receptacle	152.2 ± 16.2	128–179.2	115.2 ± 12.8	102.4–128	181.5 ± 25.8	140.8–207.4	183.6 ± 75.9	76.8–307.2
Width of seminal receptacle	81.7 ± 12.0	64–102.4	56.3 ± 0	56.3	72.2 ± 20.9	38.4–89.6	62.4 ± 12.9	38.4–84.5
Length of ovary	353.3 ± 38.4	268.8–396.8	273.1 ± 19.5	256–294.4	393.9 ± 110.3	294.4–550.4	364.6 ± 89.3	102.4–486.4
Width of ovary	139.1 ± 18.9	102.4–166.4	130.6 ± 13.5	115.2–140.8	103.8 ± 18.6	76.8–140.8	128.3 ± 16.8	115.2–353.3
Vagina length	248.9 ± 26.5	204.8–294	156.2 ± 9.2	148.5–166.4	233.8 ± 37.9	184.3–294.4	229.5 ± 20.2	204.8–268.8
Length of vitellarium	140.5 ± 15.4	115.2–161.3	157.0 ± 13.1	145.9–171.5	156.4 ± 28.6	128–204.8	162.4 ± 17.1	128–192
Width of vitellarium	48.4 ± 7.3	38.4–58.8	68.3 ± 9.0	58.9–76.8	61.1 ± 13.5	43.5–89.6	60.6 ± 4.6	51.2–64
Length of eggs	54.3 ± 4.8	50.5–60.6	—	—	50.0 ± 1.7	47.5–51.5	45.9 ± 1.0	45.4–47.5
Width of eggs	45.2 ± 4.1	41.4–50.5	—	—	42.2 ± 4.4	36.4–46.5	39.9 ± 1.7	37.4–41.4
Length of oncosphera	29.5 ± 0.9	28.3–30.3	—	—	28.3 ± 1.8	26.3–30.3	22.2 ± 1.6	20.2–24.2
Width of oncosphera	20.7 ± 1.0	20.2–22.2	—	—	20.7 ± 1.0	20.2–22.2	17.2 ± 0.8	16.2–18.2
Length of internal lateral hooklets	9.6 ± 0.6	9.1–9.9	—	—	9.5 ± 0.7	8.7–9.7	8.9 ± 0.1	8.8–9.0
Length of external lateral hooklets	9.3 ± 0.3	9.0–9.3	—	—	8.6 ± 0.1	8.4–8.9	8.6 ± 0.1	8.5–8.7
Length of central oncosphera hooklets	10.1 ± 0.05	10.1–10.2	—	—	9.9 ± 0.2	9.6–10.1	9.1 ± 0.1	9.0–9.2

* Cestodes studied: 2 from *C. glareolus*, 1 from *M. gerbei*, 15 from *M. arvalis*, and 10 from *M. agrestis*.

TABLE III. Statistical differences in metrical characters in the level of mature segments between individuals from different host species, host localities, and the same host in different localities.*

	P values						
	Hosts			Localities			Host (localities)
	Cg–Ag	Cg–Ar	Ag–Ar	M/E	M/V	E/V	Ar (M/V)
Length of cirrus sac	<0.001	<0.001	<0.05		<0.05	<0.05	
Width of cirrus sac		<0.001	<0.05		<0.001	<0.001	<0.001
Length of internal seminal vesicle		<0.001	<0.05				
Width of internal seminal vesicle	<0.05	<0.001	<0.001		<0.001	<0.001	<0.001
Length of external seminal vesicle		<0.05	<0.05		<0.05	<0.05	
Width of external seminal vesicle							
Length of seminal receptacle		<0.05			<0.05		
Width of seminal receptacle						<0.001	<0.001
Length of ovary							<0.001
Width of ovary		<0.001	<0.05		<0.05	<0.001	
Length of vitellarium	<0.05					<0.05	<0.001
Width of vitellarium	<0.001	<0.05		<0.001	<0.001		
Vagina length				<0.05	<0.05		
Maximal diameter of testes	<0.05	<0.001			<0.001	<0.05	<0.001
Minimal diameter of testes	<0.05			<0.05	<0.001	<0.05	<0.001

* Abbreviations: Cg, *C. glareolus*; Ar, *M. arvalis*; Ag, *M. agrestis*; M, Moulis; V, Vall d'Aran; E, Eugi.

deed, a unique allele was encountered at each locus investigated for all the individuals.

DISCUSSION

Identification of *Rodentolepis* species is difficult because of morphological polymorphism, wide geographical distribution, and, in several cases, apparent low host specificity. Consequently, the present host range of these species is unclear because of dubious faunistic data and incomplete morphologic and metric descriptions. The most recent studies of *R. asymmetrica* (Baer and Tenora, 1970; Tenora and Murai, 1972) redefined the species. This species differs from other armed hymenolepidids from rodents, in morphologic and morphometric characters and in its life cycle (Murai, 1989). Confusion regarding the status of *R. straminea* (Goeze, 1782), *R. microstoma* (Dujardin, 1845), *H. diminuta* Rudolphi, 1810, *Hymenolepis arvicolae* Galli-Valerio, 1930, and *Hymenolepis ampla* Erhardova, 1955 have been discussed and resolved (Baer and Tenora, 1970; Tenora and Murai, 1972).

In myomorph rodents, *R. asymmetrica* was reported from *C. glareolus*, *M. arvalis*, *M. agrestis*, *M. nivalis*, *M. subterraneus*, *M. tatricus*, *M. gerbei*, *Arvicola terrestris*, and *Apodemus flavicollis* (Schmidt, 1986). The metric data for *R. asymmetrica* in a range of hosts and also in the same host but in several localities are highly variable. Tenora and Murai (1972) studied the variability of *R. asymmetrica* in *M. agrestis*, *M. arvalis*, and *M. subterraneus* from 3 different localities, and they reported that the sexual structures of this worm vary greatly between different hosts and localities, between different hosts in the same locality, and also between the same host in different localities. These authors, however, considered data ranges only, without statistical comparisons.

Rodent hymenolepidids, like other cestode families, exhibit a high degree of morphological variability. The sources of this variability are poorly known, except for the crowding effect (Smyth and Mc Manus, 1989). Because parasite intensities were low, this effect was not considered further. A few studies have combined morphological and biochemical approaches (De Vos et al., 1990; Reversat et al., 1991; Snabel et al., 1994; Hanzelová, Snabel et al., 1995). Most of these have led to the differentiation of biological species, which do not show a clear discriminant morphology. The relation between morphological variability and genetic heterogeneity is controversial. Baverstock et al. (1985) found that both characteristics are uncorrelated in *Progamotaenia festiva* (Rudolphi, 1819), whereas De Vos et al. (1990), using isoenzyme analysis of *Dipyllobothrium dendriticum* (Nitzsch, 1824), detected a relationship between morphology and isoenzyme patterns of malate dehydrogenase and esterase. In *Proteocephalus exiguus* La Rue, 1911, a cestode with remarkable morphological variability (Hanzelová, Scholtz et al., 1995), genetic flexibility was suggested by the polymorphism detected in several loci by starch electrophoresis (Snabel et al., 1994) and DNA studies (Siles-Lucas et al., 1995). *Rodentolepis asymmetrica* showed an absence of polymorphism in all the loci tested. The individual intensities detected in the hosts in the present study, with generally 1 specimen per host (Table I), indicate that self-fertilization should be the most common form of reproduction. Moreover, the electrophoretic study of more than 1 individual from a single host revealed the same pattern of genetic uniformity. *Rodentolepis asymmetrica* appears to be self-fertilizing from the time the single gravid specimens occurred commonly in the host sample, and the same isoenzyme patterns were observed in all the individuals from a single host. It has been proposed that self-fertilization in *Echinococcus* spp. reduced the global genetic variability observed (Smyth and Smyth, 1964).

In a biochemical study of *Progamotaenia* spp. cestodes from marsupials, Baverstock et al. (1985) reported little evidence of coespeciation of parasites and their hosts and postulated the existence of host switching in the past, with the differentiation

of new evolutionary linages and a possible role of intermediate hosts in *Progamotaenia* speciation. In the present study *R. asymmetrica* does not occur in a specific host, with full development occurring in several species of Arvicolidae (Tenora and Murai, 1972). Although fully gravid *R. asymmetrica* were not found in *M. gerbei*, this species has been reported as a potential host for the complete development of the parasite (Feliu et al., 1997).

The apparent absence of genetic variation of *R. asymmetrica* at the species and population levels is unusual according to the isozyme variability in other species of Hymenolepididae (Novak et al., 1989). Likewise, the present results contrast with the level of allozyme diversity observed in populations of other self-fertilizing species, with an apparent loss of genetic diversity in laboratory conditions (Okamoto et al., 1997).

At the species level the absence of variation could be explained by the fact that the populations sampled in this study originated from only 1 part of the whole distribution area of *R. asymmetrica* in Europe. Other studies comparing geographically widespread and geographically restricted groups of cestode species have found a positive correlation between the amount of genetic variation and the size of geographical range (Snabel et al., 1994). Also, it could be expected that central populations of widespread species are usually more variable than marginal ones. Given that the sampled populations are marginal for the whole distribution area of the species, and are restricted to the Pyrenean mountains, the absence of variation in *R. asymmetrica* is less surprising.

At the population level the fixed allozyme pattern observed may be a consequence of a high degree of inbreeding as a result of its highly autogamous breeding system (Trouvé et al., 1996). Although no studies of the present outcrossing rate have been made in *R. asymmetrica*, the most common intensity of 1 individual per host indicated that outcrossing in this species is extensive. In the present morphological study all hymenolepidids exhibited self-fertilization in the same segment and in adjacent segments. This indicates that the self-fertilizing mode of reproduction is common and that there is an absence of morphological barriers to prevent outcrossing.

The amount of genetic variation in the founding individuals, the length of time the effective population remained small, and the levels of gene flow could have determined the invariant pattern we observed for *R. asymmetrica*. At present, there is not enough information to conclude which of these factors is the most important. It can only be concluded that stochastic factors, selection pressures, or a combinations of these forces have caused the absence of variation in the *R. asymmetrica* populations sampled in the Pyrenean mountains.

Finally, these results on *R. asymmetrica* of several arvicolid species confirm that the morphological and metrical criteria reported by Tenora and Murai (1972) are reliable discriminant features for identifying *R. asymmetrica*.

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