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Whole-Body Enantiomorphy and Maternal Inheritance of Chiral Reversal in the Pond Snail *Lymnaea stagnalis*

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Sinistral and dextral snails have repeatedly evolved by left–right reversal of bilateral asymmetry as well as coiling direction. However, in most snail species, populations are fixed for either enantiomorph and laboratory breeding is difficult even if chiral variants are found. Thus, only few experimental models of chiral variation within species have been available to study the evolution of the primary asymmetry. We have established laboratory lines of enantiomorphs of the pond snail *Lymnaea stagnalis* starting from a wild population. Crossing experiments demonstrated that the primary asymmetry of *L. stagnalis* is determined by the maternal genotype at a single nuclear locus where the *dextral* allele is dominant to the *sinistral* allele. Field surveys revealed that the *sinistral* allele has persisted for at least 10 years, that is, about 10 generations. The frequency of the *sinistral* allele showed large fluctuations, reaching as frequent as 0.156 in estimate under the assumption of Hardy–Weinberg equilibrium. The frequency shifts suggest that selection against chiral reversal was not strong enough to counterbalance genetic drift in an ephemeral small pond. Because of the advantages as a model animal, enantiomorphs of *L. stagnalis* can be a unique system to study aspects of chirality in diverse biological disciplines.

Most animals exhibit bilateral symmetry in external body plans. However, internal organs such as digestive tracts, kidneys, and lungs are generally asymmetric (Odhner 1919; Wood 1997; Levin 2005; Hozumi et al. 2006). The direction of visceral asymmetry corresponds to the polarity of the primary asymmetry which appears in early development (Levin 2005; Hozumi et al. 2006), whereas, for example, the mouth of scale-eating fish (Hori 1993) and snake mandibles (Hoso et al. 2007) exhibit the secondary asymmetry which develops independently of the primary asymmetry. Mutation of the primary asymmetry is known to produce whole-body chiral dimorphism (enantiomorphs) by left–right reversal throughout development (Freeman and Lundelius 1982; Félix et al. 1996; Levin 2005; Hozumi et al. 2006). However, "mirror-image" species, which are reversed in bilateral visceral asymmetry, have not evolved in most groups of animals. In contrast, both dextral and sinistral species have repeatedly evolved in gastropods by left–right reversal of the primary asymmetry (Vermeij 1975; Gittenberger 1988; Robertson 1993; Asami et al. 1998; Ueshima and Asami 2003). Coiling direction is secondary asymmetry, which does not necessarily correspond to the primary asymmetry in polarity (Robertson 1993). In several species of pulmonates, however, the polarity of spiral cleavage corresponds to both the bilateral handedness of visceral mass and the direction of coil developing later (Crampton 1894; Camey and Verdonk 1970; Freeman and Lundelius 1982). Thus, in pulmonates, clockwise and anticlockwise coils are likely to indicate dextral and sinistral whole-body enantiomorphs reversed in the primary asymmetry.

Despite the relatively frequent evolution of chiral reversal, enantiomorphy is rarely found within species of snails (Johnson 1982; Gould et al. 1985; Asami et al. 1998). Because of left–right reversal in the location of the genital orifice on the body side and mating behaviors, copulation between enantiomorphs is expected to be physically difficult and has been shown to be the case in several terrestrial pulmonates (Lipton and Murray 1979; Johnson 1982; Asami et al. 1998; Gittenberger 1988; Ueshima and Asami 2003; but see Schilthuizen et al. 2007; Sutcharit et al. 2007). Thus, the less common morph suffers difficulties in mating with the common morph, which results in positive frequency-dependent selection (Johnson 1982; Asami et al. 1998; Ueshima and Asami 2003) and explains chiral monomorphism within populations of internally fertilizing gastropods. However, externally fertilizing snails and animals other than gastropods also exhibit little chiral variation in the primary asymmetry, despite the theoretical easiness of interchiral mating. In attempts to explain the generality of directional primary asymmetry, developmental or phylogenetic constraint has been proposed (Vermeij 1975; Gould and Lewontin 1979;

Gould et al. 1985). However, the constraint or purifying selection against chiral reversal has been little substantiated and remains controversial (Johnson 1987). Similarly relative difficulties of interchiral mating remain untested in most snail groups, primarily because of technical difficulties of breeding and the rarity of chiral variation in natural populations. For those reasons, chiral variants of gastropods that are easily bred in captivity are precious resources for studies of chiral evolution in animals.

Breeding experiments have shown in 4 phylogenetically independent families of pulmonates (Wade et al. 2006) that either sinistral or dextral form is dominant in maternal inheritance (Toyama 1913) at a single nuclear locus (Sturtevant 1923; Boycott et al. 1930; Degner 1952; Murray and Clarke 1976; Ueshima and Asami 2003, Figure 1). In *Lymnaea stagnalis* (L.), before the rediscovery of Mendel's law, chiral reversal has been reported to have a genetic basis, based on the production of sinistral progeny by one of sinistral variants collected in the wild (Anonymous 1872; Collin 1872/3). In *L. peregra* (Müller), the *sinistral* allele is recessive to the *dextral* allele because only the latter allele produces the chiral determinant, according to the results of cytoplasmic transplantation between dextral and sinistral eggs (Freeman and Lundelius 1982). In that system, siblings produced by the same mother should typically be identical in chiral phenotype because of determination by the maternal nuclear genotype. However, some laboratory lines of *L. peregra* were producing mixed broods of enantiomorphs, which were consistently either mostly dextral or sinistral (Boycott and Diver 1923; Boycott et al. 1930). Freeman and Lundelius (1982) explained it by somatic recombination within a chiral-determinant gene, prompting many questions for further research. Meanwhile, all the laboratory lines of enantiomorphs of *L. peregra* descending from the lines established by Boycott and Diver (1923) have been lost (Freeman G, personal communication). Until now, no equivalent model system of enantiomorphs has been available. We have established laboratory lines of enantiomorphs using sinistral variants discovered in a natural population of dextral *L. stagnalis* (Hierck et al. 2005). An earlier study using the present system has suggested that homologues of 5 genes that are involved in the development of vertebrate asymmetry are differentially expressed in reproductive organs between enantiomorphs in *L. stagnalis* (Hierck et al. 2005).

Here, we document the mode of chiral inheritance and persistence of enantiomorphy in a natural population in *L. stagnalis*. Considering the long history of dextral *L. stagnalis* as a model animal (Morrill 1982; Meshcheryakov 1990; Endo et al. 2004; Hierck et al. 2005), our study provides a critical basis for using the enantiomorphy of *L. stagnalis* for further research of biology and evolution of chirality.

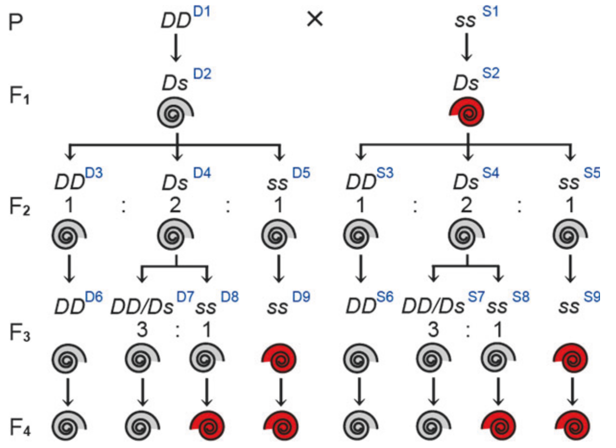

Figure 1. Prediction of chiral inheritance according to the model of maternal inheritance determined by the dominant *dextral* (*D*) and recessive *sinistral* (*s*) alleles at a single nuclear locus. Red and gray spirals indicate sinistral and dextral phenotypes, respectively. Superscripts of each genotype correspond to the genotype number in Table 1. The model predicts that *Ds* and *DD* genotypes expected in F_2 generation can be identified by the presence and absence of sinistral hatchlings in F_4 descendants. However, *Ds* and *DD* genotypes in F_3 generation are not distinguishable without F_5 generation.

Materials and Methods

Crossing Experiment

We used the dextral and sinistral laboratory lines of *L. stagnalis*, which originate from 10 adult dextrals and 7 sinistral individuals collected from a natural pond (48°48′57″N, 11°44′10″E) in Neustadt an der Donau, Germany in 2000. No sinistral has appeared in descendants of the dextrals kept in a single aquarium, which suggested that this line is homozygous of the *dextral* allele (*D*). However, all 7 sinistrals produced only dextrals, suggesting that *D* is dominant to the *sinistral* allele (*s*) and that the wild caught sinistrals were heterozygotes, following the maternal inheritance model (Toyama 1913; Sturtevant 1923; Boycott et al. 1930, Figure 1). Because of probable insemination before being collected, we kept the sinistrals in individual isolation and discarded their progeny for 2 months, during which each individual laid 10–20 clutches. After this, we collected newly hatching dextrals produced by presumable selfing of each of the 7 sinistrals and let those offspring also self in individual isolation. We pooled sinistrals produced by some of those dextrals and found no dextral variants in their later generations. We fed them on lettuce at 25 °C in aquaria with limestone gravel, where the generation time was ordinarily less than 2 months. Thus, for the present crossing experiments in 2003, we used descendants later than 12 generations because chiral variation had disappeared from the sinistral line.

We confirmed that the enantiomorphs of *L. stagnalis* used in the present study are reversed in left–right polarity of the primary asymmetry by examinations of visceral bilateral asymmetry and of spiral cleavage at the 4-cell and 8-cell stages of embryos. Based on one-to-one correspondence between the polarity of the primary asymmetry and the coiling direction, we identified individual chiral phenotypes by shell coiling direction in the subsequent crossing experiments.

Table 1. Maternal inheritance of chiral reversal in interchiral-crossing experiments in *Lymnaea stagnalis*

Genotype number in Figure 1	Generation	Cross type	Phenotype	Hypothetical genotype	Expected offspring phenotype	Observed offspring phenotype		Expected ratio of offspring genotype			Confirmed offspring genotype			χ^2 test probability
						D	S	*DD*	*Ds*	*ss*	*DD*	*Ds*	*ss*	
D1	P	D × S	D	*DD*	D	356	0	0	1	0	0	19	0	
D2	F_1	Selfed	D	*Ds*	D	415	0	1	2	1	19	28	16	0.588
D3	F_2	Selfed	D	*DD*	D	468	0	1	0	0	36^a		0	
D4	F_2	Selfed	D	*Ds*	D	434	0	1	2	1	54^a		21	0.549
D5	F_2	Selfed	D	*ss*	S	0	406	0	0	1	0^a		48	
D6	F_3	Selfed	D	*DD*	D	358	0	1	0	0	—	—	—	
D7	F_3	Selfed	D	*DD* or *Ds*	D	503	0	3	2	1	—	—	—	
D8	F_3	Selfed	D	*ss*	S	0	357	0	0	1	—	—	—	
D9	F_3	Selfed	S	*ss*	S	0	285	0	0	1	—	—	—	
S1	P	D × S	S	*ss*	S	0	367	0	1	0	0	23	0	
S2	F_1	Selfed	S	*Ds*	D	490	0	1	2	1	11	33	18	0.399
S3	F_2	Selfed	D	*DD*	D	499	0	1	0	0	48^a		0	
S4	F_2	Selfed	D	*Ds*	D	400	0	1	2	1	59^a		15	0.347
S5	F_2	Selfed	D	*ss*	S	0	509	0	0	1	0^a		35	
S6	F_3	Selfed	D	*DD*	D	363	0	1	0	0	—	—	—	
S7	F_3	Selfed	D	*DD* or *Ds*	D	461	0	3	2	1	—	—	—	
S8	F_3	Selfed	D	*ss*	S	0	414	0	0	1	—	—	—	
S9	F_3	Selfed	S	*ss*	S	0	417	0	0	1	—	—	—	

D, dextral; S, sinistral.

[a] Either *DD* or *Ds* according to the dextral phenotype of their offspring (F_4).

To test the prediction of the genetic model (Figure 1), we crossed enantiomorphs in 2 pairs. To minimize the chance of selfing, we raised each pair from 3 weeks after hatching to maturation in a single container. We confirmed their outcrossing by genotyping parents and F_1 individuals at 1 (D5) of the 9 microsatellite loci developed by Knott et al. (2003). We chose this locus because the 2 individuals of both pairs were homozygous for different alleles. Thus, all offspring produced by outcrossing would be heterozygotes. We used the forward primer ATCATCGGCTATCCATC-CAT and the reverse primer CAGACAGAAAGTCAA-TCCG, which were redesigned based on the sequence registered in GenBank (AY225963). For that purpose, we sliced off the posterior tip (about 1 mm) of the foot from each animal after we confirmed its successful oviposition and extracted DNA from that tissue. After F_1 production by outcrossing, F_2 to F_4 generations were all produced by selfing. To test Mendelian segregation of the expected 3 genotypes in the F_2 generation by the χ^2 test, we identified *DD* and *Ds* by the absence and presence of sinistrals in their F_4 descendants, respectively, according to the prediction in Figure 1. We ended the experiment with the F_4 generation. Thus, *DD* and *Ds* of the F_3 generation were not separable from each other but only from *ss*, which was identified by the production of sinistral progeny. For that reason, the segregation ratios between *ss* and the other 2 genotypes were tested in F_3 generation.

Field Survey

Following the initial discovery of sinistral variants of *L. stagnalis* in 26 September 1991 (Hierck et al. 2005), we examined possible persistence of enantiomorphy in 25–26 August 2000, 11–12 August 2001, 19–20 September 2002, 24 August 2003, and 12 July 2007. The pond is a slender oval located 25 m from the Danube riverbank and was about 30 × 5 m at the largest size recorded in 2000 and 2007. According to the observed changes of water quantity and clay residues on plants around the pond, the pond fluctuated considerably in size with periods of drought and floods. We used a landing net of 5-mm mesh to collect samples of *L. stagnalis*. In 2000, we roughly estimated the relative abundance of *L. stagnalis* by counting the numbers of individuals obtained from the bottom to the surface in three 1-m^2 quadrates. Because the pond was small in 2001, we counted all the individuals in the entire pond by repeatedly sieving the pond from bottom to surface until no additional specimen were caught.

Results and Discussion

Maternal Inheritance of the Primary Asymmetry

We found no evidence against the prediction of maternal inheritance of chiral reversal at a single nuclear locus, where the dextral is dominant to the sinistral (Table 1, Figure 1). Siblings produced by each parent all developed the same phenotype, either dextral or sinistral. We found no significant differences in the segregation ratios of the genotypes in either the F_2 or F_3 generation from the dextral or sinistral parent in either of the replicate crosses (data not shown) and thus pooled the outcome of the replicates. We detected no significant deviations from Mendelian segregation ratios of genotypes expected at a single locus with the dominant *D* and recessive *s* alleles, in either the F_2 or the F_3 generation (Table 1, χ^2 test; $P = 0.588$ and 0.549 in the dextral lineage and $P = 0.399$ and 0.347 in the sinistral lineage, respectively). The genetic model also predicts that the dextral and the sinistral lineages exhibit identical patterns of chiral inheritance from the F_2 generation in Figure 1. We found no significant differences between the chiral lineages (Fisher exact test; $P = 0.281$ in F_2 and $P = 0.339$ in F_3). Pooling the dextral and sinistral lineages also revealed no significant deviations (χ^2 test; $P = 0.856$ in F_2 and 0.813 in F_3, respectively).

The present results demonstrate that the sinistrality of the primary asymmetry is recessive to the dextrality, determined by a maternal effect of a single nuclear gene. It is similar to the genetic system of chiral reversal in *L. peregra* (Sturtevant 1923; Boycott et al. 1930). We have not, however, found exceptional lineages that produce mixed broods of dextrals and sinistrals as known in *L. peregra* (Boycott and Diver 1923; Freeman and Lundelius 1982) throughout our breeding records since the collection of the original specimens of *L. stagnalis* in 1991. Our crossing experiments showed that dextrals and sinistrals of *L. stagnalis* are capable of interchiral mating in spite of the left–right reversal of the genital orifice at the body side. Interchiral copulation has been shown to be possible in 4 phylogenetically independent groups of pulmonates, *Lymnaea* (Boycott and Diver 1923), *Laciniaria* (Degner 1952), *Partula* (Lipton and Murray 1979), and *Amphidromus* (Sutcharit et al. 2007), whereas *Helix pomatia* (L.), *Arianta arbustorum* (L.) (Gittenberger 1988), and *Bradybaena similaris* (Férussac) (Asami et al. 1998) cannot or with much difficulty copulate. Interchiral mating success, however, does not indicate the absence of physical difficulties, even if buoyancy may make it easier in aquatic habitats. To understand the strength of possible positive frequency-dependent selection against chiral reversal, we need to evaluate the relative success of interchiral mating compared with intrachiral mating.

In the ordinary mode of inheritance, deviation from the Mendelian segregation ratio of genotypes could result from unequal survival rates between phenotypes that those genotypes express. In the present tests of Mendelian segregation, even if the sinistral phenotype expressed in progeny survives less than the dextral phenotype, it does not affect the observed ratio of parental genotypes that are identical in phenotype, unless any pleiotropy or linked gene affects the survival of genotype regardless of their phenotype. Thus, the present agreement with the Mendelian ratios of genotypic segregation does not assure equal survival rate of dextrals and sinistrals. To examine possible differences in fitness between the genotypes of left–right polarity, life-history traits of their progeny need to be compared between enantiomorphs.

Persistence of Enantiomorphy

One of us (G.F.) found 3 sinistral variants of *L. stagnalis* in the pond during a survey of zoobenthic diversity in the proximity along the Danube in September 1991, but not in a similar survey in 1984. Those 3 sinistrals and several dextrals were collected to establish dextral and sinistral laboratory lines (Hierck et al. 2005). In August 2000, we found 7 sinistrals at the same locality by searching the entire pond. In average, 16.7 (±5.1 standard deviation) dextrals were collected in a 1-m^2 quadrate. The total area of water surface was 112.8 m^2 and the highest depth 1.1 m. Thus, we roughly estimated the total number of dextrals collectable by the present method at 1884. We only counted snails that were sieved by a net of 5 × 5 mm mesh and thus clearly underestimated their abundance. However, both dextrals and sinistrals were collected in the same manner. Thus, 0.0037 (7 out of 1884) would be a rough but reasonable estimate of the phenotypic frequency of sinistrals, which represents the frequency of parental *ss* genotype unless sinistrals suffer reduced survival. By assuming no viability selection and based on the genetic model supported by crossing experiments, we could estimate the frequency of parental *s* allele at 0.061. Those 7 sinistrals and 10 dextrals were collected to maintain chiral variation in additional laboratory lines.

In 2001, the water surface in the pond measured 38.4 m^2 and the highest depth 0.30 m. We repeatedly sieved the entire range of pond water till no additional specimens showed up. We found a total of 5 sinistrals and 201 dextrals. Taking the same assumptions as in the above calculations, the frequencies of sinistrals and the *s* allele can be estimated at 0.024 and 0.156, respectively. In 2002, the pond surface area was 55.4 m^2 and the highest depth 0.61 m. We found 349 dextrals but no sinistral individuals from the entire range of the pond. However, the absence of the sinistral phenotype in 2002 does not indicate the total loss of the *s* allele from the population because heterozygotes could have remained without producing sinistral offspring and thus without suffering possible negative selection. At the time of the survey in 2003, the moist clay bottom of the pond was exposed with no free water available and no living specimens were found. In 2007, the area of water surface in the pond was equivalent to that observed in 2001. However, we found no *L. stagnalis*, although other species of freshwater pulmonates such as *Planorbis planorbis* (L.), *Planorbarius corneus* (L.), *Stagnicola turricula* (Held), and *Aplexa hypnorum* (L.) were abundant as before in the pond.

Our results show that enantiomorphy had persisted in a population of *L. stagnalis* for at least 10 years from 1991 to 2001. In the climate of the region, approximately 6 months would be available for their yearly growth and reproduction. Then, there could have been one generation per year (Brown 1979). Thus, the *s* allele has persisted more than about 10 generations after the first discovery in the population. In *L. stagnalis*, only similar case has been recorded that 7 sinistrals and 23 dextrals were collected in 1871 and 20 sinistrals (number of dextrals not mentioned) in 1872, from a pond less than 100 m^2 (Pelseneer 1920, p. 737) near Aerschot, Belgium (Collin 1872/3; Anonymous 1872). Because of maternal inheritance, the frequency of recessive phenotype allows us to estimate the frequency of recessive allele in parental generation, by assuming the equal proportions of the *ss* genotype in parents and of the sinistral phenotype in offspring. Thus, if sinistrals are under viability selection, their phenotypic frequency underestimates the parental frequency of the *ss* genotype. Sinistrals could also be under positive frequency-dependent selection if interchiral mating is difficult, as known in terrestrial snails. Their selfing ability, however, would relax frequency-dependent selection, although the antagonistic effect of inbreeding depression cannot be ignored. In the present case, however, the frequency of the *s* allele has increased from 0.061 to 0.156 in one year after 10-year persistence. Thus, selection against sinistral variants may not be strong enough to counterbalance stochastic fluctuations of allelic frequency in a small population of *L. stagnalis*. It is a further subject of importance to quantify the strength of selection against chiral reversal in aquatic gastropods.

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