

How to achieve internal fertilization without a vagina: the study case of the genus Archilina Ax, 1959 (Platyhelminthes, Proseriata) from Canary Islands

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1 **How to achieve internal fertilization without a vagina: the study case of the genus *Archilina***
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3 **Ax, 1959 (Platyhelminthes, Proseriata) from Canary Islands**
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27 **Abstract**

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5 29 Five new species of Monocelididae (Platyhelminthes, Proseriata) are described from Eastern
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7 30 Atlantic. Three new *Archilina* species came from the Canary Islands. Two of them have the cirrus
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9 provided with a stylet, cylindrical in *A. regina* sp. nov., and spike-like in *A. imperatrix* sp. nov.
10 31
11 Both species lack a vaginal pore: ventrally to the bursa, an area of **vacuolar** parenchyma is present.
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14 33 *A. coronata* sp. nov. lacks a stylet, and has a vaginal pore. The three species are sister taxa to
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17 34 *Duploperaclistus circocirrus* and *Duplominona miranda* sp. nov., from Brittany (France),
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19 35 characterized by an extremely long, tubular stylet, about 150 µm in length. A further *Archilina* is
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21 described from Cabo Verde Islands, *A. regisfilia* sp. nov., with a funnel-shaped cirrus, and without
22 36
23 a vaginal pore. The finding in the Canary Islands of closely related species, occurring abundantly in
24 37
25 the same habitats, and differing for presence/absence of stylet and vaginal pore, suggests a role of
26 38
27 the stylet in allowing internal fertilization in species without external vagina. The phylogeny
28 39
29 presented, based on rDNA 18S and 28S genes, does not reflect present systematics of the
30 40
31 Monocelididae, and the **monophyly** of most genera is not supported.
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34 42
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39 43 *Key words:*

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41 44 Meiofauna; Phylogeny; Taxonomy; Specie delimitation; Hypodermic impregnation
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53 Introduction

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3 54 Platyhelminthes are hermaphrodites with internal fertilisation and complex reproductive
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5 55 systems, **often occupying most of the body** (Cannon 1986). In the Proseriata, one of the major
6
7 56 groups of meiobenthic flatworms, an array of copulatory structures **can be** present, **consisting of**
8
9
10 57 spines, needles **and/or** stylets, **combined with** complex female genital organs, usually including a
11
12 58 bursa, where allosperm are maintained till their migration to oviducts and fertilisation of oocytes
13
14 59 (Curini-Galletti 2001). The number of congeneric species of Proseriata occurring in sympatry and
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16
17 60 differing for the morphology of sclerotized pieces of the copulatory organ (e.g. Martens and
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19 61 Curini-Galletti 1993; Delogu and Curini-Galletti 2009; Casu et al. 2014), suggests a role of these
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21
22 62 structures in preventing interspecific crossings. It may be hypothesized, in fact, that the variously
23
24 63 constructed vaginas may be receptive only of a particular, species-specific morphology and size of
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26
27 64 sclerotized structures. However, in many species and even within whole genera of Proseriata (see
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29 65 e.g. Martens and Curini-Galletti 1999), no vagina could be detected in any life-stage, and a role for
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31
32 66 hypodermic impregnation, similarly to what observed in other groups of Platyhelminthes (Janssen
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34 67 et al. 2015 and citations therein) could not be discounted. However, hypodermic impregnation has
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36 68 so far never been observed in Proseriata. Furthermore, even in species lacking vagina and vaginal
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39 69 pore, an otherwise complete female reproductive system, inclusive of a bursa, often containing
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41 70 sperm, is present. Since self-fertilisation appears to be exceedingly rare in Proseriata, and is
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44 71 accompanied by clear indications of connections between male and female genital ducts (Casu et
45
46 72 al. 2012), the presence of presumptive allosperm in the bursa suggests that, should hypodermic
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49 73 impregnation be present in these animals, this may not happen at random through the body, as is
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51 74 the rule in Platyhelminthes (e.g. Michiels and Newman 1998), but, rather, in specific places,
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54 75 allowing sperm to be injected into the female ducts, if not directly into the bursa.

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56 76 Here we present the case of a group of new species of Proseriata Monocelididae, **belonging**
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58 77 **to** the genus *Archilina*, found in the Canary and Cabo Verde Islands. Although **closely** related, they
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61 78 differ **considerably in** the morphology of **the** copulatory structures, as well as for presence/absence
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of an external vagina. We aimed to produce a solid phylogenetical framework for these species. Molecular analyses were performed by means of rDNA 18S and 28S genes, which have been extensively used both to reconstruct their phylogeny and to detect species boundaries (Litvaitis et al. 1996; Littlewood et al. 2000; Curini-Galletti et al. 2010; Casu et al. 2011, 2014; Girstmair et al. 2014; Scarpa et al. 2016a, 2017a, b, c). This, together with morphological reconstructions, helped to shed light on the possible pathways to achieve internal fertilization and proper placement of sperm within the bursa, without a vaginal pore.

Materials and Methods

Sampling

Samples were collected manually by scooping up the superficial layer of sediment. No specific permits were required for sampling sites, which were not privately owned or protected, nor are the animals object of this study protected or endangered. Flatworms are not regulated in directive 2010/63/EU of the European Parliament. **Sampling of the newly sequenced species occurred in October 2011 (from Lanzarote), September 2016 (from Asinara, Sardinia - Italy) and in June 2008 (from Punta Ala, Tuscany - Italy).** Specimens were extracted from the sediments using the MgCl₂ decantation method (see Schockaert 1996). Once isolated, individuals were first studied alive by slight squeezing under the coverslip. Specimens were then retrieved and processed for further analysis. Whenever possible, the rear part of the organism, where most of the critical features allowing identification are located, was cut and mounted in lactophenol, and preserved as part of the type series or as voucher; the front part was stored in ethanol 95° for molecular studies.

Morphological analysis

After relaxation in an isotonic MgCl₂ solution, specimens were fixed in cold Bouin's fluid and embedded in Paraplast at 56°C. Serial sections were cut 3 to 4 µm thick, stained in Mayer's haematoxylin and eosin, and mounted in Depex. In species descriptions, cirrus length refers to the

104 spiny portion of cirrus; proximal and distal spines are described, if not indicated otherwise, as seen
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 105 in cirrus in inverted conditions.
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4
 106 Abbreviations used in figures: b, bursa; ci, cirrus; cm, circular muscle; co, copulatory organ;
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 107 fd, female duct; fg, female glands; fp, female pore; gid, genito-intestinal duct; gl, gut lumen; iml,
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 9
 108 inner muscle layer; ma, male antrum; mp, male pore; oml, outer muscle layer; ov, ovaria; ph,
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 109 pharynx; po, prostatic organ; pop, pore of prostatic organ; pos, stylet of prostatic organ; pp,
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 110 “parenchymatous plug”; pr, “parenchymatous ring”; pv, prostate vesicle; sp, sperm; sph, sphincter;
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 111 st, stylet; sta, statocist; sv, seminal vesicle; t, testis; v, vagina; vi, vitellaria; vp, vaginal pore.
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21 22 *DNA extraction, amplification and sequencing*

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 244 Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue
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 2615 (MACHEREY-NAGEL GmbH & Co. KG) according to the supplier’s instructions. After
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 2916 extraction, DNA was stored as a solution at 4 °C. Complete 18S and partial 28S (spanning variable
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 3117 domains D1-D6) were analyzed for a total of 112 individuals, 30 of which newly sequenced for this
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 3418 work and 82 taken from GenBank, 81 of which already sequenced by us for previous papers (for
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 3619 details about specimens and sampling localities see Supplementary Material S1). Overall, the
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 39120 molecular dataset includes one specimen of Archimonocelididae and 111 belonging to the family
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 4121 Monocelididae. PCRs for 18S and 28S regions were carried out using the following primers: 18S:
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 4422 A (forward) GCG AAT GGC TCA TTA AAT CAG, and B (reverse) CTT GTT ACG ACT TTT
 45
 46
 4623 ACT TCC (Littlewood and Olson 2001); 28S: for (forward) GCG GAG GAA ARG AAA CTA
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 48
 4824 ACA AGG A, and rev (reverse) AAC TCT TCC GGG AAC CAT CGC CGA C (Scarpa et al.
 49
 50
 51
 5125 2016b); 28S D1-D6: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and
 52
 53
 5326 LSUD6-3 (reverse) GGA ACC CTT CTC CAC TTC AGT C (Littlewood et al. 2000). PCRs were
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 5527 carried out in a total volume of 25 µl containing 25 ng of total genomic DNA (5 ng/µl) on average,
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 5828 1.0 U of Taq DNA Polymerase (Euroclone), 1× reaction buffer, 3.5 mM of MgCl₂, 0.32 µM of
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 60
 6029 each primer, and 200 µM of each dNTP. PCR amplifications were performed in a MJ PTC 200
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130 Thermal Cycler (Biorad) programmed as follows: 1 cycle of 2 min at 94°C, 35 cycles of 1 min at
 1 94°C, 1 min at 54°C (18S/28S D1-D6 primers' annealing temperature), and 1 min and 30 s at 72°C.
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Phylogeny, species delimitation and dating

The 18S and 28S D1-D6 sequences were aligned separately using the algorithm Q-INS-I implemented in Mafft 7.187 (Kato and Standley 2013), which is appropriate for non-coding RNA as it considers RNA secondary structure.

In order to test the phylogenetic signal (Schmidt and von Haeseler 2012), the likelihood-mapping analysis of 10,000 random quartets was performed singularly by means of TreePuzzle (Schmidt et al. 2002), singularly for both genes. The best probabilistic model of sequence evolution was determined independently for each gene using jModeltest 2.1.3 (Darriba et al. 2012), with a maximum likelihood optimized search, and both the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). Both criteria selected the GTR+I +G (Tavaré 1986) as the best fitting model for both 18S and 28S D1-D6 datasets.

Phylogenetic relationships among taxa were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the concatenated 18S and 28S D1-D6 sequences. ML analysis was carried out using the software RAxMLGUI version 1.3 (Silvestro and Michalak 2012), setting the analysis option to 'ML+ thorough bootstrap', which consists in a thorough bootstrap analysis followed by a maximum likelihood search. Then, the bootstrap support values

156 are drawn on the most likely tree. Analysis was carried out with 10 runs and 1000 bootstrapping
 1
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 3 157 replicates using the GTRGAMMAI model. The consensus tree and the bootstrap support values
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 5 158 were visualized by means of the software FigTree 1.4.0 (available at
 6
 7 159 <http://tree.bio.ed.ac.uk/software/figtree/>). BI was carried out using the software MrBayes 3.2.6
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 9
 10 160 (Ronquist et al. 2012), specifying a partitioned model in which 18S and 28S genes were deemed as
 11
 12 161 distinct partitions. Setting as model parameters: NST = 6, rates = invgamma, ngammacat = 4, we
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 14 162 allowed each partition to have its own set of parameters and a potentially different overall
 15
 16
 17 163 evolutionary rate. Two independent runs, each consisting of four Metropolis-coupled MCMC
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 20 164 chains (one cold and three heated chains), were run simultaneously for 5,000,000 generations,
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 22 165 sampling trees every 1000 generations. The first 25% of the 10,000 sampled trees was discarded as
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 24 166 burnin. Runs, carried out at the Cipres Phylogenetic Portal (Miller et al. 2010), were checked for
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 26
 27 167 the convergence of chains ensuring that the Average Standard Deviation of Split Frequencies
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 29 168 (ASDSF), approached 0 (Ronquist et al. 2012), and the Potential Scale Reduction Factor (PSRF)
 30
 31
 32 169 was around 1 (Gelman and Rubin 1992). Nodes with a percentage of posterior probability lower
 33
 34 170 than 95% were considered as not highly supported. Phylogenetic tree was visualized using FigTree
 35
 36
 37 171 1.4.0 (available at <http://tree.bio.ed.ac.uk/software/figtree/>).

38
 39 172 The time-based tree was been obtained by means of the software package BEAST 1.8.3
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 42 173 (Drummond and Rambaut 2007), following as described in Scarpa et al. (2015). In order to verify
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 44 174 the taxonomic assessment of the specimens included in the dataset, several methods of species
 45
 46 175 delimitation were used. First we applied two methods **inspired by** the PSC (Phylogenetic Species
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 49 176 Concept), which are able to discriminate between population and speciation patterns on a given
 50
 51 177 species tree: i) the ST-GMYC (Single Threshold-Generalized Mixed Yule Coalescent) method by
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 53
 54 178 Pons et al. (2006), and ii) the PTP (Poisson Tree Processes) model, its Bayesian implementation,
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 56 179 the bPTP (Zhang et al. 2013). We also applied the ABGD (Automatic Barcode Gap Discovery)
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 58
 59 180 (Puillandre et al. 2012) method on the combined dataset (18S + 28S D1-D6) by using K2P genetic

181 distances (Kimura 1980). All of the used species delimitation methods have been set according to
 182 Scarpa et al. (2017a,c).

183 For the new species *A. coronata* sp. nov., *A. regina* sp. nov., *A. imperatrix* sp. nov. and *D.*
 184 *miranda* sp. nov. the molecular pure diagnostic characters (see Jörger and Schrödl 2013), based on
 185 the 18S and 28S genes, were detected within the family Monocelididae by means of the SPIDER
 186 package (SPecies IDentity and Evolution in R) (Brown et al. 2012) implemented in the R statistical
 187 environment (available at <http://r-forge.rproject.org/projects/splits/>). This analysis allowed us to
 188 obtain for each tested taxon a list of the diagnostic nucleotides in each marker (i.e. those
 189 nucleotides that are fixed within species and different from all other species within their family)
 190 (Brown et al. 2012).

191 In order to explore the morphological shifts between the presence/absence of stylet, vagina
 192 and prostatoid organ, the Maximum Likelihood based Ancestral State Reconstruction (ASR) was
 193 performed by means of the software Mesquite 3.31 (Maddison and Maddison 2017). The history of
 194 character evolution were reconstructed on the 18S+28S phylogeny by using the “Markov k-state 1
 195 parameter model” (Mk1 model) which is a k-state generalization of the Jukes-Cantor model, and
 196 corresponds to the Mk model of Lewis (2001). Computations were performed using Likelihood
 197 Ancestral state in Trace Character History implementing a binary matrix on the occurrence of the
 198 examined morphological features.

200 Results

201 *Molecular analyses*

202 After the alignment, 1539 and 1565 bp-long sequences were obtained for the 18S and 28S
 203 D1-D6 regions, respectively (see Supplementary Material S1 for the GenBank accession numbers).
 204 The likelihood map (Fig. 1) indicated a strong phylogenetic signal, with a percentage of points in
 205 the network-like areas (Schmidt et al. 2002) of 4.0% (Fig. 1A) and 0.8% (Fig. 1B) for the 18S and
 206 28S respectively. Accordingly, both datasets were reliable for phylogenetic and taxonomic

207 inferences (Schmidt and von Haeseler 2012). Both ML and BI analyses converged on the same
 1 topology, thus only the Bayesian tree was reported (Fig. 2). In our group of interest, i.e. the three
 208 species of *Archilina* from Canary Islands, divergence times were about 7.97 mya, ranging 4.94-
 4
 209 12.38 mya for node D1 (*A. coronata* + *A. regina* + *A. imperatrix*) and 4.20 mya, ranging 1.95-7.32
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 210 mya for node D2 (*A. regina* + *A. imperatrix*). [The ancestral state reconstruction finds the likelihood](#)
 8
 211 [of each state at each of the nodes. The likelihood state assignment maximized the probability to](#)
 11
 212 [arrive at the observed states in terminal taxa, given the evolution model, and allowing the states at](#)
 13
 213 [all other nodes to vary](#) (see Fig. 3 for details on the likelihood values of each node).
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1714
 18
 19 The species delimitation methods yielded slightly different results. The ST-GMYC model
 20 identified 36 entities (CI = 29–44), 15 of which represented by singletons and 21 by clusters ($P <$
 21 0.001). The PTP/bPTP model found 37 entities (CI = 35–47), 17 of which represented by
 2216
 23
 24 0.001). The PTP/bPTP model found 37 entities (CI = 35–47), 17 of which represented by
 25
 26 singletons and 20 by clusters. The ABGD method, checked at the prior maximal distance ($P =$
 2718
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 29 0.001), identified 34 entities. It is noteworthy that all the methods used detected the new species
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 31 here described as well defined molecular taxonomic entities. Further details on the species
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 33 delimitation results are shown in Supplementary Material S1.
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 35

36 Molecular diagnostic pure character have been detected for both analyzed genes (see
 37
 38 Supplementary Material S2 for details).
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41 Alignment and phylogenetic tree files were deposited and made available in TreeBase with the
 42
 43 accession number TB2:S22149.
 4425
 45

46 47 48 49 *Taxonomic account*

50
 5128 The following nomenclatural acts have been registered in Zoobank, the online registration
 52
 53 system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the
 54
 55 associated information viewed through any standard web browser by appending the LSID to the
 5630
 57
 58 prefix <http://zoobank.org/>. The LSID for this publication is urn:lsid:zoobank.org:pub:28C72E2E-
 59
 60 EF97-4ADF-8136-B1F3EDB804C6.
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233 The authorship of the new taxa reflects the actual involvement of the participants of this
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 234 research in the taxonomic description. Types have been deposited at the Swedish Museum of
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 235 Natural History (SMNH) (Stockholm, Sweden) and in the Collections of the Zoological Museum
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 236 (CZM), University of Sassari (Italy).
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Order Proseriata Meixner, 1938

Family Monocelididae Hofsten, 1907

Genus *Archilina* Ax, 1959

***Archilina regina* Curini-Galletti, Casu & Scarpa sp. nov.**

urn:lsid:zoobank.org:act:13BC28C2-1422-4B03-84B3-F344FC1B0B0B

(Figs. 4A, B and 5A, B and 6G, H and 7D, E; Supplementary Material S2)

Holotype. Lanzarote (Canary Islands): Mala, medium-fine sand at –20 m. (08.10.2011) (Lat.

29.0834811, Long: –13.4497494). [One whole mount](#) (SMNH-Type 8965)

Paratypes. Same data as holotype, [one specimen sagittally sectioned \(CZM 741\)](#); [three whole mounts \(CZM 742-744\)](#).

Other material: Lanzarote: Mala, sand patch in front of small cavern, under loose macroalgae, coarse shell gravel at –12 m (08.10.2011) (Lat. 29.0834811; Long. –13.44974935): four whole

mounts (one with two specimens) (CZM 745-748). Mala, inside a marine cave, very fine/silty sand at –11 m (08.10.2011) (Lat. 29.0834811; Long. –13.44974935): three whole mounts (one with two

specimens) (CZM 749-751). Mala, medium-coarse calcareous sand at –48 m (08.10.2011) (Lat. 29.0834811; Long. –13.44974935), two specimens sagittally sectioned (CZM 752, 753); four

whole mounts (one with two specimens) (CZM 754-757). Charcos de Luis, tidal pool, very silty fine sand at –0.5 m (04.10.2011), (Lat. 29.16294536; Long. –13.43172222), four specimens

sagittally sectioned (CZM 758-761), two whole mounts (CZM 762, 763); beach south of Orzola, medium-coarse sand at –0.5 m (0.6.10.2011) (Lat. 29.21666149; Long. –13.4422231), one whole

mount (CZM 764); tidal pools near Orzola, coarse sand at –0.5/-2 m (14.10.2011) (Lat.

259 29.22225159; Long. -13.4494328), two whole mounts (one with 5 specimens) (CZM 765-766);
1
260 Puerto del Carmen, Playa Chica, inside the marine cave ‘La Catedral’, silty shelly gravel at -29/-
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261 31 m (14.10.2011) (Lat. 28.9190394; Long. -13.6685264), four whole mounts (CZM 767-770);
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7
262 Puerto del Carmen, Playa Chica, in front of the marine cave ‘La Catedral’, silty shelly gravel at -33
8
9
263 m (14.10.2011) (Lat. 28.9190394; Long. -13.6685264), one whole mount (CZM 771); Puerto del
11
12
264 Carmen, Playa Chica, fine sand at -11 m (14.10.2011) (Lat. 28.9190394; Long. -13.6685264), one
13
14
265 whole mount (CZM 772); Punta Jameos del Agua, coarse sand at -12 m, (15.10.2011) (Lat.
15
16
266 29.15697257; Long. -13.427192), (CZM 773, 774).
18
19
267 Gran Canaria (Canary Islands): Bahia de Santa Agueda, medium-coarse sand at -0.5 m
20
21
268 (21.10.2011) (Lat. 27.750529; Long. 15.642610), two specimens sagittally sectioned (CZM 775,
22
23
269 776); one whole mount with two specimens (CZM 777). Maspalomas, beach close to the
24
25
270 Lighthouse, coarse sand with granules at -0.5 m (24.10.2011) (Lat. 27.735015; Long. -15.598073),
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27
271 three whole mounts (CZM 778-780).
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31
272 *Etymology.* From Latin *regina*: queen. The specific epithet refers to the morphology of the
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273 cirrus, shaped like a crown.
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274 *Description.* A medium-sized moncelid: fixed specimens up to 1.7 mm. Unpigmented. Anterior
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38
275 tip rounded, without sensory stereocilia. Caudal tip with numerous adhesive glands. Epidermis of
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40
276 the insunk type, ciliated all over the body, except for the caudal tip. With numerous small, rod-
41
42
277 shaped rhabdoids, about 4 μ m long. Longitudinal subepidermal musculature particularly strong
43
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278 ventrally.
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279 Pharynx nearly midbody (Fig. 4A), up to 130 μ m long in fixed specimens. Longitudinal and
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50
280 circular musculature well developed around the inner lumen. Pharynx ciliated apart from a small
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52
281 area at tip, where glands discharge. With two types of pharyngeal glands, extending only at a short
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54
282 distance outside the pharynx. Oesophagus well developed, about $\frac{1}{4}$ the length of pharynx.
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283 *Male genital system.* With 15-50 testes in two irregular, medio-ventral rows. Copulatory organ of
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284 the duplex-type, with an outer and an inner muscular layer. The outer muscular layer wraps the
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285 entire copulatory bulb. With a nearly spherical seminal vesicle, separated by a weakly muscular
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 286 septum from a glandular area (the ‘prostatic vesicle’) (Figs. 5A and 7D, E). Distally, a spiny cirrus
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 287 is present, lined, as most of the prostatic vesicle, by the inner muscular layer. The cirrus, 35-42 μm
 6
 288 long and 37-45 μm broad in squeezed conditions, is cup-shaped in living animals. Ejaculatory duct
 8
 289 is partly everted: on the outer side of this everted duct the epithelium is absent, and the basal
 11
 290 lamina is thickened, producing a hard tube-like structure (Figs. 4B and 6G). This tubular stylet
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 14
 291 ranges 22-38 μm in length ($\bar{X} = 30.14 \pm 5.21 \mu\text{m}$; $N = 22$), with a circular proximal aperture, 15-
 16
 17
 292 20 μm across ($\bar{X} = 17.5 \pm 1.55 \mu\text{m}$; $N = 22$) and an oblique distal aperture, 6-12 μm across ($\bar{X} =$
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 19
 293 $9.14 \pm 2.11 \mu\text{m}$; $N = 9$), difficult to notice in most specimens. The not-everted part of the
 21
 22
 294 ejaculatory duct forms a cup lined with pseudocuticula (*i.e. thickened basal lamina*) in its proximal
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 24
 295 part, and is provided with numerous spines. In most specimens, the pseudocuticula shows several
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 27
 296 furrows at the basis of the stylet. With about 8 rows of 30-75 spines ($\bar{X} = 51.2 \pm 15.5$; $N = 22$).
 28
 29
 300 Proximal spines are few (5-10), large, up to 18 μm long (most frequently about 13-14 μm), pointed,
 31
 32
 301 almost straight, with a basis 4-8 μm across. More distally, spines are denser, range between 5-10
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 34
 302 μm in length, and are straight or slightly recurve. Near the tip of the cirrus, spines are smaller, 2-4
 36
 37
 303 μm and are more strongly curved. These distal spines are lacking in many specimens, accounting
 38
 39
 400 for the large divergence in spine number observed. Their presence may be related to the attainment
 41
 42
 401 of full male maturity. The most proximal spines appear attached to the basal lamina. Many spines
 43
 44
 402 show a longitudinal furrow.
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 403 By eversion of the complete cirrus, the internal stylet becomes the most distal part of the
 48
 49
 404 copulatory organ, and forms a functional stylet, supported by the sclerotized basal lamina. In the
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 51
 405 everted cirrus, spines appear clearly arranged in decreasing size (Figs. 5B and 6H).
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 54
 406 The few specimens studied from Gran Canaria fall almost exactly in the range of variation of
 55
 56
 407 specimens from Lanzarote: the stylet ranges 22-35 μm long and 16-20 μm broad at basis ($N = 5$);
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309 spines are 30-75 in number, range 3.5-14 μm in size, decreasing in size distally in inverted
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 310 condition. Also in this case, specimens with fewer spines lacked the smallest ones.

311 The copulatory organ opens into a small male atrium, which is provided with a non-ciliated
 6
 7
 312 epithelium.

313 *Female genital system.* Ovaries ventro-lateral in front of the pharynx. Vitellaria dorso-lateral,
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 314 extending from in front of the ovaries till the copulatory organ (Fig. 4A). The female duct is lined
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 315 with a non-ciliated epithelium, and runs to the pre-penial bursa, situated just in front of the
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 17
 316 copulatory organ. The bursa is made from several large vacuoles, lined with a **resorbient** tissue
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 19
 317 (Figs. 5B and 7E). Dorsally bursal tissues appear to merge with the gut lining, and a connection
 20
 21
 318 with the gut is plausible. The bursal canal is surrounded by a **sphincter consisting of several thick**
 23
 24
 319 **muscle fibers**. Similar sphincters occur in the female duct, close to its connection to the bursal
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 26
 320 canal. Without external vagina. Ventrally to the bursal canal, a thick, toroid-shaped ring of
 28
 29
 321 parenchymatous tissue, 20-30 μm across, is present (Fig. 5B). It surrounds a core of less dense,
 30
 31
 322 somewhat vacuolar, parenchyma, often protruding to the outside as a convex ‘plug’ (Figs. 5B and
 33
 34
 323 **7D, E**). Below this core area, there are no muscular fibres, which run laterally to it. **The female duct**
 35
 36
 324 **continues posteriorly, lined with an irregular, vacuolated epithelium, and opens behind the male**
 37
 38
 325 **pore though the female pore**. The distalmost portion of the female duct is surrounded by numerous
 40
 41
 326 female glands. Numerous glands (“kittdrusen”) are present behind the female pore.

327 *Karyotype.* The few plates observed had $n = 3$, with a basic karyotype for the genus *Archilina*
 45
 46
 328 (Curini-Galletti and Martens 1990), with two large pairs of metacentric chromosomes and a smaller
 47
 48
 329 pair of heterobrachial chromosomes.

330 *Diagnosis.* Species of *Archilina* with up to 50 testes. Copulatory organ with a cup-shaped cirrus,
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 53
 331 provided with about 8 rows of 30-75 spines and a stylet. Proximal spines are large (13-18 μm); they
 54
 55
 332 decrease progressively distally, to 2-4 μm . Stylet ranges 22-38 μm in length. With a vacuolar bursa
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 333 in front of the copulatory organ. Without an external vagina; the bursal duct ends in an area of
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 334 vacuolar parenchyma, surrounded by a ring of denser tissue. Karyotype with $n = 3$.

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***Archilina imperatrix* Curini-Galletti, Casu & Scarpa sp. nov.**

urn:lsid:zoobank.org:act:E4E227E0-DB07-4D28-BC71-4337E24976E3

(Figs. 4C and 5C, D and 6E, F and 7H; Supplementary Material S2)

Holotype. Gran Canaria (Canary Islands): Maspalomas, beach close to the Lighthouse, medium-fine sand at –2 m (24.10.2011) (Lat. 27.734964; Long. –15.597904). **One whole mount** (SMNH-Type 8966).

Paratypes. Same data as holotype, **one specimen sagittally sectioned (CZM 799); two whole mounts (CZM 800-801).**

Etymology. From Latin *imperatrix*: empress. The specific epithet is coined after *A. regina*, and refers to the crown-shaped cirrus of the two species.

Description. Animals 1 mm long in sections. Habitus as the previous species. Pharynx just after midbody, 65 µm long.

Male genital system. With 10 testes in one median row. Copulatory bulb similar to *A. regina*. The ejaculatory duct is partly everted: on its outer side, the epithelium is absent, and the basal lamina is thickened, producing an acutely-pointed, funnel-shaped, spike-like stylet, 26-31 µm long, with a U-shaped basis 7-10 µm across ($N = 3$) (Figs. 4C and 6E, F). The non-everted part of the ejaculatory duct forms a cup, lined with a thick pseudocuticula for most of its length. Three large (25-35 µm long, about 4 µm wide at basis), acutely pointed, spines and two, more distal, falcate spines 7-12 µm long, appear connected basally to this pseudocuticula. Additional, very small spines (1-2 µm) may be present at the outer edge of the thickened pseudocuticula (Fig. 5D).

Female genital system. Topography of organs similar to *A. regina*. In the sectioned specimen, no bursal canal nor obvious connection to the gut was observed. External vagina lacking. Similarly to *A. regina*, a ‘plug’ of vacuolar, loose tissue, surrounded by a much denser, toroid-shaped ring of parenchymatous tissue, was present ventrally to the bursa (Figs. 5C and 7H).

360 *Diagnosis.* Species of *Archilina* with about 10 testes. Copulatory organ with a cup-shaped cirrus,
 1 provided with few (about 5) large spines, 7-35 μm long, and a spine-like stylet, 26-31 μm long.
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 361
 3 provided with few (about 5) large spines, 7-35 μm long, and a spine-like stylet, 26-31 μm long.
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 362 Additional, very small spines may be present. With a vacuolar bursa in front of the copulatory
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 363 organ. Without an external vagina; with an area of vacuolar parenchyma, surrounded by a ring of
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 364 denser tissue, ventrally to the bursa.
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***Archilina regisfilia* Curini-Galletti sp. nov.**

urn:lsid:zoobank.org:act:FD6430A0-16A7-478B-B8F0-1D7249C73C44

(Figs. 4D and 6I and 7A)

Holotype. Cabo Verde Is.: Boa Vista Is.: Sal Rei, Playa de Cruz (Lat. 16.187799, Long. –
 22.918332), lower intertidal in coarse sand among rocks (09.02.2013); whole mount of posterior
 half of body (SMNH-Type 8967); front half used for molecular study.

Etymology. From Latin *regisfilia*: princess. The specific epithet is coined after *A. regina* and
A. imperatrix, and refers to the crown-shaped cirrus of these species.

Description. Limited observations were made on a single living specimen, before its fixation (Fig.
 6A). General habitus, size (about 1.5 mm), topography of internal organs, morphology of rhabdoids
 and gg2 glands, as well the post-median position of the short pharynx, very similar to the previous
 species.

Male genital system. The specimen had 12 testes, in one single line. Copulatory bulb similar to *A.*
regina nov. sp. (Fig. 7A). With a narrow cirrus, about 52 μm high and 20 μm wide, provided with a
 stylet, surrounded by pseudocuticula, and with numerous spines. The stylet is a distinctly funnel-
 shaped structure, 24.5 μm high, with a proximal opening 19 μm across, rapidly narrowing to about
 2.5 μm at 7 μm from the basis. The distal portion of the stylet is a straight, narrow tube, distinctly
 furrowed in its middle, with an oblique distal opening about 5 μm across, and provided with a
 acutely pointed distal tip (Figs. 4D and 6I). The stylet is surrounded by a sclerotized
 pseudocuticula, appreciable at least in the proximal third of the cirrus. There are at least 12 rows of

386 more than 80, densely-packed spines. Uncertainties in the number are due to the partly everted
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 387 condition of the cirrus, which made observations difficult. Proximal spines are less densely packed,
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 388 acuminate, 2-3 μm long, and slightly **curved**. Size of spines increases distally, with median spines
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 389 6-9 μm long, and distalmost spines 10-11 μm long, as well as their shape, which becomes
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 390 progressively more slender.

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Female genital system. Arrangement of vitellaria and ovaria as the previous two species. No external vagina seen. Instead, a ‘plug’ of vacuolar tissue, surrounded by a ring of more homogeneous parenchyma, was observed (Fig. 7A). Bursa vacuolar; at least one vacuole contained sperm. The female duct was observed from the bursa to the female pore, posterior to the copulatory organ.

Diagnosis. Species of *Archilina* with a cup-shaped cirrus, provided with about 80, densely packed, spines and a funnel-shaped stylet, 24.5 μm long. Proximal spines are small, 2-3 μm long; spines increase in size distally, with distalmost spines 10-11 μm long. With a vacuolar bursa in front of the copulatory organ. Without an external vagina; with an area of vacuolar parenchyma, surrounded by a ring of denser tissue, ventrally **from** the bursa.

***Archilina coronata* Curini-Galletti, Casu & Scarpa sp. nov.**

urn:lsid:zoobank.org:act:6E1A6CC2-C3CB-4503-AB79-BE1F9500A636

(Figs. 4E and 5E, F and 6B, C and 7B, C; Supplementary Material S2)

Holotype. Lanzarote (Canary Islands): Mala, sand patch in front of small cavern, under loose macroalgae, coarse/shell gravel at -12 m (08.10.2011) (Lat. 29.0834811; Long. -13.44974935).

One whole mount (SMNH-Type 8968).

Paratypes. Same data as holotype, two specimens sagittally sectioned (CZM 781, 782); three whole mounts (CZM 783-784).

Other material: Lanzarote: Mala, medium-fine sand at -20 m. (08.10.2011) (Lat. 29.0834811, Long. -13.4497494): one whole mount (CZM 785); Mala, inside a marine cave, very fine/silty

412 sand at -11 m (08.10.2011) (Lat. 29.0834811; Long. -13.44974935): one whole mount (CZM
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 413 786); Mala, medium-coarse calcareous sand at -48 m (08.10.2011) (Lat. 29.0834811; Long. -
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 414 13.44974935), one whole mount (CZM 787); tidal pools near Orzola, coarse sand at -0.5/-2 m
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 415 (14.10.2011) (Lat. 29.22225159; Long. -13.4494328), one whole mount (CZM 789); Puerto del
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 416 Carmen, Playa Chica, inside the marine cave 'La Catedral', silty shelly gravel at -29/-31 m
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 417 (14.10.2011) (Lat. 28.9190394; Long. -13.6685264), one whole mount (CZM 790); Puerto del
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 418 Carmen, Playa Chica, in front of the marine cave 'La Catedral', silty shelly gravel at -33 m
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 419 (14.10.2011) (Lat. 28.9190394; Long. -13.6685264), three whole mounts (two with three
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 420 specimens each) (CZM 791-793); Puerto del Carmen, Playa Chica, fine sand at -11 m (14.10.2011)
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 421 (Lat. 28.9190394; Long. -13.6685264), the whole mounts (CZM 794-796).
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422 Gran Canaria (Canary Islands): Bahia de Santa Agueda, medium-coarse sand at -0.5 m
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 423 (21.10.2011) (Lat. 27.750529; Long. -15.642610), two whole mounts (CZM 797, 798).
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424 *Etymology.* From Latin *coronata*: crowned. The specific epithet is coined after *A. regina*, *A.*
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 425 *imperatrix*, and *A. regisfilia*, and refers to the crown-shaped cirrus of these species.
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426 *Description.* Size, general habitus and topography of internal organs indistinguishable from co-
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 427 occurring individuals of *A. regina* sp. nov.
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428 *Male genital system.* Morphology of the copulatory bulb nearly identical with *A. regina* (Figs. 5E
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 429 and 7C). However, the cirrus (30-37 μm broad, 32-40 μm high, $N = 9$) is not provided with a stylet,
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 430 nor with any appreciable thickening of the basal lamina (Figs. 4E and 5F and 6B, C). With 5-7
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 431 irregular rows of 30-70 conoidal spines ($N = 9$). Proximally, a few spines 4-10 μm long, are
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 48
 432 present. The second row includes the largest spines, up 22 μm (most commonly 14-18 μm) long.
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 433 Spines progressively decrease in size distally: distal-most spines are 3-4 μm long. Some of the
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 434 specimens **have** additional, very small, distal spines, 1-2.5 μm long. Only two specimens from
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 435 Gran Canaria could be studied: they had 46 and 70 spines respectively, with proximal spines up to
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 436 14 μm long. Size of spines decreased distally, and distal-most spines were 2-3 μm long. In both
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 437 populations, the longest spines **are** mostly straight, whereas medium-sized and small spines **are**
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438 **mostly curved**. Similarly to *A. regina* sp. nov., specimens with the higher count of spines were
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 439 those where the small distal spines were present.
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440 *Female genital system*: topography of the system nearly identical to *A. regina*. The female duct,
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 441 prior to its connection to the bursa, appears swollen and vacuolar (Fig. 5E). With an ovoid bursa,
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 442 lined with a vacuolar epithelium, especially dorsally, where direct connection with the gut could be
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 443 traced in a few specimens. Ventrally, the bursa is connected to the outside via a short vaginal duct,
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 444 which opens to the outside through a vaginal pore, surrounded by a strong muscular sphincter
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 445 (Figs. 5E and 7B). The female duct opens to the outside posterior to the copulatory organ through
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 446 the female pore, surrounded with female glands. ‘Cement glands’ are present caudally.
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Diagnosis. Species of *Archilina* with a cup-shaped cirrus, provided with 5-7 rows of 30-70 spines. Proximal spines are large (up to 22 μm); they decrease progressively distally, to 1-4 μm . Without a stylet. With a vacuolar bursa in front of the copulatory organ. With a short vaginal duct and an external vagina in front of the copulatory organ.

452 ***Duplominona miranda* Curini-Galletti, Casu, Delogu & Scarpa sp. nov.**

453 urn:lsid:zoobank.org:act:F936F6ED-20D0-437D-8DFA-973308DDDFE8

454 (Figs. 4F and 5G, H and 6D and 7F, G, I, J; Supplementary Material S2)

455 *Holotype*. Roscoff (France): medium sand at -16 m. (23.05.2013) (Lat. 48.732686, Long: -
 46 3.984793). One specimen sagittally sectioned (SMNH-Type 8969).

457 *Paratypes*. Same data as holotype, four whole mounts (CZM 802-805).

458 *Etymology*. From Latin *mirandus*: **admirable** The specific epithet refers to the exceptional
 49 length of the stylet of the new species.

460 *Description*. Fixed specimens about 1.1 mm long. Unpigmented. Anterior tip rounded, without
 55 sensory stereocilia. Epidermis of the insunk type, ciliated all over the body, except for the caudal
 56 tip. With numerous rod-shaped rhabdoids all over the body; caudally, a few very large glands
 57 (gg2), at least 30 μm long, are present. Longitudinal subepidermal musculature particularly strong

464 ventrally. Pharynx nearly midbody, up to 150 μm long in fixed specimens. Pharynx ciliated apart
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 465 from a small area at tip, where glands discharge. With two types of pharyngeal glands, extending
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 466 greatly outside the pharynx. Oesophagus well developed, about 40 μm long; pre-oesophageal area
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 467 unciliated, very short (about 2 μm).
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 1468 *Male genital system.* With about 10 testes in front of pharynx. The duplex-type copulatory bulb is
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 11
 1469 surrounded by an extremely thick musculature: a thick layer of circular fibres (8 μm thick)
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 1470 externally, and few but very strong fibres, orthogonal to the previous, internally (Figs. 5G and 7I,
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 1471 J). The comparatively small seminal vesicle is separated by the prostatic vesicle by a muscular
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 1972 septum (Fig. 7I); prostatic tissue poorly developed, surrounded by a thick layer of orthogonal
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 2473 muscular fibres, to which the sclerotized, basal rim of the stylet appears connected. Distally, the
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 2474 outer muscular layer becomes thinner and irregularly pleated and extends to about 2/3 the length of
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 2475 the stylet, till the level of the connection of the bulb with a long male atrium. The rest of the stylet
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 28
 2976 lies in a pocket, not surrounded by musculature (Fig. 5G). Inner muscle layer comparatively thin.
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 3477 Cirrus with an extremely long and slender stylet, surrounded by a sclerotized pseudocuticula and,
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 3478 more distally, by very many small spines (Figs. 4F and 5H and 6D). The stylet is 148-155 μm long
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 3479 ($N = 4$), about 10 μm wide at its basis, which is surrounded by a sclerotized ring 4-5.5 μm thick.
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 3980 The stylet is about 6 μm across at its middle, narrowing to 3.5 μm distally. The distalmost tip, for a
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 4481 length of about 10-15 μm , appears slit into very numerous, parallel threads. Due to the length of the
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 4482 stylet, it appears variously curved and deformed in the squashed whole mounts; it is regularly
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 4683 arcuate in the sectioned specimen. The stylet is surrounded by sclerotized pseudocuticula, and,
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 4484 distally, by about 30 rows of densely crowded small spines. Basal-most spines are about 2 μm long,
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 5185 recurve; in the middle they become more slender and **more straight**; distally, spines are straight and
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 53
 5486 slender, about 3 μm long. Distal-most spines are less sclerotized, and appear less acute. The male
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 55
 5687 atrium is long, lined with a high, intra-nucleated epithelium, and provided proximally with strong
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 5888 muscular sphincters (Figs. 5G and 7I).
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489 With a globular 'prostatoid' organ, about 20 μm across, containing fine glandular secretion, and
 1 provided with a slender, pen-nib shaped stylet 18 μm long (Fig. 5G). The prostatoid organ opens to
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492 *Female genital system.* Arrangement of vitellaria and ovaria as the previous species. Bursa very
 493 small, consisting of few vacuoles surrounded by resorbiens tissue, separated from the gut by a
 494 strong muscular sphincter (Figs. 5G and 7F). With a narrow vaginal duct, about 120 μm long, lined
 495 by a high, intensely stained, apparently glandular epithelium, and surrounded by very strong
 496 circular muscular fibres. The vaginal duct opens to the exterior through a vaginal pore (Figs. 5G and
 497 7G). The female duct continues posteriorly, and opens caudal to male and prostatoid pores through
 498 the female pore, surrounded by female glands. Cement glands present caudally.

499 *Diagnosis.* Species of *Duplominona* with a copulatory bulb surrounded by thick muscle layers.
 500 Cirrus provided with a narrowly tubular stylet, 148-155 μm long and about 10 μm wide at its basis,
 501 which is surrounded by a sclerotized ring 4-5.5 μm thick. Distal tip split into fine, parallel threads.
 502 With about 30 rows of densely crowded small spines, 2-3 μm long. With a prostatoid organ
 503 provided with a stylet 18 μm long, opening with its own pore. With a small vacuolar bursa, a
 504 vaginal duct about 120 μm long surrounded by circular muscles, and an external vagina.

506 Discussion

507 *Species justification*

508 Three new species of *Archilina* described here have a stylet. *A. regina* sp. nov. and *A. regisfilia* sp.
 509 nov. are clearly distinct for the morphology of their stylets, as well as for number and arrangement
 510 of spines (see Figs. 4B and 5B and 4D respectively). *A. imperatrix* sp. nov. has a peculiar spike-like
 511 stylet (Figs. 4C and 5D), quite unlike the other two species. Presence of a stylet is a rare character
 512 in *Archilina*, only present in *A. endostyla* Ax, 1959 from the Mediterranean, and *A. stylifera* Curini-
 513 Galletti & Martens, 1996 from the Caribbean. *Archilina endostyla* has a cylindrical stylet 30-60 μm
 514 long; the distal-most portion of its ejaculatory duct continues beyond the stylet and forms an apical

515 ‘crown’ provided with numerous, small spines. In addition, it has a broad vagina, opening through
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 516 a vaginal pore (Ax 1959; Martens and Curini-Galletti 1994). *Archilina stylifera* has a slender,
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 517 cylindrical stylet, 25 µm high, about 6 µm wide at basis, which is not tapering significantly distally;
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 518 cirrus spines are small (2-4 µm). In addition, it has a vaginal pore, and a bursa placed lateral the
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 519 copulatory organ (Curini-Galletti and Martens 1996).
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Most of the numerous species of *Archilina* without stylet have a cirrus which is much longer than wide (Martens and Curini-Galletti 1994), unlike the nearly equilateral cirrus of *A. coronata* sp. nov. (Fig. 6C). *Archilina palestina* Martens & Curini-Galletti, 1994 from eastern Mediterranean, with a cirrus 50-60 µm high, and 25-30 µm wide, is most similar to the new species. The arrangement of spines is also similar, with the longest spines, up 16-22 µm, sub-proximally, and the shortest spines, 3-6 µm long, distally (Martens and Curini-Galletti 1994). However, in *A. palestina* spines are arranged in more rows (8-10), are more acutely triangular in shape, and decrease in size distinctly more abruptly than in *A. coronata* sp. nov. (see Martens and Curini-Galletti 1994 fig. 5J, p. 138). *Archilina palestina*, furthermore, appears restricted to the Levant Sea, where it is quite common in shallow waters, and has never been found in the rest of the Mediterranean (Curini-Galletti, pers. obs.), hence the two species appear geographically separated.

Quite a few *Duplominona* species have a cirrus provided with a stylet similar to the situation in *D. miranda* sp. nov. Among them, *D. axi* Martens & Curini-Galletti, 1989 from Sulawesi, and *D. sieversi* Ax & Ax, 1977 from Galapagos, have a spine-less cirrus (Ax and Ax 1977; Martens and Curini-Galletti 1989). Others (*D. galapagoensis* Ax & Ax, 1977; *D. karlingi* Ax & Ax, 1977; *D. krameri* Ax & Ax, 1977, all from Galapagos; *D. brasiliensis* Curini-Galletti, 2014; and *D. septentrionalis* Martens 1983 from north-western Europe) have a stylet ranging 20-45 µm (Ax and Ax 1977; Curini-Galletti 2014; Martens 1983), much shorter than the stylet of the new species. Only three species, all from the Pacific Ocean, have a longer stylet, although still noticeably shorter than the new species: *D. samaloniae* Martens & Curini-Galletti, 1989 from Sulawesi, *D. japonica* Ax, 2008, from northern Japan, and *D. stylifera* Sopott-Ehlers and Ax, 1985, from north-western

541 USA. *D. samaloniae* has slender stylet, about 100 μm long, and its spines are in the same size range
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 542 (1 μm proximally to 3 μm distally) as **those of** the new species. However, the cirrus of *A.*
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 543 *samaloniae* appears spiny **all over its** length, while it is spiny only **in its distal part** in the new
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 544 species. Furthermore, the copulatory organ of *A. samaloniae* is not surrounded by any noticeable
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 545 muscle layer (Martens and Curini-Galletti 1989). *D. japonica* has a narrow stylet, 68-70 μm long
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 546 (**Ax 2008**), and a cirrus that is spiny over its entire length, with spines about 7 μm long, much
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 547 longer than **those of** the new species. *Duplominona stylifera* has a **recurved** stylet, 62 μm long, and
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 548 a cirrus provided with few large spines, 7-8 μm long, distally. This species is particularly
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 549 interesting because, besides the obvious differences in the sclerotized pieces of the copulatory
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 550 organ, it has a female genital system nearly identical to **the situation present in** *D. miranda* sp. nov.,
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 551 with a very small bursa connected to a long vagina surrounded by strong muscular fibres (Sopot-
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 552 Ehlers and Ax 1985). Given the rarity of the characters and the occurrence of *D. stylifera* and *D.*
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 553 *miranda* sp. nov. in different Oceans and biogeographical provinces, it is unlikely that these shared
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 554 characters **may support** a sister-species relationships. Rather, it is plausible that these traits have
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 555 been evolved convergently, in species with a very long and thin stylet. Unfortunately, no
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 556 morphological reconstructions are available for *D. samaloniae* and *D. japonica*, and the existence of
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 557 a clear pattern cannot be confirmed.
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42 *A Canarian lineage?*

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 560 According to our results, the common ancestor of the three new **species of** *Archilina* from
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 561 Canary Islands is dated about 8 million years (myr) ago (ranging 5-12 myr, Fig. 3). The stylet-
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 562 bearing sister species *A. regina* sp. nov. and *A. imperatrix* sp. nov. diverged about 4 myr ago
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 563 (ranging 2-7 myr). Since the emergence of both Gran Canaria and Lanzarote is dated about 15 myr
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 55
 564 ago (Van den Bogaard 2013), divergence times of the **Canarian species of** *Archilina* are not
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 565 conflictual with the hypothesis that their lineage may have evolved within the Archipelago.
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566 It is worth of mention that while the genus *Archilina* is quite well represented in [the](#) central and
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 567 eastern Mediterranean, with [nine](#) species described (Martens and Curini-Galletti 1994; Curini-
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 4
 568 Galletti and Martens 1995) and many more still undescribed (Curini-Galletti, unpubl. data), species
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 569 of the genus progressively disappear westwards, and none of them reaches the extreme Western
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 570 Mediterranean. Furthermore, extensive research in Southern Portugal failed to reveal any [species of](#)
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 571 *Archilina* (Curini-Galletti, unpubl. data). So far, the only report [of a species of *Archilina*](#) in [the](#)
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 572 Eastern Atlantic is a [record](#) of *A. papillosa* (Ax & Ax, 1977) for Northern Spain (Noreña et al.
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 573 2007). *Archilina papillosa* has been described from Galapagos Islands (Ax and Ax 1977), and its
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 574 presence in the Atlantic, let alone North-Eastern Atlantic, seems quite unlikely. [In any case, *A.*](#)
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 575 [papillosa has a long cirrus with very minute spines, all of the same size, and therefore appears quite](#)
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 24
 576 [distinct from the new species described here from the Canary Islands.](#)
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 577 [Present data thus suggest the existence of a hiatus between the species composition of *Archilina*](#)
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 578 [between the Mediterranean and Canary Islands.](#) The clear relationship, at least on morphological
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 579 grounds, of the Canarian species with *A. regisfilia* sp. nov. from Cabo Verde Islands, suggests
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 580 instead the existence [of a lineage of *Archilina*](#) peculiar to West Africa. It is generally recognized
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 581 that the marine fauna of Cabo Verde Islands is linked to that of tropical West Africa, the
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 582 Senegalian Province, while the Canary Islands belong to the Atlantic-Mediterranean fauna
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 583 (Spalding et al. 2007; Bianchi et al. 2012). However, due to their geographical position, [the](#) Canary
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 584 Islands host a not negligible fraction of [West African macrofaunal species](#) (Rolán 2011). This
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 585 case seems to apply to our new species, which appear related to West-African rather than
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 586 Mediterranean faunas. It may be questioned whether the biogeographical subdivisions mentioned
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 587 above, based solely on macrofauna, may indeed apply to meiofaunal organisms, or whether a
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 588 ‘meiofauna-based biogeography’ may apply to all interstitial phyla. Unfortunately, present
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 589 knowledge on spatial distribution of meiofauna is particularly scanty, and these questions are
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 590 destined to remain unanswered for the moment.

592 “How the worm got its stylet”

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3 593 A copulatory stylet is found in species of most genera of the Monocelididae with a cirrus,
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5 594 which constitute the “Duplomonocelidinae” Litvaitis, Curini-Galletti, Martens & Kocher, 1996, a
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7 595 paraphyletic taxon (see Litvaitis et al. 1996 and Fig. 2 in present paper). However, it is not a
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10 596 particularly common character, and, in most genera, species without stylet predominate. In our
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12 597 phylogeny, the ancestral-state reconstruction is not conclusive whether or not absence of stylet is
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14 598 the ancestral condition for the Monocelididae, although a higher likelihood of absence is suggested
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17 599 (Fig. 3). In our group of interest, i.e. the three Canarian species of *Archilina*, which include two
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19 600 species with a stylet (*A. regina* sp. nov. and *A. imperatrix* sp. nov.) and one species without stylet
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22 601 (*A. coronata* sp. nov.), the analysis suggests a slight prevalence for the presence of a stylet as the
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24 602 ancestral state, basing on an ‘anecdotal’ strength of resolution (0.61 and 0.39 for presence and
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27 603 absence respectively). Indeed, in our phylogeny, the ancestral-state reconstruction for the character
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29 604 suggests the occurrence of multiple, independent events of loss/gain of a stylet within
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32 605 Monocelididae.

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34 606 The Canarian lineage may help to understand possible pathways of the process. In
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36 607 Lanzarote, the most intensely studied area *A. regina* sp. nov. and *A. coronata* sp. nov. were the
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38
39 608 most common proseriate species. They were often extremely abundant, showing a remarkable
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41 609 tolerance to depth and nature of sediment, and they were found together in most samples. They
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44 610 appear identical in size, behavior, general anatomy and construction of genital organs, and could be
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46 611 recognized only in squashed conditions, where the presence/absence of a stylet becomes apparent -
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49 612 although with difficulty even in this condition. The different construction of the vaginal duct (open
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51 613 in *A. coronata* sp. nov., closed with a ‘plug’ surrounded by a ring of thicker parenchyma in *A.*
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53 614 *regina* sp. nov.) might thus minimize the chance of inter-specific copulation. In fact, the stylet-less
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56 615 cirrus of *A. coronata* sp. nov. may face a mechanical block when trying to copulate with specimens
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58 616 of *A. regina* sp. nov, while the stylets of *A. regina* sp. nov. and *A. imperatrix* sp. nov. have the
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617 possibility to pierce the trabecular parenchyma of the ‘plug’, and inject sperm directly into the
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 618 bursa.
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 619 Hypodermic impregnation is known to occur, although rarely, in Platyhelminthes (Janssen et al.
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 620 2015 and citations therein). However, spermatozoa appear to be injected at random in the
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 621 parenchyma, and move actively towards the ovaria (Michiels and Newman 1998). What is seen in
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 622 our species, where the stylet is hypothesized to inject sperm through a loose barrier directly into the
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 623 female system, seems thus uniquely evolved in this lineage. Although sections are missing,
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 624 observations on the living individual of the Cabo Verde species, *A. regisfilia* sp. nov., revealed an
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 625 area of loose parenchyma surrounded by a ring of thicker tissue, identical to *Archilina* from Canary
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 626 Islands and this stylet-bearing species *seems* to present the same strategy. We hypothesize that
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 627 selective pressure aimed to minimize inter-specific crossings may be responsible of the different
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 628 morphology of the cirrus in these species. The stylet of *A. imperatrix* sp. nov. is particularly
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 629 interesting, as its morphology *hints to* its close relationships with otherwise ‘normal’ spines of the
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 630 cirrus. The modification of one of them into a funnel-shaped structure, capable to convey sperm,
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 631 may thus happen without major re-organisations of the cirrus itself.

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 632 *It may thus be asked whether the presence of a stylet is correlated with absence of a vagina.*
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 633 *This does not seem to be the case. Our analyses (Fig. 3), in fact revealed to a strong support for the*
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 634 *presence of both a vagina and a stylet as the ancestral state in the common ancestor of the family*
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 43
 635 *Monocelididae in almost any node of interest. It should be noted that the molecular sample is*
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 636 *limited, and whole genera whose members lack a vagina, such as *Promonotus* Beklemischev, 1927,*
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 637 *have not been sequenced. An interesting outcome of the analysis (Fig. 3) is that, in the common*
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 51
 638 *ancestor of the three Canarian *Archilina* species, presence of vagina as ancestral state (0.91 and*
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 639 *0.09 for presence and absence respectively) is strongly supported, and the ‘plug’ blocking a direct*
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 640 *access to the bursa appears then as a clearly derived character in *A. regina* sp. nov. and *A.**
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 641 **imperatrix* sp. nov.*

In any case, a role of the stylet in preventing inter-specific fertilisation cannot be

discounted. *Duplominona miranda* sp. nov. is of particular interest in this regard. *Duplominona* species are typical of tropical to warm-temperate habitats, with very few species occurring in higher latitudes (see Martens and Curini-Galletti 1989 and references therein). Only two species are known from Western Europe: *D. miranda* sp. nov. and *D. septentrionalis*. This latter species has a very short stylet, and an extremely short vaginal duct, leading to a bursa very close to the ventral surface of body (Martens 1983). Given the different construction of the female genital system of the two species, only the extremely long stylet of *D. miranda* may transfer sperm in bursa of a conspecific, which is quite far from the ventral surface. The two species may thus occur together, but be mechanically incapacitated to copulate. It is then not unrealistic to assume that the shape of stylet may be strongly influenced by past or present sympatry with closely related species. Finally, species without a vagina and with a stylet should be carefully examined morphologically, with the aim to assess the presence of a ‘plug’ of vacuolar parenchyma ventral to the bursa, and assess whether the strategy here described is unique to the West African/Canarian *Archilina* lineage or is more widespread in Monocelididae.

Phylogeny and Systematics

Traditionally, definition of genera of “Duplomonocelidinae” (clades A and C in Fig. 2) has given particular weight to presence/absence of a stylet-bearing accessory male-organ, known as “prostatoid organ”, and secondarily, to the position and structure of the bursa-vagina complex. On these bases, combination of diagnostic features of the genera present in our phylogeny (based on Karling 1966; Martens 1983; Martens and Curini-Galletti 1994) are as follows:

Duplominona Karling, 1966, with prostatoid organ, and pre-penial bursa;

Duploperaclistus Martens, 1983: with prostatoid organ, and post-penial bursa;

Archilina Ax, 1959; without prostatoid organ; with pre-penial bursa, and external vaginal pore;

667 *Archilopsis* Meixner, 1938: similar to *Archilina*; with vagina interna, opening together with the
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 668 male pore into a common atrium;
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669 *Archiloa* de Beauchamp, 1910: similar to *Archilopsis*; without direct connection of the female duct
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 670 with the prepenial bursa; with an accessory cirrus.
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1071 The rigidity of this system is apparent, and molecular-based phylogenies have not supported
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 1272 the **monophyly** of most of the genera above (Litvaitis et al. 1996; Curini-Galletti et al. 2010). This
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 1573 is particularly evident in the phylogeny presented here, where species morphologically attributable
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 1774 to *Archilina* are spread in the tree, as sister taxa of *Archilopsis* (clade A), or nested among species
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 2075 of *Duplominona* and *Duploperaclistus* (Clade C). In particular, the three Canarian species of
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 2276 *Archilina* form a clade that is the sister taxon of *Duploperaclistus circocirrus*, while the
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 2577 Mediterranean species of *Archilina* are external to this clade. In addition, the Caribbean *Archilina*
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 2778 *stylifera* is instead related to species of *Duplominona* from the same geographical area (Fig. 2) The
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 3079 close relationship of species, distinct for the presence or absence of a prostatoid organ, suggests
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 3279 that the phylogenetic value of this character has been greatly over-estimated. Previous studies
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 3581 suggested indeed multiple, independent events of loss of the prostatoid organ in
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 3782 “Duplomonocelidinae” (Litvaitis et al. 1996). Our analysis supports the presence of the prostatoid
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 3983 organ in common ancestor of the clade *Duplominona miranda* + *Duploperaclistus circocirrus* + the
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 41
 4284 Canarian species of *Archilina*, suggesting the loss of the character in the Canarian lineage, an event
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 4585 apparently independent from its loss in the species attributed *Archilina* from the Mediterranean
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 4786 and the Caribbean (Fig. 3). It is thus apparent that present taxonomic arrangement of the
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 4987 Monocelididae does not reflect phylogeny. The merit of the existing system was to **single out** from
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 5188 a very large number of species, subunits of species with comparable morphology, making
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 5489 taxonomic comparisons easier. The option to join the existing genera of “Duplomonocelidinae”
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 5690 into a few, if not one single genus, could be thus phylogenetically sensible, but would make
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 5991 taxonomy intractable, and create a number of synonyms, such as *Duplominona japonica* Ax, 1988
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 6192 and *Archilina japonica* Ax, 1988. We therefore postpone a thorough revision of the systematics of
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693 “Duplomonocelidinae”, until a more extensive dataset of species is available, and the delineation of
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 694 lineages, which may be phylogenetically and morphologically sensible, possible.
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696 *Acknowledgments*

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1056 FIGURE LEGENDS

1057 **Figure 1.** Likelihood mapping of the 18S (a) and 28S D1–D6 (b) genes. The likelihood-mapping
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 1058 method (Strimmer and von Haeseler 1996) partitions the area of the equilateral triangle into seven
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 1059 regions. The three trapezoids at the corners represent the areas supporting strictly bifurcating trees,
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 1060 that is the presence of a tree- like phylogenetic signal. The three rectangles on the sides represent
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 1061 regions where the decision between two trees is not obvious. The centre of the triangle represents
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 1062 sets of points P (posterior probabilities of the unrooted trees) where all three trees are equally
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 1063 supported (see Schmidt and von Haeseler 2012). In this case it is depicted a strong phylogenetic
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 1064 signal.
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1065 **Figure 2.** Phylogenetic tree. Tree obtained by BI showing the interrelationships of the species
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 1066 based on combined 18S+28S D1–D6. The branch length scale refers to the number of substitutions
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 1067 per site. Nodal supports are indicated for both ML (Bootstrap Values - BV) and BI (Posterior
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 1068 Probability - PP) in the legend at the bottom right. The collapsed clade named “*Monocelis lineata*
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 1069 species complex” refers to the ingroup of the study case presented in Scarpa et al. 2016a.
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870 **Figure 3.** Maximum Likelihood based Ancestral State Reconstruction (ASR) of the three discussed
 1 morphological features, i.e. from left to right, stylet, vagina and prostatoid organ. The Likelihood
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 371 reconstruction method **finds ancestral states** that maximize the probability that the observed state
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 572 would evolve under a stochastic model of evolution (Schluter et al. 1997; Pagel 1999). Values on
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 773 internal nodes represent likelihoods of the different character states. Internal pattern of branches
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 974 indicates the potential dichotomy for presence/absence of the character. Grey branches indicate
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 1175 unknown character state. Nodes labeled D1 and D2 have been dated to 7.97 mya, ranging 4.94-
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 1376 12.38, and 4.20 mya, ranging 1.95-7.32 mya, respectively.
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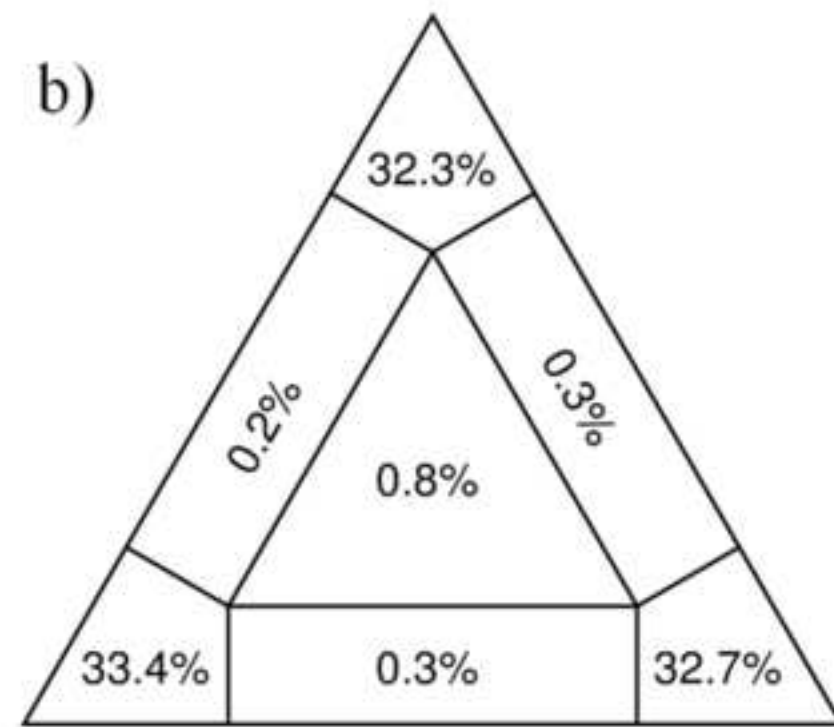
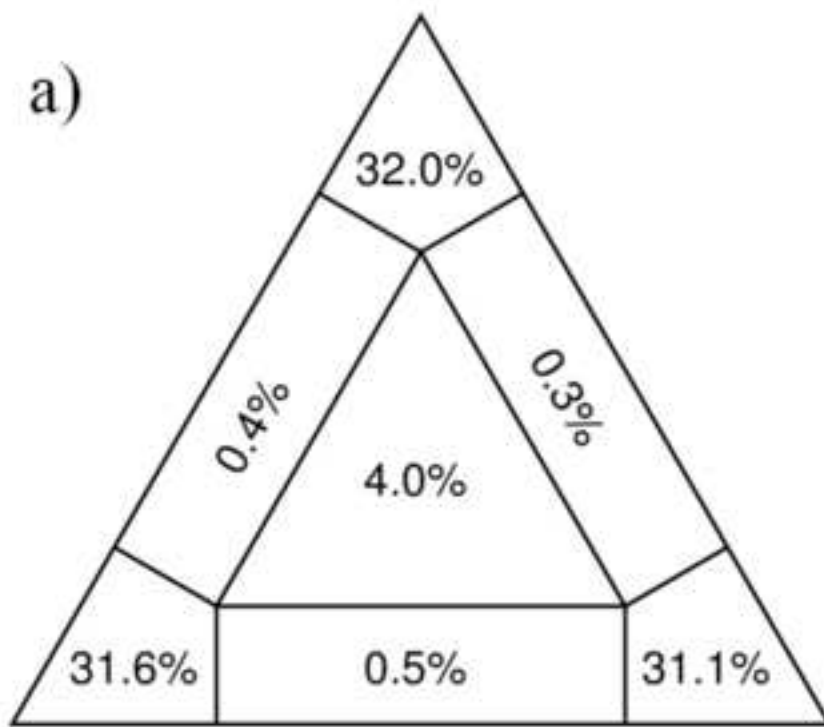
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 1778 * More internal nodes present the same Likelihood values.
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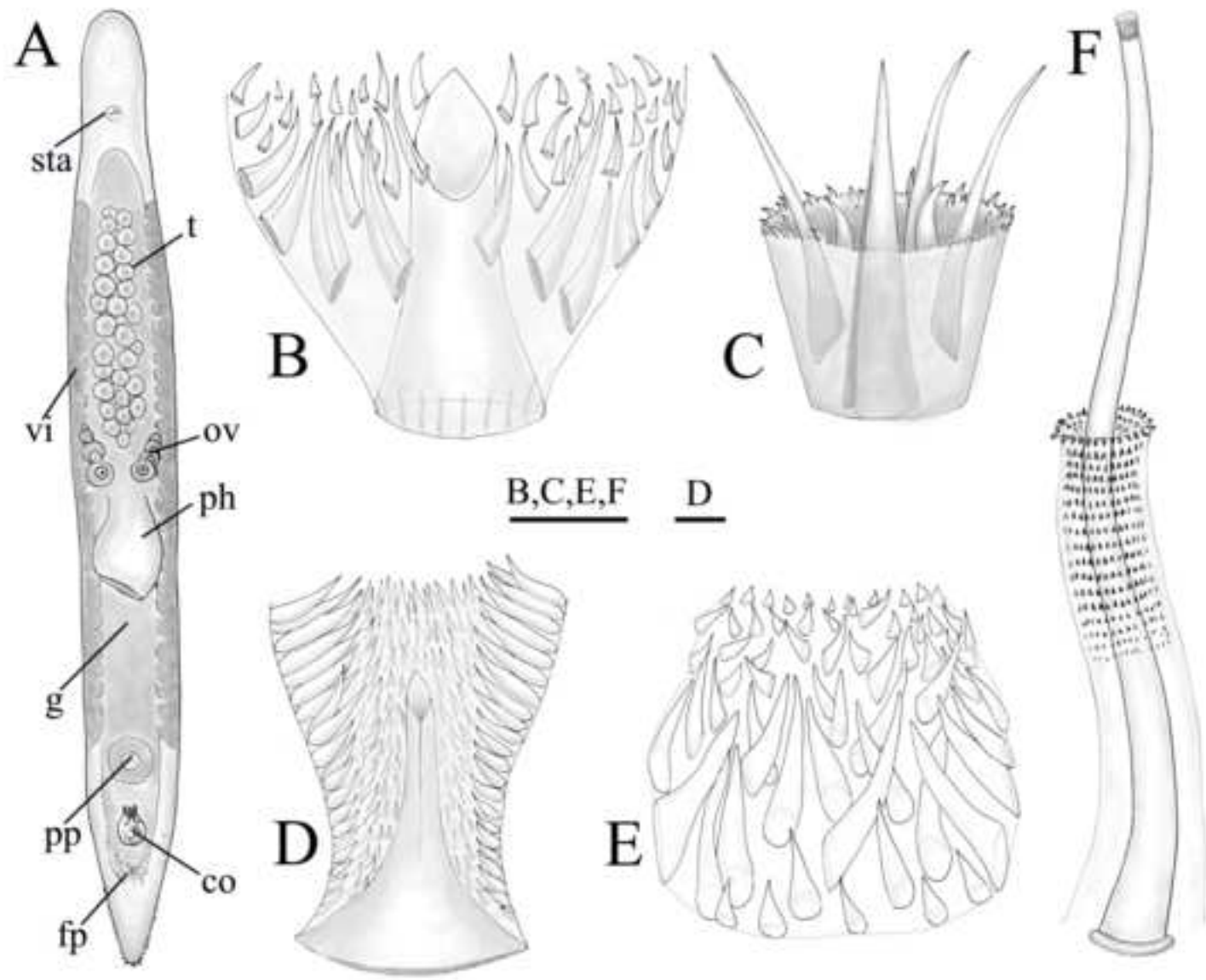
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 2279 **Figure 4.** General organization of a live specimen of *Archilina regina* sp. nov. (A). Cirrus in
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 2580 inverted condition of *A. regina* sp. nov. (B); *A. imperatrix* sp. nov. (C); *A. regisfilia* sp. nov. (D); *A.*
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 2781 *coronata* sp. nov. (E); *Duplominona miranda* sp. nov. (F). Scale bar = 10 μ m.
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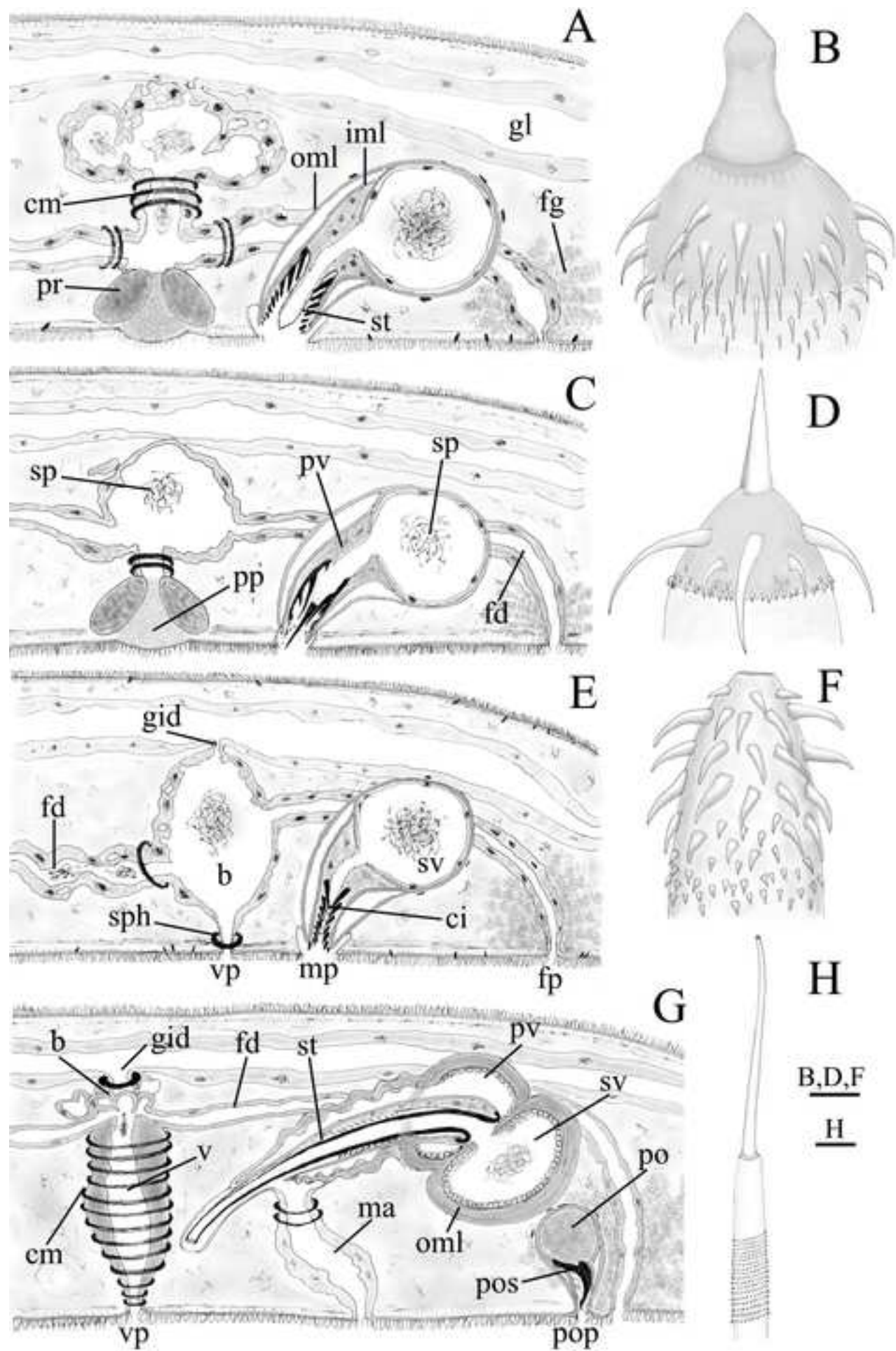
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 3082 **Figure 5.** Sagittal reconstruction of the genital area (on the left) and cirrus in everted condition (on
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 3283 the right) of *Archilina regina* sp. nov. (A, B), *A. imperatrix* sp. nov. (C, D); *A. coronata* sp. nov.
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 3484 (E, F); *Duplominona miranda* sp. nov. (G, H). Scale bars: B, D, F = 10 μ m; H = 25 μ m.
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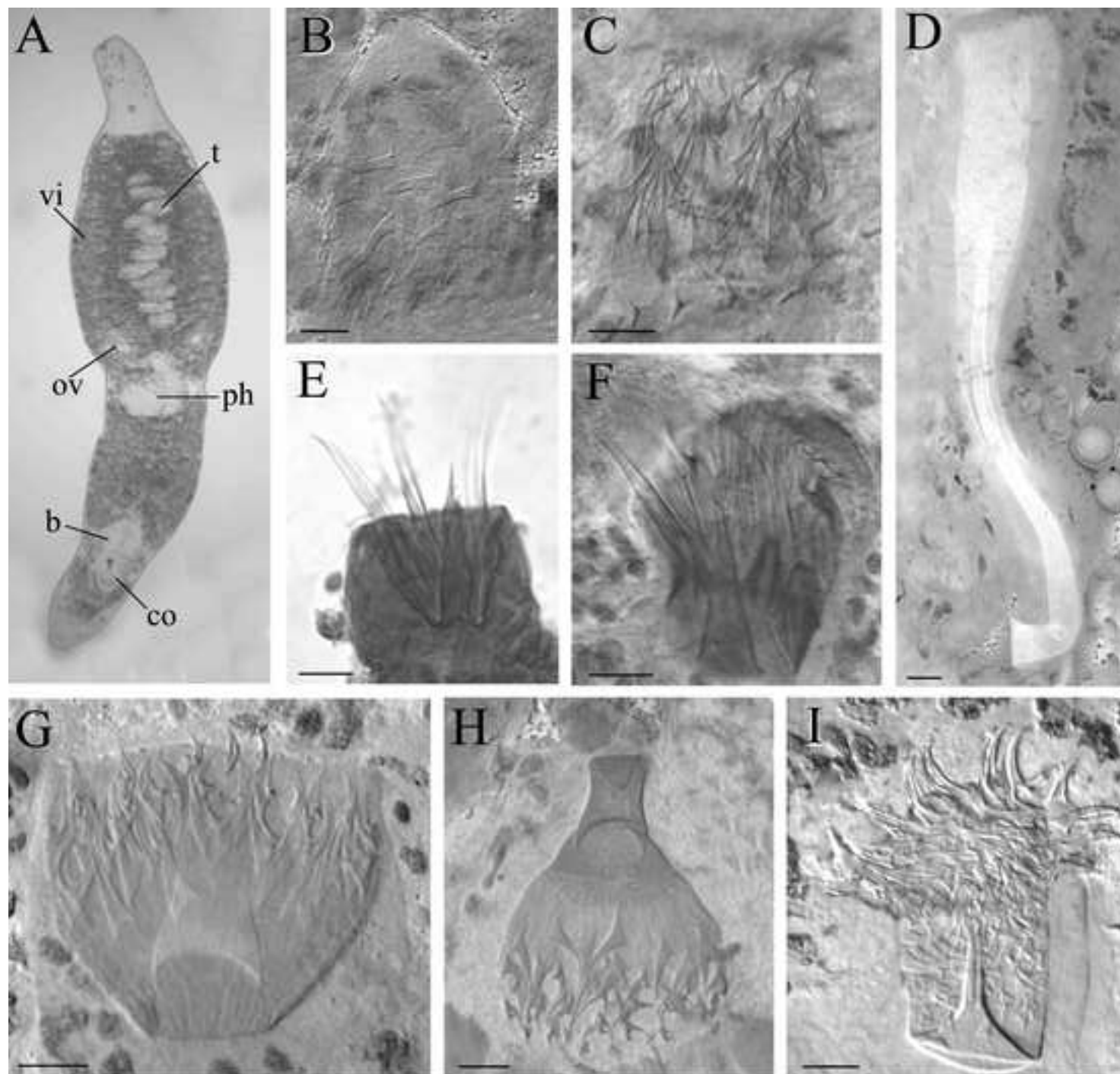
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 3785 **Figure 6.** Microphotograph of a living specimen of *Archilina regisfilia* sp. nov. (A). Cirrus of *A.*
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 3986 *coronata* sp. nov. in everted (B) and inverted (C) condition; Cirrus of *Duplominona miranda* sp.
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 4187 nov. in inverted condition (D); Cirrus of *A. imperatrix* sp. nov. in inverted conditions (E, F); Cirrus
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 4388 of *A. regina* sp. nov. in inverted (G) and everted (H) condition; Cirrus of *A. regisfilia* sp. nov. in
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 4589 inverted condition (I). Scale bar = 10 μ m.
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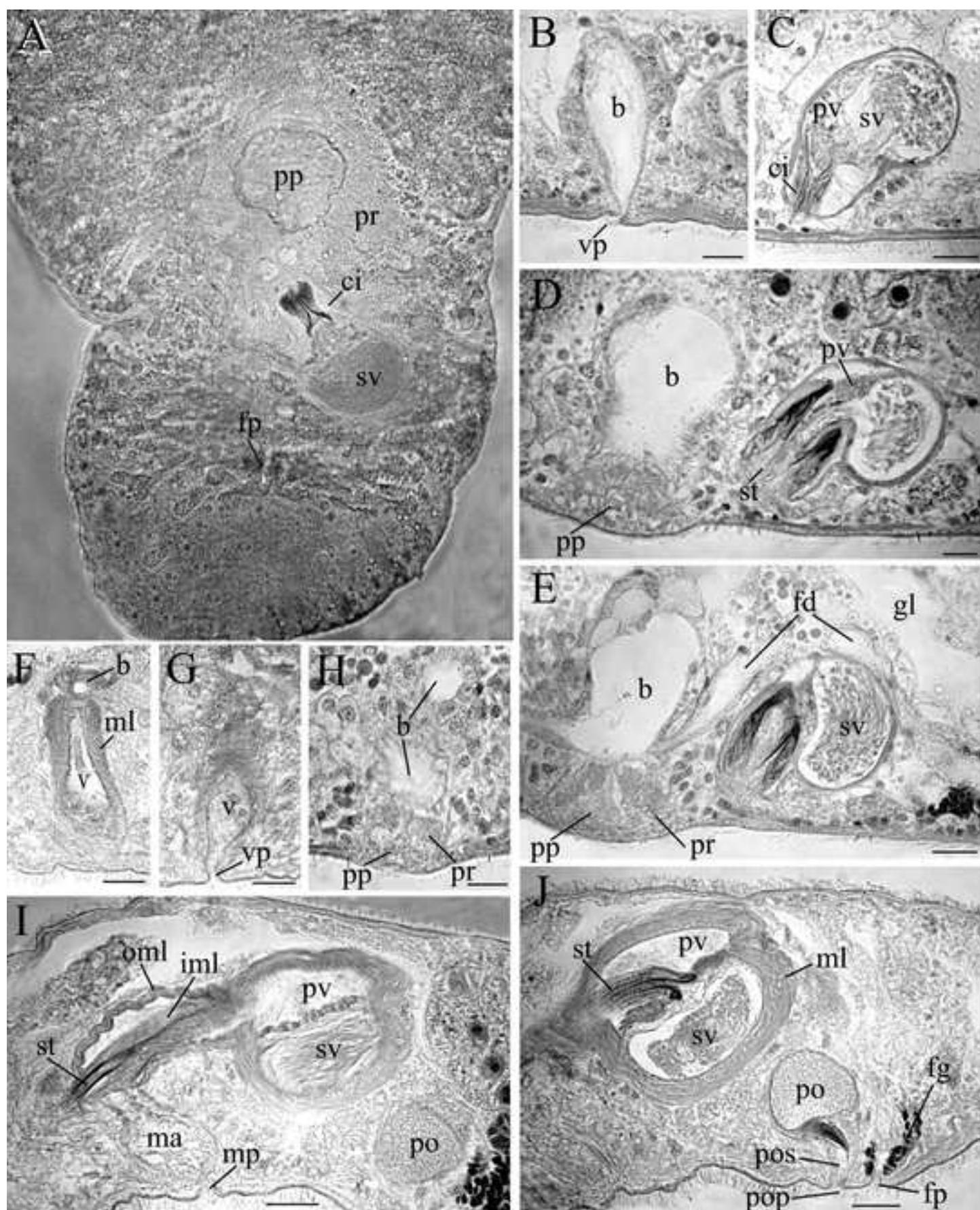
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 4990 **Figure 7.** Microphotograph of the genital area of a living specimen of *Archilina regisfilia* sp. nov.
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 5191 (A); *A. coronata* sp. nov.: sagittal section of female (B) and male (C) genital areas; *A. regina* sp.
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 5392 nov: sagittal section of genital area of specimens from Lanzarote (D) and Gran Canaria (E);
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 5593 *Duplominona miranda* sp. nov: sagittal section, at different focus, of female (F, G) and male (I, J)
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 5794 genital areas; H: *A. imperatrix*: sagittal section of female genital area. Scale bar = 10 μ m.
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How to achieve internal fertilization without a vagina: the study case of the genus *Archilina* Ax, 1959 (Platyhelminthes, Proseriata) from Canary Islands

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Supplementary Material S1. List of populations sampled. Accession numbers of sequences refer to GenBank codes. Accession numbers of newly sequenced taxa are in bold. The last three columns indicate results obtained by the several species delimitation methods used. Specimens with the same number within the same column has been ascribed to the same species. Note that numbers across columns and thus across methods do not represent the same species.

Species	Locality	18S	28S D1-D6	ST-GMYC	PTP/bPTP	ABGD
<i>Archilina coronata</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 66	MG7785 96	1	1	1
<i>Archilina coronata</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 67	MG7785 97	1	1	1
<i>Archilina coronata</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 70	MG7786 00	1	1	1
<i>Archilina coronata</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 56	MG7785 86	1	1	1
<i>Archilina coronata</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 57	MG7785 87	1	1	1
<i>Archilina coronata</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 58	MG7785 88	1	1	1
<i>Archilina regina</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 52	MG7785 82	2	2	2
<i>Archilina regina</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 53	MG7785 83	2	2	2
<i>Archilina regina</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 54	MG7785 84	2	2	2
<i>Archilina regina</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785 55	MG7785 85	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 59	MG7785 89	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 60	MG7785 90	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 61	MG7785 91	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 62	MG7785 92	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 63	MG7785 93	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 64	MG7785 94	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 65	MG7785 95	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 68	MG7785 98	2	2	2
<i>Archilina regina</i> sp. nov.	Lanzarote (Canary Islands, Spain)	MG7785 69	MG7785 99	2	2	2
<i>Archilina imperatrix</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	MG7785	MG7785	3	3	3

<i>Archilina imperatrix</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	48 MG7785 49	78 MG7785 79	3	3	3
<i>Archilina imperatrix</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	50 MG7785 51	80 MG7785 81	3	3	3
<i>Archilina imperatrix</i> sp. nov.	Gran Canaria (Canary Islands, Spain)	51 MG7785 76	81 MG7785 06	3	3	3
<i>Archilina endostyla</i> Ax, 1959	Punta Ala (Tuscany, Italy)	76 MG7785 77	06 MG7785 07	4	4	4
<i>Archilina endostyla</i> Ax, 1959	Punta Ala (Tuscany, Italy)	77 MG7785 74	07 MG7785 04	4	4	4
<i>Archilina deceptoria</i> Martens & Curini-Galletti, 1994	Asinara (Sardinia, Italy)	74 MG7785 75	04 MG7785 05	5	5	5
<i>Archilina deceptoria</i> Martens & Curini-Galletti, 1994	Asinara (Sardinia, Italy)	75 MG7785 71	05 MG7785 01	5	5	5
<i>Archilina styliifera</i> Curini-Galletti & Martens, 1996	Playa La Angosta (Colón, Panama)	71 MG7785 72	01 MG7785 02	6	6	6
" <i>Archiloa</i> " sp. 1	Playa La Angosta (Colón, Panama)	72 MG7785 73	02 MG7785 03	7	7	7
" <i>Archiloa</i> " sp. 2	Playa La Angosta (Colón, Panama)	73 KR36464	03 KR36469	8	8	7
<i>Monocelis algicola</i> Curini-Galletti & Casu, 2016	Cala Rossa (Sardinia, Italy)	6	1	9	9	8
<i>Monocelis algicola</i> Curini-Galletti & Casu, 2016	Cala Rossa (Sardinia, Italy)	7	2	9	9	8
<i>Monocelis algicola</i> Curini-Galletti & Casu, 2016	Cala Rossa (Sardinia, Italy)	8	3	9	9	8
<i>Monocelis algicola</i> Curini-Galletti & Casu, 2016	Cala Rossa (Sardinia, Italy)	9	4	9	9	8
<i>Monocelis algicola</i> Curini-Galletti & Casu, 2016	Cala Rossa (Sardinia, Italy)	0	5	9	9	8
<i>Monocelis exquisita</i> Curini-Galletti & Casu, 2016	Porto Puddu (Sardinia, Italy)	1	6	10	10	9
<i>Monocelis exquisita</i> Curini-Galletti & Casu, 2016	Porto Puddu (Sardinia, Italy)	2	7	10	10	9
<i>Monocelis exquisita</i> Curini-Galletti & Casu, 2016	Porto Puddu (Sardinia, Italy)	3	8	10	10	9
<i>Monocelis exquisita</i> Curini-Galletti & Casu, 2016	Porto Puddu (Sardinia, Italy)	4	9	10	10	9
<i>Monocelis exquisita</i> Curini-Galletti & Casu, 2016	Porto Puddu (Sardinia, Italy)	5	0	10	10	9
<i>Monocelis lineata</i> OF Müller, 1774	Colostrai (Sardinia, Italy)	8	3	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Colostrai (Sardinia, Italy)	9	4	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Colostrai (Sardinia, Italy)	0	5	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Al Iskandariyya (Egypt)	KR36463	KR36467	11	11	10

<i>Monocelis lineata</i> OF Müller, 1774	Casaraccio (Sardinia, Italy)	1 KR36465	6 KR36469	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Casaraccio (Sardinia, Italy)	1 KR36465	6 KR36469	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Pilo (Sardinia, Italy)	2 KR36462	7 KR36467	11	11	10
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	7 KR36461	2 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	9 KR36462	4 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	0 KR36462	5 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	1 KR36462	6 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	2 KR36462	7 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	3 KR36462	8 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	4 KR36462	9 KR36466	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Charaki (Rhodes, Greece)	5 KR36462	0 KR36467	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Charaki (Rhodes, Greece)	6 KR36462	1 KR36467	11	11	11
<i>Monocelis lineata</i> OF Müller, 1774	Ferrol (Galicia, Spain)	2 KR36463	7 KR36467	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Ferrol (Galicia, Spain)	3 KR36463	8 KR36467	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Kilmore Quay (Ireland)	4 KR36463	9 KR36467	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Kilmore Quay (Ireland)	5 KR36463	0 KR36468	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Keflavik (Iceland)	8 KR36463	3 KR36468	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Tjärnö (Sweden)	3 KR36465	8 KR36469	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Tjärnö (Sweden)	4 KR36465	9 KR36469	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Tjärnö (Sweden)	6 KR36465	1 KR36470	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Tjärnö (Sweden)	6 KR36463	1 KR36468	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Tjärnö (Sweden)	7 KR36463	2 KR36468	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Ardrossan (Scotland)	5 KR36465	0 KR36470	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Roscoff (France)	KR36465	KR36470	12	12	12

<i>Monocelis lineata</i> OF Müller, 1774	Roscoff (France)	7 KR36465	2 KR36470	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Helsingor (Denmark)	8 KR36463	3 KR36468	12	12	12
<i>Monocelis lineata</i> OF Müller, 1774	Helsingor (Denmark)	9 KR36464	4 KR36468	12	12	12
<i>Monocelis fusca</i> Örsted, 1843	Ardrossan (Scotland)	0 KR36461	5 KR36465	13	13	13
<i>Monocelis fusca</i> Örsted, 1843	Ardrossan (Scotland)	4 KR36461	9 KR36466	13	13	13
<i>Monocelis fusca</i> Örsted, 1843	Ardrossan (Scotland)	5 KR36461	0 KR36466	13	13	13
<i>Monocelis fusca</i> Örsted, 1843	Ardrossan (Scotland)	6 KR36461	1 KR36466	13	13	13
<i>Monocelis fusca</i> Örsted, 1843	Ardrossan (Scotland)	7 KR36461	2 KR36466	13	13	13
<i>Minona ileanae</i> Curini-Galletti, 1997	Great Bitter Lake (Egypt)	JN22490 5	JN22491 0	14	14	14
<i>Monocelis longiceps</i> (Duges, 1830)	Al-Maharas (Tunisia)	KC97106 4	KC97108 7	15	15	15
<i>Monocelis longistyla</i> Martens & Curini-Galletti, 1987	La Maddalena (Sardinia, Italy)	KR36461 8	KR36466 3	16	16	16
<i>Minona</i> cf <i>trigonopora</i> Ax, 1956	Palau (Sardinia, Italy)	KJ682346	KJ682408	17	17	17
<i>Minona</i> sp. nov. 1	Faro (Portugal)	KJ682359	KJ682421	18	18	18
<i>Minona</i> sp. nov. 1	Faro (Portugal)	KJ682360	KJ682422	18	18	18
<i>Minona</i> sp. nov. 2	Playa La Angosta (Colón, Panama)	KJ682344	KJ682406	19	19	14
<i>Minona</i> sp. nov. 2	Playa La Angosta (Colón, Panama)	KJ682345	KJ682407	19	19	14
<i>Minona gemella</i> Ax & Sopott-Ehlers, 1985	Playa La Angosta (Colón, Panama)	KJ682347	KJ682409	20	20	19
<i>Minona gemella</i> Ax & Sopott-Ehlers, 1985	Playa La Angosta (Colón, Panama)	KJ682348	KJ682410	20	20	19
<i>Minona gemella</i> Ax & Sopott-Ehlers, 1985	Playa La Angosta (Colón, Panama)	KJ682349	KJ682411	20	20	19
<i>Minona</i> cf <i>gemella</i> Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	KJ682350	KJ682412	21	21	20
<i>Minona</i> cf <i>gemella</i> Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	KJ682351	KJ682413	21	21	20
<i>Minona</i> cf <i>gemella</i> Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	KJ682352	KJ682414	21	21	20
<i>Minona</i> sp. nov. 3	Playa de Cruz (Boa Vista Island, Cape Verde)	KJ682353	KJ682415	22	22	21
<i>Minona</i> sp. nov. 3	Playa de Cruz (Boa Vista Island, Cape Verde)	KJ682354	KJ682416	22	22	21
<i>Minona</i> sp. nov. 4	Lanzarote (Canary Islands, Spain)	KJ682361	KJ682423	23	23	22
<i>Minona</i> sp. nov. 4	Tenerife (Canary Islands, Spain)	KJ682362	KJ682424	23	23	22
<i>Minona</i> sp. nov. 4	Tenerife (Canary Islands, Spain)	KJ682363	KJ682425	23	23	22
<i>Duplominona brasiliensis</i> Curini-Galletti, 2014	Ilhabela (Brazil)	KJ682367	KJ682429	24	24	23
<i>Duplominona miranda</i> sp. nov.	Roscoff (France)	KJ682376	KJ682438	25	25	23
<i>Duplominona</i> sp. nov. 1	Lanzarote (Canary Islands, Spain)	KJ682364	KJ682426	26	26	24
<i>Duplominona</i> sp. nov. 2	Faro (Portugal)	KJ682365	KJ682427	27	27	25
<i>Duplominona</i> sp. nov. 2	Faro (Portugal)	KJ682366	KJ682428	27	27	25
<i>Duplominona</i> sp. nov. 3	Naos Island (Panama)	KJ682374	KJ682436	28	28	26
<i>Duplominona</i> sp. nov. 3	Naos Island (Panama)	KJ682375	KJ682437	28	28	26

<i>Duplominona</i> sp. nov. 4	Naos Island (Panama)	KJ682368	KJ682430	29	29	27
<i>Duplominona</i> sp. nov. 4	Naos Island (Panama)	KJ682369	KJ682431	29	29	27
<i>Duplominona</i> sp. nov. 4	Naos Island (Panama)	KJ682370	KJ682432	29	29	27
<i>Duplominona</i> sp. nov. 5	Naos Island (Panama)	KJ682373	KJ682435	30	30	28
<i>Duplominona</i> sp. nov. 6	Blanes (Spain)	KJ682379	KJ682441	31	31	29
<i>Duplominona tridens</i> (Marcus, 1954)	São Sebastião (Brazil)	KJ682371	KJ682433	32	32	30
<i>Duplominona tridens</i> (Marcus, 1954)	São Sebastião (Brazil)	KJ682372	KJ682434	32	32	30
<i>Duploeraclistus circocirrus</i> Martens, 1983	Roscoff (France)	KJ682377	KJ682439	33	33	31
<i>Duploeraclistus circocirrus</i> Martens, 1983	Roscoff (France)	KJ682378	KJ682440	33	34	31
<i>Archilopsis spinosa</i> (Jensen, 1878)	Roscoff (France)	KJ682380	KJ682442	34	35	32
<i>Archilopsis arenaria</i> Martens, Curini-Galletti & Pucinelli, 1989	Roscoff (France)	KJ682381	KJ682443	35	36	33
<i>Archimonocelis staresoi</i> Martens & Curini-Galletti, 1993	Porto Cesareo (Lecce, Italy)	AJ270152	AJ270166	36	37	34

How to achieve internal fertilization without a vagina: the study case of the genus *Archilina* Ax, 1959 (Platyhelminthes, Proseriata) from Canary Islands

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Supplementary Material S2. Molecular pure diagnostic characters based on the 18S and 28S genes for the new species *Archilina coronata* sp. nov., *Archilina regina* sp. nov., *Archilina imperatrix* sp. nov. and *Duplominona miranda* sp. nov.

Nucleotide positions refer to the sequences in GenBank, whose accession numbers are within brackets near to each species.

Marker	Molecular Pure Diagnostic Characters
<i>Archilina coronata</i> sp. nov. (GenBank accession numbers: MG778566-67, MG778570 and MG778556-58 for the 18S; MG778596-97, MG778600 and MG778586-88 for the 28S)	
18S	23 (T); 30 (A); 104 (C); 338 (A); 339 (A); 388 (C); 501 (A); 502 (C)
28S	635 (T); 639 (T); 666 (A); 832 (A); 834 (C); 836 (A); 963 (A); 1004 (T); 1029 (A); 1172 (G); 1476 (G); 1499 (C)
<i>Archilina regina</i> sp. nov. (GenBank accession numbers: MG778552-55, MG778559-65 and MG778568-69 for the 18S; MG778582-85, MG778589-95 and MG778598-99 for the 28S)	
18S	554 (C)
28S	623 (C); 628 (C); 863 (A); 864 (C)
<i>Archilina imperatrix</i> sp. nov. (GenBank accession numbers: MG778548-51 for the 18S; MG778578-81 for the 28S)	
18S	556 (C); 575 (G)
28S	493 (G); 573 (C); 627 (G); 658 (A); 871 (T); 1278 (T); 1497 (G)
<i>Duplominona miranda</i> sp. nov. (GenBank accession numbers: KJ682376 for the 18S; KJ682438 for the 28S)	
18S	15 (T); 36 (A); 65 (A); 69 (T); 71 (T)
28S	none