Zoological Journal of the Linnean Society, 2020, **XX**, 1–18. With 5 figures.

Toward a resolution of the cosmopolitan *Botryllus schlosseri* species complex (Ascidiacea, Styelidae): mitogenomics and morphology of clade E (*Botryllus gaiae*)

RICCARDO BRUNETTI[1$], FRANCESCA GRIGGIO[2,$†], FRANCESCO MASTROTOTARO[3], FABIO GASPARINI[4], and CARMELA GISSI[5,6,*]

[1]*Natural History Museum of Venice, Santa Croce 1730, 30135 Venezia, Italy*
[2]*Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, Milano, Italy*
[3]*Dipartimento di Biologia and CoNISMa LRU, Università degli Studi di Bari "Aldo Moro", Via E. Orabona 4, 70125 Bari, Italy*
[4]*Dipartimento di Biologia, Università di Padova, Via Ugo Bassi 58/B, 35131 Padova, Italy*
[5]*Dipartimento di Bioscienze, Biotecnologie and Biofarmaceutica, Università degli Studi di Bari "Aldo Moro", Via E. Orabona 4, 70125 Bari, Italy*
[6]*IBIOM, Istituto di Biomembrane, Bioenergetica e Biotecnologie Molecolari, CNR, Via G. Amendola 165/A, 70126 Bari, Italy*

Received 19 September 2019; revised 26 January 2020; accepted for publication 24 February 2020

Botryllus schlosseri is a model colonial ascidian and a marine invader. It is currently recognized as a species complex comprising five genetically divergent clades, with clade A globally distributed and clade E found only in Europe. This taxon has also been recently redescribed by designation of a clade A specimen as the neotype. To clarify the taxonomic status of clade E and its relationship to clade A, we examine the entire mitochondrial genome and study the morphology of clade E. The mitogenome of clade E has an identical gene order to clade A, but substantially differs in the size of several non-coding regions. Remarkably, the nucleotide divergence of clade A-clade E is incompatible with the intraspecies ascidian divergence, but similar to the congeneric one and almost identical to the divergence between species once considered morphologically indistinguishable (e.g. the pair *Ciona intestinalis* (Linnaeus, 1767)-*Ciona robusta* Hoshino & Tokioka, 1967, and the pair *Botrylloides niger* Herdman, 1886-*Botrylloides leachii* (Savigny, 1816)). Clade E differs morphologically from the *Botryllus schlosseri* neotype mainly in the number and appearance of the stomach folds, and the shape of the anal opening, the first intestinal loop and the typhlosole. Our integrative taxonomical approach clearly distinguishes clade E as a species separate from *Botryllus schlosseri*, with unique morphological and molecular characters. Therefore, we here describe clade E as the new species ***Botryllus gaiae* sp. nov.**

ADDITIONAL KEYWORDS: ascidian – Botryllinae – complete mitochondrial genome – cryptic species – cytochrome oxidase 1 – gene order – integrative taxonomy – morphology – nonsynonymous substitution rate.

INTRODUCTION

Botryllus schlosseri (Pallas, 1766), a colonial ascidian of subfamily Botryllinae, has in the last decades become a reference organism, not only in fields such as evolutionary developmental biology and immunobiology, but also for studying biological processes such as stem cell migration, apoptosis, regeneration and ageing (Sabbadin, 1982; Manni *et al*., 2007, 2019; Rosengarten & Nicotra, 2011; Voskoboynik & Weissman, 2015). *Botryllus schlosseri* colonies propagate asexually by cyclical waves of blastogenesis (i.e. palleal budding) and during colony growth processes such as apoptosis and stem cell migration

*Corresponding author. E-mail: carmela.gissi@uniba.it
[$]These authors contributed equally to this work
[†]Current address: Centro Piattaforme Tecnologiche, Università di Verona, Piazzale A. Scuro 10, 37134 Verona, Italy
[Version of record, published online 31 March 2020; http://zoobank.org/ urn:lsid:zoobank.org:pub: DB18D5A7-0E96-458E-B3C0-5E93327359AC]

© 2020 The Linnean Society of London,

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

occur synchronously and massively (Rinkevich *et al.*, 2013; Rosner *et al.*, 2013; Franchi *et al.*, 2016; Manni *et al.*, 2019). In addition to its role as a model organism, *Botryllus schlosseri* is a cosmopolitan fouling species and one of the most widespread marine invaders (Berrill, 1950; Carlton, 2005; Ben-Shlomo *et al.*, 2010; Bock *et al.*, 2012; Lord, 2017), and is potentially able to damage the industry of shellfish aquaculture (Carman *et al.*, 2010) and other marine industrial activities (Carver *et al.*, 2006).

In the context of the above-mentioned importance of this species, its taxonomy needs to be unequivocally and fully resolved, and the identification of the relative specimens has to occur beyond doubt based on clear diagnostic characters. However, in the last decade molecular data has shown that *Botryllus schlosseri* is a "species complex" consisting of five genetically highly divergent clades, named from A to E, each corresponding to a cryptic species (Lopez-Legentil *et al.*, 2006; Bock *et al.*, 2012). Clade A is globally distributed and clade E is present only in European waters, whereas the other clades are geographically restricted to a few localities (Stach & Turbeville, 2002; Lopez-Legentil *et al.*, 2006; Perez-Portela *et al.*, 2009; Bock *et al.*, 2012; Yund *et al.*, 2015; Nydam *et al.*, 2017; Reem *et al.*, 2017). The five clades have been identified for the first time by Lopez-Legentil *et al.* (2006) based on a short fragment of the mitochondrial (mt) gene *cox1,* but they were not described as cryptic species but just as clades characterized by high nucleotide diversity, low haplotype diversity and restricted gene flow. Using both *cox1* and nuclear microsatellites, Bock *et al.* (2012) have put forward the hypothesis that these clades correspond to cryptic species. Indeed, Bock *et al*. observed a high interclade (10.8–16.5%) and a comparatively low intraclade (0.8–3.8%) *cox1* divergence and, at microsatellite level, a complete lack of contemporary gene flow between clades, suggestive of reproductive isolation (Bock *et al.*, 2012). Later on, studies on the entire mitochondrial genome (mtDNA) identified three surprisingly distant taxa/subclades within clade A, that have been explained as further cryptic species or as the outcome of ongoing speciation events (Griggio *et al.*, 2014). A Pacific specimen "sc6ab" from clade A has been the target of a genome sequencing project, thus leading to the publication of both the nuclear and mitochondrial genomes (Voskoboynik *et al.*, 2013). Moreover, a Mediterranean clade A specimen having the same *cox1* haplotype of the mtDNA of the "VE" specimen (Griggio *et al.*, 2014) has been designated as the neotype for *Botryllus schlosseri* when it was redescribed by Brunetti *et al.* (2017). The sc6ab and the VE specimens belong to two of the three divergent taxa/subclades identified by the mtDNA within clade A (Griggio *et al.*, 2014).

The exact phylogenetic relationships between the five clades remain elusive, because the few published *cox1* trees are not fully resolved, due to the presence of unreliably supported nodes (Lopez-Legentil *et al.*, 2006; Yund *et al.*, 2015) and/or were reconstructed with unusual and potentially inappropriate methodologies for the *cox1* nucleotide sequences (i.e. assuming a strict molecular clock (Yund *et al.*, 2015) or analysing the translated sequences (Lopez-Legentil *et al.*, 2006)). However, without considering node reliability/methodology anomalies, all trees agree in recognizing clade E as sister to all other clades (Lopez-Legentil *et al.*, 2006; Bock *et al.*, 2012; Yund *et al.*, 2015).

Population genetic studies have also started investigating the geographic distribution, origin and dispersal history of the two widespread cryptic species, i.e. clade A and clade E (Bock *et al.*, 2012; Yund *et al.*, 2015; Nydam *et al.*, 2017; Reem *et al.*, 2017). Although the picture is far from complete, these studies have revealed that clades A and E are both successful invaders and have shown traces of recent range expansion of clade E (Nydam *et al.*, 2017). Moreover, hypotheses on the geographic origin and dispersal history of the species complex/clades have also been presented (Berrill, 1950; Carlton, 2005; Nydam *et al.*, 2017; Reem *et al.*, 2017).

Due to the relevance of this species complex, the need for a resolution on the taxonomic crypsis of this organism is growing. In particular, the *Botryllus schlosseri* taxonomic re-evaluation should lead not only to a more accurate discrimination of this species from other botryllids, but also to the identification of clear morphological and molecular diagnostic characters able to distinguish the various cryptic species. An accurate taxonomic description will help the studies on this model ascidian, with a better interpretation of possible differences in the outcomes of experiments carried out on different clades, as well as the finding of possible differences in the invasive potential of the various cryptic species, with consequences in the setting of the most appropriate monitoring and management strategies. For this purpose, and considering the lack of a type specimen and the previous unclear morphological descriptions, in 2017 we designated a neotype of *Botryllus schlosseri,* as reference point for future studies (Brunetti *et al.*, 2017). Following an integrative taxonomy approach, this neotype was described both at the morphological and molecular level, allowing to associate a precise morphology to clade A (Brunetti *et al.*, 2017) and in particular to a *cox1* haplotype identical to that of the previously published mtDNA of the VE specimen (European Nucleotide Archive [ENA] Accession Number: FM177702) (Griggio *et al.*, 2014). We believe that an integrative taxonomy approach should be applied to all genetically identified clades of the *Botryllus schlosseri* species complex with the aim of elucidating their taxonomic status.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

Here, in order to clarify the taxonomic status of clade E and its relationship to clade A, we analysed clade E specimens according to an integrative taxonomy approach that accounts for morphological and molecular characters. Then, we compared the clade E features to those of the *Botryllus schlosseri* neotype (Brunetti *et al.*, 2017). In particular, we analysed the colony and zooid morphology, sequenced the entire mtDNA of a clade E specimen and compared several genome-level mitochondrial features (i.e. sequence divergence, gene order, and non-coding regions). The differences observed between clade E and clade A/*Botryllus schlosseri sensu* Brunetti (2017) at both the mitogenomic and morphological level show that clade E *sensu* Bock *et al.* (2012) can be recognized as a distinct species, which is described below.

MATERIAL AND METHODS

Sampling and morphological analyses

Colonies morphologically similar to *Botryllus schlosseri sensu* Brunetti *et al.* (2017), as evaluated morphologically in the field, were sampled in different years at various localities in Italy in the Adriatic and northern Ionian Sea (Central Mediterranean Sea). Table 1 contains a list of the specimens assigned to clade E by means of *cox1* amplification and sequencing (see "Molecular analyses" information in the "Mitochondrial genome analyses" section) together with the sampling details. As reported in Table 1, specimen collection was carried out also during samplings not specifically devoted to the study of the *Botryllus schlosseri* species complex. Each of three colonies BT2, BT4 and P14a was split in two fragments (Table 1): one piece was narcotized with menthol and fixed in 10% formalin in seawater for morphological analyses, while another piece was preserved in 90–99% ethanol for DNA extraction. For morphological analyses, colonies were dissected, and zooids were stained with haemalum and mounted on slides. Only BT2 and BT4 were in good relaxation and fixation conditions for morphological analyses, whereas P14a was not well relaxed and contained such a large number of pigmented blood cells that morphological analyses were impractical. The taxonomic characters here analysed are in accordance with the informative traits commonly used to identify species of the Botryllinae: zooid appearance, test surface, number of rows of stigmata (with particular attention to the shape of the second row), presence/absence of muscles in the pharynx, number and shape of stomach folds, shape of pyloric caecum, gonad position and presence/absence of incubation pouch (Brunetti & Mastrototaro, 2017). In addition, we consider the opening of the anus and the shape of both the typhlosole and the intestinal loop.

Table 1. Clade E colonies identified along the Italian coasts

Colony no.	*cox1* haplotype*		Sampling locality	Coordinates	Depth; method; substrate	Study on:	Date
	No.	Name	AC number				
2 †	1	BT2 = BT4 †	LR745518 LR743465†	Southern Adriatic Sea, Barletta, Italy	41°22'55.646"N, 16°10'06.586"E	2.5–4.0 m; clam dredge; sandy bottom populated by *Cymodocea nodosa*	Clam sampling	20 Feb. 2013
2	2	1T, 2T	LR743459-60	Northern Ionian Sea, Mar Grande, Taranto Gulf, Italy	40°26'20.6"N, 17°14'37.1"E	2–3 m; scuba diving; on ropes and submerged iron chains	Benthonic fauna	27 Mar 2013
1§	1	P14a	LR743463	Northern Ionian Sea, Mar Piccolo, Taranto Gulf, Italy	40°29'32.5"N, 17°14'14.8"E	2–3 m; scuba diving; on ropes and submerged iron chains	Benthonic fauna	7 Nov. 2018
9	4#	10, 14, 16, BT2#	LR743461-62–64	Northern Adriatic Sea, Venice Lagoon, Italy	45°13'03.2"N, 12°17'21.7"E	1 m; manual collection; on *Zostera* leaves, ropes and floats	*Botryllus schlosseri*	17 Nov. 2016

* = defined by the dinF/Nux1R amplicon. The haplotype name corresponds to the name of one of the colonies.
† = BT2 and BT4 colonies, i.e. the syntypes used for both morphological and molecular analyses. They have the same *cox1* haplotype, however two sequences have been deposited at the ENA database: LR745518, corresponding to the complete mtDNA of BT2, and LR743465, corresponding to the dinF/Nux1R amplicon of BT4.
§ = preserved both in 10% formalin and in 99% ethanol.
# = one haplotype is identical to that found in Barletta.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

Developmental stages were expressed according to Berrill (1941). Drawings were electronically processed from originals obtained by camera lucida. Types were deposited at the Natural History Museum of Venice (Italy) (MSNVE).

Mitochondrial genome analyses

Total DNA was extracted from a small piece of colony or from isolated zooids using a modified CTAB method (Hirose & Hirose, 2009). Assignment of the specimens to clade E was based on a *cox1* fragment of about 850 bp, amplified and sequenced as described in Brunetti *et al.* (2017) using the primer pairs dinF/Nux1R (Table 2). These *cox1* sequences were then compared to *Botryllus schlosseri* homologues available in the nucleotide nr/nt database of NCBI on 1 May 2019.

The sequence of the entire mtDNA was determined for the BT2 specimen, collected in Barletta (Table 1), following the strategy of Griggio *et al.* (2014). Briefly, the whole genome was amplified in four overlapping fragments using heterologous and species-specific primers, and it was sequenced with the Sanger method. Depending on size and yield, amplicons were completely sequenced by primer walking, or used as template in nested/semi-nested PCRs able to produce short sequenceable fragments. In order to reduce the risk in introducing nucleotide substitutions during the amplification steps, all amplicons were obtained with high fidelity DNA polymerases, i.e. LA Taq polymerase or PrimeSTAR HS DNA polymerase (Takara Bio Inc.). The first PCR reactions were carried out hypothesizing a mitochondrial gene order identical to that of the four already sequenced mtDNAs of clade A (Griggio *et al.*, 2014). As described in Table 2, the mtDNA of BT2 was amplified in two long fragments of 7.4 and 5 kb, plus two short fragments of about 2 kb: the longest amplicons were obtained using heterologous primers designed on highly conserved ascidian mt regions, whereas the shortest amplicons were obtained using species-specific primers designed on the sequences at the ends of the long amplicons. Regions with long A or T homopolymeric tracts (≥ 10 nt), producing low-quality sequences, were also cloned with the CloneJET PCR (Fermentas) or the Zero Blunt TOPO PCR (Invitrogen) cloning kits, and their final sequence was set to the consensus of three clones. Sanger sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

The sequences of the complete mtDNA and of the *cox1* fragments were deposited at the European Nucleotide Archive, ENA (see the accession numbers [AC] numbers, in Table 1 and Table 3). Genes for tRNAs, proteins and rRNAs were annotated as in Griggio *et al.* (2014).

Comparative analyses at intraspecies and congeneric levels were carried out on the 29 mtDNAs listed in Table 3. This dataset includes the public sequences of *Botrylloides leachii,* recently suggested as actually belonging to *Botrylloides diegensis* Ritter & Forsyth, 1917, based on the comparison of *cox1* sequences of new specimens (Viard *et al.*, 2019). However, in that study the assignment of the new specimens to *Botrylloides diegensis* is based only on the colour pattern of the colonies, without checking anatomical traits discriminating *Botrylloides leachii* from *Botrylloides diegensis* (i.e. the number of stigmata rows, the presence/absence of muscle in the branchial sac and the number of stomach folds; see Brunetti & Mastrototaro (2017)). Therefore, here we have not changed the assignment of all previously published sequences to *Botrylloides leachii*, pending further studies on this matter.

The 13 mt protein-coding genes (PCGs) were aligned at the amino acid level with MATTF v.6 (Katoh & Toh, 2008). Single alignments were then manually optimized, back-translated to nucleotides using TranslatorX (http://translatorx.co.uk/) and concatenated with Geneious (http://www.geneious.com/). The non-synonymous (dN) and synonymous (dS) pairwise substitution rates

Table 2. Amplicons used for clade E identification or covering the entire mtDNA of clade E, with primer sequences

Fragment	kb	Aim	Primer	Sequence (5'–>3')	Reference
dinF/nux1R	0.8	Barcoding	dinF*	CGTTGRTTTATRTCTACWAATCATAARGA	Brunetti *et al.* (2017)
			nux1R*	GCAGTAAAATAWGCTCGRGARTC	Brunetti *et al.* (2017)
dinF/ucbR	7.4	mtDNA sequencing	dinF*	CGTTGRTTTATRTCTACWAATCATAARGA	Brunetti *et al.* (2017)
			ucbR*	GGAATASAYCGTAAAATVGCATARGC	Iannelli *et al.* (2007)
zx3F/u16R	5	mtDNA sequencing	zx3F*	TGTKCTATTTGRTATTGRCATTTTGTBGA	Griggio *et al.* (2014)
			u16R*	TAARYCGACATCGAGGTCRTAA	Griggio *et al.* (2014)
zcbF/sen3R	1.8	mtDNA sequencing	zcbF*	AGTTTTTGRRGGGCSACMGTWATTAC	Griggio *et al.* (2014)
			sen3R	CTAATTCCTAATAAAGATAAAGCGG	This study
bs16F/nux1R	2.2	mtDNA sequencing	bs16F	ACTGATGGGGCCTGCTCTATG	Griggio *et al.* (2014)
			nux1R*	GCAGTAAAATAWGCTCGRGARTC	Brunetti *et al.* (2017)

* = heterologous primers.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

© 2020 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2020, **XX**, 1–18

Table 3. Ascidian mitochondrial genomes analysed in this study

Classification	Species	Sample name	Locality[†]	AC number
Stolidobranchia				
Botryllinae	*Botryllus schlosseri* clade A	VE-″neotype″[§]	Venice Lagoon, Northern Adriatic Sea, Italy	FM177702
Botryllinae	“	TR	Taranto Gulf, Northern Ionian Sea, Italy	HF548550
Botryllinae	“	sc6ab	Santa Cruz Harbour, Pacific Ocean, California	HF548551
Botryllinae	“	EA	Els Alfacs Bay, Ebre Delta, Western Mediterranean, Spain	HG931923
Botryllinae	*Botryllus gaiae*, former clade E	BT2	Barletta, Southern Adriatic Sea, Italy	LR745518
Botryllinae	*Botrylloides leachii*[#]	Ble L2-VE*	Venice Lagoon, Northern Adriatic Sea, Italy	HF548553[#]
Botryllinae	“	Ble BA-TR*	Taranto Gulf, Northern Ionian Sea, Italy	HG931921[#]
Botryllinae	*Botrylloides giganteus*	Bgi VI-IT*	Taranto Gulf, Northern Ionian Sea, Italy	HF548554
Botryllinae	“	Bgi PE-CA*	Harbour Island, San Diego Bay, Pacific Ocean, California	HG931922
Botryllinae	*Botrylloides niger*[‡]	Bni		HF548559
Botryllinae	*Botrylloides violaceus*	Bvi		HF548552
Styelidae	*Styela clava*	Scl		HG931920
Styelidae	*Styela plicata*	Spl		AM292601
Pyuridae	*Halocynthia papillosa*	Hpa		FM177863
Pyuridae	*Halocynthia roretzi*	Hro		AB024528
Pyuridae	*Halocynthia spinosa*	Hsp		HF548558
Phlebobranchia				
Cionidae	*Ciona intestinalis*	Cin		AM292218
Cionidae	*Ciona robusta*	Cro*	Gulf of Naples, Italy	AJ517314
Cionidae	“	Cro CAhm*	Half Moon Bay, California	AABS01001113
Cionidae	*Ciona savignyi*	Csa JPmb*	Mutsu Bay, Japan	AB079784
Cionidae	“	Csa CAsf*	San Francisco Bay, California	AACT01048180
Ascidiidae	*Phallusia fumigata*	Pfu		AM292602
Ascidiidae	*Phallusia mammillata*	Pma		AM292320
Aplousobranchia				
Polyclinidae	*Aplidium caeruleum*[¥]	Aca		Griggio *et al.* (2014)
Polyclinidae	*Aplidium conicum*	Aco		FN313538
Polyclinidae	*Aplidium tabarquensis*	Ata		HF548555
Clavelinidae	*Clavelina lepadiformis*	Cle ITgl*	Gulf of La Spezia, Italy	AM292603
Clavelinidae	“	Cle SEgu*	Gullmarsfjord, Sweden	FJ839918
Clavelinidae	*Clavelina phlegraea*	Cph		AM292604

* = sequences of the intraspecies pairs of Fig. 2.
† = reported only for specimens of the same species.
§ = *cox1* haplotype identical to that of the *Botryllus schlosseri* neotype (Brunetti *et al.*, 2017).
# = possible reassignment to *Botrylloides diegensis* based on the data published in Viard *et al.*, (2019). See discussion in *Material and Methods*.
‡ = sequence originally reported as belonging to *Botrylloides* affinis *leachii* (Rubinstein *et al.*, 2013; Griggio *et al.*, 2014).
¥ = partial genome, assembled in eight contigs.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

were calculated on the overall concatenated alignment only for intraspecies and congeneric pairs. The codeml program of the PAML v.4.4b package (Yang, 1997) was used according to the Goldman and Yang's model of codon substitution, that accounts for differences in the transition–transversion rates and for codon usage bias (Goldman & Yang, 1994). Codeml was therefore run with the options CodonFreq = 2, icode = 9 (ascidian mt) and runmode = -2 (pairwise comparison).

Pairwise uncorrected distances were calculated with PAUP* (Swofford, 2003) separately for the various gene categories as well as for the first plus second codon positions (P12), the third codon position (P3) and the non-coding regions (NCR).

Phylogenetic reconstructions within the Botryllinae were performed according to Maximum Likelihood (ML) and Bayesian methods on the concatenated alignment of the 13 mitochondrial-encoded proteins. Poorly aligned positions were identified and removed with Gblocks v.0.91b (Castresana, 2000), using the standard web options for block selection (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The final alignment consists of 3487 sites, i.e. 95% of the original 3667 positions. The mtDNA of *Polycarpa mytiligera* (Savigny, 1816) (ENA Accession Number: HF548556), belonging to the subfamily Styelinae, was used as outgroup. Indeed, several ascidian phylogenies recognized the genus *Polycarpa*, rather than *Styela,* as more closely related to the Botryllinae (Rubinstein *et al.*, 2013; Shenkar *et al.*, 2016; Alie *et al.*, 2018). The best Botryllinae outgroup should belong to the subfamily Polyzoinae, recognized as the sister group of the Botryllinae based on both morphological and molecular data (Berrill, 1950; Zeng *et al.*, 2006; Alie *et al.*, 2018). However, mtDNA sequences of the Polyzoinae are not available.

The ML tree was inferred by the online PhyML v.3.0 software (Guindon & Gascuel, 2003), which includes the automatic model selection by Smart Model Selection (SMS) (Lefort *et al.*, 2017) (http://www.atgc-montpellier.fr/phyml-sms/). The substitution model was set to MtZoa + G + I + F (with I = invariant sites; F = observed amino acid frequencies), i.e. to the substitution model best fitting to the alignment data under the Akaike Information Criterion (AIC). Bootstrap values, indicating node reliability, were based on 100 replicates.

Bayesian inference (BI) was carried out with PhyloBayes v.3.2f (Lartillot *et al.*, 2009) under the CAT + GTR + G mixture model. The site-heterogeneous CAT mixture model (Lartillot & Philippe, 2004) accounts for site-specific amino-acid replacement preferences and is less prone to long branch attraction artefacts, thus it is well suited for phylogenomic studies. Three Markov chains Monte Carlo (MCMC) were run and sampled every ten cycles. Chain convergence was verified with the bpcomp and tracecomp utilities included in PhyloBayes, checking that the values of maximum discrepancy across bipartitions were less than 0.1 and the minimum effective size of the summary variables was higher than 100. Each chain was stopped after sampling 23 590 trees. The first 4 718 trees of each MCMC were treated as the burn-in step and thus excluded, therefore the majority-rule consensus tree was computed from the remaining 3 × 18 872 combined trees. Trees were graphically edited with the TreeGraph 2 software (Stover & Muller, 2010).

RESULTS

Presence of clade E in the Mediterranean sea

Our samplings identify for the first time the presence of clade E in the Ionian Sea and in both the southern and northern Adriatic Sea (Table 1). For the first time we have also found clade E in the Venice Lagoon (northern Adriatic Sea), but only in 2016 (Table 1): our previous samplings carried out in 2010, 2013 and 2015 at exactly in the same site (unpublished data), as well as other published records never identified clade E in Venice (see *Discussion*). Moreover, in most investigated localities, clade E has been identified in sympatry with clade A (data not shown).

Mitogenome comparison

The mtDNA of the BT2 specimen, a clade E representative, has been completely sequenced and carefully compared to the four available mtDNAs of clade A (Table 3), including that of the specimen VE, hereafter indicated as VE-″neotype″, since it shows a *cox1* haplotype identical to that of the *Botryllus schlosseri* neotype (Brunetti *et al.*, 2017).

The mtDNAs of clade E and clade A have almost the same length (14 890 bp against 14 935 ± 7 bp), identical gene order (Fig. 1) and identical position of the longest NCRs (with size ≥ 25 bp) (Fig. 1). Moreover, the mtDNA of clade E shows a tRNA-like structure in three long NCRs (see K2, K3 and L2, in Fig. 1), as found also in clade A (Griggio *et al.*, 2014).

A significant difference between the two clades concerns the size of the longest NCRs (i.e. NCRs ≥ 25 bp in at least one specimen, reported as filled blocks in Fig. 1). Indeed, seven of the ten longest NCRs show a substantial length difference between clade E and clade A, ranging from 9 to 58 bp (see bp of red boxes in Fig. 1). No comparable variation in the size of the longest NCR is observed within clade A. Indeed, among the four available clade A mtDNAs, only one long NCR has a length difference of a maximum 12 bp (see the red NCR between "W" and "Lc" in Fig. 1, comparing the bp separated by asterisks).

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

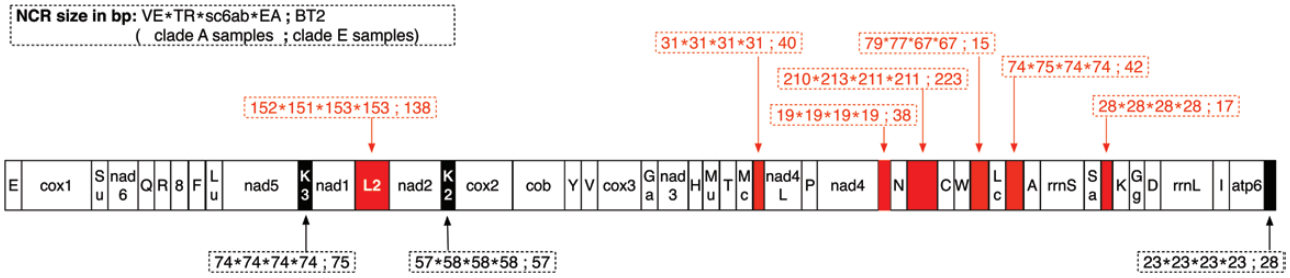


Figure 1. Mitochondrial genome organization of clade A and clade E specimens, with position of the longest non-coding regions (NCR). Filled black and red boxes: NCRs ≥ 25 bp in at least one specimen; red boxes: NCRs with size differences ≥ 9 bp between clade A and clade E; black boxes: NCRs with size differences < 9 bp between clade A and clade E; asterisk: separator between NCR size of the four clade A specimens; semi-colon: symbol preceding the NCR size of the only clade E specimen (BT2); L2, K2, K3: NCRs containing the L2, K2 and K3 tRNA-like structures. tRNAs are named by the transported amino acid, except for: Ga, tRNA-Gly(AGR); Gg, tRNA-Gly(GGN); Lu, tRNA-Leu(UUR); Lc, tRNA-Leu(CUN); Mc, tRNA-Met(CAU); Mu, tRNA-Met(UAU); Sa, tRNA-Ser(AGY); Su, tRNA-Ser(UCN); 8, atp8. The AC numbers of the analysed mtDNAs are listed in Table 3.

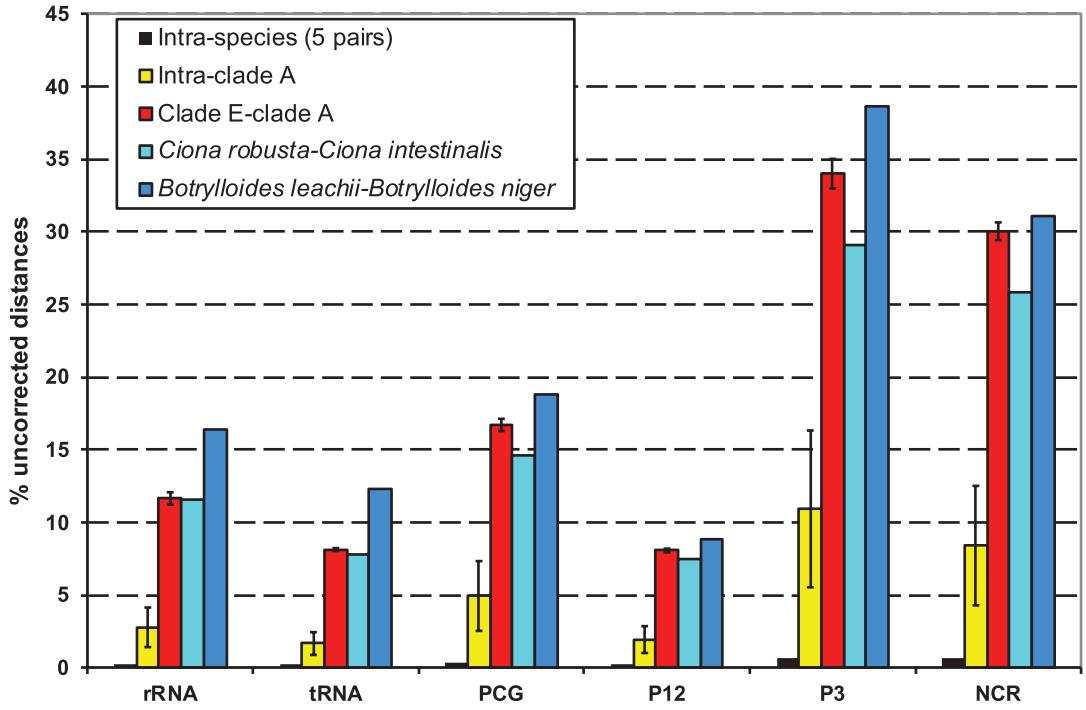


Figure 2. Uncorrected distances for intraspecies and congeneric ascidian pairs, calculated on different regions of the mitochondrial genome. NCR: Non-Coding Regions; PCG: 13 Protein-Coding Gene; P12: first and second codon positions; P3: third codon position; rRNA: 2 rRNA genes; tRNA: 24 tRNA genes. The five intraspecies pairs belong to the species: *Botrylloides giganteus, Botrylloides leachii*, *Ciona robusta*, *Ciona savignyi* and *Clavelina lepadiformis*. The respective mtDNAs analysed in intraspecies pairs are indicated with an asterisk in Table 3.

Figure 2 shows the uncorrected distances calculated on six different functional mt regions for: a) all pairwise comparisons within the *Botryllus schlosseri* species complex, b) all available intraspecies ascidian pairs, c) two peculiar congeneric pairs, *C. robusta-C. intestinalis* and *Botrylloides leachii-Botrylloides niger*, consisting of species morphologically hard to discriminate, exactly as is the case of the clade A-clade E pair. In particular:

- The *C. robusta-C. intestinalis* pair consists of two ex-cryptic species (formerly known as *C. intestinalis* type A and type B, respectively) for which the diagnostic

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

morphological traits were identified only after great efforts (Brunetti *et al.*, 2015; Pennati *et al.*, 2015).

- The *Botrylloides leachii-Botrylloides niger* pair consists of two morphologically highly similar and hard-to-discriminate *Botrylloides* species (see discussion in Brunetti (2009)), that were often confused with each other (see for example Griggio *et al.* (2014)).

In addition, it was already shown that these two congeneric pairs have the lowest substitution rate among all available ascidian congeneric pairs (Griggio *et al.*, 2014), so they are appropriate to represent the lowest known interspecies ascidian divergence.

Depending on the regions/pairs considered, the clade E-clade A distances are one or two orders of magnitude higher than the intraspecies distances, and from 2.3 to 195 times higher than the intraclade A distances (Fig. 2). Noteworthy, the clade E-clade A distances are almost identical to the distances found in the two congeneric pairs *C. robusta-C. intestinalis* and *Botrylloides leachii-Botrylloides niger* (ratio range: 0.7–1.2 times). As expected, in all pairwise comparisons P3 and NCR are the fastest evolving regions (Fig. 2).

To further investigate the evolutionary distances between the two *Botryllus schlosseri* clades, we have compared the more accurate non-synonymous nucleotide substitution rates, dN, between all available intraspecies and congeneric ascidian pairs (Fig. 3). It should be noticed that the analysed dataset consists of 17 congeneric pairs and seven other genera, including the genus *Botrylloides*, i.e. the one phylogenetically closest to *Botryllus*. Figure 3 shows the presence of a clear-cut gap between the dN values calculated at the congeneric and at intraspecies level (see dot and triangle symbols, respectively, in Fig. 3): excluding all *Botryllus schlosseri* pairs, the minimum congeneric value is equal to 0.052 substitutions/sites, while the maximum intraspecies value is equal to 0.0018. As observed for the uncorrected distances (Fig. 2), the dN value of clade E-clade A pair is almost identical to that of the pairs *C. robusta-C. intestinalis* and *Botrylloides leachii-Botrylloides niger* (0.051 against 0.052 and 0.057, respectively; see Fig. 3). These results indicate clade E-clade A as a pair consisting of two different (congeneric) species. As for the intraclade A distances, it is striking to note that only the dN value of the EA-sc6ab pair is in the range of those observed at the intraspecies level, whereas the dN values of the remaining intraclade A pairs are from five up to 42 times higher than the intraspecies distances (compare diamond to triangle symbols in Fig. 3).

Phylogenetic reconstructions within the Botryllinae

As shown in Fig. 4, both the Bayesian and the ML phylogenetic trees recognize the *Botryllus schlosseri* species complex as a monophyletic group and identify clade E as the sister group of clade A (bootstrap: 100%; and Bayesian Posterior Probability (BPP): 0.99). Within clade A, only the clustering of sc6ab with EA is significantly supported (bootstrap: 100%; BPP: 0.99), whereas the basal node consists in a polytomy in both the ML and Bayesian trees. The exact phylogenetic position of the VE-"neotype" within clade A remains unresolved (clustering VE-TR: bootstrap 51%; BPP < 0.50).

As for the genus *Botrylloides*, it is recognized as monophyletic with moderate support in the ML tree (bootstrap: 82%), but the relative node remains unresolved in the Bayesian tree (BPP < 0.50). Inconsistences between the ML and the Bayesian trees concern even the position of *Botrylloides niger* and *Botrylloides violaceus* Oka, 1927. Indeed their relative nodes are fully or moderately supported in the ML tree (bootstrap: 100% for the *Botrylloides niger-Botrylloides leachii* clade; 76% for the clade consisting of *Botrylloides giganteus* (Pérès, 1949) and *Botrylloides violaceus*), but poorly supported in the Bayesian reconstruction (BPP < 0.77; Fig. 4).

Morphological analyses and taxonomy

Botryllus gaiae Brunetti sp. nov.

LSID:urn:lsid:zoobank.org:pub:DB18D5A7-0E96-458E-B3C0-5E93327359AC

Etymology: From Gaia, the name of the first granddaughter of the author of this species, R. Brunetti.

Material examined: Syntypes: MSNVE 25086, BT2, colony fragment in 10% formalin; MSNVE 2508, BT4, colony fragment in 10% formalin; MSNVE 25088, BT2A, another fragment of the BT2 colony preserved in 99% ethanol; MSNVE 25089, BT4A, another fragment of the BT4 colony preserved in 99% ethanol. All collected by F. Mastrotorato during clam sampling.

Type locality: Barletta (Italy), 41°22'55.646"N, 16°10'06.586"E, 2.5–4.0 m depth on *Cymodocea nodosa* (Ucria) Asch. Seagrass meadow.

Description: In both colonies from Barletta, labelled BT2 and BT4, the test surface is hard but, once it is torn, zooids are easily pulled out. Sample BT4 consists of two pieces of colony, each of them with 2–3 systems at the beginning of the generation change (developmental stage 9–11/8–9/6–7/1) (Berrill, 1941). Therefore, the older generation is at the beginning of regression (stage 9–11) and the new generation presents syphons still closed and all internal organs completely developed (stage 8–9). The regressing zooids are clearly arranged in *Botryllus schlosseri*-type

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

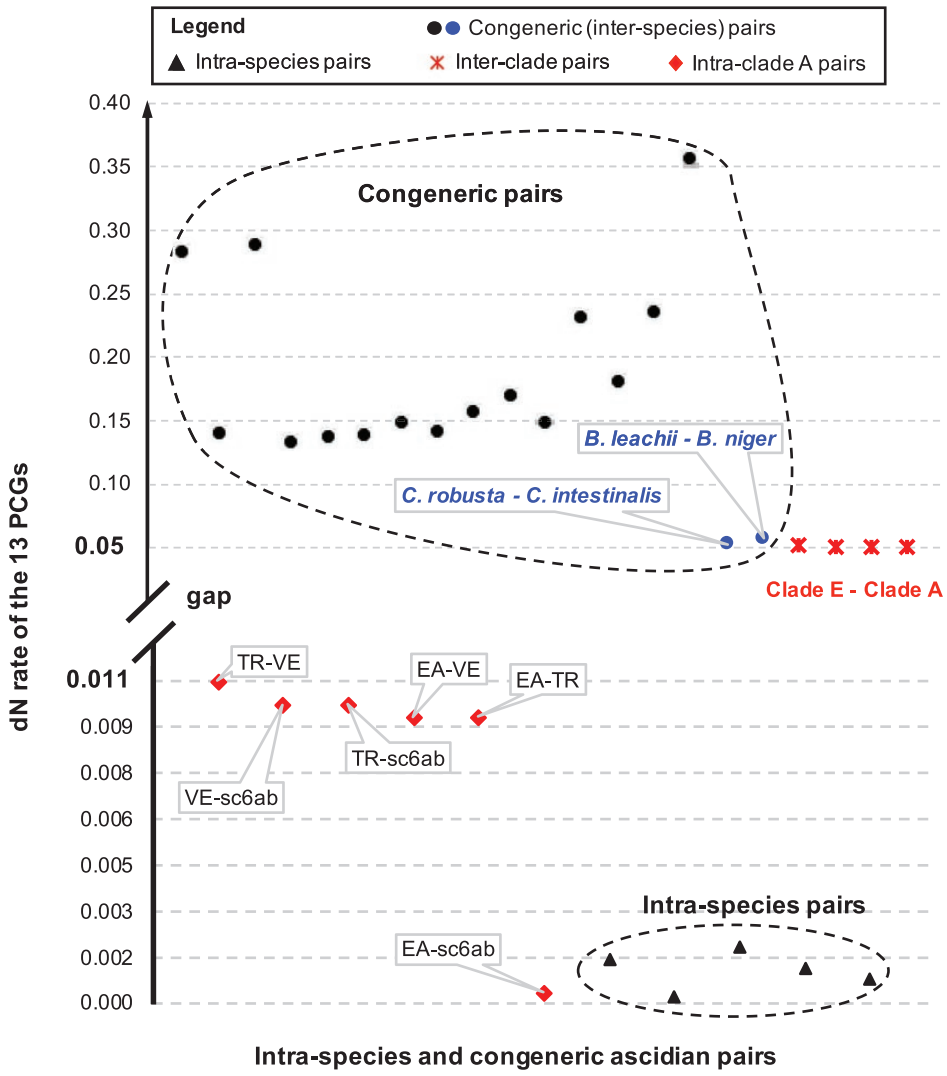


Figure 3. Non-synonymous substitution rates (dN) of intraspecies and congeneric ascidian pairs. Red: intra and interclade comparisons between specimens of the *Botryllus schlosseri* species complex; blue: comparisons between ex-cryptic or morphologically hard-to-discriminate species; black: comparisons between all other analysed ascidians. The AC numbers of the analysed mtDNAs are listed in Table 3.

systems. Sample BT2 contains a piece of colony of about 1 cm^2 of surface with two systems consisting of 15 zooids. As shown in Figure 5A-B, in life these two colonies are green (BT4) and green-yellow (BT2), whereas in preservative there are whitish.

The following description is chiefly based on the observation of the first order buds of sample BT2, which have virtually finalized their development (only the syphons have to be completed) (Fig. 5C–E). The adult regressing zooids are about 1.5 mm high and the same height is recorded in first order buds. Therefore, it is justifiable to deduce a height up to 2 mm for the filtering zooid, because an increase in size is observed when the bud opens the syphons. The body wall presents fine muscle fibres running from the anterior-dorsal side to the ventral one. In the buds there are eight tentacles: four large and four intercalated short. The atrial syphons, still visible in some regressing zooids, is conic with a dorsal tongue. The branchial sac has nine rows of stigmata on the right and eight on the left side; the second row is dorsally incomplete (Fig. 5D–E). The zooid has a cylindrical shape having about 20 stigmata in the first half row and about 15 in the seventh one. The branchial formula at the level of fourth row of stigmata is usually E 5.5.5.5 DL. Along the transversal branchial vessels, no muscle fibres are detected. A simple edged dorsal lamina rises from the dorsal vessel at level of fourth to fifth row of stigmata and reaches a height of three times the diameter of the dorsal vessel (Fig. 5D–E) at the oesophageal opening which is at level of the last

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

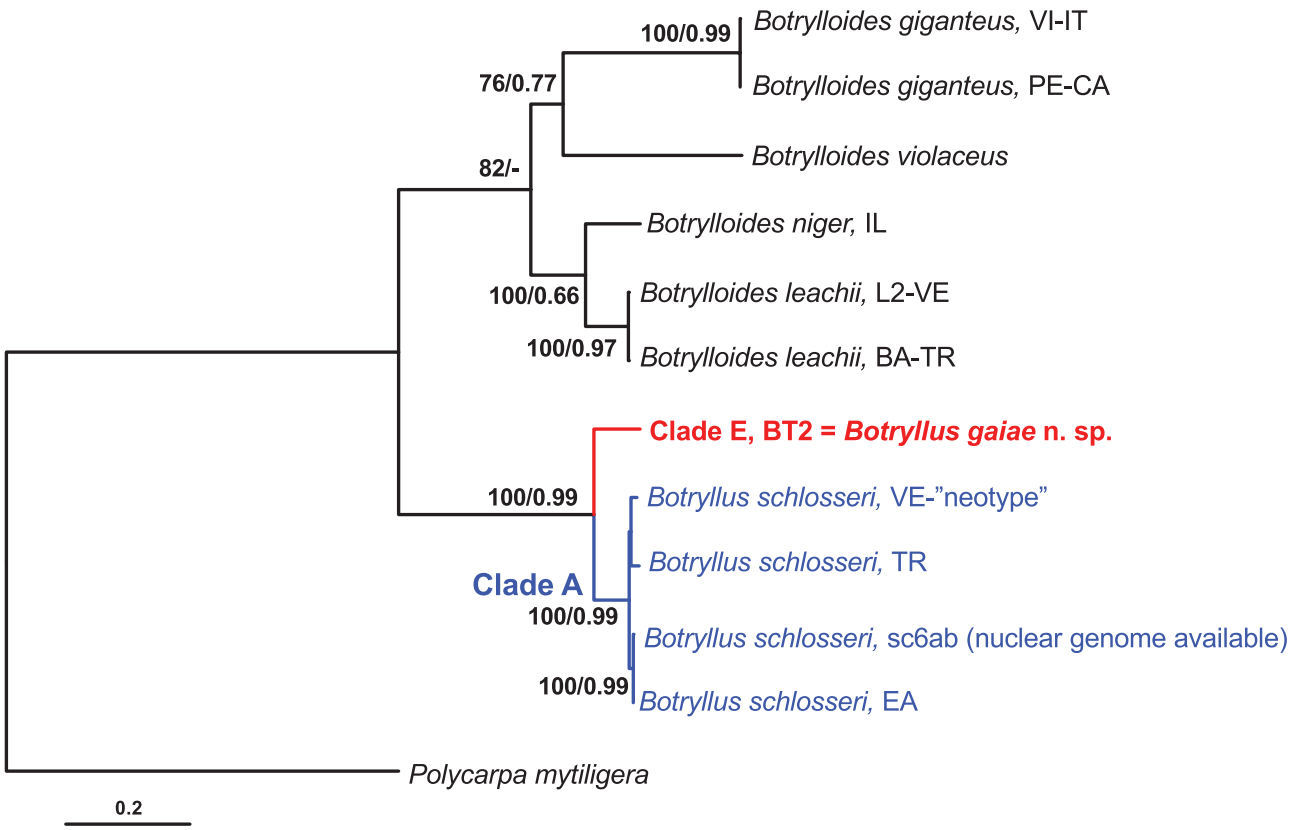


Figure 4. Phylogeny of the Botryllinae inferred from the concatenation of the 13 mitochondrial-encoded proteins. Node values correspond to ML bootstrap and BPP. Nodes having both ML bootstrap ≤ 55% and BPP ≤ 0.50 have been collapsed.

row of stigmata. The intestinal loop is horizontally arranged (Fig. 5D). The stomach is almost totally below the branchial sac. It is slightly cylindrical, with nine folds slightly spirally arranged and all posteriorly closed. These folds have a smooth surface (Fig. 5F, I–L), without the longitudinal groove observed in *Botryllus schlosseri* (Brunetti *et al.*, 2017: fig. 1). They are longer on the mesial side (folds 5–8 in Fig. 5L) and without swellings at their cardiac end. The pyloric caecum is long, about half of the stomach, and has a rounded dilated tip; it rises from about the posterior third part of the typhlosole, the anterior part of which is much larger than the posterior one (Fig. 5F, I). The intestine has a peculiar shape, with a hairpin bend (indicated with an arrow head in Fig. 5C–D) at level of the first intestinal loop. This aspect is not due to pressure of the gonads, as it is present also in zooids without developed gonads. The rectum is strongly stretched by a connection with the branchial sac through a trabecula at the level of the transversal vessel between the seventh and eighth rows of stigmata (Fig. 5D–E, H). The anus opens at level of the seventh row of stigmata, that is one or two rows above the oesophageal opening. The anal opening has four lobes, each of them splits into three minor ones (Fig. 5G). On the rectum surface, there are four grooves going from the anus to the pyloric gland. In sample BT4, well-developed gonads are present only in buds of the first order, which are ready to substitute the parents. There are up to four eggs (diameter of about 140 μm) on each side, anterior and dorsal to the testis follicles, which partially embrace them as a cup (Fig. 5E). Gonadal primordia are present also in buds of the second order. No larvae and embryos have been found.

Remarks: Based on the structure of the cloaca, this species clearly belongs to the genus *Botryllus,* as defined by Brunetti (2009). The main morphological features distinguishing *Botryllus gaiae* from *Botryllus schlosseri* are reported in Table 4 and include several traits currently considered valid diagnostic features to discriminate species in the genus *Botryllus* (Brunetti, 2009; Brunetti & Mastrototaro, 2017). These traits, highlighted in bold in Table 4, concern peculiarities of the stomach and the intestine, such as the shape of the first and second intestinal curve, the shape and position of the anus opening, the number and surface appearance of the stomach folds and the shape of the typhlosole. All these morphological differences are highly significant and support the hypothesis that clade E is a new species, distinct from *Botryllus*

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

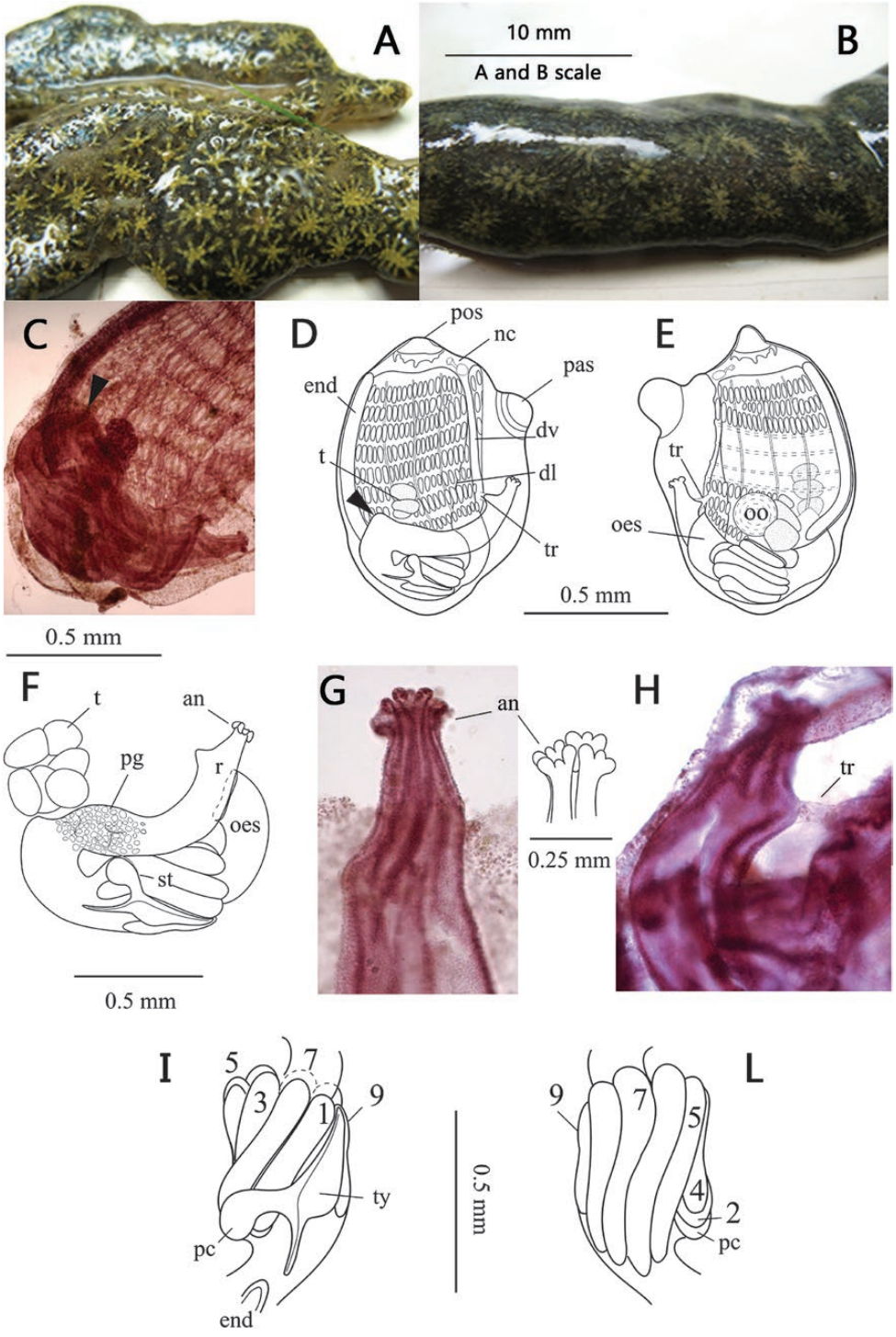


Figure 5. *Botryllus gaiae*. A, BT2 living colony, syntype; B, BT4 living colony, syntype; C–E, first order bud ready to substitute the regressing parental zooid (stage 8→9); C–D, left side; E, right side; F, intestinal loop; G–H, rectum and anus; I, parietal side of the stomach; L, mesial side of the stomach. Symbols: arrow head, hairpin bend of the first intestinal loop; an, anus; dl, dorsal lamina; dv, dorsal vessel; end, endostyle; nc, neural complex (dorsal tubercle and neural gland); oes, oesophagus; oo, oocyte; pas, primordia of the atrial syphon; pc, pyloric caecum; pg, pyloric gland; pos, primordia of the oral syphon; r, rectum; st, stomach; t, testis; tr, trabecula; ty, typhlosole.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

schlosseri sensu Brunetti *et al.* (2017) and therefore from clade A.

DISCUSSION

THE NEW SPECIES

Both morphological and mitogenomic data agree in demonstrating that clade E has unique features clearly distinguishing it from *Botryllus schlosseri sensu* Brunetti *et al.* (2017) and thus from clade A. The main diagnostic characters discriminating clade E from *Botryllus schlosseri sensu* Brunetti *et al.* (2017) are: a) the position and shape of the anal opening with its four lobes, each trilobed, b) the number and the surface appearance of the stomach folds, c) the shape of the typhlosole, and d) the hairpin shape of the first intestinal loop (Table 4; compare also Fig. 5D–F of the present article with Brunetti *et al.*, 2017: figure 1A–D). The future finding of ripe colonies with larvae will allow verifying the possible existence of differences also in the size and morphology of eggs, embryos and larvae, all well-known discriminant characters in the genus *Botrylloides* (Saito *et al.*, 1981; Saito & Watanabe, 1985) and in other ascidians (Pennati *et al.*, 2015).

The junior synonym list of *Botryllus schlosseri* reported in the World Register of Marine Species (WoRMS) database (Shenkar *et al.*, 2019) consists of 24 species that were described from 1774 to 1912 (see Supporting information, Table S1): we excluded from the original list the misspelling of *Botryllus badius* Alder & Hancock, 1912 and *Botryllus gascoi* Della Valle, 1877, because this last species was originally described by Della Valle as *Botrylloides*. All these synonyms are characterized by poor morphological descriptions focussing mostly on external characters and colouration of the colony, considered at that time fundamental discriminant traits. For example, based

Table 4. Comparison between the new species *Botryllus gaiae* and the neotype of *Botryllus schlosseri* (Brunetti *et al.*, 2017). The discriminant characters are reported in bold

Character	*Botryllus gaiae*	*Botryllus schlosseri* neotype
ZOOID		
Height	1.5 mm	
Tentacle number	Usually 8 (4 large + 4 small)	
Tentacles shape	?	Spoon shaped
Presence of oral islands	?	Yes
BRANCHIAL SAC		
General shape	cylindrical	conical
Rows of stigmata	9 on right; 8 on left	~8
Second row	Incomplete	
Number of stigmata per half row	About 15	
Branchial sector width*	D = L = V	
Branchial cell islands	?	At level of rows of stigmata
Branchial formula*	Ds ≈ Vs > Ls	
INTESTINE		
First curve shape	**With a hairpin bend**	**Rounded**
Second curve shape	**Open**	**Almost absent**
Rectum arrangement	Connected to pharynx	Connected to pharynx and body wall
Anus opening (position)	**Above the oesophageal opening**	**At level of oesophageal opening**
Anus opening (shape)	**With four lobes, each trilobed**	**Smooth edged**
STOMACH		
Outline	Slightly cylindrical	Oval or cylindrical
Position	Almost totally below the branchial sac	
Folds number†	**9**	**8**
Folds shape	Closed at both ends	
Folds surface	**Smooth**	**With a longitudinal groove**
Typhlosole shape	**Wide in the anterior part**	**Constant width**
Pyloric caecum length	~1/2 of the stomach	
Pyloric caecum width	Less wide than the typhlosole	Wide as the typhlosole

* = D = dorsal; L = lateral; V = ventral.† = typhlosole not counted.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

almost exclusively on the colour of the colony, Giard (1872) described six new *Botryllus* species and up to four varieties for each species, all currently listed as junior synonyms of *Botryllus schlosseri*. Only since the second decade of the 20th century a greater attention was paid to the anatomy of internal organs, whose peculiarities were thereafter reported in botryllid descriptions. The contribution of Michaelsen (in Hartmeyer & Michaelsen, 1928) was fundamental to the introduction of this new method of species description. Given these preconditions, there is no possibility to ascertain whether clade E corresponds to one of the old species synonymized under *Botryllus schlosseri*, because their descriptions did not report on the internal characters here identified as diagnostic for *Botryllus gaiae* Moreover, the main discriminant morphological traits of clade E have not been reported in other previously described *Botryllus* species, so it is likely that it is a previously undescribed species.

As for the mitochondrial genome, the substantial differences observed between *Botryllus gaiae* (clade E) and *Botryllus schlosseri* (clade A) are incompatible with intraspecific variability. These differences concern the size of many longest NCRs (Fig. 1) and the nucleotide divergence (Figs 2,-3). The NCR size differences between the two clades are too high compared to those found within clade A (compare the size of red NCRs in Fig. 1), suggesting an interspecies variability. Likewise, as shown in Figures 2 and 3, the nucleotide divergence between *Botryllus gaiae* and *Botryllus schlosseri* clade A is incompatible with the ascidian intraspecies variability, but shows the same order of magnitude of the divergence observed between ascidian congeneric species. Even more interestingly, the clade E-clade A divergence is almost identical to that of species once considered morphologically indistinguishable and recently reconsidered as valid species, that is:

- two ex-cryptic species, i.e. *C. robusta-C. intestinalis*, for which the identification of discriminant diagnostic characters was problematic and debated for a long time (Brunetti *et al.*, 2015; Pennati *et al.*, 2015; Gissi *et al.*, 2017);
- the *Botrylloides leachii-Botrylloides niger* pair: these two *Botrylloides* species are morphologically so similar that they were considered by some authors as a single species (see discussion in Brunetti (2009)). Moreover, when only the morphology was considered, they were often misidentified. For example, the *Botrylloides niger* mtDNA was initially published as *Botrylloides* affinis *leachii* (Rubinstein *et al.*, 2013) and only a subsequent morphological re-analysis of the sample allowed its correct assignment to *Botrylloides niger* (Griggio *et al.*, 2014) (Brunetti R, personal communication).

Therefore, it seems that, in our ascidian dataset, lowest mt nucleotide divergences are associated with the smallest morphological differences. Although we did not attempt to quantify the morphological distances for the species pairs here analysed, this observation suggests that congeneric ascidian datasets could be a good target for studying the old debated issue of the correlation between the morphological and the molecular evolutionary rate.

The existence of a high nucleotide divergence between *Botryllus gaiae* and *Botryllus schlosseri* clade A does not contradict the observation of an identical mt gene order. Although the mt gene order is generally hypervariable in ascidians (Gissi *et al.*, 2010), it appears more stable within the Styelidae, where we have observed that: the gene order is identical in the four studied species of *Botrylloides* (*Botrylloides giganteus*, *Botrylloides leachii*, *Botrylloides niger* and *Botrylloides violaceus*); *Styela plicata* (Lesueur, 1823) and *Styela clava* Herdman, 1881, differs only for the position of one tRNA gene; and only moderate gene order rearrangements are present between the genera *Botryllus* and *Botrylloides* (data not shown). Therefore, in the Styelidae and even more in the Botryllinae we can expect that different species have an identical mt gene order.

The mitochondrial phylogenetic reconstructions identify clade E, alias *Botryllus gaiae*, as the sister taxon of the entire clade A and therefore also of the VE-″neotype″ (maximal support in both ML and BI trees, see Fig. 4). Unfortunately, some nodes within clade A and the relationships between *Botrylloides* species remain unresolved (Fig. 4), as they also were in a previous mt phylogeny of the entire Tunicata group (Shenkar *et al.*, 2016). In particular, the polytomy within clade A between VE-″neotype″, TR and the EA + sc6ab subclade, based on amino acid characters (Fig. 4 and Shenkar *et al.* (2016)), reflects the strange dN values observed between the same three taxa/subclade at the nucleotide level (Fig. 3). These dN values are exactly in between those observed in unequivocal intraspecies and congeneric pairs (Fig. 3 and Griggio *et al.* (2014)). As we already hypothesized in Griggio *et al.* (2014), this result supports the existence in clade A of three genetically well-distinct clusters (VE-″neotype″, TR and EA + sc6ab), corresponding to additional cryptic species or arising from ongoing speciation events. The identification of morphological diagnostic characters allowing the description of clade E as a new species (*Botryllus gaiae*) prompts the detailed investigation of the morphological (and also the molecular) variability within clade A, as well as the possible existence of morphological discriminant characters for the rare B–D clades.

We have here associated the morphological description of *Botryllus gaiae* to a reliable molecular

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

marker consisting of the entire mtDNA. This mtDNA sequence will facilitate future identification of this species by genetic analyses, for example by setting new molecular markers/fast PCR tests based on the NCRs differentiating *Botryllus schlosseri* from *Botryllus gaiae*, and will help performing population genetic studies (for example, based on mitochondrial regions with the highest evolutionary rate).

We think that the here applied integrated approach, combining molecular and morphological data, provides sufficient evidence to conclude that clade E is a separate species. The analysis of additional *Botryllus gaiae* specimens, both morphologically and molecularly, is needed to properly assess the intraspecific variability of this species and to increase the confidence in its validity. Although the examination of several specimens coming from different populations is certainly the best practice for species identification, it should be considered that, for several reasons, not only in the past but even in recent years a single specimen has been often used by taxonomists for the description of a new species or by evolutionary biologists for proposing DNA-based "species hypotheses" (see discussion of this issue in Lim *et al.* (2012)). Even though clade E can be found throughout European waters, its collection for concurrent molecular and morphological analyses poses several problems and is not straightforward: there are no external characters to distinguish clades in the fields, so in many cases we sampled clade E just by chance or after collecting a high number of colonies; colonies are delicate and sensitive to the relaxation and fixation steps; there is no standardized protocol for colony relaxation, the success of which seems to be related to the colony size/developmental stage and is crucial for subsequent morphological analyses; the fixation step needs to be carefully carried out as soon as an optimal relaxation has been achieved, therefore the colonies need to be continuously monitored; the frequent abundance of large pigmented blood cells hinders the fine description of the zooid anatomy, making the haemalum staining problematic and rendering the whole sample inadequate for morphological studies. Based on these considerations, we think that, even in the absence of an accurate estimation of intraspecies morphological/molecular variability, the publication of our results is timely and appropriate, because it will allow other researchers to pay attention to the variability of the morphological characters here defined as diagnostic for *Botryllus schlosseri*/*Botryllus gaiae*. Moreover, it will be fundamental for the taxonomic re-evaluation of the *Botryllus schlosseri* species complex and for addressing future morphological studies aimed at identifying the discriminant characters of the rare clades B–D.

As final remark, we stress that the cryptic speciation of *Botryllus schlosseri*, and probably even its cosmopolitism, could be partially due to the original inaccurate definition of this species (Brunetti *et al.*, 2017), that has to be traced back also to the intrinsic difficulty in the identification of diagnostic characters in the Botryllinae. In particular, an overconservative systematics of the genus *Botryllus* led in the past to include most species in the single taxon *Botryllus schlosseri*. More recent molecular systematic studies, introducing the more easily quantifiable genetic characters, have revealed a high genetic variability within this taxon, addressing its taxonomic re-analysis.

THE DISTRIBUTION OF *BOTRYLLUS GAIAE*

Clade E, here named *Botryllus gaiae* was so far identified only in European waters, both in the Atlantic and Mediterranean (Lopez-Legentil *et al.*, 2006, 2015; Lejeusne *et al.*, 2011; Yund *et al.*, 2015; Nydam *et al.*, 2017; Reem *et al.*, 2017). Along the European Atlantic coasts, it is particularly abundant in the English Channel, where it occurs almost always in sympatry with *Botryllus schlosseri* clade A, while it is less abundant in the Bay of Biscay (Nydam *et al.*, 2017). As summarized in Table 5, in the Mediterranean Sea, *Botryllus gaiae* was hitherto found only in the western region, the most sampled area, but it was also recorded from one of the only two sampled localities of the eastern region (Glyfada, see Table 5). Here, we record its presence in the central Mediterranean region. Indeed, we have identified *Botryllus gaiae* in localities of the Ionian and south Adriatic Seas, which were never investigated before, and in 2016 in the Venice Lagoon in the north Adriatic Sea, where only clade A had been previously found during the numerous samplings between 2004 to 2015 (Table 5). In general, the Adriatic Sea has been incompletely sampled, with only two other localities in the north and central Adriatic Sea investigated previously (Rovinj & Ancona, see Table 5), identifying only clade A there (Reem *et al.*, 2017). However, it should be noted that clade E was identified in 2000 in the Greek Aegean Sea (Glyfada, see Table 5; Reem *et al.* (2017)), which is adjacent to the Ionian Sea where *Botryllus gaiae* was found in more recent years (Tables 1 and 5). Although the data of the Mediterranean distribution of *Botryllus gaiae* are highly incomplete, both for sampling localities and dates (Table 5), it is noteworthy that *Botryllus gaiae* has been identified in the central and eastern Mediterranean only recently and/or in localities strongly influenced by human activities (high maritime traffic in Glyfada, Venice and Taranto; aquaculture in Taranto, etc.). Therefore, we could hypothesize a recent human-mediated spread of *Botryllus gaiae* in new regions of the Mediterranean

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

Table 5. Sampling localities of clade E, alias Botryllus gaiae, and clade A in the Mediterranean Sea. The numerous localities of France and Spain are not listed

Locality	Year	Colony number			Reference
		Total	Clade A	Clade E	
CENTRAL					
Northern Adriatic Sea					
Venice Lagoon, Italy	2004*	8	8		Yund *et al.* (2015)
"	2005	2	2		Griggio *et al.* (2014); personal data
"	2010	6	6		Personal data
"	2010	28	28		Bock *et al.* (2012)
"	2013	4 + 14	4 + 14		Brunetti *et al.* (2017); personal data
"	2015	9	9		Personal data
"	2016	32	23	9	This study
Rovinji, Croatia	1996†	35	35		Reem *et al.* (2017)
Central Adriatic Sea					
Ancona, Italy	2007†	32	32		Reem *et al.* (2017)
Southern Adriatic Sea					
Barletta, Italy	2013	5	3	2	This study
Ionian Sea					
Taranto, Mar Piccolo, Italy	2011	1	1		Griggio *et al.* (2014)
"	2018	4	3	1	This study
Taranto, Mar Grande, Italy	2013	2		2	This study
EASTERN					
Aegean Sea					
Glyfada, Greece	2000†	23	14	9	Reem *et al.* (2017)
Israel coasts					
Michmoret	2006†	39	39		Reem *et al.* (2017)
WESTERN					
Naples, Tyrrhenian Sea	2010	7	7		Bock *et al.* (2012)
Palermo, Tyrrhenian Sea	2005†	18	18		Reem *et al.* (2017)
Carrara, Ligurian Sea	2006†	25	18	7	Reem *et al.* (2017)
Loano, Ligurian Sea	2006†	26	26		Reem *et al.* (2017)
Gibraltar, Spain	1998†	15	15		Reem *et al.* (2017)
Montril, Spain	1998†	8	8		Reem *et al.* (2017)
France and Spain coasts (> 10 localities)	2005–2014§	-	present	present	Lopez-Legentil *et al.* (2006, 2015); Lejeusne *et al.* (2011); Bock *et al.* (2012); Nydam *et al.* (2017); Reem *et al.* (2017)

* = P. Yund (pers. comm).
† = E. Reem (pers. comm).
§ = partial range of time, since the sampling dates are reported only in few papers. This range includes personal communications from D. Bock and M. Nydam.

Sea. This tendency to expansion of *Botryllus gaiae* also agrees with population genetic studies by Nydam *et al.* (2017). Therefore, we suggest monitoring the presence and possible spread of *Botryllus gaiae* mainly in the central and eastern Mediterranean regions.

The list of the clade E *cox1* sequences present in public nucleotide databases and that have to be reassigned to *Botryllus gaiae* is reported in Supporting information (Table S2).

CONCLUSIONS

The identification of clade E as *Botryllus gaiae*, as a distinct but closely related species to clade A, i.e. *Botryllus schlosseri sensu* Brunetti *et al.* (2017), is here based on an integrated approach consisting of the combined analysis of morphological characters and mitochondrial genomic data. We hope that our results will trigger further investigations aimed not only at confirming the validity of these two species, but

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

also at a more accurate characterization of the entire *Botryllus schlosseri* species complex. Indeed, missing data concerns several aspects of the biology (such as the reproductive isolation, the ecological preferences and the degree of invasiveness of the species here validated of *Botryllus gaiae*, *Botryllus schlosseri* and of the geographically restricted B–D clades). In any case, an integrated approach is essential to unravel the taxonomy of the *Botryllus schlosseri* species complex and, in general of Botryllinae species, as also highlighted in a recent study on *Botrylloides* (Rocha *et al.*, 2019).

ACKNOWLEDGEMENTS

This work was partially supported by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR): PRIN2015; and by Consiglio Nazionale delle Ricerche (CNR): Molecular Biodiversity Laboratory of the Italian node of Lifewatch. The authors thank Marika Salonna and Federica Montesanto for help in sampling and DNA barcoding; Lucia Manni and Dorothée Huchon for helpful discussions and four anonymous reviewers for suggestions. The authors declare that they have no conflict of interest.

REFERENCES

Alie A, **Hiebert LS**, **Simion P**, **Scelzo M**, **Prunster MM**, **Lotito S**, **Delsuc F**, **Douzery EJP**, **Dantec C**, **Lemaire P**, **Darras S**, **Kawamura K**, **Brown FD**, **Tiozzo S. 2018.** Convergent acquisition of nonembryonic development in Styelid ascidians. *Molecular Biology and Evolution* **35:** 1728–1743.

Ben-Shlomo R, **Reem E**, **Douek J**, **Rinkevich B. 2010.** Population genetics of the invasive ascidian *Botryllus schlosseri* from South American coasts. *Marine Ecology Progress Series* **412:** 85–92.

Berrill NJ. 1941. The development of the bud in *Botryllus*. *Biological Bulletin* **80:** 169–184.

Berrill NJ. 1950. *The Tunicata with an account of the British species*. London: The Ray Society.

Bock DG, **MacIsaac HJ**, **Cristescu ME. 2012.** Multilocus genetic analyses differentiate between widespread and spatially restricted cryptic species in a model ascidian. *Proceedings of the Royal Society B: Biological Sciences* **279:** 2377–2385.

Brunetti R. 2009. Botryllid species (Tunicata, Ascidiacea) from the Mediterranean coast of Israel, with some considerations on the systematics of Botryllinae. *Zootaxa* **2289:** 18–32.

Brunetti R, **Gissi C**, **Pennati R**, **Caicci F**, **Gasparini F**, **Manni L. 2015.** Morphological evidence that the molecularly determined *Ciona intestinalis* type A and type B are different species: *Ciona robusta* and *Ciona intestinalis*. *Journal of Zoological Systematics and Evolutionary Research* **53:** 186–193.

Brunetti R, **Manni L**, **Mastrototaro F**, **Gissi C**, **Gasparini F. 2017.** Fixation, description and DNA barcode of a neotype for *Botryllus schlosseri* (Pallas, 1766) (Tunicata, Ascidiacea). *Zootaxa* **4353:** 29–50.

Brunetti R, **Mastrototaro F. 2017.** *Ascidiacea of the European waters*. Milan: Edagricole - Edizioni Agricole di New Business Media Srl.

Carlton JT. 2005. *Setting ascidian invasions on the global stage*. Woods Hole: Woods Hole Oceanographic Institution.

Carman MR, **Morris JA**, **Karney RC**, **Grunden DW. 2010.** An initial assessment of native and invasive tunicates in shellfish aquaculture of the North American east coast. *Journal of Applied Ichthyology* **26:** 8–11.

Carver CE, **Mallet AL**, **Vercaemer B. 2006.** Biological synopsis of the colonial tunicates, *Botryllus schlosseri* and *Botrylloides violaceus*. *Canadian Manuscript Report of Fisheries and Aquatic Sciences* **2747:** 1–42.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17:** 540–552.

Della Valle A. 1877. Contribuzioni alla storia naturale delle Ascidie Composte del Golfo di Napoli. *Napoli, Tipografia dei Comuni*, 48.

Franchi N, **Ballin F**, **Manni L**, **Schiavon F**, **Basso G**, **Ballarin L. 2016.** Recurrent phagocytosis-induced apoptosis in the cyclical generation change of the compound ascidian *Botryllus schlosseri*. *Developmental & Comparative Immunology* **62:** 8–16.

Giard A. 1872. Recherches sur les Ascidies composées ou Synascidies. *Archives de Zoologie Expérimentale et Générale*. **1:** 501–687.

Gissi C, **Hastings KEM**, **Gasparini F**, **Stach T**, **Pennati R**, **Manni L. 2017.** An unprecedented taxonomic revision of a model organism: the paradigmatic case of *Ciona robusta* and *Ciona intestinalis*. *Zoologica Scripta* **46:** 521–522.

Gissi C, **Pesole G**, **Mastrototaro F**, **Iannelli F**, **Guida V**, **Griggio F. 2010.** Hypervariability of ascidian mitochondrial gene order: exposing the myth of deuterostome organelle genome stability. *Molecular Biology and Evolution* **27:** 211–215.

Goldman N, **Yang Z. 1994.** A codon-based model of nucleotide substitution for protein-coding DNA sequences. *Molecular Biology and Evolution* **11:** 725–736.

Griggio F, **Voskoboynik A**, **Iannelli F**, **Justy F**, **Tilak MK**, **Turon X**, **Pesole G**, **Douzery EJ**, **Mastrototaro F**, **Gissi C. 2014.** Ascidian mitogenomics: comparison of evolutionary rates in closely related taxa provides evidence of ongoing speciation events. *Genome Biology and Evolution* **6:** 591–605.

Guindon S, **Gascuel O. 2003.** A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52:** 696–704.

Hartmeyer R, **Michaelsen W. 1928.** Ascidiae Dictyobranhiae und Ptychobranchiae. *Fauna Südwest-Australiens* **5:** 251–460.

Hirose M, **Hirose E. 2009.** DNA barcoding in photosymbiotic species of *Diplosoma* (Ascidiacea: Didemnidae), with the description of a new species from the southern Ryukyus, Japan. *Zoological Science* **26:** 564–8.

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

Iannelli F, **Pesole G**, **Sordino P**, **Gissi C. 2007.** Mitogenomics reveals two cryptic species in *Ciona intestinalis*. *Trends in Genetics* **23:** 417–422.

Katoh K, **Toh H. 2008.** Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* **9:** 286–298.

Lartillot N, **Lepage T**, **Blanquart S. 2009.** PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* **25:** 2286–2288.

Lartillot N, **Philippe H. 2004.** A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Molecular Biology and Evolution* **21:** 1095–1109.

Lefort V, **Longueville JE**, **Gascuel O. 2017.** SMS: smart model selection in PhyML. *Molecular Biology and Evolution* **34:** 2422–2424.

Lejeusne C, **Bock DG**, **Therriault TW**, **MacIsaac HJ**, **Cristescu ME. 2011.** Comparative phylogeography of two colonial ascidians reveals contrasting invasion histories in North America. *Biological Invasions* **13:** 635–650.

Lim GS, **Balke M**, **Meier R. 2012.** Determining species boundaries in a world full of rarity: singletons, species delimitation methods. *Systematic Biology* **61:** 165–169.

Lopez-Legentil S, **Legentil ML**, **Erwin PM**, **Turon X. 2015.** Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. *Biological Invasions* **17:** 1623–1638.

Lopez-Legentil S, **Turon X**, **Planes S. 2006.** Genetic structure of the star sea squirt, *Botryllus schlosseri*, introduced in southern European harbours. *Molecular Ecology* **15:** 3957–3967.

Lord J. 2017. Temperature, space availability, and species assemblages impact competition in global fouling communities. *Biological Invasions* **19:** 43–55.

Manni L, **Anselmi C**, **Cima F**, **Gasparini F**, **Voskoboynik A**, **Martini M**, **Peronato A**, **Burighel P**, **Zaniolo G**, **Ballarin L. 2019.** Sixty years of experimental studies on the blastogenesis of the colonial tunicate *Botryllus schlosseri*. *Current Directions in Tunicate Development* **448:** 293–308.

Manni L, **Zaniolo G**, **Cima F**, **Burighel P**, **Ballarin L. 2007.** *Botryllus schlosseri*: a model ascidian for the study of asexual reproduction. *Developmental Dynamics* **236:** 335–352.

Nydam ML, **Giesbrecht KB**, **Stephenson EE. 2017.** Origin and dispersal history of two colonial ascidian clades in the *Botryllus schlosseri* species complex. *PLoS One* **12:** e0169944.

Pennati R, **Ficetola GF**, **Brunetti R**, **Caicci F**, **Gasparini F**, **Griggio F**, **Sato A**, **Stach T**, **Kaul-Strehlow S**, **Gissi C**, **Manni L. 2015.** Morphological differences between larvae of the *Ciona intestinalis* species complex: hints for a valid taxonomic definition of distinct species. *PLoS One* **10:** e0122879.

Perez-Portela R, **Bishop JD**, **Davis AR**, **Turon X. 2009.** Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **50:** 560–570.

Reem E, **Douek J**, **Paz G**, **Katzir G**, **Rinkevich B. 2017.** Phylogenetics, biogeography and population genetics of the ascidian *Botryllus schlosseri* in the Mediterranean Sea and beyond. *Molecular Phylogenetic and Evolution* **107:** 221–231.

Rinkevich Y, **Voskoboynik A**, **Rosner A**, **Rabinowitz C**, **Paz G**, **Oren M**, **Douek J**, **Alfassi G**, **Moiseeva E**, **Ishizuka KJ**, **Palmeri KJ**, **Weissman IL**, **Rinkevich B. 2013.** Repeated, long-term cycling of putative stem cells between niches in a basal chordate. *Developmental Cell* **24:** 76–88.

Rocha RM, **Salonna M**, **Griggio F**, **Ekins M**, **Lambert G**, **Mastrototaro F**, **Fidler A**, **Gissi C. 2019.** The power of combined molecular and morphological analyses for the genus *Botrylloides*: identification of a potentially global invasive ascidian and description of a new species. *Systematics and Biodiversity* **17:** 509–526.

Rosengarten RD, **Nicotra ML. 2011.** Model systems of invertebrate allorecognition. *Current Biology* **21:** R82–R92.

Rosner A, **Moiseeva E**, **Rabinowitz C**, **Rinkevich B. 2013.** Germ lineage properties in the urochordate *Botryllus schlosseri* – from markers to temporal niches. *Developmental Biology* **384:** 356–374.

Rubinstein ND, **Feldstein T**, **Shenkar N**, **Botero-Castro F**, **Griggio F**, **Mastrototaro F**, **Delsuc F**, **Douzery EJ**, **Gissi C**, **Huchon D. 2013.** Deep sequencing of mixed total DNA without barcodes allows efficient assembly of highly plastic ascidian mitochondrial genomes. *Genome Biology and Evolution* **5:** 1185–1199.

Sabbadin A. 1982. Formal genetics of ascidians. *American Zoologist* **22:** 765–773.

Saito Y, **Mukai H**, **Watanabe H. 1981.** Studies on Japanese compound styelid ascidians. II. A new species of the genus *Botrylloides* and redescription of *B. violaceus* Oka. *Publications of the Seto Marine Biology Laboratory* **26:** 357–368.

Saito Y, **Watanabe H. 1985.** Studies on Japanese compound styelid ascidians IV. Three new species of the genus *Botrylloides* from the vicinity of Shimoda. *Publications of the Seto Marine Biology Laboratory* **30:** 227–240.

Shenkar N, **Gittenberger A**, **Rius M**, **Rocha RM**, **Swalla BJ**, **Turon X. 2019.** Ascidiacea World Database. Available at: http://www.marinespecies.org/ascidiacea. Accessed 29 October 2019.

Shenkar N, **Koplovitz G**, **Dray L**, **Gissi C**, **Huchon D. 2016.** Back to solitude: solving the phylogenetic position of the Diazonidae using molecular and developmental characters. *Molecular Phylogenetics and Evolution* **100:** 51–56.

Stach T, **Turbeville JM. 2002.** Phylogeny of Tunicata inferred from molecular and morphological characters. *Molecular Phylogenetic and Evolution* **25:** 408–428.

Stover BC, **Muller KF. 2010.** TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics* **11**: 7.

Swofford DL 2003. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland: Sinauer Associates.

Viard F, **Roby C**, **Turon X**, **Bouchemousse S**, **Bishop J. 2019.** Cryptic diversity and database errors challenge non-indigenous species surveys: an illustration with *Botrylloides* spp. in the English Channel and Mediterranean Sea. *Frontiers in Marine Science* **6:** 615.

Voskoboynik A, **Neff NF**, **Sahoo D**, **Newman AM**, **Pushkarev D**, **Koh W**, **Passarelli B**, **Fan HC**,

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020

Mantalas GL, **Palmeri KJ**, **Ishizuka KJ**, **Gissi C**, **Griggio F**, **Ben-Shlomo R**, **Corey DM**, **Penland L**, **White RA**, **Weissman IL**, **Quake SR. 2013.** The genome sequence of the colonial chordate, *Botryllus schlosseri*. *eLife* **2:** e00569.

Voskoboynik A, **Weissman IL. 2015.** *Botryllus schlosseri*, an emerging model for the study of aging, stem cells, and mechanisms of regeneration. *Invertebrate Reproduction & Development* **59:** 33–38.

Yang Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Bioinformatics* **13:** 555–556.

Yund PO, **Collins C**, **Johnson SL. 2015.** Evidence of a native northwest Atlantic *COI* haplotype clade in the cryptogenic colonial ascidian *Botryllus schlosseri*. *Biological Bulletin* **228:** 201–216.

Zeng L, **Jacobs MW**, **Swalla BJ. 2006.** Coloniality has evolved once in stolidobranch ascidians. *Integrative and Comparative Biology* **46:** 255–268.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Table S1. List of the junior synonyms of *Botryllus schlosseri* with reference details and notes
Supplementary Table S2. *Cox1* sequences of clade E available in the NCBI nucleotide database at August 2019, with sampling locality, n.r. = not reported

Downloaded from https://academic.oup.com/zoolinnean/advance-article-abstract/doi/10.1093/zoolinnean/zlaa023/5813975 by guest on 01 June 2020