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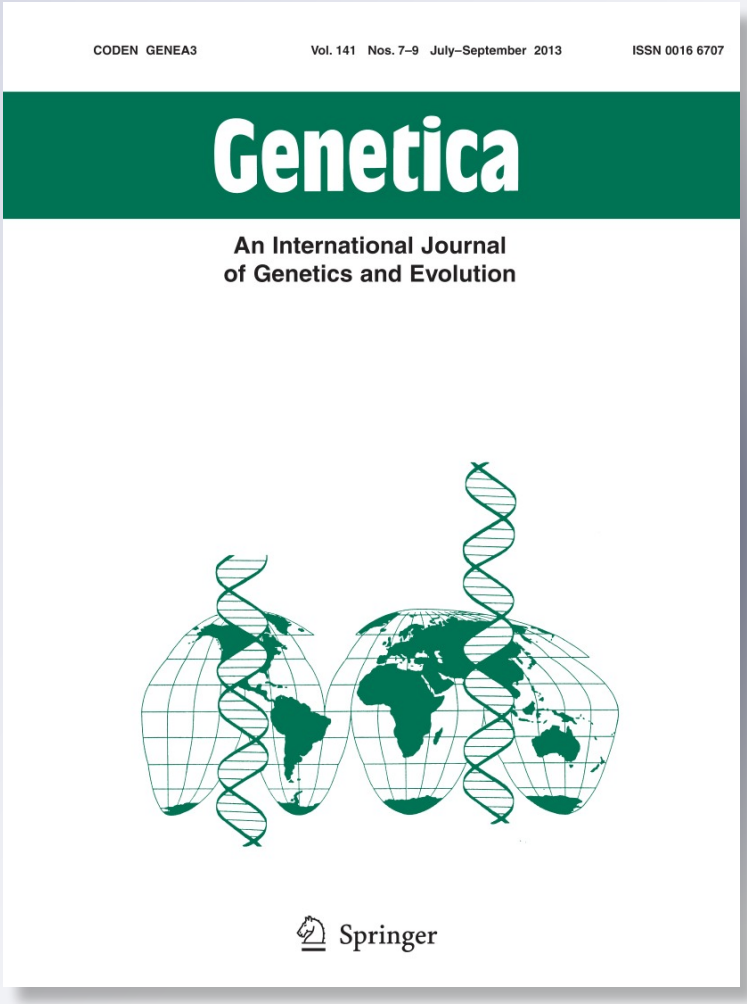

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Abstract Populations of periwinkles *Littorina saxatilis* (Olivi 1792) and *L. arcana* Hannaford Ellis, 1978 are well suited for microevolutionary studies, being at the same time closely related and intraspecifically diverse. The divergence between these two sibling species, sympatric over large parts of their distribution areas, is small, the only morphological difference being the pallial gland complex structure in females. Molecular identification is possible with the use of a RAPD nuclear marker (cloned A2.8 DNA fragment) typical for *L. arcana*. However, in some individuals from sympatric populations molecular and morphological criteria suggest conflicting species affiliation, which may be explained either by hybridization or by shared ancestral polymorphism. We tested the hybridization hypotheses examining the micro-spatial distribution of these two species across the intertidal zone in two distant sites at the Barents Sea. We found that (a) the frequency of putative hybrids in sympatric populations was proportional to the frequency of *L. arcana*; (b) *L. saxatilis* bearing A2.8 DNA fragment were almost absent in the lower part of the intertidal zone, where *L. arcana* was absent too; (c) there was a close positive correlation between the distribution of potential parent molluscs and putative hybrids. Moreover, logistic regression models showed a good agreement between the distribution of putative hybrid frequencies and that of parental species frequencies. All our observations taken together support the hypothesis of hybridization between *L. saxatilis* and *L. arcana*. Elucidating the mechanisms that support the species status of these sympatric populations is necessary.

Keywords Littorinidae · Sibling species · Sympatric population · Parental species frequencies · Hybrid frequencies · Microevolution

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Introduction

Populations of the "saxatilis" species complex (*Littorina saxatilis* (Olivi 1792), *L. compressa* Jeffreys, 1865 and *L. arcana* Hannaford Ellis, 1978) are attractive models for microevolutionary studies, including those of situations preceding and following speciation events (Grahame et al. 2006; Rolán-Alvarez 2007; Johannesson et al. 2010; Doellman et al. 2011). On the one hand, the intraspecific diversity is high, as exemplified by the ecotype formation in *L. saxatilis* populations from distant areas (NE England, NW Sweden, and NW Spain) (Johannesson 2003). On the other hand, these three species (*L. saxatilis*, *L. compressa* and *L. arcana*) are so closely related that their phylogeny has not been unambiguously resolved yet (Small and Gosling 2000; Wilding et al. 2000a, b; Reid et al. 2012).

The three periwinkles of the "saxatilis" complex are very similar morphologically and can be regarded as sibling species, but *L. compressa* can be distinguished from

the other two by fine conchological features (Reid 1996). Moreover, both males and females of *L. compressa* possess certain species-specific features in the anatomy of the reproductive system. Discrimination between *L. saxatilis* and *L. arcana* is more challenging, the only, though reliable, diagnostic character being the structure of the pallial gland complex of the females (Reid 1996). In addition (and closely related to the differences in the pallial gland), *L. arcana* is egg-laying while *L. saxatilis* gives birth to small crawl-away juveniles.

Littorina arcana and *L. saxatilis* are sympatric over large parts of their distribution areas (Reid 1996). These species may hybridize under laboratory conditions, and the hybrid frequency of 2 % was predicted to be present in the field (Warwick et al. 1990). However, field studies in a UK site failed to reveal any ongoing hybridization (Ward and Janson 1985). Earlier we have revealed a RAPD nuclear marker (cloned A 2.8 DNA fragment) typical for *L. arcana*, which is always absent in *L. saxatilis* from allopatric populations but present in about 20 % of *L. saxatilis* individuals in populations sympatric with *L. arcana* (Mikhailova et al. 2009). The presence of the analyzed fragment in such populations may be explained either by hybridization, if we suggest that it is specific for *L. arcana* (Mikhailova et al. 2009), or, alternatively, by shared ancestral polymorphism (see e.g. Wilding et al. 2000a, b).

In order to critically assess the hybridization hypothesis, we analyzed the micro-spatial distribution of the two periwinkles species in sympatric sites. Our aim was to test whether the actual occurrence of the hybrids was in accordance with the predictions made on the basis of the distribution of parental species across the intertidal. If the observed distribution of the RAPD marker is due to hybridization, the ratio of *L. saxatilis* individuals bearing this fragment should be the highest in the intertidal zones with the greatest abundance of *L. arcana*. On the contrary, in the intertidal zones where *L. arcana* is absent, *L. saxatilis* individuals may be expected to lack this fragment. By the same token, the ratio of RAPD-negative *L. arcana* individuals should be the highest in the zones with the densest overlapping of *L. arcana* and *L. saxatilis* populations. Alternatively, if the distribution of the fragment is due to the shared ancestral polymorphism, the ratio of RAPD-positive *L. saxatilis* individuals and RAPD-negative *L. arcana* individuals would not depend, respectively, on the distribution of *L. arcana* and *L. saxatilis* across the intertidal. In this case, we may expect an even spatial distribution of RAPD-negative and RAPD-positive individuals with a species-specific frequency.

Materials and methods

General research outline

Periwinkles were collected at two distant (more than 1,000 km apart) intertidal sites of the Barents Sea (Fig. 1): the Yarnyshnaya Inlet (Eastern Murman, Russia) and the Telegraph Inlet on Tromso Island (Western Barents Sea,

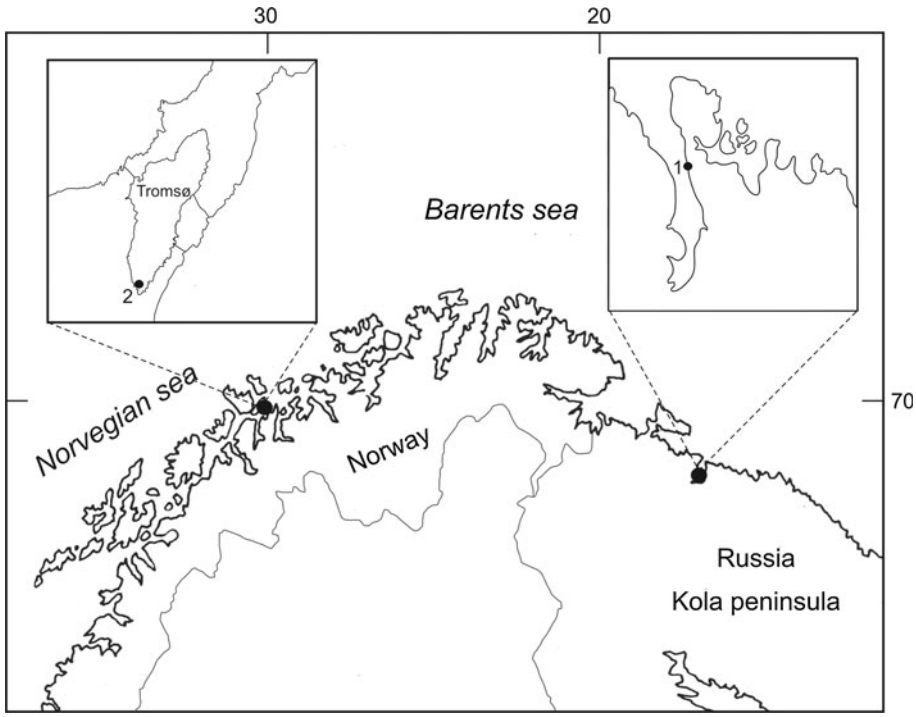

Fig. 1 The map showing macro- and micro-geographic location of the transects. Population 1 (Yarn08) located in Yarnyshnaya Inlet (Eastern Barents Sea). Population 2 (Troms09) located in Telegraph Inlet on Tromso Island (Western Barents Sea)

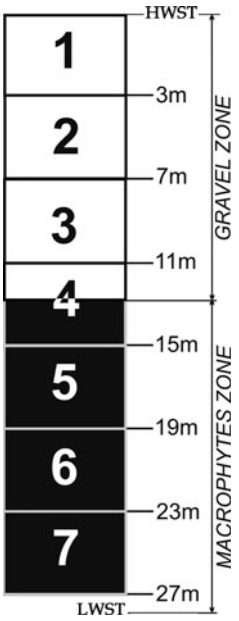

Fig. 2 The transect's diagram showing the levels (1–7, from High Water Spring Tide (HWST) down to Low Water Spring Tide (LWST) line), their width (m) and position of macrophyte zone and gravel zone

Norway). The sampling was carried out in August, 2008 and in August, 2009, respectively. At both sites we chose for sampling a 28-m-wide sheltered zone of a gently sloping stony shore. Its lower part (12 m wide) was covered with macrophytes, while the upper part was represented by gravel.

Snails were collected at low tide along a transect from the splash zone (High Water Spring Tide level) down to the Low Water Spring Tide level. In accordance with the site and the year of sampling, the transects were designated as Yarn08 and Troms09. Both transects were divided into seven equal sections 4 m wide ("levels") (Fig. 2). At each level quantitative samples (snails from one 1/40 m^2 area each) were taken (see Table 1 for information about the number of samples and their distribution across levels along the transects).

All snails were dissected under MBI-10 binocular microscope at $7^{\times}$–$84^{\times}$ magnifications; their sex and, when possible, species was identified and the degree of parasitic infection was assessed. Morphological identification was made on the basis of species-specific features of the reproductive system (Reid 1996). Three periwinkles species were found in the samples: *L. saxatilis*, *L. arcana* and *L. compressa*. Only the former two periwinkles were involved in the analysis; *L. compressa* snails, which inhabited the lower intertidal at both study sites, were ignored.

Based on the morphology of reproductive traits, we identified the species of mature females (*L. saxatilis* or *L. arcana*) and registered, without identifying, immature females (those with the pallial glands yet unformed) and infected females (those with the pallial glands reduced due to parasitic castration by trematodes) of these two species (see Table 1). All females whose species was identified at the morphological level were later examined for the presence of the molecular marker. As there is no reliable morphological criterion for male periwinkles, their species was not identified morphologically. Mature males were scored for the presence of the molecular marker A2.8 (Mikhailova et al. 2009), while immature and infected males were not. Infected females and males were excluded from the following correspondent modelling analysis (see below).

DNA was extracted from the soft tissue (head and foot) of mature non-infected snails individually preserved in 70 % ethanol, and amplified with the A 2.8 primers. Genotypes were scored following the method described previously (Mikhailova et al. 2009).

Based on the results of morphological and molecular examination, the collected snails were distributed into the following categories (see Table 1):

1. Females, *L. saxatilis*, PCR-negative (F_{LS-});
2. Females, *L. saxatilis*, PCR-positive (F_{LS+});
3. Females, *L. arcana*, PCR-negative (F_{LA-});
4. Females, *L. arcana*, PCR-positive (F_{LA+});
5. Females, immature or infected, not tested molecularly ($F_{L?}$);
6. Males, PCR-negative ($M_{L?-}$);
7. Males, PCR-positive ($M_{L?+}$);
8. Males, immature or infected, not tested molecularly ($M_{L?}$);

The number of snails in all the categories and their distribution across the intertidal zone at both sites are presented in Table 1.

To describe the zonal distribution of any given group/category of periwinkles, we used the number of snails from this group/category divided by the total number of snails collected from this level. We also calculated exact binomial confidence intervals for every proportion investigated (using STATISTICA 6.0).

The distribution of *L. saxatilis* and *L. arcana* was assessed in two sympatric populations with similar population density but different proportion of sibling species. Zonal distribution of PCR + molluscs was assessed in Yarn08 and in Troms09 there the frequency of such individuals among *L. saxatilis* was very low and the population density of *L. arcana* was low too. The deviation of the observed distribution of PCR + molluscs from the average value in the population was assessed in three intertidal level groups: the upper (data from levels 1 and 2), the middle (levels 3 and 4) and the lower (levels 5, 6 and 7). The analysis of data from all the levels (1–7) was hampered by the fact that the values of the expected frequencies were often less than 5, which could distort the statistic Chi square assessment.

Table 1 The number of collected and analyzed molluscs of various categories in different levels of the intertidal zone in the study sites

Morphological criterium:		*L. saxatilis*, females		*L. arcana*, females		Females, species not identified ($F_{L?}$)		Males, species not identified ($M_{L?}$)				SUM 1 All collected molluscs	SUM 2 All collected females	SUM 3 All females used for PCR and morphological identification
Molecular marker:		$F_{LS}-$	F_{LS+}	$F_{LA}-$	F_{LA+}	$F_{L?}$ immat.	$F_{L?}$ infected	$M_{L?}-$	$M_{L?+}$	$M_{L?}$ immat.	$M_{L?}$ infected			
Levels	Samples	Number of individuals in different categories										SUMs 1–3		
Yarnyshnaya Inlet, transect Yarn08														
1 level	8	14	5	1	0	18	4	23	10	6	1	82	42	20
2 level	8	12	8	4	4	16	8	12	7	9	4	84	52	28
3 level	8	18	7	13	7	9	8	16	7	16	7	108	62	45
4 level	4	6	1	24	3	20	15	17	5	9	10	110	69	34
5 level	4	28	0	6	4	17	14	5	2	13	17	106	69	38
6 level	4	16	0	1	1	26	13	11	0	5	7	80	57	18
7 level	4	21	1	0	0	5	18	13	0	1	12	71	45	22
Totally	40	115	22	49	19	111	80	97	31	59	58	641	396	205
Used for PCR		137		68		Not used		128		Not used				
Tromse island, transect Troms09														
1 level	3	13	0	0	0	9	4	13	0	1	1	41	26	13
2 level	3	8	1	0	0	2	2	6	0	0	0	19	13	9
3 level	3	17	0	1	4	5	8	12	2	0	4	53	35	22
4 level	3	13	1	1	1	8	17	20	3	4	10	78	41	16
5 level	3	26	1	0	2	4	10	21	0	4	8	76	43	29
6 level	3	7	0	0	0	6	13	8	0	1	14	49	26	7
7 level	3	8	0	0	0	6	7	10	3	7	11	52	21	8
Totally	21	92	3	2	7	40	61	90	8	17	48	368	205	104
Used for PCR		95		9		Not used		98		Not used				

F_{LS}, *Littorina saxatilis* females; F_{LA}, *L. arcana* females; $F_{L?}$, females, species not identified; $M_{L?}$, males, species not identified

"+", PCR-positive individuals; "−", PCR-negative specimens

Different SUMs (1–3) are used in Figs. 3, 4, 5 (see below)

Correspondence modelling for the spatial frequency distribution

Previous studies (Mikhailova et al. 2009) suggest that the marker used in molecular diagnostics is typical for *L. arcana* (see Introduction). Therefore, we used the data on the zonal distribution of females with conflicting morphological and genetic identification (F_{LS+} and F_{LA-}) as an estimate of the number of putative "hybrid" snails in the investigated locations.

We used correlation analysis (Spearman correlation coefficient, Myers and Well 2003) to test the association between the predicted and the actual number of "hybrids" at both sites. Confidence limits for correlation coefficients were estimated using Fisher's z-transformation (Choi 1977). The following predicted frequencies of hybrids were assessed:

a. based on relative number (N) of morphologically identified females *L. saxatilis* and *L. arcana*: $N F_{LS} * N F_{LA}/(N F_{total})^2$;
b. based on relative number (N) of females with and without the molecular marker:

$$N F_- * N F_+/(N F_{total})^2.$$

As it was still possible to identify the genotypic status of male snails, we could use them for estimation of predicted number of hybrids, which yielded another estimate of predicted number of hybrids:

c. based on relative number of snails (males and females) with and without the molecular marker: $(N F_- + N M_-) * (N F_+ + N M_+)/(N_{total})^2$.

Correlations were estimated separately for Yarn08 and Troms09 and together for the pooled data from both locations.

To improve our understanding of associations between the predicted and the observed frequencies of putative "hybrids", we built regression models with the mollusc status ("hybrid" or not) as a dependent variable and predicted frequencies of hybrids as independent variables. Unfortunately, data from Troms09 were inappropriate for model construction (insufficient number of *L. arcana* found), and only data from Yarn08 were used for modelling. The predicted frequencies of hybrids were derived by multiplying the frequencies of potential parental individuals in separate levels of the intertidal zone (all samples within one level where pooled together). As we needed to model a binary outcome, the logistic regression was the most appropriate type of model. Scaled Deviance and Pearson Chi square were used to assess goodness of fit (Long 1997). As the results were essentially the same, we only present Scaled Deviance in this paper.

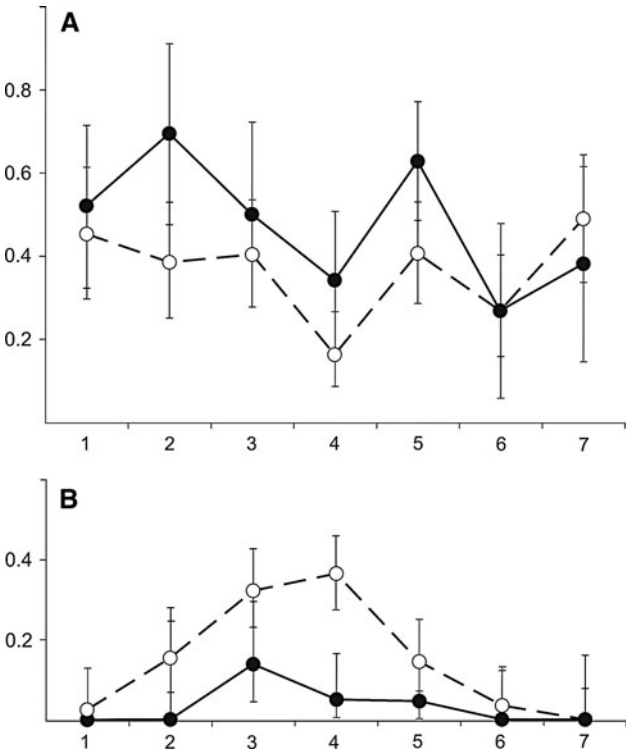

Fig. 3 Zonal patterns of *Littorina saxatilis* females (**a**) and *L. arcana* females (**b**) distribution in Yarn08 populations (*empty circles*) and Troms09 populations (*filled circles*). 1–7—levels of intertidal zone from HWST down to LWST. 0.95 % confidence limits are shown. SUMs 2 from Table 1 are used for calculations

The data yields only 7 observations (as we pooled together all molluscs from a given intertidal level, we ended up with a single data point for each of the level), which imposes strict limits in terms of the number of covariates which may be included into the model. Thus, we omitted models including more than 4 independent variables from this analysis. As we built our models consequently and as the parameters included in the model were chosen based on the results of the previous one, the detailed description of all the models is given in "Results" section.

Results

The combined average population density of *L. saxatilis* and *L. arcana* in both locations Yarn08 and Troms09 was similar, 641 and 697 snails per square meter (Table 1). The total percentage of immature and infected females and males was also similar (48.5 % in Yarn08 and 45.1 % in Troms09). Both immature and infected individuals were excluded from further analysis. Despite the same combined density, a specific proportion of *L. saxatilis* and *L. arcana* varies considerably in sympatric populations (our data). In accordance with the morphological species identifications

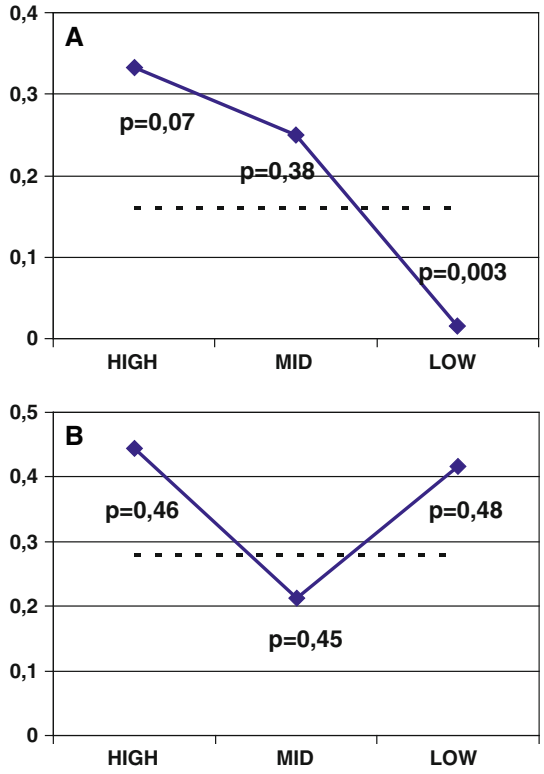

Fig. 4 Zonal patterns distribution of PCR-positive *Littorina saxatilis* (**a**) and PCR-positive *L. arcana* (**b**) in Yarn08 populations. Zones of the intertidal: HIGH (levels 1 and 2), MID (levels 3 and 4, LOW (levels 5, 6 and 7). Parts of PCR-positive mollusks are given on the Y axes. SUMs 3 from Table 1 are used for calculation. Average population value of PCR-positive molluscs is given by dotted line. Deviations from average value, tested by Chi square tests are given as *p*-values for every zone

of females, the proportion of *L. arcana* was about 50 % in Yarn08 and only 10 % in Troms09.

Distribution of morphologically identified *Littorina saxatilis* and *L. arcana*

The ratio (relative densities) of *L. saxatilis* and *L. arcana* females varied across the zones (Fig. 3a,b). Relative number of female *L. saxatilis* was higher in Troms09, but the zonal distribution of the females of this species was similar, and quite uniform, in both locations (Fig. 3a). The only substantial difference was observed in the lowermost intertidal (level 7), where the share of female *L. saxatilis* was slightly higher for Yarn08 population.

On the contrary, *L. arcana* females were unimodally distributed, with their maximum in the mid-intertidal zone (level 4) (Fig. 3b). The share of female *L. arcana* was higher in Yarn08. On the whole, the patterns of distribution of female *L. arcana* were similar in two populations, with the maximum values being observed at levels 3–5.

Distribution of individuals bearing the tested DNA fragment A2.8 (PCR + molluscs)

The frequency of PCR + molluscs was higher in *L. arcana* populations than in the sympatric *L. saxatilis* populations ($\chi^2_{(1)} = 4.01$, $p = 0.045$ for Yarn08 populations and $\chi^2_{(1)} = 52.67$, $p < 0.0001$ for Troms09 populations). At the same time, the ratio of PCR + molluscs among *L. saxatilis* individuals was much higher in Yarn08 population as compared to *L. saxatilis* from Troms09 (16 and 3 %, respectively, $\chi^2_{(1)} = 9.71$, $p = 0.002$). On the contrary, the ratio of PCR + molluscs among *L. arcana* was lower in Yarn08 population as compared to *L. arcana* from Troms09 (28 and 78 %, respectively, $\chi^2_{(1)} = 8.83$, $p = 0.003$).

The distribution of PCR + *L. saxatilis* in the upper, the middle and the lower part of the intertidal is very uneven ($\chi^2_{(2)} = 4.01$, $p = 0.004$). In the upper part of the intertidal (levels 1–2) the number of the found PCR + *L. saxatilis* is somewhat above the populational average. In the lower part of the intertidal (levels 5–7), on the contrary, the ratio of such individuals is considerably lower (Fig. 4a).

The distribution of PCR + *L. arcana* across the intertidal zone did not deviate from the populational average (Fig. 4b). No indications of the spatial unevenness of their distribution were revealed.

Table 2 Spearmen correlations (confidences are given in parentheses) of the number of putative hybrid females $F_{hybr} = (N\ F_{LS+} + N\ F_{LA\ -})$ with the relative number in the same littoral level of: —morphologically identified females *L. saxatilis* and *L. arcana* (column 1); —females with and without the molecular marker (column 2); —males and females with and without the molecular marker (column 3)

Site	F_{hybr} versus $N\ F_{LS} * N\ F_{LA}/(N\ F)^2$	F_{hybr} versus $N\ F_{-} * N\ F_{+}/(N\ F)^2$	F_{hybr} versus $(N\ F_{-} + N\ M_{-}) * (N\ F_{+} + N\ M_{+})/(N\ F + M)^2$
	1	2	3
Yarn08	**0.643 [0.170; 0.875]**	**0.750 [0.364; 0.916]**	**0.750 [0.364; 0.916]**
Troms09	**0.613 [0.123; 0.863]**	**0.885 [0.667; 0.963]**	0.449 [−0.107; 0.791]
Both sites	**0.726 [0.317; 0.907]**	**0.795 [0.457; 0.932]**	**0.748 [0.360; 0.915]**

Significant values are in bold

Table 3 The parameters and results of five consecutive logistic models

	Parameter	ν	Estimate	SE	χ^2	*p*	Deviance
Model 1	$E(\text{logit}(p(H))) = k*NF_- * NF_+/N^2$						
	Intercept	1	−1.527	0.338	20.4	<.0001	1.2568
	k	1	6.213	2.021	9.46	0.0021	
Model 2	$E(\text{logit}(p(H))) = k_1* NF_- * NM_{?+}/N^2 + k_2* NM_{?-} *NF_+/N^2$						
	Intercept	1	−3.491	0.6478	29.04	<.0001	1.0752
	k_1	1	65.081	12.644	26.5	<.0001	
	k_2	1	−1.38	8.586	0.03	0.872	
Model 3	$E(\text{logit}(p(H))) = k_1* NF_{LS-} * NM_{?+}/N^2 + k_2* NF_{LA-} * NM_{?+}/N^2$						
	Intercept	1	−2.997	0.631	22.53	<.0001	1.0635
	k_1	1	44.882	16.389	7.5	0.006	
	k_2	1	66.673	11.626	32.89	<.0001	
Model 4	$E(\text{logit}(p(H))) = k * NF_{LS-} * NM_{?+}/N^2$						
	Intercept	1	−0.981	0.287	11.7	0.0006	1.2944
	k	1	14.9	10.145	2.16	0.142	
Model 5	$E(\text{logit}(p(H))) = k * NF_{LA-} * NM_{?+}/N^2$						
	Intercept	1	−1.592	0.233	46.52	<.0001	1.1064
	k	1	55.071	9.76	31.84	<.0001	

Data on Yarn08 *Littorina saxatilis* and *L. arcana* populations

Correlation analysis

The results of correlation analysis are shown in Table 2. We observed positive and statistically significant associations between the number of putative "hybrid"-individuals and all the assessed predictors for Yarn08 data. The picture was very much the same for Troms09 and for the pooled data from both sites, the only exception being the correlation between the predicted hybrid frequency based on male and female data and the actual number of putative hybrid individuals for Troms09 data.

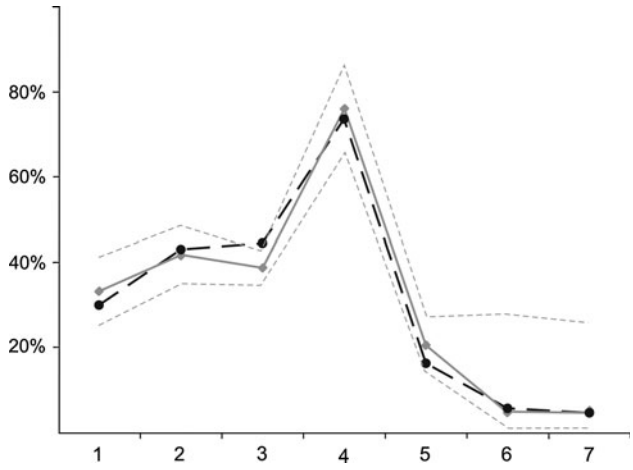

Fig. 5 Zonal patterns of predicted (*solid line*, *gray*) and observed (*dotted line*, *black*) percentages of putative hybrids (sum of PCR-positive *Littorina saxatilis* and PCR-negative *L. arcana*). Data of Logistic Model 3 (see Table 3); Yarn08 populations. 1–7—levels of littoral zone from HWST down to LWST. SUMs 3 from Table 1 are used for calculation of observed frequencies

The logistic models

The first model took into account only the data on the distribution of females because there were both morphological and molecular data on this sex. We assumed that the numbers of PCR+ and PCR− females within each intertidal level determines the numbers of discrepant (F_{LS+} and F_{LA-}) individuals inhabiting this level (Table 3, Model 1). The distribution predicted by the model did not conform to the observed one accurately enough. The k value was statistically significant, but the deviance was high. In other words, the model was poorly compliant with the observed distribution.

In the second model, as in the first one, no morphological criteria were taken into account, but the observed distribution was modelled basing on both male and female genotypes frequencies (Table 3, Model 2). Low deviance values indicated the significant compliance of the calculated and the observed distributions. Moreover, of the two factors used, only the one estimating $F_-*M_{?+}$ combination showed a high significance. ($F_+*M_{?-}$) combinations described by the second factor were not connected with the observed frequencies of females in different littoral levels ($p = 0{,}872$).

Using the selected significant ($F_-* M_{?+}$) factor from the previous model, we specified the model taking into account the females' morphological criterion. The obtained model estimated both possible combinations, ($F_{LS-} * M_{?+}$) and

(F_{LA-} * $M_{?+}$) (Table 2, Model 3). This third model described the observed values better than the previous ones, with the deviance being minimal (1.0635). The plot of the predicted and the observed percentages of "hybrids", taken from Model 3, is presented in Fig. 5. Both of its components were statistically significantly connected with the observed distribution. However, the (F_{LA-} * $M_{?+}$) factor contributed to the model's compliance much more significantly.

The two factors from model 3 were further analyzed in models 4 and 5 (Table 3). The data from model 4 showed that (F_{LS-} * $M_{?+}$) factor estimated by itself did not comply with the observed variation of frequencies. (F_{LA-} * $M_{?+}$) factor (model 5) was significant by itself, but the compliance of this model with the observed distribution was much weaker than that of the model 3, where this factor was estimated together with (F_{LS-} * $M_{?+}$) factor. Thus, each of the factors from model 3 taken by itself described the observed frequencies distribution worse than the combination of these factors.

Discussion

Because of morphological and ecological similarity of *L. saxatilis* and *L. arcana*, their status as different species has long been questioned. Only recently, the species status of *L. arcana* species has been reliably confirmed by molecular data using five mitochondrial and nuclear genes (Reid et al. 2012). Although the phylogeny within the"saxatilis" species complex has not been resolved, it is shown that the three species (*L. saxatilis, L. arcana, L. compressa*) have a common ancestor and the there is recent or incipient speciation. At the same time, genetic population studies of sister species demonstrated that haplotypes can be shared between *L. saxatilis* and *L. arcana* because of incomplete lineage sorting or introgression (Small and Gosling 2000; Wilding et al. 2000a, b; Kemppainen et al. 2009; Doellman et al. 2011). This fact led to the assumption that these species (besides, having morphologically identical males) can hybridize in nature. This assumption is confirmed by the data (Warwick et al. 1990) about successful laboratory crossing experiments with *L. saxatilis* and *L. arcana*. However, the presence of the hybrid individuals in natural populations has not been analyzed in detail until now. Our data provide the first information on the quantitative individual-based characteristics of two sibling species from sympatric populations.

Our results confirm that the *L. saxatilis* population is distributed across the whole intertidal zone. On the contrary, *L. arcana* population is associated with the middle part of the intertidal, that is, the upper border of the macrophytes' belt. These distributions are observed in two geographically distinct places, on the background of *L. arcana* different populational densities, high (Yarn08) as well as low (Troms09). Thus, the greatest overlapping of *L. arcana* and *L. saxatilis* populations is observed in the upper part of the belt of macrophytes.

As in other sympatric populations of *L. saxatilis* and *L. arcana* (Mikhailova et al. 2009), we found PCR-positive *L. saxatilis* and PCR-negative *L. arcana* specimens. These so-called "discrepant" molluscs were relatively numerous in Yarn08 population (34.6 %) and quite rare in Troms09 population (4.8 %). This difference corresponds to that in the relative number of "morphological" *L. arcana* females (21.3 and 6.2 % in Yarn08 and Troms09, respectively). Depending on the hypothesis, the "discrepant" molluscs are (a) representatives of ancient polymorphism as to A2.8 DNA fragment, their frequency being specific for each population of *L. saxatilis* or *L. arcana*; or (b) putative interspecific hybrids.

The test of spatial evenness of the distribution of PCR + molluscs showed that in case of *L. arcana* the distribution of such molluscs did not differ from even. At the same time, in case of *L. saxatilis* the distribution of PCR + molluscs was very uneven. In the lower part of the macrophyte belt, where the periwinkles of this species almost never encounter *L. arcana* individuals, PCR + snails are few. Thus, the spatial distribution of the marker in the *L. saxatilis* population supports the hybridization hypothesis. However, this hypothesis is not supported from the side of *L. arcana* because of the zonal "evenness" of polymorphism by this fragment in its population. Strictly speaking, the frequency of the fragment in *L. arcana* does not depend on the number of *L. saxatilis* individuals at a given level. It should be noted, however, that, contrary to *L. saxatilis*, the zonal distribution of *L. arcana* on the intertidal falls completely within the limits of *L. saxatilis* population area. It is this factor that may promote the levelling of the marker's frequency, "masking" the consequences of hybridization.

Therefore: (a) the frequency of putative hybrids is proportional to the frequency of *L. arcana* in sympatric populations *L. saxatilis–L. arcana*; (b) their distribution in different zones of the intertidal zone is significantly uneven (*L. saxatilis*); (c) *L. saxatilis*, PCR-positive (F_{LS+}) are near absent in the lower part of the intertidal where *L. arcana* is absent, too. In general, these arguments support the hypothesis of interspecies hybridization between *L. saxatilis* and *L. arcana*. However, the even distribution of the markers' frequencies in the population of *L. arcana* somewhat undermines this argumentation.

One more argument in favour of hybridization comes from the logistic models. The point is that the frequency of the supposedly hybrid individuals (the observed distribution) complies wonderfully with that of the presumably parental individuals (the predicted distribution) in the relevant intertidal level (whose frequency was the source for calculating the frequency of predicted hybrids).

The structure of the best fitted model shows that the distribution of several groups of individuals contributes significantly to the precise correspondence of the model. These are PCR-positive males, all *L. saxatilis* females (regardless of their molecular status) and *L. arcana* PCR-negative females. That is the specified groups are probably more involved in the gene exchange. On the contrary, morphologically identified *L. arcana* females (PCR-positive), as well as PCR-negative males, according to the model, play no significant role in hybridization. From the point of view of interspecific hybridization this means that all "hybrid" and "pure" *L. saxatilis* females can widely hybridize, but only "hybrid" *L. arcana* females can do so. We can call it the "asymmetrical" character of hybridization between the two species, which should be reflected in the asymmetric interspecific gene exchange.

Importantly, in the only lab experiment on *L. saxatilis* and *L. arcana* hybridization (Warwick et al. 1990) the "asymmetry" of the supposed gene flow has also been reported. Successful reciprocal interspecific crosses were obtained only between male *L. saxatilis* and female *L. arcana*. In our study PCR-positive males (presumably *L. arcana*) sired with both *L. saxatilis* and hybrid *L. arcana* females, while PCR-negative males (presumably *L. saxatilis*) would only intercross with conspecific females. Unfortunately, a more detailed comparison of our data with the experimental ones is impossible. In the cited paper species were identified by morphological characters and the authors could not control the experimental molluscs according to DNA markers in order to reveal their possible hybrid nature.

To sum up, the pattern of micro-spatial distribution of *L. saxatilis* and *L. arcana* in sympatric populations revealed in this study indicates that these periwinkles may hybridize. In an earlier study involving an RAPD nuclear marker we arrived at the same conclusion (Mikhailova et al. 2009). So, all our observations taken together support the hypothesis of hybridization between *L. saxatilis* and *L. arcana*. Taking into account that *L. arcana* is always lives in the sympatry with *L. saxatilis* we can suggest that there are effective isolation mechanisms that support the species status of populations in spite of wide hybridization. The study of isolation between these species would bring about an insight into the mechanisms of "ecological speciation".

Though we cannot totally rule out the hypothesis of lineage sorting based on ancient polymorphism, we find it hard to reconcile it with our findings of the differences in the molecular marker frequencies observed in the parts of the population of a given species occupying adjacent intertidal levels. To do so, one would have to assume that in both *L. saxatilis* and *L. arcana* populations there is a constant selection in favour of the species-specific differentiation of the marker frequencies at the few meters' scale. Such an assumption seems to us rather far-fetched.

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