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Description of *Spadella valsalinae* sp. nov., a neo-endemic benthic chaetognath from Northern Adriatic Sea (Croatia) with remarks on its morphology, phylogeny and biogeography

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Abstract We describe a new chaetognath species, *Spadella valsalinae* sp. nov., discovered and collected in the Northern Adriatic Sea (Pula, Croatia). *S. valsalinae* sp. nov. is a benthic species living at 10–12 m depth attached to the surface of roughly sorted sediment. The cosmopolitan, seagrass-inhabiting species *Spadella cephaloptera* Busch, 1851 was also recorded in the sampling area. Characters from comparative morphology and molecular phylogeny based on SSU rRNA (18S) sequences support a sister group relationship of *S. valsalinae* sp. nov. with *Spadella lainezi* and *Spadella ledoyeri*. Thus, *S. valsalinae* sp. nov. represents the first member in the 'Spadella ledoyeri' complex that is not a troglophilic species. By comparison with the various known European *Spadella* species, we defined a specific set of morphological characters, such as the number, shape and mutual proportions of the teeth, the lateral fins, the corona ciliata, the seminal vesicles, as well as the presence of ventral adhesive papillae, which is unique for *S. valsalinae* sp. nov. The biogeography of *Spadella* members in the Mediterranean Sea and the Eastern Atlantic as well as

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their phylogenetic relationships are discussed, with the focus on processes of neo-endemism in an epeiric area like, for instance, the Northern Adriatic Sea.

Introduction

Chaetognaths (*chaete*=bristle, *gnathos*=jaw) are a group of marine animals that live in various habitats of all oceans, from polar to tropical regions, at all depths (Bone et al. 1991). The majority are pelagic but members of the Spadellidae, represented by the subtaxa *Bathyspadella* Tokioka, 1939, *Paraspadella* Salvini-Plawen, 1986, *Hemispadella* Casanova, 1996, *Calispadella* Casanova and Moreau, 2006, and *Spadella* Langerhans, 1880, are all benthic animals. They can be found in all seas from hallow water to the bathyal zone, but most *Spadella* species populate the neritic and photic zone. Recently, cave-inhabiting (Casanova et al. 2006) as well as interstitial *Spadella* species have been described (Casanova and Perez 2000; Kapp and Giere 2005). These observations shed new light on the hitherto underestimated diversity of ecological niches formed by the various European *Spadella* species.

During summer 2009, we surveyed benthic and bentho-planktonic animals populating sea weeds and sediments in the shallow waters of the Valsaline Bay of Pula (Istria, Croatia, Northern Adriatic Sea). One objective of this study was to evaluate and sample the population in this area of *Spadella cephaloptera* Busch, 1851—a widespread benthic, seagrass-inhabiting species of Chaetognatha. While analysing the samples, however, about 90 specimens of the herewith described new benthic species, *Spadella valsalinae* sp. nov., were discovered on roughly sorted sediment at 10-12 m depth. This paper provides the first record and description of this new species using a multi-methodological approach. In addition, we discuss several evolutionary scenarios that may explain the puzzling geographic and ecological separation of cold-water adapted *Spadella* species populating the Eastern Atlantic and Mediterranean Sea. These boreal species are pooled due to our analyses of both molecular and morphological data and form the so-called '*ledoyeri*' complex.

Material and methods

Material examined

All 93 individuals of *Spadella valsalinae* sp. nov. were found in the Bay of Valsaline, Pula (44°50′59, 42″ N; 13°49′57, 77″ E), which is located in the southwest of the Istrian Peninsula (Croatia) in the Northern Adriatic Sea (Fig. 1). Sampling

Fig. 1 **a** Sampling location of *Spadella valsalinae* sp. nov. in the Northern Adriatic Sea (*box*). *Black arrowheads* (see also *inset*) indicate type locality of *S. valsalinae* sp. nov. in the Bay of Valsaline (Croatia). **b** Substrate diversity and horizontal zonation of the infralittoral in the Bay of Valsaline. **c** Detailed vertical zonation of the infralittoral in the Bay of Valsaline. Altogether eight zones are defined here according to substrate structure, depth and macrozoobenthic community. The zonation follows a scuba diving course of almost 50 m distance starting from the landing stage close-by the Marine Biological Station "Meeresschule" (zone VIII) and ending, by following a straight western direction, at the type locality (*cross*), which coincides with the upper border of a slowly dropping plateau covered by fine sediment (zone I). The collection of underwater pictures give an impression of sediment structure including some typical macroalgae and macrozoobenthic inhabitants. Please also see the authors' movie of the type locality available as supplementary material

occurred from May to July 2009 on roughly sorted *Branchiostoma-Venus verrucosa*-sediment in 10–12 metres depth by scuba diving (Fig. 1b,c; see also supplementary movie 1 and related text 1). In order to collect the specimens, a dip net with a mesh diameter of 1 mm was pulled across the sediment in a way that only the top layer (a few millimetres) was scooped into the net. Afterwards, samples were placed in transparent tanks to supply the specimens with fresh sea water. During this time the specimens moved from the sediment to attach themselves to the side wall of the tank. Subsequently, the specimens were collected with a pipette for further investigation using light microscopy.

Histology

Three individuals of *S. valsalinae* sp. nov. were fixed in Bouin's fixative solution containing saturated picrinic acid, formaldehyde, glacial acetic acid at the ratio 15:5:1. The paraffin embedding procedure involved dehydration of the individuals in a series of graded alcohol solution (70–100 % ethanol), then xylene, followed by two consecutive infiltrations of paraffin (1 h and 2 h). During the latter process, paraffin was kept at 59 °C–61 °C. Finally, specimens were removed from the heated paraffin and embedded in fresh paraffin. Serial thick cross-sections were made at a thickness of 6 μm using a Leica RM2145 histomicrotome. After removing the paraffin, by chemical dissolution using xylene, thick sections were stained with Azan solution. Selected cross-sections of the trunk were viewed with a Nikon Eclipse 90i microscope and documented with a digital camera (Nikon DS2-MBWc) mounted on the microscope.

Scanning electron microscopy

For scanning electron microscopy, ten specimens of *Spadella valsalinae* sp. nov. were transferred from paraformaldehyde (4 % PFA in 0.1 M PBS, pH 7,4) in a graded series of ethanol for dehydration. After critical-point-drying and gold-sputtering, they were analysed under a scanning electron microscope DSM 940A (Zeiss, Kochen, Germany).
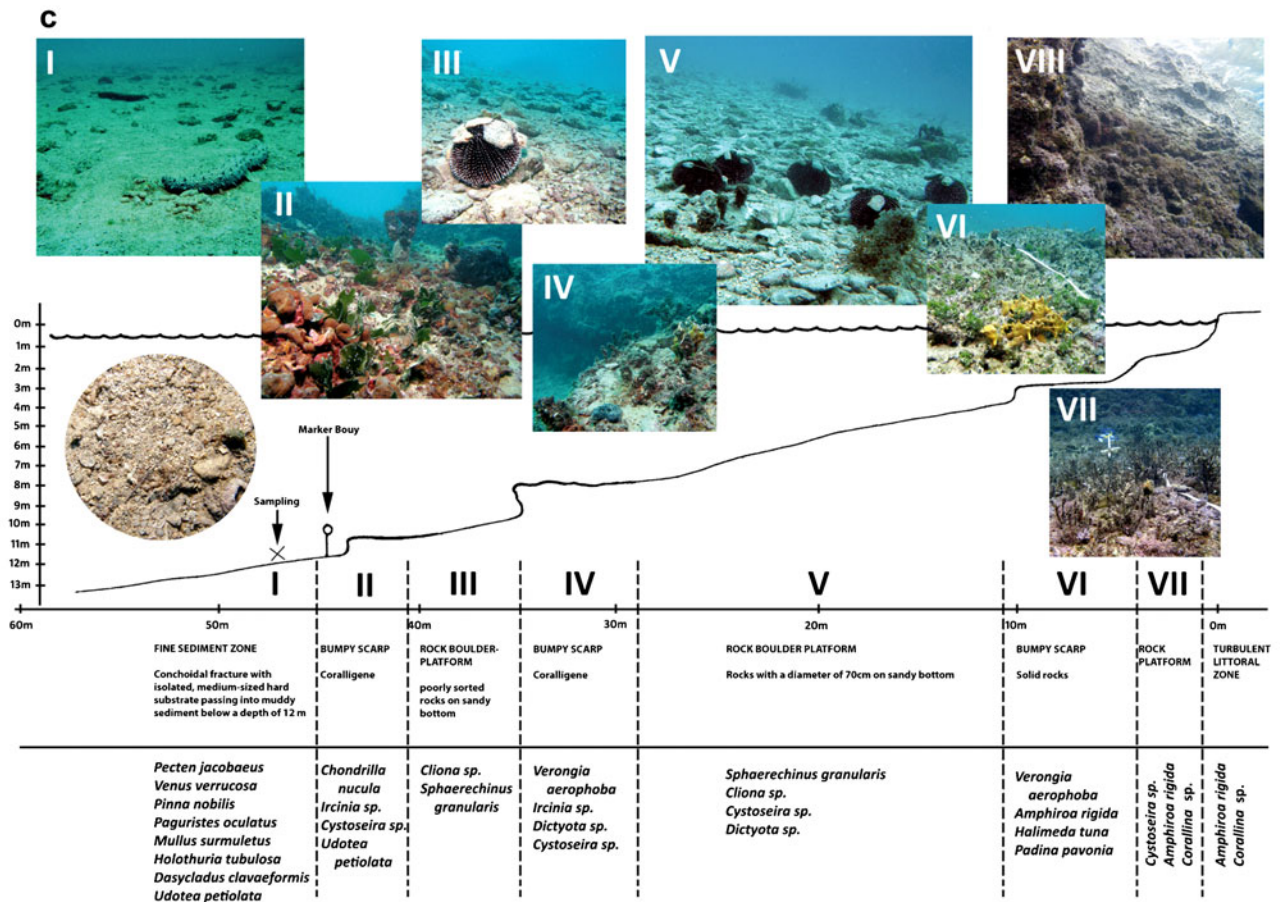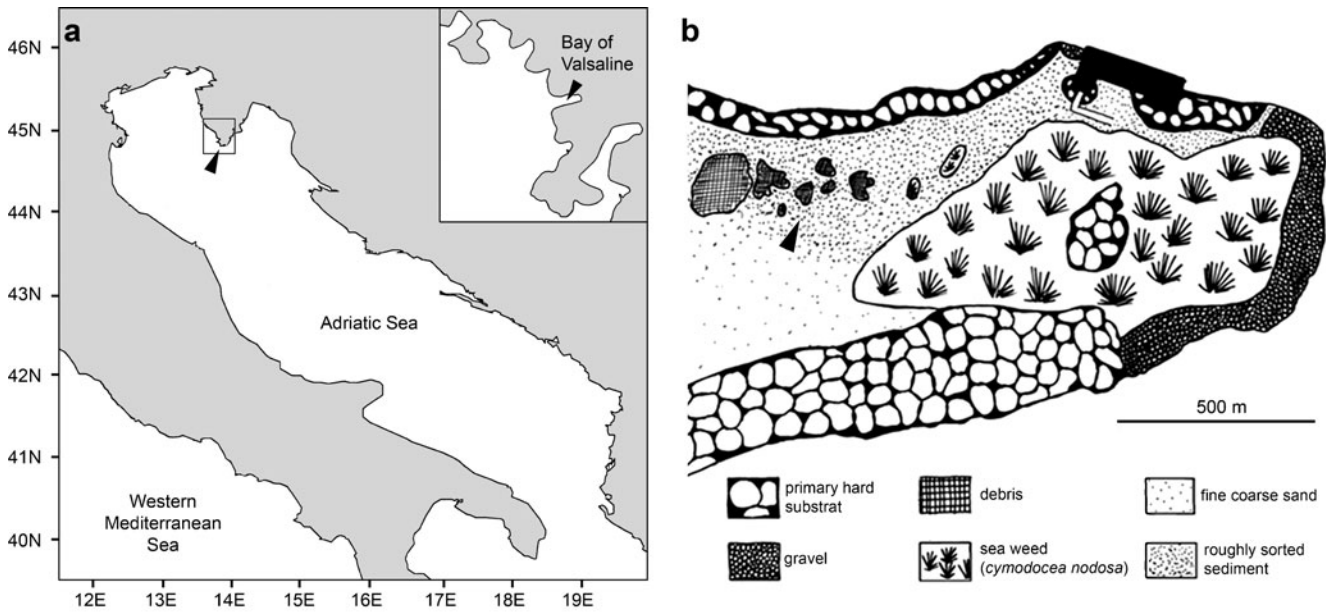
DNA extraction and cloning of SSU rRNA genes

Twenty specimens were fixed placed in 80 % ethanol. DNA was extracted from adults devoid of alimentary bolus to prevent contamination by ingested prey. Previous molecular studies showed that the chaetognath genome contains two different classes of the large (Telford and Holland 1997) and small (Papillon et al. 2006) subunit ribosomal RNA genes (respectively LSU rRNA and SSU rRNA). The wide distribution of each class across the phylum suggests an ancestral duplication of the whole ribosomal gene cluster in Chaetognatha. The SSU rRNA genes of *Spadella valsalinae* sp. nov. were amplified according to the method described in Papillon et al. (2006). Because of the duplication of the ribosomal cluster, two sets of specific primers for each paralogous gene were used to amplify sequences of 1,600 bp (class I: 18SCI5' TTGATGAAACTCTGGATAACTC and 18SCI3' GGACCTCTCTACATCGTTCG) and 1,200 bp (class II: 18SCII'5 TCGTCGGGGTCTCATCC and 18SCII3' AGA TACCTCGCAAAATCG), respectively. PCR fragments were cloned into pGemT-easy vector (Promega, Madison, WI) before sequencing.

Sequence alignment and phylogenetic analysis

SSU rRNA sequences of chaetognath were aligned automatically using Clustal in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and were then adjusted manually. Phylogenetic trees were inferred with the maximum likelihood (ML) method using PHYML 3.0 (Guindon and Gascuel 2003) and the Bayesian method using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) with a HKY model of substitution and four substitution rate categories (HKY+I+Γ4). The robustness of nodes in the ML analysis was estimated by running bootstrap tests with 100 replicates. The Bayesian analysis was run for 100,000 generations, with four simultaneous chains. Trees were sampled every 100 generations, starting after a burn-in of 2,500 generations.

The analyses were conducted using all sequences belonging to Phragmophora species available in GenBank (listed in Table 1). The sequence generated for this study has been in part deposited in GenBank and given accession numbers listed in Table 1. Alignments are available upon request.

Results

Deposition of holo- and paratypes

The holotype (ZMH C854) and three paratypes (ZMH C855–C857) were deposited in the Zoological Museum of the University of Hamburg, Germany, while another five paratypes (ZIMG II/28088–28092) are deposited in the Zoological Museum of the University of Greifswald, Germany. The holotype as well as the paratypes were collected in the Bay of Valsaline, Pula (Croatia), in July 2009 (Fig. 1a).

Etymology

The specific name refers to the Bay of Valsaline from which the specimens were collected.

Diagnosis

The anatomical features described here are based on mature adult specimens.

Type locality

The sea bottom in the Bay of Valsaline is characterized by primary and secondary hard substrates, expanded sandy

Table 1 Phragmophora species used in the phylogenetic analysis and GenBank accession numbers for SSU rRNA sequences (paralogy groups: classI and II)

Species	Paralogy group	Accession number	Family
Xenokrohnia sorbei	Class I	DQ351888	Heterokrohnidae
Eukrohnia bathypelagica	Class I	DQ351886	Eukrohnidae
Eukrohnia hamata	Class I	DQ351887	Eukrohnidae
Paraspadella gotoi	Class I	D14362	Spadellidae
Spadella ledoyeri	Class I	DQ351883	Spadellidae
Spadella cephaloptera	Class I	DQ351884	Spadellidae
Spadella valsalinae sp. nov.	Class I	JX458821	Spadellidae
Xenokrohnia sorbei	Class II	DQ351902	Heterokrohnidae
Eukrohnia fowleri	Class II	DQ351889	Eukrohnidae
Eukrohnia bathypelagica	Class II	DQ351896	Eukrohnidae
Spadella cephaloptera	Class II	DQ351897	Spadellidae
Spadella ledoyeri	Class II	DQ351899	Spadellidae

sediments of various composition and sorting degrees as well as by seagrass beds (Fig. 1b). In the type locality, *Spadella valsalinae* sp. nov. is found on roughly sorted sandy, gravely and rocky sediment at a depth of about 10 m. The type locality represents the higher end of an expanded sandy flat that is bordered to the shore-sided rocky boulder field by a bumpy scarp (2-3 m in height) overgrown by various macroalgae (see inset picture II and list in Fig. 1c). The rocky boulder fields extend to and pass into a *Cystoseira* phytal zone close to the shore (see inset pictures and zones III–VIII in Fig. 1c). Below a depth level of 12 m, the sandy, heterogenous sediment more and more passes into a mud flat interspersed with sprouts of the sea grass species *Cymodocea nodosa* (see inset picture I and zone I in Fig. 1c). The sediment particles in the type locality vary in diameter from 1 mm to approximately 20 cm. Due to proximity to the mud zone, the surface of this sediment usually appeared covered widely by a thin layer of fine sand and silty particles with a diameter smaller than 200 μm. The sediment also contains exo- and endo-skeletal remains of various benthic invertebrates, such as Bivalvia, Gastropoda, Crustacea, and Echinoidea.

Ecology

The Bay of Valsaline has been revealed already to accommodate some fascinating and unusual hard substrate and phytal biocoenoses (Müller and Schubart 2007). The absence of a vast *Posidonia* seagrass zone, in combination with a generally lenitic nature, causes extensive converging of silty or muddy and rocky shores in the shallow waters of the Bay of Valsaline. *Spadella valsalinae* sp. nov. belongs to a soft bottom facies combining elements of the 'Branchiostoma' (also called 'Amphioxus') sand and 'Venus verrucosa' type. The reader may obtain a more detailed insight into the structure and macro- and megazoobenthic inhabitants of the type locality of *S. valsalinae* sp. nov., by watching our supplementary movie 1. Detailed information about the community structure is provided in the movie description.

Spadella valsalinae sp. nov. lives epibenthically on roughly sorted sandy sediment at a depth that just represents the upper margin of the mud flat zone, expanding from the upper infralittoral down to the shelf region of the Northern Adriatic Sea (Fig. 1b,c). A clear endobenthic lifestyle is improbable because of the sampling method applied. Likewise, the considerable size of the animals (4–5 mm in length) basically precludes permanent occupation of the interstitial spaces.

To date, neither prey nor predators that may be specifically linked to *S. valsalinae* sp. nov. could be found at the type locality. Feeding on meiozoobenthic organisms or zooplankton of the benthic boundary layer is, however, likely. Individuals of the seagrass-inhabiting species *Spadella cephaloptera* were captured in thalli of macroalgae overgrowing larger rocks, which overlie the sediment. At least in the vicinity of those rocks, both *Spadella* species appear to live syntopically.

Eidonomy and internal anatomy of *Spadella valsalinae* sp. nov.

The body looks transparent and rigid. It reaches a total length of 4–5 mm when mature, excluding the tail fin (Fig. 2a,b). The broadest part of the body is at the trunk–tail septum, close to the anterior end of the lateral fins. The ventral nerve centre lies ventrally in the middle of the trunk and makes up about 40 % of the total trunk length. The tail region takes up 53.7 % of the whole body, not including the tail fin.

The triangular head is almost as wide as the broadest part of the body. It is covered by a hood (praeputium) that exhibits dorsally on each side a small, cylindrical, brown protuberance, the functional significance of which is unknown (Fig. 2c). The 9–10 grasping spines (hooks) are smooth with a tip at the edge (Fig. 3a,b). The 2–4 anterior teeth are long, curved and thin with the second inner being the longest (Figs. 2d and 3c,d). The 1–3 shorter posterior teeth are located laterally of the anterior teeth. All teeth are ornamented and display several longitudinal rows of indentations: three on posterior teeth and four on anterior teeth. Indentations are pointed distalwards, vary in size and are distanced unequally to each other. The eyes are large, with a pigmented area divided by a big pigmented cell into a large cup ocellus and five smaller ocelli (Fig. 2e). The neck is surrounded by a well-developed collarette, which is less marked around the trunk and tail regions. The corona ciliata is slightly oval. The gut is whitish with two small anterior diverticula (Fig. 2c). Two bilateral bands of oblique muscles (phragms) are spanned through the trunk general cavity over its entire length (Fig. 2f–h). Mature ovaries extend to the middle of the trunk containing oval immature and polyhedral mature oocytes. The body surface is covered ventrally with adhesive gland papillae, especially aggregated at the distal border of the tail (Fig. 3e,f). Ciliary fence receptors cover the entire body. Lateral fins are short, narrow and range from about the trunk-tail septum to about 53–55 % of the tail. Tail fin looks spatulated and are, like lateral ones, completely rayed. Neither has any contact with the seminal vesicles. Seminal vesicles appear slightly reniform and display a specific shape by forming a little bulb at the level of the median opening (Fig. 3f,g). Further phenotypical details, as for instance on the pigmentation of the integument, can be depicted from supplementary movie 2 which shows one specimen of *S. valsalinae* sp. nov. in living state.

Molecular phylogeny

The set of primers we used enabled us to amplify and sequence about 1,600 nucleotides for the classI paralogy group. The alignment of all SSU rRNA sequences consists of 1,577 aligned positions (Table 1). As shown previously, Chaetognatha display two classes of SSU rRNA gene (ClassI and II, Fig. 4) allowing

Fig. 2 **a–h** *Spadella valsalinae* sp. nov. **a** Semi-schematic drawing showing the habitus, dorsal view. *Bar* 1 mm. **b** Light micrograph of a specimen fixed with 4 % paraformaldehyde (PFA). Sectioning levels for **f, g** and **h** are indicated. Details of the head and neck region of a living specimen (dorsal view). Note the two little brown and cylindrical protuberances on either side of the head (*arrows*). *Bar* 250 μm. **d** Detail of the anterior and posterior teeth. *Bar* 30 μm. **e** Detail of the eye and the darkly pigmented cell separating the photoreceptor cells. **f–g** Series of thick histological cross-sections through different parts of the trunk (ordered from anterior to posterior): **f** anterior trunk region, **g** median trunk region at the level of the ventral nerve centre, **h** posterior trunk region with ovaries. Note the bilaterally symmetrical oblique muscles (phragms) extending from ventromedian to lateral inner margin of the extracellular matrix (ecm). In **h**, the dorsal epidermis is ripped off along with a breakage of the dorsal longitudinal musculature, which is due to sectioning artefact. Thick sections have been stained with haematoxylin-eosin. *at* anterior teeth, *c* colarette, *cc* corona ciliata, *e* eye, *ecm* extracellular matrix, *ep* epidermis, *lf* lateral fin, *lm* longitudinal musculature, *ov* ovary, *pt* posterior teeth, *sv* seminal vesicle, *tc* trunk coelom, *tf* tail fin, *ts* trunk-tail septum, *vnc* ventral nerve center, *asterisk* midgut
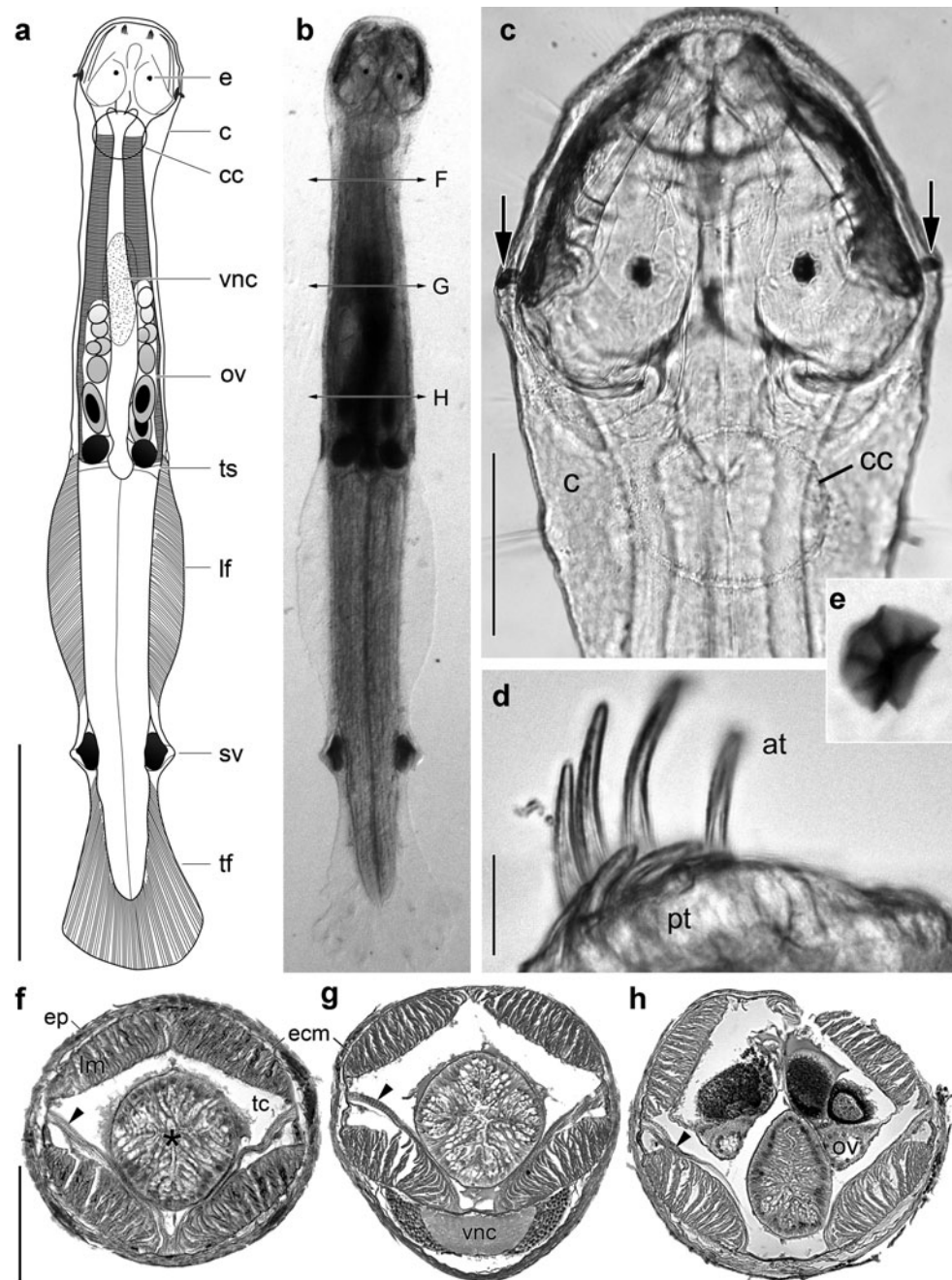
the rooting of one class of paralogy by another in phylogenetic analyses. The best ML and Bayesian trees resulting from the SSU rRNA analysis show similar topologies and are well resolved and contain few low supported nodes. From the traditional view of chaetognath phylogenetic relationships, Phragmophora order is defined by the presence of oblique muscles (phragms) in the tail and trunk or in the trunk only and is represented here by the Spadellidae, Eukrohnidae and Heterokrohnidae families. The monophyly of the Eukrohnidae and Spadellidae families is corroborated by high bootstrap support values and posterior probabilities. Among the four Spadellidae species represented for the class I SSU rRNA gene,

the sequence of *Spadella valsalinae* sp. nov. groups together with the sequence of the cave species *Spadella ledoyeri*.

Discussion

Comparison with related species and phylogenetic relationships: the 'ledoyeri' complex

The genus *Spadella* currently includes 13 species (for a review, see Casanova and Moreau 2004 and Casanova et al. 2006), 7 of them being observed in the Mediterranean

Fig. 3 a–g. Habitus of *Spadella valsalinae* sp. nov. Scanning electron (**a–f**) and light (**g**) micrographs. **a** Frontoventral view of the head. **b** Detail of a hook (grasping spine). Note the tip at the edge. **c, d** Detailed view of the ventral side of the head around the mouth region showing the elongated teeth. **e** Ventral view of the tail. *Arrow* marks the ventral adhesive papillae more aggregated in the transition zone from trunk to tail. **f, g** Higher magnification and detail of the seminal vesicle. *Arrows* point to ciliary fence receptor organs, whereas *arrowheads* mark some adhesive papillae. *at* anterior teeth, *ho* hood (praeputium), *oep* expelled oesophageal glandular epithelium, *pt* posterior teeth, *vo* vestibular organ, *vp* vestibular pit
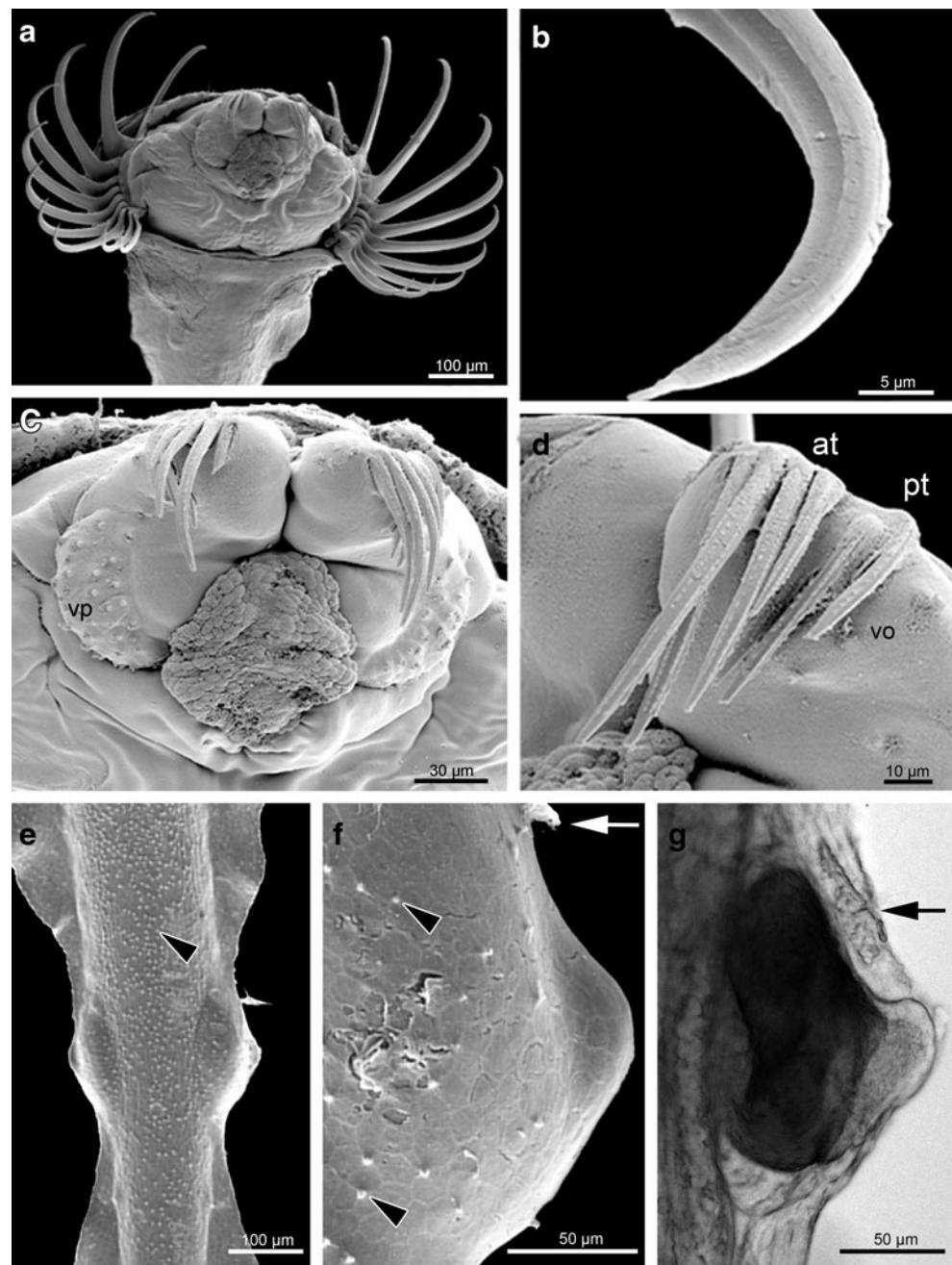
and Eastern Atlantic Sea. Comparative morphology indicates that *Spadella valsalinae* sp. nov. considerably resembles the Canarian cave species *Spadella lainezi* (Casanova et al. 2006) and *Spadella ledoyeri* (Casanova 1986, 1992). Altogether, the teeth shape, the proportion of lateral fins, the corona ciliata, and the shape of seminal vesicles constitute a set of highly valuable characters supporting a close relationship between *S. valsalinae* sp. nov., *S. lainezi* and *S. ledoyeri* (Table 2). Phylogenetic relationships within European Spadellidae based on SSU rRNA sequences likewise strongly support a close affinity of *S. valsalinae* sp. nov. to *S. ledoyeri*. Unfortunately, it was not possible to include *S. lainezi* in our molecular study, but this will be followed up in a future study.

Interestingly, *S. valsalinae* sp. nov. differs from its sister species *S. ledoyeri* by the presence of adhesive papillae on the ventral body surface and two thin protuberances on each side of the head. However, *S. valsalinae* sp. nov. shares these characters with *Spadella cephaloptera*. Interestingly, *S. valsalinae* sp. nov. and *S. cephaloptera* occur syntopically in the investigation area. The two species differ from each other in the shape of the corona ciliata, the proportion of the lateral fins and the type of seminal vesicles. In contrast to *S. valsalinae* sp. nov., *S. cephaloptera* moreover

Fig. 4 Phylogenetic analysis based on SSU rRNA sequences from eight species of chaetognaths belonging to Phragmophora with maximum likelihood (ML) and Bayesian methods. The topology presented is the ML reconstruction. The class II sequences are used as outgroups. Superscript *asterisks* indicates nodes with bootstrap support values (bs) and posterior probabilities (pp) lower than 70
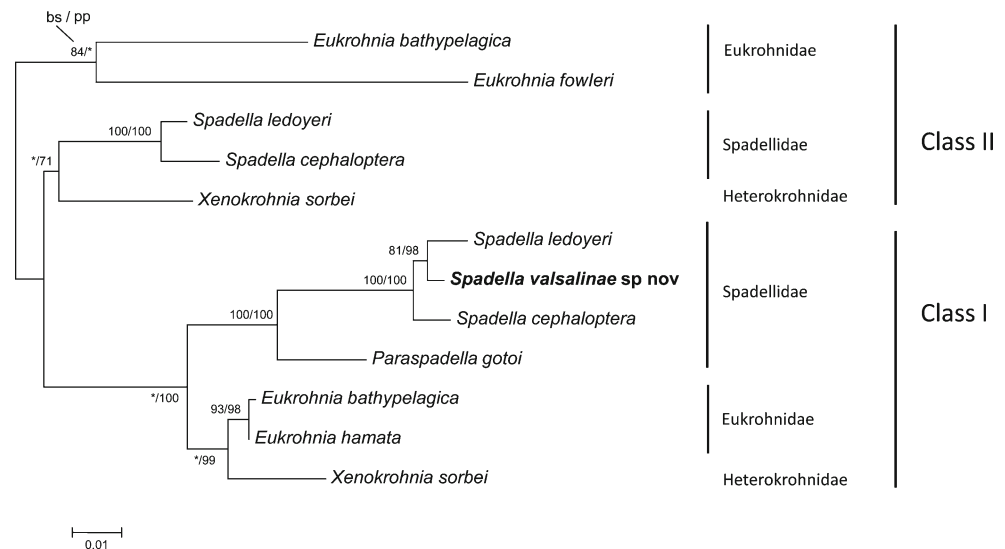
has rounded seminal vesicles that touch the lateral fins as well as the tail fin (Fig. 5). Moreover, *S. cephaloptera* displays a conspicuous oval (John 1933) to elliptic (Scaccini and Ghirardelli 1941) corona ciliata, contrasting with the roundish one displayed by *S. valsalinae* sp. nov. (Table 2).

In terms of general morphology, the species most closely related to *S. valsalinae* sp. nov. is beyond doubt the recently described *S. lainezi* (Casanova et al. 2006). The main difference is the lack of ventral adhesive papillae on the body of *S. lainezi*, which were, however, unequivocally observed and documented in *S. valsalinae* sp. nov. Besides that, *S. lainezi* is a little bit smaller than *S. valsalinae* sp. nov., reaching 3–4.5 mm, and the tail makes up 48.7–52 % of the total length (excluding the tail fin). When compared to *S. valsalinae* sp. nov., the head of *S. lainezi* is more rectangular. However, our observations on living and fixed specimens of *S. valsalinae* sp. nov. and *S. cephaloptera* show that such a difference in shape may be due to fixation artefacts, which often induce muscular contraction. The number of hooks is about the same in *S. valsalinae* sp. nov. and *S. lainezi* but the latter species bears reddish to light brown hooks.

The second, phenotypically closely related species, *S. ledoyeri*, grows larger than *S. valsalinae* sp. nov. and differs from the latter in the absence of intestinal diverticula (Casanova 1986). In living specimens of *S. ledoyeri*, the gut shows a red coloration (Casanova et al. 2006), whereas it is whitish in *S. valsalinae* sp. nov. However, the seminal vesicles of *S. ledoyeri* appear longish-rounded with a smaller bulb, but the gap between the lateral and the tail fins is considerably longer (cf. Fig. 5a and c).

Finally, the Atlantic deep-sea species *Spadella birostrata* (Casanova 1987) with a body length of maximum 7 mm and a body:tail ratio of 51.2 % is larger than *S. valsalinae* sp. nov. and differs from the latter in the absence of the intestinal diverticula, longer lateral fins, which reach the seminal vesicles, and in a reniform corona ciliata with an additional,

finger-formed protuberance towards the head region (Table 2). However, while *S. birostrata* has been described as a member of the '*cephaloptera*' complex (Casanova 1987), once again it is interesting to note a feature shared with *S. valsalinae* sp. nov. Indeed, in *Spadella birostrata*, the anterior teeth appear long, thin and curved, and the posterior ones are shorter. This pattern is highly similar to that found in *S. valsalinae* sp. nov.

Comparative ecology and biogeography

*Ecological niche of Spadella valsalinae sp. nov.*

The three species suggested so far to belong to the '*ledoyeri*' complex differ not only in their habitat but also strongly in their geographical range. They are all endemic, either to the Mediterranean Sea or the Canary Islands. Furthermore, *S. ledoyeri* and *S. lainezi* are restricted to submarine caves with entries ranging from approximately 10 m to 30 m in depth, the former being located at the Gulf of Lyon nearby Marseille (Casanova 1992), while the latter is found southeast of the coast of the Canary Island Tenerife (Casanova et al. 2006). Both type localities are far distant and isolated from each other, given the recent pattern of eastern Atlantic and Mediterranean surface currents. In contrast, in Pula, the type locality of epipsammic *S. valsalinae* sp. nov. seems to be restricted to roughly sorted sandy and silty sediments in the photic zone at 12 m depth. As the Mediterranean Sea in general and the Northern Adriatic Sea in particular is one of the most intensively investigated and faunistically monitored seas in the world (e.g. Travizi and Vidaković 1997; Štěvčić 2002; Por and Dimentman 2006), given the present state of knowledge, we do not expect that *S. valsalinae* sp. nov. necessarily inhabits areas much further south to Pula. In spite of some conserved morphological characters, species of the '*ledoyeri*' complex show a clear geographical separation. This fragmented pattern of species ranges may be explained by the

Table 2 Overview of diagnostic characters used in discriminating the *Spadella* species known to occur in the Mediterranean Sea and the Eastern Atlantic Ocean

Group	'ledoyeri'			'cephaloptera'			'nunezi'	
Species	*Spadella valsalinae* sp. nov.	*Spadella lainezi*	*Spadella ledoyeri*	*Spadella birostrata*	*Spadella equidentata*	*Spadella cephaloptera*	*Spadella nunezi*	*Spadella interstitialis*
Body Length	4–5 mm	3–4.5 mm	4.8–7 mm	up to 7.6 mm	7.2 mm	4–5 mm	up to 2.3 mm	up to 1.8 mm
Trunk/tail ratio	51.3–53.7 %	48.7–52 %	50–53.4 %	47.3–53.1 %	51.4 %	50–60 %	47–50 %	50–55 %
Grasping spines	9–10	10–11	10–11	8–11	8	8–11	8–9	6–9
Anterior teeth	2–4 long, thin and curved, the second is the longest. Ornamented	2–4 long, thin and curved, the second is the longest. Smooth	3–5 long, thin and curved, the second is the longest.	2–8 long, thin and curved, the first is the longest	6 short and ornamented	0–5 short and ornamented	1–2 short and ornamented	2–4 short and ornamented
Posterior teeth	1–4 shorter than anterior teeth. Ornamented	3–4 shorter than anterior teeth. Smooth	3 shorter than anterior teeth	4–6 shorter than anterior teeth	4–5 Same size as the anterior teeth	1–3 lack of posterior teeth not unusual	3–4 Same size as the anterior teeth Ornamented	1–3 Very short
Seminal vesicles	Bulb-like shaped. Slightly separated from the lateral and tail fins	Bulb-like shaped. Separated from the lateral and tail fins.	Bulb-like shaped. Distant from the lateral and tail fins. Closer to the tail fin than the lateral ones.	Roundish. In contact with the lateral and tail fins	Elongated. In contact with the lateral and tail fins	Roundish. In contact with the lateral and tail fins	Conical and elongated with a posterior opening. In contact with the lateral and tail fins	Conical and elongated with a posterior opening. In contact with the lateral and tail fins
Corona ciliata	Slightly oval	Rounded	Slightly oval	Reniform with a protrusion anteriorly	Not observed	Oval to elliptic	Small and slightly oval	Oval, with or without a protrusion anteriorly
Intestine diverticula	Two small diverticula	Two small diverticula	Absent	Absent	Absent	Two diverticula	One unpaired diverticula	Absent
Lateral fins	Short and narrow Extended to 53-55 % of the tail	Short and narrow Extended to ?-?% of the tail	Very short and triangular Extended to ?-?% of the tail	Long In contact with seminal vesicles	Long In contact with seminal vesicles	Long In contact with seminal vesicles	Long and narrow In contact with seminal vesicles	Long and narrow In contact with seminal vesicles
Adhesive papillae	On the ventral side of trunk and tail	Absent	Absent	Not observed	Not observed	On the ventral side of trunk and tail	On the ventral side of trunk and tail	Absent
Depth	10–11 m	16 m	15 m	150–555 m	452 m	Photic zone	22 m	12 m
Habitat	Roughly sorted sediment	Cave	Cave	Bathyal zone	Bathyal zone	Sea weed and microalgae	Organogenous sands	Calcareous sands
Distribution	Northern Adriatic Sea Bay of Valsaline	East Atlantic Ocean (Teneriffe)	Western Mediterranean line	Alboran Sea and Western side of Gibraltar	Western side of Gibraltar	Cosmopolitan distribution	La Graciosa (Atlantic Sea)	Islands of Elba and Pianosa

Fig. 5 **a–d.** Comparative morphology of the posterior part of the body showing the shape and position of the seminal vesicles (*arrows*) as well as the posterior end of the lateral fins (*arrowheads*). Light micrographs of *Spadella valsalinae* sp. nov. (**a**), *Spadella lainezi* (**b**, picture montage after Casanova et al. 2006), *Spadella ledoyeri* (**c**) and *Spadella cephaloptera* (**d**). *Bar* 0.5 mm
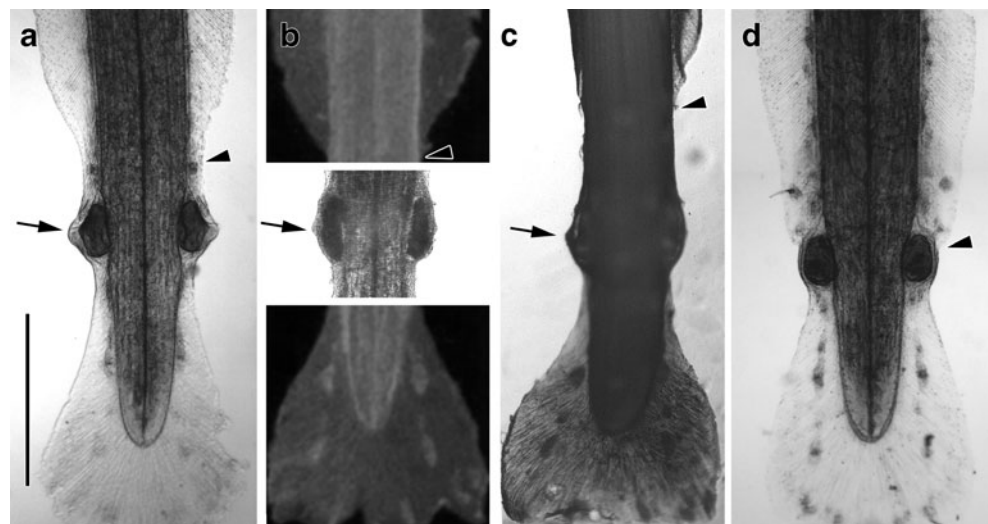
fact that not only the Gulf of Lyon but also the Northern Adriatic Sea are known to be the coldest parts of the Mediterranean Sea (e.g. Klein and Roether 2002). As we will point out further below, these areas could serve as a 'cul-de-sac' for infra- and circalittoral chaetognaths endemic to the Mediterranean Sea and adapted to cold-temperate waters.

*The dramatic history of the Mediterranean Sea—a source of endemism*

The Mediterranean basin in its present topographic state is the result of enormous tectonic movements and glacial abrasions that started in early Mesozoic, when the famous tropical Tethys Sea became ever more transformed, and ended preliminarily in the Tyrrhenian, approximately 100,000 years ago. These massive geological and climatic incidents had a dramatic impact on the immigration, speciation and survival of fossil and extant marine faunal elements in the Mediterranean, especially in the Adriatic Sea. The last known and undisputed dramatic event was the Messinian Salinity Crisis (7.2–5.3 Ma; Hsü et al. 1973; Hsü 1974) and the various Pleistocene ice ages that happened thereafter (see Fig. 6b and supplementary text A). Since then, the bulk of the present Mediterranean fauna has been recruited from Eastern Atlantic populations.

The present Mediterranean Sea is known to be a hotspot for marine biodiversity (Boudouresque 2004; Coll et al. 2010; Ben Rais Lasram et al. 2010). The Western Mediterranean basin displays the highest values of species richness, likely owing to the Atlantic influx. It has been calculated that approximately 2,000 (= 28 %) of all hitherto described species in the Mediterranean Sea have to be considered endemic (e.g. Giaccone 2003, Por and Dimentman 2006). On the global scale, an extraordinary 8–18 % of the total number of marine species is strictly

Mediterranean depending on the authors consulted (Fredj et al. 1992; Bianchi and Morri 2000; Zenetos et al. 2002; Por and Dimentman 2006).

Por and Dimentman (2006) distinguished four classes of Mediterranean endemics: (1) 'palaeo-endemics' of tropical Mesozoic or early Cenozoic origin, famously known as Tethys relics that survived the Messinian crisis; (2) cold-water adapted 'neo-endemics' of boreal Atlantic origin that evolved during Pleistocene in the, then much colder, Mediterranean Sea; (3) mostly highly mobile 'antechamber endemics' that radiated from thermophilic Atlantic (Lusitanian), subtropical or even tropical ancestors and found shelter in (and returned from) Middle Eastern Atlantic Sea areas during (after) Pleistocene glacial cooling; and (4) 'pseudo-endemics' comprising species whose Eastern Atlantic presence has been discovered just recently and, thus, whose Mediterranean endemic status must be questioned or revoked.

The Adriatic Sea is the area with the second highest species richness and shows a large number of endemics, possibly owing to its higher isolation and hydrological characteristics. According to Tortonese (1985), 10 % of marine species within the Adriatic Sea are endemic. Only a few of them are 'palaeo-endemic' and likely evolved from old Paratethyan stock (Băcescu 1985; Por and Dimentman 2006). Nowadays, the Adriatic Sea has relatively low winter temperature values and low salinity (Artegiani et al. 1997; Zavatarelli et al. 1998), which makes it different from, and peculiar, compared to the other Mediterranean areas (see Por 1978; Por and Dimentman 2006). Moreover, Por and Dimentman (2006) concluded that the class of Mediterranean 'neo-endemics' "should contain taxa known for their rapid recent evolutionary rate and reduced mobility, especially those living in localized, near shore habitats" (p. 175). The new species *S. valsalinae* fits perfectly into the category of 'neo-endemic' species. Many European spadellid species seem to be restricted to a local area, which
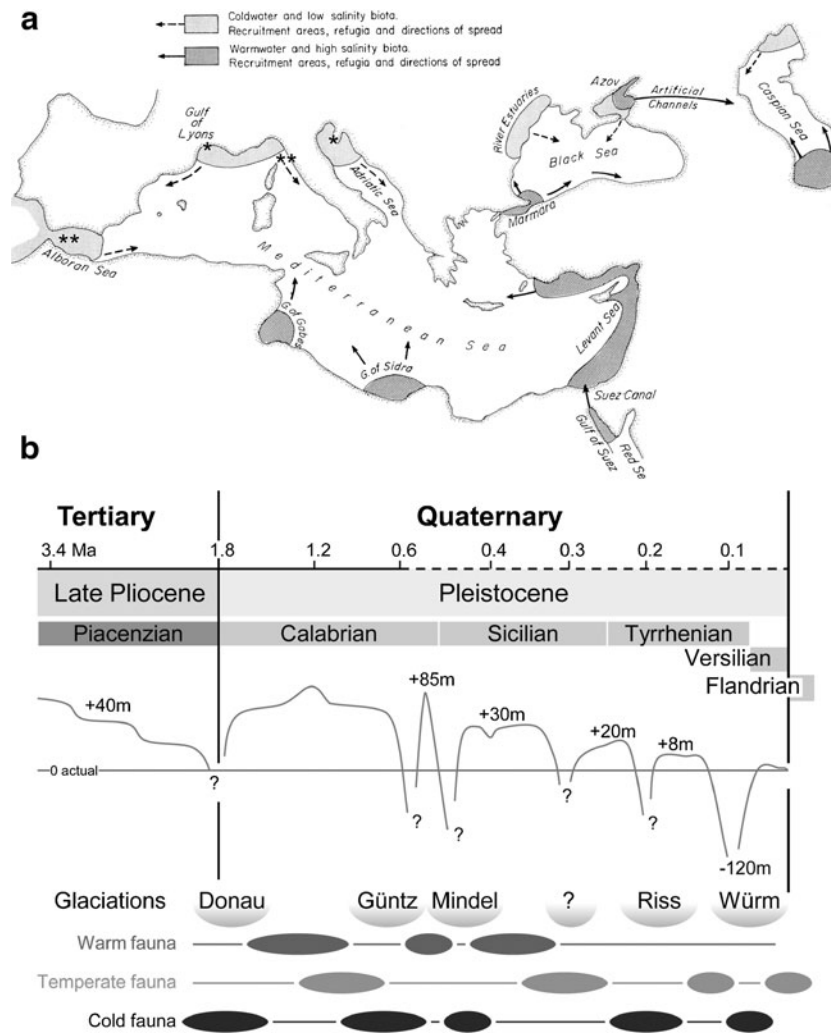
Fig. 6 a-b. Post-Messinian hydrography, climatology and biogeography of Mediterranean biota. **a** Present Mediterranean topography showing the clash of glacial (cold-water adapted taxa; tolerating low salinity) versus interglacial (moderately warm-water adapted, subtropical or even tropical taxa; tolerating high salinity) faunal elements. Type localities of Mediterranean spadellid chaetognaths, now known to belong to the boreal '*ledoyeri*' complex, are marked by a single asterisk: the cave-inhabiting *Spadella ledoyeri* from Gulf du Lyon, *Spadella valsalinae* sp. nov. from Pula (Northern Adriatic Sea). The Canarian species *Spadella lainezi* is not shown. Those Mediterranean spadellids suspicious of being further members of the '*S. ledoyeri*' may be the result of reproduction through benthic larvae. Furthermore, it is also well accepted that Chaetognatha in general exhibit a rapid and recent evolutionary rate (Telford and Holland 1997; Papillon et al. 2006; Marlétaz et al. 2008). Interestingly, Spadellidae have not been discussed yet in the context of Mediterranean paleobiogeography, probably because of the predominant attention on pelagic Sagittidae, the species of which usually have a wide area of distribution.

complex because of their occurrence in or close-by Pleistocene cold-water relict areas are highlighted by *double asterisks*: the deep-sea species *Spadella birostrata* from Alboran Sea and coast of Western Gibraltar and the mesopsammic *Spadella interstitialis* from Elba (illustration modified from Por 1978 as well as Por and Dimentman 2006). **b** Schematic model demonstrating the drastic changes of climatic and hydrographic conditions during Pleistocene in the Mediterranean Sea. At the lower end of the scheme, various periods of immigration of different faunal elements of the Atlantic Sea are exhibited corresponding to interglacial events (modified from Emig and Geistdoerfer 2004; Por and Dimentman 2006)

Certainly, there are many more macrozoobenthic species populating the Mediterranean Sea that may be assigned to the class of 'neo-endemics' (see compilation in supplementary text B). However, it often remains difficult to identify them and to reconstruct ancestral speciation patterns. The situation in the Northern Adriatic Sea is particularly complex, as genuine paratethyan palaeo-endemics, Ponto-Caspian immigrants, and still ongoing invasion by alien species hamper a reliable identification of true boreal 'neo-endemics'. Without comparative population genetics, it is virtually impossible to determine which of the Northern Atlantic or Northern Adriatic populations of a given cold-water adapted species is the more recent.

Evolution of Mediterranean Spadellidae with emphasis on 'ledoyeri' complex

The re-opening of the Strait of Gibraltar after the Messinian Salinity Crisis enabled various pelagic as well as infra- and circalittoral benthic chaetognaths to enter the Mediterranean Sea. This immigration (or immigrations) took place sometime in the late Pliocene or during Pleistocene. During at least five glaciation events at the beginning and within the Pleistocene epoch (Donau, Güntz, Mindel, Riss, Würm), these post-Messinian Mediterranean biota of subtropical or moderate cold-water Atlantic origins, among them certainly also many spadellid taxa, became either impoverished by extinction or have actively migrated from western to southern and/or eastern basin (Vermeij 1978). On the contrary, the last common ancestor of the 'ledoyeri' complex, among many other typical cold-temperate (boreal) Atlantic species, have colonised the Mediterranean Sea several times independently (cf. Vermeij 1978). At the end of the last ice age (end of Versilian subepoch), sea water became continuously warmed, which led to gradual replacement by thermophilic species (Mediterranean-Atlantic, Lusitanian faunal elements), such as for instance *Spadella cephaloptera*. Subsequently, this replacement caused a general northbound shift of boreal species more or less restricted to a cold-water environment (Fig. 6a). A similar scenario of successive replacement has been described in terrestrial organisms as an upward altitudinal response to warming temperature (Ben Rais Lasram et al. 2010). Hence, some *Spadella* populations ended up trapped in the colder parts of the northern Mediterranean coast, e.g. the Adriatic Sea and Gulf of Lyon. Nowadays, these cold-water influenced areas must be considered highly fragmented and isolated refugial areas that give rise potentially to regionally restricted endemism on various infra- and circalittoral substrates (Fig. 6a). The discovery of *Spadella valsalinae* sp. nov. strongly suggests that species belonging to the 'ledoyeri' complex developed by allopatric 'neo-endemism' not only in caves of Canary Islands and Gulf of Lyon but also in the photic zone of the Northern Adriatic Sea. This idea is at first glance incongruent with the literature as, for instance, Por and Dimentman (2006) suggested that the so-called 'Macaronesian Islands' (Canaries, Azores, Madeira) were a temporary refugial location for Lusitanian 'antechamber endemics' during Pleistocene glacial stages. However, submarine caves are surely much colder environments than surrounding, sun-exposed areas. Therefore, the concept of 'antechamber endemics' of Por and Dimentman (2006) may require modification. At least, it seems necessary to regard and specify the infralittoral microhabitats in which these animals were able to survive.

The northern part of the Adriatic Sea has average depths of only 30 m and was most certainly dried up during the last glacial event (Würm), when the sea level dropped approximately 100 m compared to current levels (Emig and Geistdoerfer 2004; Por and Dimentman 2006; cf. Fig. 6b). Therefore, at least two principal scenarios may be considered regarding the isolation and allopatric 'neo-endemic' speciation history of *S. valsalinae* sp. nov. The first scenario implies a most recent speciation that took place directly after the Würm glaciation in the Flandrian epoch or sometime thereafter. Then, *S. valsalinae* sp. nov. might have radiated from its related stem species somewhere in the Northern Adriatic Sea (Northern Adriatic 'neo-endemism'). The species then evolved probably under hydrological conditions resembling those found today. Alternatively, speciation may have taken place during or after glaciation events prior to the Würm event. Subsequently, the location at which the speciation of *S. valsalinae* sp. nov. occurred, does not necessarily have to have been the Northern Adriatic Sea, but may also have been the entire Adriatic region or even areas southern to that (e.g. Ionian Sea). If we assume the second scenario to be true, then the type locality at Pula has to be interpreted as a refugial or recruitment place populated by *S. valsalinae* sp. nov. after having been displaced from original habitat by thermophilic Lusitanian, atlanto-tropical and/or, most recently, Lessepsian immigrants (for this latter aspect, see review of Por 1978). Scenario 2 may be evaluated by carefully checking cold-water and low-salinity areas in Mediterranean Sea areas other than the Northern Adriatic Sea, such as the Alboran Sea, the North-western and -eastern Black Sea, the Northern Aegean Sea as well as even the Gulf of Lyons and the Ligurian Sea (compare zones identified and drawn by Por 1978, Fig. 6a). However unlikely, an occurrence of *S. valsalinae* sp. nov. or further new, closely related species outside the Adriatic Sea cannot be entirely excluded.

Current status and research perspectives

Of course, the story of postglacial speciation within the 'ledoyeri' complex is far from being completely unfolded. On the one hand, the diversity of *Spadella* species in the Mediterranean Sea is not fully described. Several morphospecies have been illustrated but are still awaiting scientific description (e.g. Casanova 1992; Ghirardelli and Gamulin 2004). Among them are also some cave-dwelling species ("*Spadella* sp. 1 and 2" as listed in Ghirardelli and Gamulin 2004), potential candidates for assigning to the '*Spadella ledoyeri*' complex. On the other hand, molecular sequencing data are still not present for some spadellids, despite their pivotal role in the discussion, as for instance the Atlantic deep-sea species *Spadella birostrata*, which shares similar elongated teeth with *S. valsalinae* sp. nov., or the interesting mesopsammic specialist *Spadella interstitialis* from the Island of Elba (Kapp and Giere 2005), which, because of

its vicinity to the colder Ligurian Sea, may also belong to the '*ledoyeri*' complex (see Fig. 6a). In our opinion, this complex should be investigated further, since it is truly qualified to be treated as a study model for understanding of 'neo-endemic' speciation in the Mediterranean Sea and reconstructing patterns of historical and recent fragmentation of the geographical range in cold-water adapted, boreal Atlantic taxa. The functional morphology, ecology as well as hunting and reproductive behaviour of *S. valsalinae* sp. nov. is worth further investigation. Like many other 'neo-endemic', cold-water (boreal) relict species, *S. valsalinae* sp. nov. may be threatened by the recent warming-up of surface Mediterranean water as induced by global climate change (see Chap. 2.5.4 in Por and Dimentman 2006 for a review).

How long might the 'cul-de-sac' habitat of *S. valsalinae* sp. nov. last in the context of still ongoing global warming? Furthermore, how do we explain certain phenotypical characters that *S. valsalinae* sp. nov. shares with *S. cephaloptera*, such as the finger-formed protuberance on either side of the head? Has allopatric speciation been followed by local hybridization with *S. cephaloptera*, which perhaps enabled, and still enables, the boreal neo-endemic species to survive in the warming Adriatic waters? These are just a few of the interesting questions to be answered by future studies on *Spadella* populations from the Mediterranean Sea and the Atlantic Ocean.

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