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Evidence for Poecilogony and Potential "Sequential" Poecilogony in Mediterranean Members of the Genus *Raphitoma* (Mollusca: Gastropoda: Conoidea: Raphitomidae)

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Abstract: Published morphometric data of the maximum diameter of protoconch I of 20 *Raphitoma* species belonging to 10 pairs of "sibling" species grouped in 12 sets were subjected to Orthogonal Regression Analysis (ORA) for congruence with the poecilogony hypothesis proposed by MANOUSIS et al. (2018). Results exclude the possibility that the obtained data are random but further investigations (e.g. DNA) will support the hypothesis.

Key words: Mediterranean Sea, poecilogony, *Raphitoma*

1. Introduction

Members of the genus *Raphitoma* usually inhabit maerl and biogenic bottoms of rather deep waters, thus making the acquisition of a substantial number of live specimens for DNA markers analysis a laborious and long-term effort. Concequently, morphometry has been the only available way to deal with systematics in the group. Until recently, the assumption that the dichotomy paucispiral protoconch/lecithotrophic development vs. multispiral protoconch/planktrotrophic development (JABLONSKI & LUTZ, 1980) can be used to identify between members of "sibling" pairs was employed on certain Mediterranean and northeastern Atlantic species of the genus (BOUCHET, 1989; OLIVERIO, 1996a, 1996b, 1997). Apparently, the implied idea that "loss of

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planktotrophy" leads *a priori* to "speciation" was not justified by any experimental results. By 2018, the ongoing revision of the genus in the Mediterranean Sea and northeastern Atlantic by PUSATERI *et al*. (2012; 2013; 2016; 2017) and GIANNUZZI-SAVELLI *et al*. (2018a; 2018b) produced a considerable number of "sibling" species the existence of which was impressive in its frequency and in need of some kind of explanation. MANOUSIS *et al*. (2018) revised the Hellenic members of the genus, had the opportunity to handle a considerable number of specimens, measure directly the morphological characters and consider modes of reproduction that could lead to such a frequent evolutionary phenomenon of kinship. As a result, they came up with and proposed the theory that the phenomenon could be attributed to a simple gene regulating the cell cycle in the gonads and functioning in conjunction with other genes and environmental factors to exhibit a discontinuous multifactorial inheritance leading to poecilogony (see also, KNOTT & McHUGH 2012, for a review on poecilogony). Below a threshold, the animals may produce fewer and larger germ cells, giving rise after meiosis to fewer and larger eggs and large lecithotrophic embryos with large paucispiral protoconch I, while above that threshold, more and smaller germ cells leading after meiosis to smaller eggs and to planktotrophic larvae with small protoconch I and large multispiral protoconch II (MANOUSIS *et al*., 2018; see also McDONALD *et al*., 2014, for relevant embryonal shell sizes due to poecilogony in the caenogastropod *Calyptraea lichen*). Preliminary measurements were in support of that hypothesis (MANOUSIS *et al*., 2018). As additional cell cycles would produce sister cells with a diameter smaller than that of the maternal cells by a factor of 0.7937, they measured the maximum diameter of protoconch I of both the lecythotrophic and the planktotrophic members in three pairs of "sibling" species to find a congruence with the expected dimensions predicted by the theory (MANOUSIS *et al*., 2018). Additional useful data were produced after GIANNUZZI-SAVELLI *et al*. (2018a, 2018b) revised 11 more pairs of "sister" cryptic species of the genus, thus giving the opportunity for the poecilogony theory to be put to additional test. As members of the genus inhabit maerl and biogenic bottoms of rather deep waters and therefore the acquisition of a substantial number of live specimens for DNA markers analysis constitutes a laborious and long-term effort, the present publication aims at combining present and previous support of the theory through a statistical analysis and, by that, draw appropriate attention and treatment of the relevant issues and encourage members of the international community to invest towards the crucial molecular analysis.

2. Methodology

The approach to the issue was to use published data as appropriate material. More specifically, the mean maximum diameter (± standard deviation) of either lecithotrophic or planktotrophic protoconch I (Table 1) was used as it has been estimated in the relevant literature (PUSATERI et al., 2012; 2013; 2016; 2017;

GIANNUZZI-SAVELLI *et al*., 2018a; 2018b; MANOUSIS *et al*., 2018). We presuppose that: i) Stem cells are spheres dividing into 2 equal spheres in each additional mitosis; ii) each successive mitosis produces 2 daughter cells with diameter = mother cell diameter X 0.7937 (Fig. 1); iii) after final mitoses cells undergo meiosis and addition of yolk to become eggs; iv) After fertilization, development of embryos leads to formation of protoconch I. All published protoconch I maximum diameter data are listed in Table 1 in an increasing size of planktotrophic protoconch I order.

For the statistical process of the data the Orthogonal Regression Analysis (ORA) (GLAISTER, 2001) was employed. In the case of *Raphitoma griseomaculata* lecythotrophic protoconch dimensions we have observed an inconsistency between the given protoconch I width in the text (225 μm) and in the corresponding SEM image (463 μm) of the GIANNUZZI-SAVELLI *et al*. (2018) relevant publication. As the lecithotrophic protoconch I is larger than that of the planktotrophic one, we understand that there must be an error and, therefore, we have taken into account the value implied in the SEM image.

3. Results

All lecithotrophic protoconches of the studied species exhibit a nearly identical morphology (Fig. 2) that commences with the embryonic shell followed by the larval one before the onset of the teleoconch. A standart pattern of morphology is also the case for all planctotrophic protoconches, with the multispiral larval shell inbetween the embryonic one and the teleoconch. All lecithotrophic (paucispiral) protoconches I are in a size group distinctly separated from that of the planktotrophic (multispiral) protoconches I, the later being nearly 2 times smaller in diameter than the former (Fig. 3). Applying the hypothesis of additional pre-meiotic divisions (2-5, prior to egg formation) on the expected and observed maximum diameter of the planktotrophic protoconch I in each pair of siblings, it seems that the observed values of the planktotrophic protoconches I statistically coincide with the observed ones (Figs 4 and 5). More specifically, the applied Deming Regression Analysis showed that in order for the estimated and the observed values of the protoconch I diameter of the studied species (in pairs) to meet the concept of randomness, the pairs of values should diferenciate each other by providing a range of variation which simulates the corresponding on the water column. For this to happen, value pairs should be considered as deriving from a bivariate distribution which must not deviate from a linear change or, otherwise, conform to an optimal fit of the pairs on a straight line with the regression coefficient being equal to the unit. In other words, both measurements provide an equivalent result. This relationship is described by the Deming regression equation which admits that every variable (observable and estimated values) maintains its own variation (variance ratio = 0.899). In the regression diagram (Fig. 4) the value pairs cover the entire extent of the line which statistically indicates the great proximity of the measurements

(R^2=92.38%, Table 2). This equation is expressed by the relationship: Observed = 12.50 + 0.948 Estimated. The b value 0.948 of regression does not statistically differ from 1 (95% Confidence Intervals of b: 0.77-1.13), so the two different measurement express the same result. Moreover, the fit values of the two variables (observed and expected in the equation) considerably approximate the actual values of the measurements (Table 2) of regression.

In the case of the "sibling" pair of *Raphitoma spadiana* - *R. contigua* it is observed (Table 1) that, although the lecythotrophic protoconch I size is almost the same in two different areas of the Mediteranean, e.g. the Central Mediterranean (PUSATERI *et al*., 2012) and the Aegean Sea (MANOUSIS *et al*., 2017), that of the planktotrophic one is different and corresponds to 4 and 3, respectively, additional pre-meiotic cell divisions prior to egg production. A similar phenomenon is observed in *R. atropurpurea* of the Aegean, in which shallow waters form bears protoconch I of 225 μm while the deep waters form of 195 μm which corresponds to an additional premeiotic division.

4. **Discussion**

General

The decisive stage of any taxonomic outcome in malacology is the critical evaluation of the shell characters with the aim to designate and exemplify reliable discriminating features. Towards this direction, in the last few years, protoconch characteristics have been almost exclusively used for the systematics of all pairs of Mediterranean and northeastern Atlantic, so called "sibling *Raphitoma* species" (PUSATERI *et al*., 2013).

When, though, someone examines the characteristics of either the paucispiral-lecithotrophic or the multispiral-planktotrophic protoconches of these members of the genus, is prompted to the conclusion that there are not fundamental differences between the protoconches of either type within the different species. This lack of significant differences together with the interspecific variability related either to the number of the whorls or the microsculpture, make rather impossible the definite characterization of a species of *Raphitoma* exclusively on the bases of its protoconch's characteristics. This resemblance is indicative of a common gene set controlling the sculpture of the protoconch either of the embryonic shell (protoconch I) of both lecithotrophic and planktotrophic *Raphitoma* species or that of the larval shell (protoconch II) of the planktotrophic ones. Apparently, a common gene set would be regulatorily responsible for a common structure of the mantle, including the secretory epithelium, the nerve, muscle and connective tissues, and thus of the almost identical protoconch structures within the genus. As a consequence, is seems rather improbable that such a set of genes is present in one member of a sibling pair and absent in the other member, especially if one

considers that the same set of genes is also essential for the production of the teleoconch and also takes into account that no "gene clearing mechanism" has been eccounderd in nature as yet. It would be more reasonable to accept that that set of genes is present both in the paucispiral protoconch and the multispiral protoconch species and only a trigger is needed to give that set of genes the opportunity to express its potential e.g. environmental factors may be ultimately responsible for the mode of the larval development. Furthermore, absolute reliance on shell characters for the distinction of these species is particularly justified by the fact that quite a few species of *Raphitoma* are from uncommon to rare and have never been encountered alive. As a result, the majority of the described species of the genus are represented by shells, thus leaving shell morphological characters the major source of evidence for their taxonomy and making difficult the use of molecular tools and DNA barcoding for the identification of potentially cryptic species or the genetic basis of poecilogony. Therefore, characterizing larval phenotypes and identifying the consequences of alternative phenotypes seems to be the remaining ways to infer poecilogony in the genus.

On the results

The overall structural similarities between all paucispiral protoconches including the size of protoconch I (Fig. 2) - directly related to the egg size - and the number of whorls are indicative of identical developmental mechanisms and genetic background and comprises an evidence for selection towards certain protoconch size and specific weight that "ensures" lack of buoyancy and, therefore, benthic residency and development after hatching. This also holds in the case of planktotrophy when buoyancy and, therefore, planktonic life and planktotrophic larval development after hatching is secured.

The grouping of the lecithotrophic protoconches I and the planktotrophic protoconches I in two distinct size ranges and the lack of intermediate forms (Fig. 3) is an evidence for either absence of Mendelian inheritage determining the size of protoconch I – and subsequently the mode of larval development - or death and elimination of all intermediate sizes and modes of development - an apparently costly proliferation strategy, or P (Planktrotrophy gene) is dominant and l (lecithotrophy gene) is recessive (PP=P, Pl=P, ll=l). If the later was the case, then all Mediterranean *Raphitoma* species should appear in both – lecithotrophic and planktotrophic – forms. This later option cannot be the case, as certain species of Mediterranean *Raphitoma* are never found in both developmental forms.

The highly restricted number of pausispiral protoconch I total whorls (Fig. 2) is also indicative of selection towards a mechanism that andows the paucispiral protoconch with the property to commence whithout delay its benthic live and the formation of the teleoconch. Otherwise, buoyancy of the paucispiral protoconch would lead the animal to planktotrophy, a rather impossible case as no planktotrophic individuals are ever reported

with protoconch I the size of that of a lecithotrophic protoconch. This also holds for paucispiral protoconch I as no individuals are ever reported with protoconch I the size of a planktotrophic protoconch I. In brief, there are either large eggs - and protoconches I heavier than the sea water leading to rather immediate bethic life - or small eggs - and protoconches I lighter than the medium and leading to planktotrophy. In other words, the size of the eggs is critical for the mode of larval development, hence, under precise control of the cell cycle in the gonads.

It would also be reasonable that a, by any means, loss of planktotrophy (OLIVERIO, 1996a, 1996b, 1997) could not automatically spread to all members of a population, thus leaving space for natural selection to act on the frequencies of the alleles responsible for the control of the cell cyrcle in the gonads prior to ova production by meiosis. Nevertheless such a selection would favour planktotrophy rather than lecithotrophy because planktotrophic mode of life with long-living planktonic larvae is considered advantageous in their dispersal as they may drift considerable distances with the currents (JABLONSKI & LUTZ, 1983; LEAL & BOUCHET, 1991).

On the possibilities

It is rather reasonable to envisage that in any case of a pair of "sibling" species, there are three possibilities: ***a***. They are, indeed, two different species, namely, two genetically isolated populations one of which bears the genetic background to produce large eggs and bear large protoconch I that leads to lecythotrophy and the other a different genetic background that leads through small eggs and small protoconch I to planctotrophy; ***b***. It is a case of interspecific variability based upon genetic variability determining the type of the protoconch, hence the mode of embryonic development, similar to the above situation but through a common gene pool; ***c***. Poecilogony, in the strict sense, as proposed by HOAGLAND & ROBERTSON (1988) and BOUCHET (1989) that the same female individual can produce offspring of either lecithotrophic or planktotrophic larval development depenting upon environmental factors.

In case *a*, although it seems possible that, despite the type of the protoconch and the different genetic background, homoplasy could lead a pair of "sibling" species to the same teleoconch morphology, it is highly improbable that the same phenomenon would independently repeat itself in a dozen of pairs of species, as it seems to be the case. If it is the *b* possibility with two alleles determining the type of the protoconch, then intermediate forms of protoconches would be expected, but this is not what is observed. If *c* is the case, then: i) Large paucispiral-lecithotrophic larvae are expected to be produced when the environmental conditions favor a limited number of mitoses of the stem cells, while small protoconch I multispiral-planktotrophic protoconch II larvae are produced when the environmental conditions favor additional mitoses of the stem cells, ii) As the

responsible for the control of the cell cycle gene(s) go along with multicellularity and the formation of gamets, they are *a priori* present in all Mediterranean *Raphitoma* species thus excluding the paradoxe that the *a* case unavoidably implies, that is, "loss of planktotrophy" is mearly a control phenomenon, applies to certain individuals and does not necessarily lead to speciation and iii) The phenomenon should be common, that is, more pairs would show poecilogony as the above statistical analysis supports.

Additional parameter

Finally, as poecilogony would occur in the same female individual confronting different environmental contingencies and that female's reproductive output would be *a priori* limited and specific, fewer meioses are expected to produce larger eggs of a specific total embryos' volume and surface, while more meioses smaller eggs of the same total volume but bigger total surface. In that later case, the shell would be thinner and relatively ligher and, therefore, would add more buoyancy to the, already small, larvae. Thus, larger and less buoyant larvae would remain on the bottom and embark for the production of the teleoconch soon after they hatch, while the smaller ones stay in the water column as planktotrophic drifters until they loose buoyancy and sink to grow to maturity and find either a lecythotrophic or a planctotrophic protoconch mate and reproduce.

Perhaps, this is the explanation for the grouping of both lecithotrophic and planctotrophic protoconches in two distinct size amplitudes that ensure, through buoyancy and nektonic abilities, the two different modes of development.

Final tips

The fact that in the case of the "sibling" pair *Raphitoma spadiana-R. contigua* the size of the planktotrophic protoconch I corresponds to eigher 3 or 4 additional pre-meiotic cell divisions (according to the poecilogony hypothesis) in two different areas of the Mediteranean Sea. This is also indicative of the influence of environmental factors on the control of the cell cycle prior to ova production in some members of the genus and points to a new type of peocilogony that we propose as "sequential peocilogony". On going research indicates that this phenomenon applies to more pairs of *Raphitoma* species. In marine invertebrates, an increase in parental investment is expected to favor positive selection in nutrient poor marine environments as prolonged planktotrophy in the water column becomes less effective (SANG *et al.*, 2018). Thus, "sequential peocilogony" could represent a fine tuning of the egg size regulating mechanism to the environmental nutrient supply.

5. Conclusions

The notions of "sibling" pairs of *Raphitoma* or interspecific phenotypic variation are not supported by evidence. "Loss of planktotrophy" must be, merely, a control phenomenon. Point mutations or even reverse mutations could be responsible for the high developmental and evolutionary plasticity. Poecilogony is supported by the statistical analysis. "Sequential Peocilogony" could represent a fine tuning of the egg size regulating mechanism to the environmental nutrient supply. A variant of Thorson's rule should be considered for deep – colder waters vs. shallow – warmer waters in temperate climates. Sequential peocilogony could be behind the so called "complexes of *Raphitoma* species" but for that DNA molecular analysis could have the last word.

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Figure legents

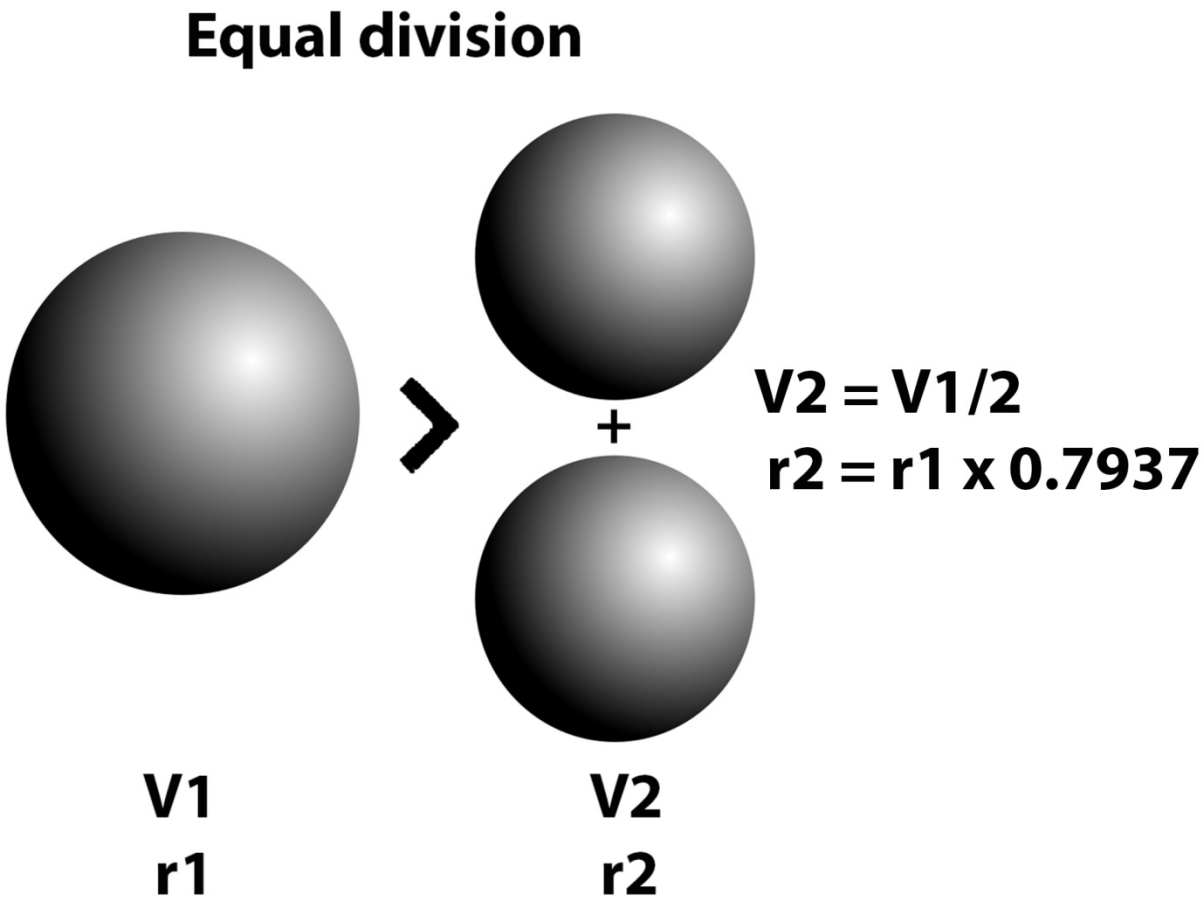

Figure 1: Relation between the mother sphere (cell) diameter and daughter spheres (daughter cells) diameter.

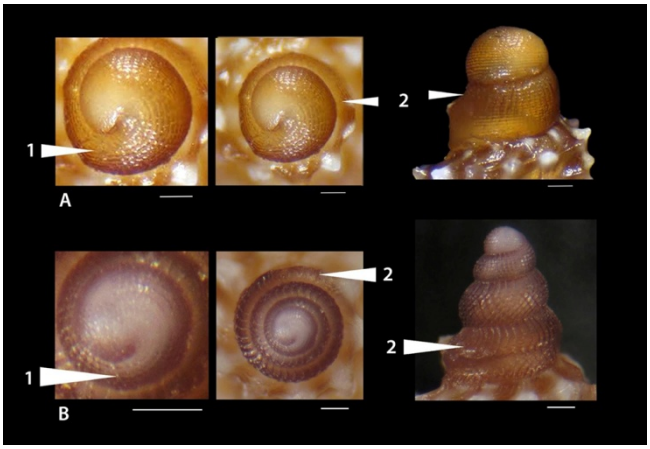

Figure 2: A. Typical lecythotrophic protoconch: 1. Limit of protoconch I (embryonal) before the onset of additional larval shell growth; 2. Limit of larval shell before the onset of teleoconch (adult); B. Typical planktotrophic protoconch: 1. Limit of protoconch I (embryonal) before the onset of protoconch II (larval); 2. Limit of protoconch II before the onset of teleoconch (adult). Barr = 100 µm.

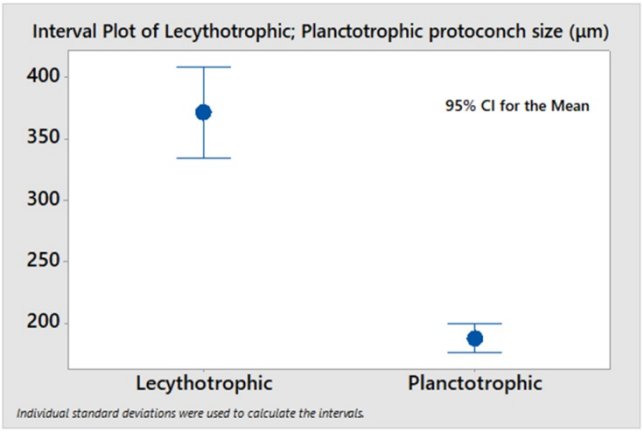

Figure 3: Interval of paucispiral protoconch I maximum diameter; multispiral protoconch I maximum diameter.

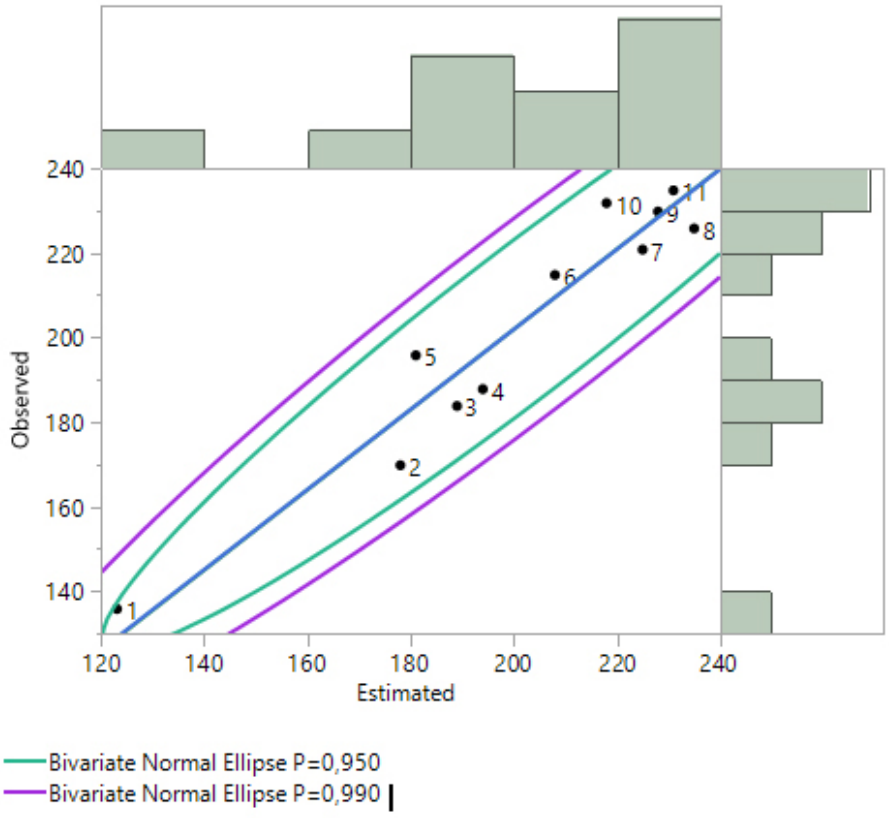

Figure 4: Bivariate fit of observed by estimated measurements.

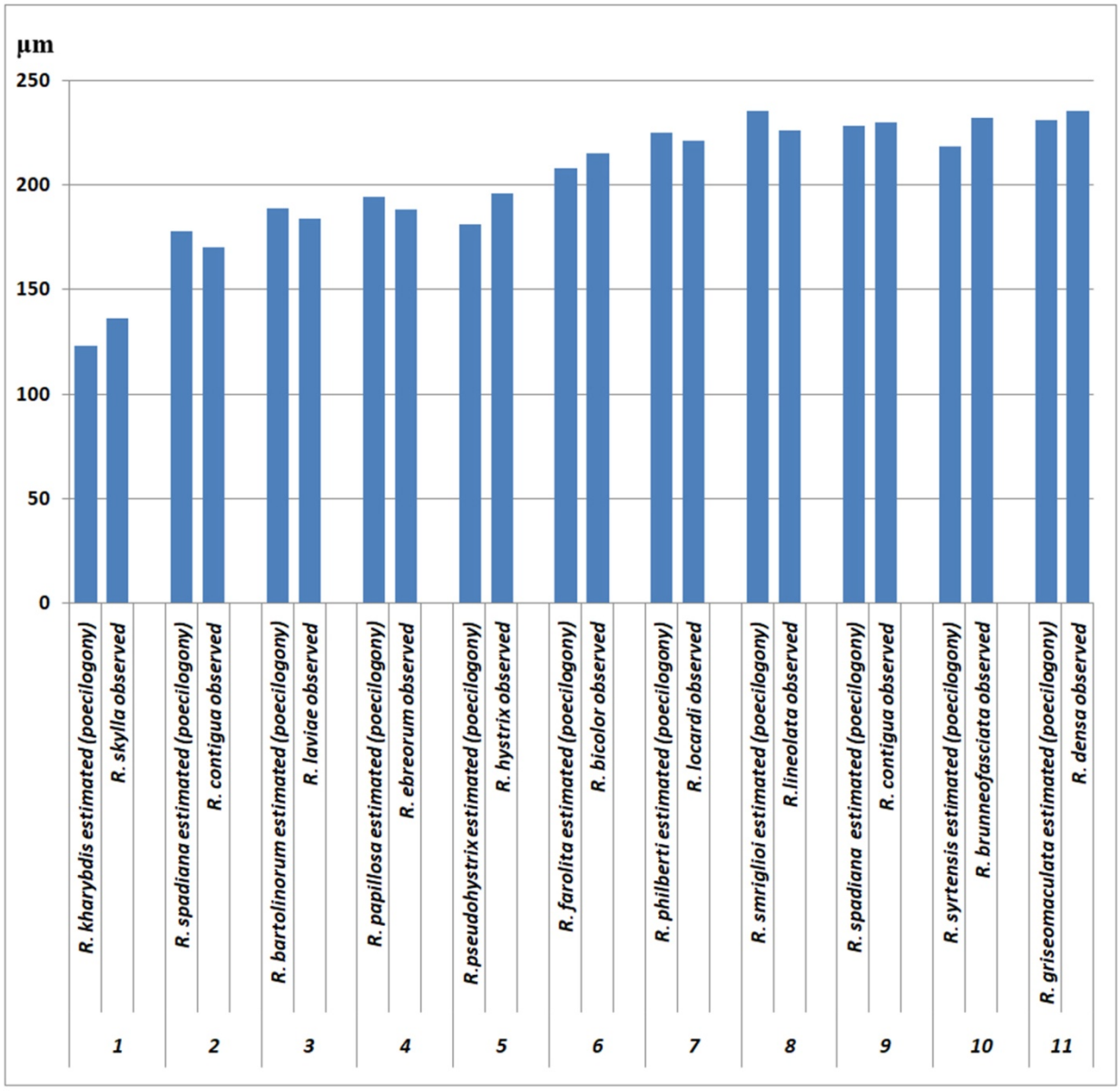

Figure 5: Comparative presentation of observed and estimated data of Table 1.

Table 1: All published protoconch I maximum diameter data in an increasing size of planctotrophic protoconch I order.

	Pair of "sibling" species	Measured lecithotrophic protoconch I maximum mean diameter (µm)	Expected planctrotrophic protoconch I maximum diameter (µm) after additional cell cycles					Measured planctrotrophic protoconch I maximum mean diameter (µm)	Corresponding additional cell cycles	Reference
			1	**2**	**3**	**4**	**5**			
1	*R. kharybdis* *R. skylla,*	*R. kharybdis* 390	309	245	195	154	**123**	*R. skylla* **136**	5	Giannuzzi-Savelli *et al*., 2018a
2	*R. spadiana* *R. contigua*	*R. spadiana* 450	357	283	225	**178**		*R. contigua* **170**	4	Pusateri *et al*., 2012
3	*R. bartolinorum.* *R. laviae*	*R. bartolinorum* 478	379	301	239	**189**		*R. laviae* **184**	4	Giannuzzi-Savelli *et al*., 2018a
4	*R. papillosa* *R. ebreorum,*	*R. papillosa* 388	307	244	**194**			*R. ebreorum* **188**	3	Giannuzzi-Savelli *et al*., 2018a
5	*R.pseudohystrix* *R. hystrix*	*R. pseudohy*. 574	456	362	287	228	**181**	*R. histrix* **196**	5	Giannuzzi-Savelli *et al*., 2018a
6	*R. farolita* *R. bicolor*	*R. farolita* 416	330	262	**208**			*R. bicolor* **215**	3	Giannuzzi-Savelli *et al*., 2018a
7	*R. philberti* *R. locardi*	*R. philberti* 358	284	**225**				*R. locardi* **221**	2	Manousis *et al*., 2017
8	*R. smriglioi* *R.lineolata*	*R. smriglioi* 375	297	**235**				*R. lineolata* **226**	2	Manousis *et al*., 2017
9	*R. spadiana* *R. contigua*	*R. spadiana* 457	363	288	**228**			*R. contigua* **230**	3	Manousis *et al*., 2017

10	*R. syrtensis* *R.brunneofasciata*	*R. syrtensis* 435	345	274	**218**			*R. brunneofa* **232**	3	Giannuzzi-Savelli *et al*., 2018a
11	*R. griseomaculata* *R. densa*	*R. griseomaculata* 463 (SEM)	367	291	**231**			*R. densa* **235**	3	Giannuzzi-Savelli *et al*., 2018b

Table 2: Statistical information of Deming Regression Analysis: Observed versus Estimated measurements.

Error Variance Ratio (Observed/Estimated): 0,899

Regression Equation

Observed = 12,50 + 0,948 Estimated

Coefficients

Predictor	Coef	SE Coef	Z	P	Approx 95% CI
Constant	12,50190	18,4663	0,6770	0,498	(-23,6914; 48,6952)
Estimated	0,94818	0,0909	10,4344	0,000	(0,7701; 1,1263)

Error Variances

Variable	Variance
Observed	38,0981
Estimated	42,3783

Fitted Values and Residuals

Obs	Estimated	Fit	Observed	Fit	Residuals	St Resid
1	123	126,624	136	132,564	6,8719	0,78723
2	178	172,053	170	175,639	-11,2780	-1,29200
3	189	184,935	184	187,854	-7,7080	-0,88302
4	194	189,545	188	192,224	-8,4489	-0,96790
5	181	187,263	196	190,061	11,8774	1,36066
6	208	210,783	215	212,362	5,2765	0,60447
7	225	222,446	221	223,421	-4,8425	-0,55475
8	235	230,083	226	230,662	-9,3243	-1,06818
9	228	228,692	230	229,344	1,3129	0,15041
10	218	224,747	232	225,603	12,7947	1,46575
11	231	232,829	235	233,266	3,4684	0,39733