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Syndesmis aethopharynx (Umagillidae, Rhabdocoela, Platyhelminthes) from the sea urchin Paracentrotus lividus: first record from the Eastern Mediterranean, phylogenetic position and intraspecific morphological variation Peer-reviewed author version

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Highlights

- *Syndesmis aethopharynx* is reported from *Paracentrotus lividus* in Greece, constituting the first record of this species from the Mediterranean.
- Previously-unreported morphological details and intraspecific variation of the species are described.
- The position of *S. aethopharynx* within Umagillidae is confirmed through Bayesian and maximum likelihood analyses (18S rDNA).



Syndesmis aethopharynx (Umagillidae, Rhabdocoela,
 Platyhelminthes) from the sea urchin *Paracentrotus lividus*: first
 record from the Eastern Mediterranean, phylogenetic position and
 intraspecific morphological variation

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Marlies Monnens¹, Tom Artois¹ and Maarten P.M. Vanhove^{1,2,3,4,5}

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¹Hasselt University, Centre for Environmental Sciences, Research Group Zoology:
 ⁹Biodiversity and Toxicology, Agoralaan Gebouw D, B-3590 Diepenbeek, Belgium.
 ¹⁰marlies.monnens@uhasselt.be_tom.artois@uhasselt.be_mvanhove@naturalsciences.be

²Hellenic Centre for Marine Research, Institute of Marine Biological Resources and Inland
 Waters, 46.7 km Athinon – Souniou Av., 190 13, P.O. Box 712, Anavyssos.
 mvanhove@naturalsciences.be

³Royal Belgian Institute of Natural Sciences, Operational Directorate Natural Environment,

Capacities for Biodiversity and Sustainable Development, Vautierstraat 29, B-1000 Brussels,
 Belgium. mvanhove@naturalsciences.be

⁴University of Leuven, Department of Biology, Laboratory of Biodiversity and Evolutionary

18 Genomics, Charles Deberiotstraat 32, B-3000 Leuven, Belgium.
 19 myanhove@naturalsciences.be

⁵Masaryk University, Faculty of Science, Department of Botany and Zoology, Kotlářská 2,

21 *611 37 Brno, Czech Republic.* <u>mvanhove@naturalsciences.be</u>

22 Abstract

Specimens of Syndesmis aethopharynx Westervelt & Kozloff, 1990 (Umagillidae, 23 Rhabdocoela, Platyhelminthes) were collected from the intestine of several specimens of the 24 sea urchin Paracentrotus lividus (Lamarck, 1816) Hansson, 2001 at the Greek coast. This 25 represents the first report of a species of Syndesmis from Greece. Our study has revealed 26 several previously-unreported morphological details and intraspecific variation, which are 27 added to the species description. The position of S. aethopharynx within Umagillidae is 28 confirmed for the first time through molecular data (based on nuclear 18S rDNA), using both 29 Bayesian and maximum likelihood analyses. 30

31 Keywords: taxonomy; phylogeny; marine microturbellarians; Echinoidea

32 **1** Introduction

With about 1700 species described, Rhabdocoela constitutes one of the most species-rich taxa among turbellarian (non-neodermatan) flatworms (Platyhelminthes), and is also one of the most diverse. While the vast majority of species within this group are free-living, several taxa have independently acquired an endosymbiotic lifestyle [3]. Umagillidae Wahl, 1910

[·] Corresponding author: marlies.monnens@uhasselt.be, +32 11 29 21 94

(Neodalyellida, Dalytyphloplanida) is by far the largest family (75 species) of symbiotic
turbellarians [5, 6]. Representatives of this group have been reported from echinoderm or
sipunculid hosts on all continents. The majority of umagillids live within the intestine or
coelomic cavity of either sea cucumbers (51%) or sea urchins (33%).

Despite the well-known ecological importance [7-9] and commercial value [10-12] of echinoids in the Mediterranean, and of the parechinid *Paracentrotus lividus* (Lamarck, 1816) Hansson, 2001 in particular, their endosymbiont fauna remains largely understudied. Occasional studies on this topic have predominantly focused on intestinal bacteria (e.g. Meziti, Kormas, Pancucci-Papadopoulou and Thessalou-Legaki [12]) and ciliates (e.g. Lynn and Strüder-Kypke [15]).

47 Until now, only three species of endosymbiotic rhabdocoels have been reported from sea urchins from the Mediterranean Sea. All of these are representatives of Syndesmis Silliman, 48 1881, the most species-rich genus of Umagillidae (see Tyler [17]). These three species are S. 49 echiniacuti Kozloff, 1997, S. echinorum François, 1886 [both described from the echinid 50 Gracilechinus acutus (Lamarck, 1816) Fell & Pawson, 1966] and S. aethopharynx Westervelt 51 & Kozloff, 1990 (known only from P. lividus). Westervelt and Kozloff [1] retrieved what is 52 presumably a fourth Mediterranean species from P. lividus, though it was not formally 53 described at the time. 54 Furthermore, hardly any molecular systematics work has been conducted on Umagillidae, or

Furthermore, hardly any molecular systematics work has been conducted on Umagillidae, or endosymbiotic rhabdocoels in general. Indeed, in the most-recently-published phylogeny of Dalytyphloplanida (based on 18S and 28S rDNA), only three of the 75 valid umagillid species were included and only one of three subfamilies (Umagillinae Stunkard & Corliss, 1951) was represented [3].

As a first step towards a better understanding of the biodiversity and occurrence of 60 Umagillidae in the Mediterranean, we examined the intestinal rhabdocoels of several 61 specimens of P. lividus from the Greek coast. Considering that both S. aethopharynx and S. 62 echinorum have been reported from P. lividus in Banyuls-sur-Mer, France [1] and given the 63 wide distribution range of several species of Syndesmis [20], we expected to find specimens 64 of at least some species of Syndesmis in local sea urchins. If this was the case, we planned to 65 sequence 18S rDNA of all specimens in order to perform the first molecular phylogenetic 66 analysis including these species of Syndesmis. 67

68 2 Material & Methods

69 2.1 Taxon sampling and microscopy

Specimens of *Paracentrotus lividus* were collected by hand in the Saronic Gulf at Anavyssos, off Mavro Lithari (37.73278°N, 23.90361°E) on February 21st, 2013. Their coelom and digestive system were inspected for flatworms under a stereomicroscope. Umagillids were fixed in analytical-grade ethanol; pictures were taken of a subset of specimens prior to fixation, slightly flattened between slide and coverslip in sea water. After fixation, all specimens were cut in half: one part was used for DNA extraction and the other to prepare whole mounts in lactophenol. The internal morphology of 25 whole-mounted specimens was studied under a Nikon Eclipse 80i compound microscope and a Leica DM 2500 microscope,

- vsing interference contrast. Internal organs were photographed and subsequently drawn with
- the aid of a camera lucida. Measurements were made along the central axis of these structures.
- 80 The pharynx turned out to be positioned in such a way that it was impossible to capture its
- 81 entirety on a single photograph. Therefore, we compiled a focus-stacked image of three
- photographs at different focal depths using Adobe Photoshop CS5 v12.1.

Voucher specimens of the flatworms and their hosts were deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Delaim) (III and XXXX XXXX and IIII best such as XXXX XXXX)

85 Belgium) (HU nos XXXX-XXXX and HU hostvoucher XXXX-XXXX).

86 2.2 DNA extraction, PCR and sequencing

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the 87 manufacturer's instructions. We amplified fragments of the nuclear 18S rDNA gene using a 88 nested PCR approach with primer combinations from Norén and Jondelius [21]. The initial 89 (5'-AMCTGGTTGATCCTGCCAG-3') 90 PCR used TimA and TimB (5'-TGATCCATCTGCAGGTTCACCT-3'). Nested PCR was carried out using the internal 91 primer combinations S30 (5'-GCTTGTCTCAAAGATTAAGCC-3') with 5FK (5'-92 TTCTTGGCAAATGCTTTCGC-3') and 4FB (5'-CCAGCAGCCGCGGTAATTCCAG-3') 93 with 1806R (5'-CCTTGTTACGACTTTTACTTCCTC-3'), respectively. Polymerase Chain 94 95 Reaction was performed in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems) 96 using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare), adding 2.5 µL of each primer (20 µM) (Sigma Aldrich) (1 µL in the nested PCR), 3 µL of template DNA and 17 µL 97 98 of double distilled, autoclaved and filter sterilized water (21 µL in the nested PCR). In the initial PCR, after an initial denaturation of 310 s at 95 °C, samples were subjected to 35 99 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C. After a final elongation of 300 s at 72 100 °C, samples were cooled to 4 °C. The nested PCR followed a similar protocol, but with 240 s 101 of initial denaturation and an annealing step at 50 °C for 30 s, an equal duration to the other 102 PCR cycle steps. Final elongation at 72° C took 600 s. PCR products were purified with the 103 QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. 104 Sequencing of both strands was performed using the internal primers with an Applied 105 Biosystems 3730 DNA analyser and BigDye version 1.1. Sequences were deposited in NCBI 106 GenBank under accession numbers xxxxxxxx. 107

108 2.3 Phylogenetic analysis

Consensus sequences were assembled from the obtained contigs in GENEIOUS v5.7.5 [22]. 109 The resulting sequences were subsequently subjected to a BLAST search [23] on the NCBI 110 website to screen for contamination. Other neodalyellid and outgroup sequences were selected 111 from Van Steenkiste, Tessens, Willems, Backeljau, Jondelius and Artois [3]. Corresponding 112 GenBank accession numbers of all used sequences are listed in Table A.1. Sequences were 113 aligned with the structural Q-INS-I algorithm in MAFFT [24], which accounts for RNA 114 secondary structure. Ambiguously-aligned sites were identified with Aliscore v2.0 [25] with 115 default sliding window settings (w = 6) and removed from the alignment using Alicut v2.3 116 [26]. The best fitting substitution model for the dataset was determined in jModeltest v2.1.6 117

118 [27] on the CIPRES Science Gateway server [28]. The GTR+G+I model was selected based119 on both the Akaike Information Criterion and the Bayesian Information Criterion.

Model-corrected pairwise genetic distances were calculated in PAUP* v4.0a152 [29]. 120 Phylogenetic trees were constructed using both maximum likelihood (ML) and Bayesian (BI) 121 approaches. ML analysis was carried out in RAxML v8.0.0 [30] on the CIPRES server: 100 122 independent runs were performed under the GTR+G+I model. Support values were assessed 123 with 1000 non-parametric bootstrap replicates. Bayesian analyses were carried out using the 124 Metropolis coupled Markov chain Monte Carlo (MC³) method under the GTR+G+I model in 125 MrBayes v3.2.6 [31]. Two independent, simultaneous runs, each including one cold and three 126 heated chains, were conducted for 10 million generations. Trees were sampled every 100th 127 generation after a burn-in of 25%. Convergence of the chains was confirmed by the average 128 standard deviation of split frequencies falling below 0.01, the potential scale reduction factor 129 approaching 1.0, and the log-likelihoods reaching a stationary distribution. A majority-rule 130 consensus tree was constructed from all retained topologies. Resulting ML and BI trees were 131 visualized in FigTree v1.4.3 [32]. 132

Intrafamilial relationships of Umagillidae were not dichotomously resolved: while Wahlia and 133 Seritia together constituted a well-supported clade (bootstrap value = 95, posterior probability 134 = 1), the exact position of *Syndesmis* within the family was left ambiguous (see results in 135 section 3 below). Therefore, we formally tested three hypotheses for the position of S. 136 aethopharynx within Umagillidae. From now on, these three topologies will be referred to as 137 topology A (which is part of the ML tree), B and C respectively (as defined in Fig. 1). Both 138 the weighted (WSH) and unweighted (SH) Shimodaira-Hasegawa test [33] and the 139 140 Approximately Unbiased (AU) test [34] were performed in Consel v0.1j [35]. These nonparametric tests use the difference in log-likelihoods of competing topologies as a test 141 statistic and apply bootstrap resampling (multiscale bootstrapping in case of the AU test) to 142 obtain its null distribution. All three tests are valid when comparing a posteriori selected 143 topologies, provided the ML tree is included [33, 34]. TREE-PUZZLE v5.2 [36] was used to 144 assess site-wise log likelihoods for the trees, which were subsequently used to construct a 145 suitable input dataset for Consel. 146

147 **3 Results**

148 3.1 Infection parameters

Out of nine specimens of *P. lividus* collected, six harboured umagillids, all being *S. aethopharynx*, with an infection intensity varying between one and seven flatworms per host.
Investigation of ten sympatric specimens of the arbaciid sea urchin *Arbacia lixula* (Linnaeus, 1758) Hansson, 2001 did not yield any turbellarians.

153 3.2 Taxonomical comments

- 154 *Syndesmis aethopharynx* Westervelt & Kozloff, 1990
- 155 New locality. Anavyssos, Mavro Lithari, Greece (37.73278°N, 23.90361°E) (21/02/2013).
- 156 **Known distribution.** Banyuls-sur-Mer, France [1].
- 157 Material. Photographs of live animals. Twenty-five specimens, half of each specimen whole-
- mounted with lactophenol and the other half used for DNA extraction.

- **Description.** The pharynx is situated in the first body half. It has a distinct bipartite structure, consisting of a proximal, bulbous part, followed by a more elongate, cylindrical portion (focus-stacked image in **Fig. 2**; original images provided in **Fig. 6** in appendix). On the only specimen on which the pharynx could be measured adequately, the bulbous part has a
- 163 diameter of 138 μ m, while the cylindrical part is 95 μ m long.

164 The paired testes form a network-like structure and are positioned laterally just behind the 165 pharynx. The sclerotized part of the male copulatory organ consists of a simple, straight stylet, 166 measuring 45 to 67 μ m ($\bar{x} = 57 \mu$ m; n = 16). It has a funnel-shaped basis and tapers distally 167 towards an open pointed end (**Fig. 3**).

- Vitelline glands are paired and confined to the middle third of the body. Each vitellarium is composed of six to nine primary trunks, each dividing dichotomously to form numerous distal branches. Ovaries are also paired and occupy the area just behind the vitellaria. Both consist of one main trunk branching into three to four distal lobes. Filamentous glands are numerous and occupy the major part of the last body third.
- 173 Six specimens contain an amber-coloured egg capsule characterized by a very long, tightly-
- 174 coiled filament (Fig. 4). The distal part of the filament shows a small expansion, as indicated
- in Fig. 4. The egg capsules measure 99 to 235 μ m in length ($\overline{x} = 176 \mu$ m, n = 6) and 72 to 164
- 176 $\mu m \ (\bar{x} = 124 \ \mu m, n = 6) \text{ in width.}$

177 3.3 Phylogenetic analysis

After processing (alignment and masking), all obtained sequences were identical to each other. Pairwise genetic distances between Umagillidae are shown in **Table 1**. Distances between all species in the analysis are summarized in **Table A.2**. The inferred Bayesian tree topology (**Fig. 5**) is identical to the ML tree. Symbols at each branch indicate posterior probabilities (pp) and bootstrap values (bp). The position of *S. aethopharynx* within Umagillidae is firmly supported (pp = 1, bp = 96), but intrafamilial relationships are not dichotomously resolved.

P-values of the SH, WSH and AU tests are listed in **Table 2**. The AU test rejected topology C (p < 0.05) and resulted in a larger p-value for topology B compared to topology A, but neither of these two hypotheses could be rejected statistically (p > 0.05). Similarly, the SH and WSH tests resulted in larger p-values for topology B, but neither topology A or C could be rejected (p > 0.05).

190 **4 Discussion**

191 4.1 Species identification and intraspecific variation

192 Traditionally, umagillid flatworms from echinoid hosts have been assigned to either 193 *Syndesmis* or *Syndisyrinx*' Lehman, 1946. The validity of the latter genus has been a topic of 194 debate. Here, we will adopt the notion of Marcus [38] that *Syndisyrinx*' is a synonym of 195 *Syndesmis*, which has been agreed upon by Stunkard and Corliss [39], Hyman [40], Jondelius 196 [41] and Doignon and Artois [42]. Our specimens display all typical features of *Syndesmis*: a 197 very long, often tightly-coiled egg filament, a simple, funnel-like stylet, numerous filament 198 glands in the rear body end, paired ovaries, testes and vitelline glands in discrete pairs and an 199 echinoid host.

The bipartite pharvnx is a unique feature for S. aethopharvnx: it has never been reported in 200 any other syndesmid, or even another umagillid species. In the original description of this 201 species [1], no measurements for the pharynx were provided, but we were able to measure 202 both distinct components of this organ on requested photographs of the paratype specimen 203 (Smithsonian National Museum of Natural History, US National Parasite Collection, USNM 204 1376373) using Fiji [43]. In the paratype, the bulbous part measures 141 µm in diameter 205 206 while the cylindrical part measured 96 µm in length, hence closely resembling the pharynx in our specimen, in which these components measure 148 µm and 95 µm respectively. 207

In their description of *S. aethopharynx*, Westervelt and Kozloff [1] stated that all their specimens possess stylets measuring "about 50 μ m". The stylets of our specimens largely correspond to this ($\bar{x} = 57 \mu$ m; n = 16). We did, however, observe a wide size range (length varying from 45 to 67 μ m).

Egg capsule size varies greatly between specimens, ranging from 99 to 235 µm in length and 212 from 72 to 164 µm in width. Two specimens have capsules that are considerably smaller than 213 the rest, measuring only 72 x 107 µm and 80 x 99 µm respectively. The remainder measure 214 between 124-158 µm in length and 187-235 µm in width. Within Syndesmis, a comparable 215 degree of variation has only been reported for S. collongistyla (Hertel, Duszynski & Ubelaker, 216 1990) Marcus, 1949 and for S. dendrastrorum Stunkard & Corliss, 1951 [45], where the 217 capsule lengths vary over a range of \pm 90 µm and \pm 100 µm respectively. Capsule width does 218 not seem to vary as much in these species (\pm 60 µm in S. collongistyla and \pm 54 µm in S. 219 dendrastrorum). 220

To the best of the authors' knowledge, the only-proposed explanation for large intraspecific variations in syndesmid capsule sizes is a difference in the number of gonads. This has been reported for the three so-called morphs of *S. dendrastrorum* [45]. However, this is not applicable to our specimens. Capsule size might also be correlated with the size of the parent worm, as is the case for the umagillid *Anoplodium hymanae* Shinn, 1983 [47]. Unfortunately, we were unable to adequately measure full body lengths in our specimens and, therefore, could not check whether this correlation also exists in *S. aethopharynx*.

No measurements for the egg capsule were specified by Westervelt and Kozloff [1]. On their 228 photograph, the paratype's egg measures 124 μ m x 65 μ m, a bit narrower than the smallest 229 egg in our specimens. Moreover, both the holotype's [as shown in Fig. 3 in Westervelt and 230 Kozloff [1]] and the paratype's (as seen on requested photographs) egg capsules are more or 231 less oval, as opposed to the rather spheroid eggs in our specimens. However, the type 232 specimens' capsule walls appear shrivelled, which may imply these structures have collapsed. 233 It has already been suggested that rhabdocoel egg capsules can sometimes deform during 234 235 fixation [48] and this might be the case for these specimens. This could also explain the fact that all our specimens' eggs were a bit wider than the paratype's egg. 236

The small expansion at the tip of the egg filament was not mentioned by the original authors.This structure may conform to the 'solidified shell secretion droplet' at the end of the filament

which von Graff [48, 49] described in his work on *S. echinorum*. A similar structure was
reported in some specimens of *S. aonikenki* Brusa, Montes, Marcotegui and Martorelli, 2017
and *S. selknami* Brusa, Montes, Marcotegui and Martorelli, 2017. It also seems to occur in *S. franciscana* (Lehman, 1946) Marcus, 1949 as shown in Fig. 1 in Shinn and Cloney [51], but
this was not discussed by these authors.

Furthermore, photographs as well as the authors' drawings of the holotype [Fig. 1 and Fig. 3 in Westervelt and Kozloff [1]] display the holotype's egg capsule in the first body half, almost at the anterior edge of the testes, but only one of our specimens conforms to this. All other specimens' egg capsules are located about midway along the body and the paratype's egg also seems to be located in the middle of the body. Since no serial sections were available, the position of the uterus in our specimens remains uncertain.

250 The position of both egg capsule and uterus have been used as diagnostic traits for several species of Syndesmis. Examples include the original descriptions of S. glandulosa Hyman, 251 1960 and S. dendrastrorum, the redescription of S. antillarum Powers, 1936 by Stunkard and 252 Corliss [39], as well as the identification keys by Hickman [53] and Cannon [54]. However, 253 both Marcus [55] and Jennings and Mettrick [56] mentioned a variable position of the egg in 254 their respective descriptions of S. evelinae Marcus, 1968 and S. franciscana, while Hertel, 255 Duszynski and Ubelaker [44] pointed out that the position of the uterus in S. collongistyla 256 varies between specimens. The position of the uterus may be dependent on whether or not an 257 258 egg capsule is present, as is suggested in the original descriptions of S. aethopharynx, S. 259 rubida Kozloff & Westervelt, 1990, S. inconspicua Westervelt & Kozloff, 1992 and S. neglecta Westervelt & Kozloff, 1992. According to these authors, the uterus may extend into 260 261 the anterior body end in cases where the egg is fully developed. Therefore, and because of the variable position of the eggs in our specimens, the positions of the uterus and/or egg capsules 262 seem at least to some degree to be determined by the stage of development. Consequently, we 263 believe these criteria are not suitable for distinguishing between species of Syndesmis and 264 should be avoided as diagnostic characters in future taxonomic work on this taxon, and 265 perhaps umagillids in general. 266

Only two other umagillids are known from P. lividus: S. echinorum and another, unnamed 267 Syndesmis sp., which may in fact represent some very aberrant specimens of S. echinorum [1]. 268 269 Over the years, many different syndesmid species have unjustifiably been attributed to S. echinorum, often with little or no notes on morphology. As a result, taxonomic literature on 270 this species has become entangled [42, 59]. Here, we will compare our specimens to the 271 272 redescription of S. echinorum by Kozloff and Westervelt [59]. Both species differ considerably in stylet length: a length of 200 µm is reported in S. echinorum [59], compared 273 274 to an average of 57 µm in our specimens. Furthermore, ovaries in S. echinorum have up to eight lobes, as opposed to the three to four terminal lobes in our specimens. Finally, S. 275 echinorum possesses a regular pharynx doliiformis, while our specimens' pharynx has an 276 atypical bipartite structure. 277

Likewise, our specimens differ notably from *Syndesmis* sp.: *Syndesmis* sp. possesses vitelline glands which extend very far anteriorly, reaching even past the testes, whereas vitellaria in our specimens are confined to the middle body third. Secondly, ovaries in *Syndesmis* sp. appear sinuous and not (or very slightly) branched, as opposed to the distinctly-lobed ovaries
in our specimens. Finally, *Syndesmis* sp. has only one well-developed testis, whereas our
specimens all possess two distinct testes [1].

From the above, it is clear that our specimens agree in morphology with the description of *S. aethopharynx* based on a combination of traits, the most important ones being the distinct shape (and size) of the pharynx, the short, straight stylet and the relative sizes and positions of testes, ovaries and vitelline glands. Furthermore, they are easily distinguished from other *P. lividus*-infesting umagillids, and hence it seems justified to attribute our specimens to *S. aethopharynx*. So far, this species had only been reported from its type locality in France, and this is the first record of a syndesmid flatworm from Greece.

291 4.2 Phylogenetic position

Previous phylogenetic analyses have demonstrated that the 18S rDNA gene generally has a sufficiently high substitution rate to differentiate between closely-related rhabdocoels (e.g. Van Steenkiste, Tessens, Willems, Backeljau, Jondelius and Artois [3]; Tessens, Janssen and Artois [60]). Because all of our obtained sequences were identical to each other, the existence of cryptic species within the morphospecies *S. aethopharynx* seems, therefore, unlikely, despite the above-mentioned intraspecific variation.

Phylogenetic inference resulted in strong support for the position of *S. aethopharynx* within Umagillidae, but intrafamilial relationships are not fully resolved. *Syndesmis* did appear as the sister group of *Wahlia* and *Seritia* in the best-scoring ML tree (**Fig. 1a**), but support values are very low (bp = 48). Likewise, topology A was more often retrieved by the Bayesian analysis (pp = 0.664) compared to the two alternate hypotheses (pp = 0.235 and pp = 0.102 respectively), but the posterior probabilities in all cases are low.

In addition, model-corrected pairwise genetic distances also lend support to topology A. Indeed, the genetic distance between *Syndesmis* and *Anoplodium* (6.6%) is considerably greater than the distance between *Syndesmis* and the *Seritia-Wahlia* group (4.0% and 3.6% respectively). Moreover, distances between *Syndesmis* and *Seritia* and between *Syndesmis* and *Wahlia* are considerably lower than the distances between *Anoplodium* and these two species (5.8% and 5.6% vs. 6.8%).

Conversely, non-parametric testing (SH, WSH and AU) generally resulted in larger p-values for topology B. However, only the AU test resulted in formal rejection of a hypothesis (topology C; p < 0.05), leaving the exact position of *S. aethopharynx* still ambiguous. Sequencing of more and more-rapidly-evolving molecular markers (e.g. COI) and inclusion of more taxa in the analysis might yield the necessary phylogenetic resolution to gain a better understanding of the phylogenetic relationships within this family.

316 4.3 Relation to morphology

No morphological evidence for either of the three hypotheses could be obtained from taxonomical literature. Our analysis did confirm the finding of Van Steenkiste, Tessens, Willems, Backeljau, Jondelius and Artois [3] that *Wahlia* and *Seritia* constitute a monophyletic clade. This result was to be expected, since the same molecular markers were used in both studies. However, the taxonomical implications of this grouping have never beendiscussed in detail.

The vast majority of proposed hypotheses on the interrelationships among Umagillidae are 323 solely based on morphology. An extensive review of Umagillidae was provided by Cannon 324 325 [54]. In that contribution, four different subgroups within the subfamily Umagillinae were defined. This classification is based on a combination of traits related to the uterus, the 326 copulatory organ and the vitelline glands. Wahlia and Seritia were assigned to the so-called 327 Cleistogamia-group, together with Ozametra Marcus, 1949 and Cleistogamia Faust, 1924. 328 329 This grouping is based on the presence of a secondary uterus, which Cannon [54] considered to be the derived character state. 330

Unfortunately, no members of *Ozametra* or *Cleistogamia* could be included in our analysis and the overall number of umagillid species in our analysis is evidently very low. However, it is worth mentioning that our results do provide some support for the hypothesis of Cannon [54] and hence a secondary uterus may indeed be an apomorphy for the *Cleistogamia*-group. Evidently, a more extensive analysis including more taxa is necessary to draw definite conclusions.

337 4.4 Conclusions and future perspectives

Syndesmis aethopharynx is reported for the first time from the Eastern Mediterranean. Until now, this species had only been reported from its type locality. The original description is supplemented with more details concerning the species' internal morphology, and previously unreported intraspecific variation is described. Moreover, the position of *S. aethopharynx* within Umagillidae is genetically confirmed, yet intrafamilial relationships remain obscure. Future research integrating more taxa and including sequence data from additional molecular markers will be mandatory to gain more phylogenetic resolution for this family.

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510

511 7 Appendix

Table A.1 Sequences used in phylogenetic analyses

Species name	GenBank accession number
Adenopharynx mitrabursalis	KC529520
Anoplodium stichopi	AF167424
Austradenopharynx sp.	KC529521
Baicalellia brevituba	KC529505
Balgetia semicirculifera	KC529503
Bresslauilla relicta	KC529515
Canetellia beauchampi	KC529504
Castrella pinguis	KC529438
Dalyellioida "houdini" sp.	KC529522
Dalyellioida sp.	KC529523
Einarella agrillophyla	AY775757
Eldenia reducta	KC529502
Gieysztoria zuluensis	KC529465
Graffilla buccinicola	AJ012521
Kytorhynchus sp.	KC529400
Neodalyellida sp. 1	KC529524
Neodalyellida sp. 2	KC529525
Pogaina sp. 1	KC529507
Pogaina sp. 2	KC529508
Pogaina sp. 3	KC529506
Provortex balticus	KC529511
Provortex karlingi	KC529510
Provortex tubiferus	AJ312269
Provorticidae sp.	KC529509
Proxenetes simplex	KC529410
Pseudograffilla arenicola	KC529514
Pterastericola australis	AJ012518
Pterastericola psilastericola	KC529516
Seritia elegans	KC529517
Syndesmis aethopharynx	XXxxxxx
Solenopharyngidae sp.	KC529519
Trisaccopharynx sp.	AY775774
Vejdovskya ignava	KC529513
Vejdovskya pellucida	KC529512
Wahlia macrostylifera	KC529518

	1 2 3 4 5 6 7 8 9 1	0 11 12 13 14 15 16 17	18 19 20 21 22 23 24	25 26 27 28 29 30	31 32 33 34 35
1 Proxenetes simplex					
2 Einarella argillophyla	9.6				
3 Vejdovskya pellucida	0.9 22.0 -				
4 Placorhynchus octaculeatus	5.5 27.9 36.0 -				
5 Wahlia macrostylifera	6.2 19.8 9.9 31.9 -				
6 Neodalyellida sp. 1	9.6 26.1 26.9 36.5 23.9 -				
7 Pterastericola australis	7.4 24.5 28.5 34.5 27.5 20.3 -				
8 Vejdovskya ignava	9.4 22.3 2.8 34.3 10.0 27.0 27.8 -				
9 Balgetia semicirculifera	2.6 24.1 26.0 30.4 24.0 18.7 16.5 27.3 -				
10 Dalyellioida sp.	5.4 22.2 22.4 31.8 21.5 16.6 14.1 23.1 11.0				
11 Seritia elegans	5.6 20.4 9.8 31.2 1.8 23.3 26.7 10.0 22.9 20	0.7 -			
12 Austradenopharynx sp.	7.2 19.1 24.4 32.8 25.4 27.4 28.4 25.8 26.1 25	5.2 26.7 -			
13 Pogaina sp. 1	6.0 23.7 27.2 30.5 24.0 18.1 14.2 26.0 13.7 14	4.0 23.5 25.4 -			
14 Kytorhynchus sp.	11.8 27.9 38.2 26.5 32.9 35.6 33.4 37.1 33.6 31	1.8 34.7 32.3 32.0 -			
15 Solenopharyngidae sp.	7.7 24.0 28.7 35.4 28.2 29.6 33.1 28.7 30.2 27	7.8 28.1 16.1 29.3 33.5 -			
16 Pterastericola psilastericola	8.4 23.7 25.4 32.8 25.2 18.6 2.9 25.3 15.0 12	2.6 24.0 27.7 13.1 35.0 32.1 -			
17 Pseudograffilla arenicola	8.7 25.1 29.9 30.7 27.1 20.9 19.5 27.6 16.1 16	5.8 27.1 26.8 16.1 33.6 29.6 17.5 -			
18 Gieysztoria zuluensis	1.9 25.8 34.0 27.1 30.1 39.1 33.5 33.7 38.8 37	7.5 31.7 36.6 37.5 21.6 41.0 33.7 41.4			
19 Provorticidae sp.	2.6 20.5 23.1 30.3 19.8 15.1 10.8 23.4 10.0 9.	.3 18.8 23.4 9.2 29.9 26.1 9.4 12.4	4 37.7 -		
20 Graffilla buccinicola	3.1 40.0 42.5 48.9 43.8 33.7 35.5 41.3 30.1 30	0.8 42.7 43.0 31.0 50.8 43.1 35.5 28.4	4 55.9 28.1 -		
21 Eldenia reducta	7.5 27.2 31.4 36.2 26.4 20.9 22.6 30.4 18.0 17	7.8 26.0 30.8 22.0 37.8 32.6 20.8 21.1	1 40.7 16.8 36.9 -		
22 Pogaina sp. 2	4.8 23.3 26.7 31.7 23.5 18.3 13.9 25.6 13.3 12	2.7 23.5 22.8 3.0 32.7 27.8 12.5 16.6	5 39.8 8.7 29.7 21.8 -		
23 Provortex karlingi	8.6 28.9 16.2 38.6 14.2 30.0 35.1 14.8 30.0 27	7.6 14.5 32.7 30.9 42.6 34.7 33.2 31.1	1 40.7 24.7 49.9 33.0 31.5 -		
24 Syndesmis aethopharynx	4.7 18.0 10.3 29.8 3.6 23.1 25.9 9.8 22.7 21	1.4 4.0 27.1 22.7 31.7 26.6 24.1 26.8	8 29.1 19.4 43.7 25.5 24.0 14.9 -		
25 Provortex tubiferus	8.2 28.9 16.1 38.9 15.0 30.8 34.5 15.1 29.5 28	8.3 14.9 32.0 32.1 43.3 33.9 33.2 31.4	4 41.5 25.4 50.4 32.9 32.8 1.3 15.2	-	
26 Neodalyellida sp. 2	5.2 28.2 30.0 40.2 26.2 7.2 23.2 30.7 21.4 20	0.4 25.5 28.5 23.5 38.4 34.5 22.0 25.6	5 41.3 18.5 42.0 25.2 22.6 34.9 25.6	5 35.2 -	
27 Provortex balticus	8.9 28.7 15.8 38.9 14.8 30.4 35.4 14.9 29.5 28	8.2 14.7 32.0 31.1 44.1 34.7 33.3 31.4	4 41.8 25.4 49.7 33.6 32.2 1.1 15.3	3 0.6 35.5 -	
28 Pogaina sp. 3	5.3 21.2 25.2 30.8 23.6 18.0 12.0 24.4 11.3 11	1.5 22.9 22.6 5.4 29.8 26.4 11.0 15.2	2 37.3 7.7 28.5 19.8 5.5 29.3 22.8	8 29.2 21.6 29.5 -	
29 Adenopharynx mitrabursalis	6.1 16.6 23.6 32.7 24.1 25.2 26.6 25.3 26.0 23	3.3 25.2 6.4 23.4 34.3 13.5 26.3 25.7	7 34.2 20.8 39.2 29.4 20.8 31.1 23.8	8 31.4 27.3 31.3 21.5 -	
30 Canetellia beauchampi	2.2 19.7 22.7 29.9 21.5 15.5 9.4 22.3 9.8 8.	.9 20.4 21.8 9.0 27.7 26.7 8.6 12.1	1 33.8 4.3 28.7 17.2 8.9 27.1 20.1	1 27.0 19.3 27.0 6.9 18.6 -	
31 Trisaccopharynx westbladi	9.4 22.4 27.4 35.6 27.3 28.1 30.2 29.1 28.9 27	7.2 27.9 14.2 27.7 35.8 7.4 29.2 28.3	3 42.5 25.4 43.3 31.1 26.0 32.9 28.0	33.1 30.6 33.3 25.4 12.6 25.2	•
32 Dalyellioida "houdini" sp.	7.3 27.9 31.5 32.7 27.3 22.7 21.7 29.6 20.3 17	7.9 28.4 25.8 18.3 35.8 28.4 20.1 8.0	43.9 15.6 32.6 24.8 17.7 33.9 28.2	2 36.2 26.9 35.1 17.6 25.3 15.0 :	27.9 -
33 Anoplodium stichopi	7.0 21.4 12.5 33.7 5.6 27.2 30.9 11.5 28.1 23	3.5 5.8 27.6 27.2 35.2 30.5 27.9 29.4	4 32.8 22.8 49.1 28.7 26.2 16.4 6.6	16.5 29.8 16.4 25.4 27.3 23.7	30.5 29.4 -
34 Castrella pinguis	$0.8 \ 24.4 \ 37.1 \ 26.4 \ 29.9 \ 37.0 \ 36.3 \ 37.1 \ 36.1 \ 33$	3.8 31.5 31.9 36.7 20.5 39.1 36.1 41.2	2 8.4 34.8 56.1 37.2 37.7 38.3 30.6	5 39.5 40.8 40.2 34.5 32.9 31.8 :	37.0 40.3 30.7 -
35 Baicalellia brevituba	1.1 19.6 22.4 30.2 21.0 14.9 8.8 21.9 9.7 8.	.6 20.1 20.5 8.7 27.7 25.6 7.9 12.5	5 34.3 3.9 27.7 17.1 8.5 26.4 19.9	9 26.1 18.9 26.3 6.5 18.3 0.3	24.2 14.7 23.5 33.0 -
36 Bresslauilla relicta	7.7 22.9 28.8 32.7 26.5 20.0 16.9 27.0 16.4 17	7.2 26.3 25.6 19.2 34.0 27.2 16.8 21.4	4 37.9 14.4 34.2 16.0 18.4 31.2 24.5	5 30.7 22.2 31.6 17.1 24.6 14.7	28.9 25.7 28.5 32.9 14.4

Tables

Table 1 GTR+G+I pairwise genetic distances (in %) between the umagillid species included in the analysis

		1	2	3
1	Anoplodium stichopi	-		
2	Seritia elegans	5.8	-	
3	Syndesmis aethopharynx	6.6	4.0	-
4	Wahlia macrostylifera	5.6	1.8	3.6

Table 2 P-values of the SH, WSH and AU tests obtained in Consel v0.1j. Significant p-values (p < 0.05) are indicated by an asterisk (*)

	Shimodaira-Hasegawa (SH)	Weighted Shimodaira- Hasegawa (WSH)	Approximately Unbiased (AU)
Topology A (ML tree)	0.302	0.400	0.297
Topology B	0.836	0.800	0.749
Topology C	0.102	0.071	0.011*

Figure legends

Fig. 1 Three hypotheses for the interrelationships of Umagillidae. **a.** Topology A, part of the ML tree as obtained by RAxML (bootstrap values are indicated above branches). **b.** Topology B. **c.** Topology C.

Fig. 2 Pharynx of *Syndesmis aethopharynx*. Focus-stacked image of three photographs at different focal depths. Photographs made on a Leica DM 2500 microscope using interference contrast. Scale bar: 50 µm.

Fig. 3 Stylet of *Syndesmis aethopharynx*. **a.** Camera lucida drawing and **b.** photograph made on a Nikon Eclipse 80i compound microscope, using interference contrast. Scale bars: 20 μm.

Fig. 4 Egg capsule of *Syndesmis aethopharynx*. **a.** Camera lucida drawing. **b.** and **c.** Photographs made on a Nikon Eclipse 80i compound microscope, using interference contrast. Filament tip expansion is indicated by an arrow. Scale bars 3A and 3B: 100 μ m; 3C: 20 μ m.

Fig. 5 Majority rule consensus tree of interrelationships within Neodalyellida, obtained from the Bayesian analysis under the GTR+G+I model. Branches with posterior probabilities below 0.97 have been collapsed. Symbols above branches indicate posterior probabilities (pp) and symbols below branches represent bootstrap support values (bp); legend is displayed in the top left. Branch lengths denote the number of expected nucleotide substitutions per site.

Appendix figure legends

Fig. A.1 Pharynx of *Syndesmis aethopharynx*. Photographs taken at three different focal depths. Scale bars: 50 µm.



















