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First record of the ascidian *Ecteinascidia turbinata* from Cyprus and its comparative morphology and genetics with *Ecteinascidia thurstoni*

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Abstract

The genus *Ecteinascidia* (family Perophoridae) comprises colonial ascidians typically found in warm seas, known for their biomedical significance. Four species have been recorded to date in the Mediterranean, but the records remain patchy due to the similar external morphology of these species and limited taxonomic data. This study presents the first record of the ascidian *Ecteinascidia turbinata* from Cyprus, extending its Mediterranean range, where it is currently regarded as cryptogenic. Here, we combine morphological and genetic data to compare *E. turbinata* from Cyprus with congeneric *Ecteinascidia thurstoni* specimens from Israel, an Indo-Pacific species introduced into the Mediterranean through the Suez Canal, highlighting the key diagnostic features and genetic divergence between the two species. Although the two species resemble each other in external appearance, *E. turbinata* and *E. thurstoni* differ in key diagnostic traits, including gut loop shape (open C vs. compact C), zooid length (15-22 mm vs. 4-7 mm), and the number of stigmata rows (17-20 vs. 12-14), respectively. Genetic data from Cypriot specimens indicate close affinity with both neighboring Mediterranean and Atlantic populations, suggesting these areas as possible sources of introduction. Based on the literature, the two species contend with similar environmental conditions, suggesting a potential niche overlap. Future monitoring of Mediterranean ascidian fauna may help to clarify this, especially in the context of global change and its influence on marine biodiversity and species distribution. Our findings also resolve past misidentifications and underscore the value of integrative taxonomy for reliable tracking of ascidian bioinvasions.

Keywords: Tunicata; bioinvasions; fouling; Levant basin; Mediterranean Sea.

Introduction

Ascidians (also known as sea squirts) are the most species-rich group within the subphylum Tunicata, comprising around 3,000 described species (Shenkar & Swalla, 2011). They are sessile filter feeders with short-lived, non-feeding motile larvae, gaining increased attention due to their invasive potential and ability to flourish in nutrient-enriched marine environments (Lambert, 2007; Zhan *et al.*, 2015). Introduced into new regions mainly as fouling organisms on marine vessels and via shellfish transport (Davidson *et al.,* 2009; Gewing & Shenkar, 2017), they threaten ecosystems by colonizing new habitats and causing physical (biofouling), economic (structural damage), and ecological (altering benthic diversity) impacts (Epelbaum *et al.*, 2009; Aldred & Clare, 2014). Ascidians thrive on submerged surfaces like concrete and plastics, marina floats, pilings, and boat hulls, especially in polluted areas such as ports and industrial zones (Carballo & Naranjo, 2002; López-Legentil *et al.*, 2015). These environments provide high concentrations of organic matter and bacteria as food resources, while offering regularly cleaned or newly submerged surfaces with a low abundance of native fouling species, thus creating ideal settlement sites for ascidian colonization (Lambert, 2007; Airoldi *et al.*, 2015).

The Mediterranean Sea, which harbors at least 229 species of ascidians (Coll *et al.*, 2010), sustains a rich diversity of marine life driven by its unique climate and geography (Izquierdo-Muñoz *et al.*, 2009; Moreno *et al.*, 2014). The biodiversity of the region has been significantly reshaped by anthropogenically introduced spe-

cies, principally those mediated by maritime traffic and the Suez Canal (Katsanevakis *et al.*, 2013), which have enabled the entry of numerous taxa, including ascidians (Rilov & Galil, 2009). Determining the origin of introduced species remains a challenge, particularly in regard to non-indigenous and cryptogenic ascidians, whose ambiguous biogeographic histories complicate efforts to understand their ecological impact and role in the Mediterranean ecosystem (Carlton, 1996; Zenetos *et al.*, 2017). This challenge is even more pronounced in Cyprus, where ascidian diversity has been poorly documented: existing studies focus almost entirely on recent non-indigenous or cryptogenic species (e.g., Rius & Shenkar 2012; Gewing *et al.*, 2016; Gerovasileiou *et al.*, 2017) and no comprehensive faunal checklist yet exists, leaving the group largely underexplored.

The genus *Ecteinascidia* Herdman, 1880 belongs to the family Perophoridae, which includes colonial ascidians found exclusively in warm seas. These species, typically, though not always, form clusters of erect zooids (Goodbody & Cole, 2006). Some species of *Ecteinascidia* have gained particular attention due to their biomedical properties such as their anti-cancer capabilities (Wright *et al.*, 1990; Guan *et al.*, 1993; Kerr & Miranda, 1995). *E. turbinata* Herdman, 1880 for example, one of the few cultured tunicates in the world, has been extensively farmed in Spain and the United States to produce sufficient material for clinical trials (López-Legentil & Turon, 2007; Marques *et al.*, 2022).

To date, 29 species of *Ecteinascidia* are known (Shenkar *et al.,* 2025) with four species recorded in the Mediterranean region: *E. herdmani* (Lahille, 1890), *E. styeloides* (Traustedt, 1882), *E. thurstoni* Herdman, 1890, and *E. turbinata* Herdman, 1880 (Moreno *et al.*, 2014; Shenkar *et al.*, 2025). Despite increasing scientific interest in the genus *Ecteinascidia*, knowledge of its current distribution in many regions in the Mediterranean Sea remains limited, due to insufficient morphological and molecular taxonomic knowledge. Notably, of the four *Ecteinascidia* species recorded in the Mediterranean, mitochondrial Cytochrome c oxidase subunit I (COI) barcode sequences were produced only for *E. turbinata* in this region (López-Legentil & Turon, 2007; Salonna *et al.*, 2021).

Ecteinascidia turbinata was originally described by Herdman (1880) from Bermuda and is considered native to the tropical Western Atlantic (Caribbean) region (Goodbody & Cole, 2006; Rocha *et al.*, 2010). Its presence in other regions has been historically questioned (Van Name, 1945), but it is now confirmed that the species occurs in West Africa (Senegal) (Pérès, 1954), the Cape Verde Islands (Collin *et al.*, 2005), the Suez Canal (Harant, 1927), and the Red Sea (Elbaz, 2009). In the Mediterranean Sea, the species has been reported from Egypt (Harant, 1939), Italy (Pérès, 1954), Tunisia (Pérès, 1954), Greece (Monniot, 1983), Spain (Ramos *et al.*, 1991; Rodríguez, 1922), and Malta (Maciver *et al.*, 2017), at depths between 0 and 42 m (Table S1 and references therein), and is treated as a cryptogenic species (Maciver *et al.*, 2017).

Ecteinascidia thurstoni is considered a non-indigenous species in the Mediterranean Sea (Galanidi *et al.,* 2023), previously recorded from Port Said, Egypt (Halim & Messeih, 2016), Israel (Shenkar & Loya, 2009), and Tunisia (Amor *et al.*, 2016; Mosbahi *et al*., 2021). It inhabits artificial and natural substrates at shallow depths and is suggested to have been introduced via the Suez Canal (Shenkar & Loya, 2009), as it occurs in the Red Sea and along the canal (Gab-Alla, 2008).

This study presents the first record of *E. turbinata* from Cyprus, offering new insights into the distribution of the genus in the region. The study also includes comparative morphological and genetic analyses with *E. thurstoni* specimens from Israel, highlighting the importance of integrative taxonomy in identifying these species.

Methodology

Sample collection and photographic evidence gathering

During field samplings conducted at Limassol Marina, Cyprus (34.66822°N, 33.040423°E) (Fig. 1), between April and October 2024, several colonies of *Ecteinascidia turbinata* were recorded on artificial substrates, including submerged pier pilings, ropes, and boat hulls at depths of 0.3-4.0 m (Fig. 2). In July 2024, multiple zooids from a single colony (CY–AS180724) were collected for morphological examination. Zooids were narcotised with menthol crystals, relaxed, and preserved in a 4 % formaldehyde-seawater solution buffered with sodium borate, following the G. Lambert protocol for ascidian fixation (pers. comm. with N. Shenkar). The fixative per liter comprised 850 mL seawater, 50 mL distilled water, 100 mL 37 % formaldehyde, and 1 g sodium borate. Additional samples from two colonies were collected on 8 October 2024 (CY–AS081024_1, CY–AS081024_2), preserved in 96% ethanol and stored at -20°C for subsequent genetic analyses. To assess the occurrence of *E. turbinata* in other locations around Cyprus, photographic records and specimen data from previous surveys, as well as images from the authors' personal archives, were reviewed. Additional sampling information for the specimens collected in Cyprus and temperature data obtained at Limassol Marina using a Conductivity-Temperature-Depth (CTD) profiler and a HOBO Pendant® data logger deployed between April and October 2024, are provided in Tables S2 and S3, respectively.

Morphological analyses

Samples of *E. turbinata* were observed using a Nikon SMZ18 stereomicroscope and a Nikon Eclipse NI-U light microscope. Samples were compared to specimens of *E. thurstoni* available at the Steinhardt Museum of Natural History, Tel-Aviv University, Israel: SMNHTAU-AS25263, SMNHTAU-AS25265, and SMNHTAU-AS25266 from Israel and from the Suez Canal (materials examined in Shenkar & Loya, 2009, Fig. 2; Table 1). The identification was performed using the original descriptions by Herdman (1880), Van Name (1945), Kott (1985), and Goodbody & Cole (2006).

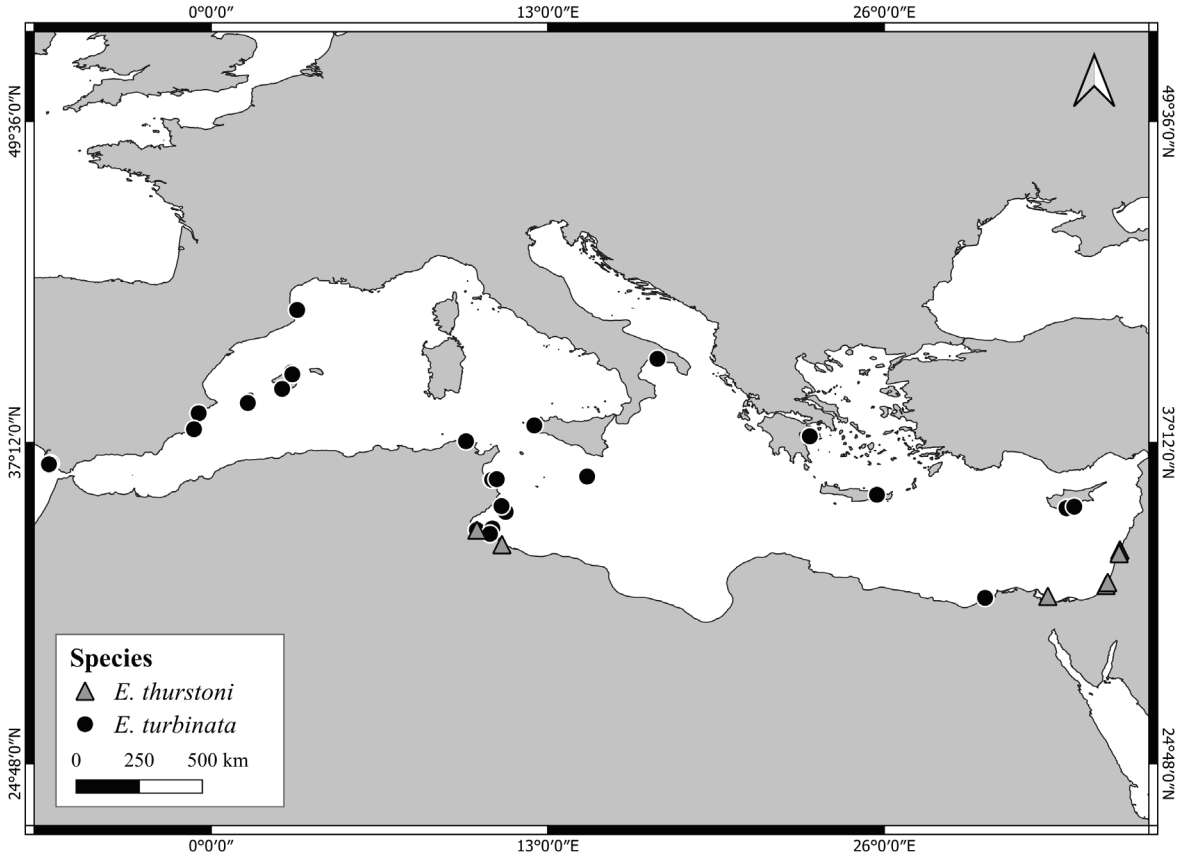

Fig. 1: Mediterranean distribution of *Ecteinascidia turbinata* and *E. thrustoni*. References are provided in Table S1.

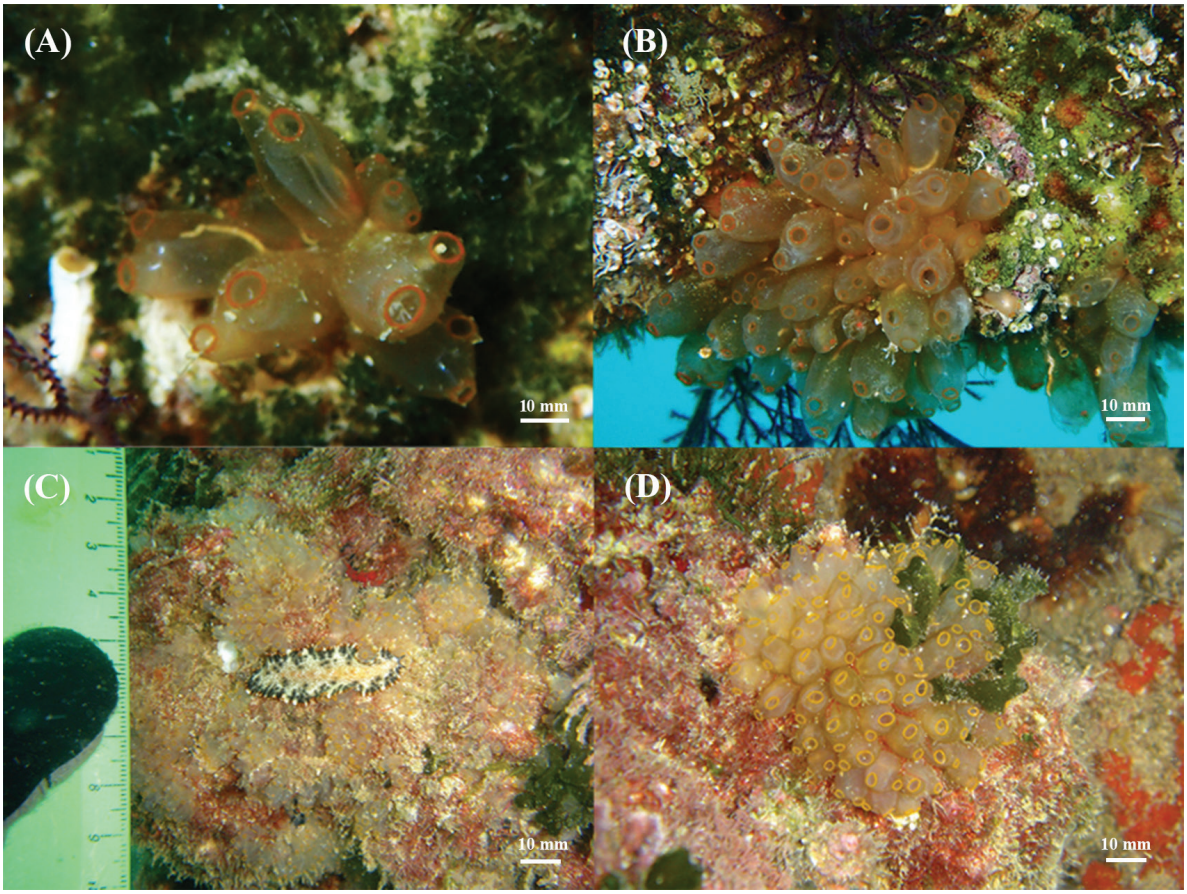

Fig. 2: (A) and (B) *Ecteinascidia turbinata* from Limassol Marina, Cyprus, photographed by C. Michail, 18/06/2024, (C) and (D) *Ecteinascidia thurstoni* preyed on by the flatworm *Maritigrella fuscopunctata* (identified by Dr. Liron Goren) in the Achziv Marine protected area, Israel, 4 m depth; photographed by N. Shenkar 28/7/2005.

Table 1. Israeli (Mediterranean Sea) and Gulf of Suez *Ecteinascidia thurstoni* samples compared in this study with colonies of *Ecteinascidia turbinata* from Cyprus. Source: Shenkar & Loya (2009). Sample AS8362 collected by Prof. Lev Fishelson and identified by Dr. Francoise Monniot as *E. thurstoni*. All samples are stored at the Steinhardt Museum of Natural History, Tel-Aviv University, Israel.

Sample #	Date	Site name	Coordinates	Depth	Substrate
AS25263	15/06/2004	Achziv National Park, 'Andarta', Israel	33.056861°N, 35.102556°E	1 m	Hard (under rocks)
AS25265	28/07/2005	'Muller' beach, Nahariya, Israel	33.041917°N, 35.097333°E	4 m	Hard (rocky reef)
AS25266, AS25316	26/06/2008	'Horses' beach, Akko, Israel	32.921389°N, 35.073167°E	0-2 m	Artificial substrate
AS8362	25/10/1971	Ras Sidr Gulf of Suez, Egypt	29.586611°N, 32.704000°E		

DNA extraction and sequencing

Total genomic DNA was extracted from two specimens (isolates S1 and S2) corresponding to colonies CY–AS081024_1 and CY–AS081024_2, respectively, following the spin column protocol for the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The mitochondrial COI gene was targeted with PCR procedures using the universal primers LCO1490 and HCO2198 (Folmer *et al.,* 1994). PCR reactions were carried out in a Veriti thermal cycler (Applied Biosystems, USA) following Dimitriou & Sfenthourakis' (2022) thermocycling profile for the respective gene. Amplicons were purified using the Qiaquick Purification Kit (Qiagen, Germany) following the manufacturer's protocol. The final products were sent for sequencing of both DNA strands at the Macrogen facilities (Amsterdam, The Netherlands). Sequencing results were sent as chromatograms, which were inspected and edited where necessary before further data elaboration. The final sequence lengths for the Cypriot samples deposited under accession numbers PV819813 and PV819814 were 610 bp and 572 bp, respectively.

For molecular comparison, an ethanol-preserved specimen of *E. thurstoni* (SMNHTAU-AS25316), collected from the Horses Beach, Akko, Israel, was used (Table 1). Genomic DNA was extracted using the lysis buffer described by Fulton *et al.* (1995), followed by Phenol:Chloroform:Isoamyl alcohol extraction (25:24:1) and ethanol precipitation (Green & Sambrook, 2018). Attempts to amplify the COI region using standard primer combinations (i.e., the Folmer *et al.* (1994) COI primers and those designed for tunicates by Salonna *et al.*, 2021) were unsuccessful. Therefore, the complete mitochondrial genome was sequenced following the approach described by Rubinstein *et al.* (2013). The extracted DNA was combined with genomic DNA from several distantly related tunicate species, and the pooled sample was submitted for library preparation and sequencing at the Technion Genome Center (Haifa, Israel). The sequencing was performed on an Illumina HiSeq 2000 platform, generating 100 bp paired end reads.

Adapter trimming was performed using Trimmomatic v0.39 (Bolger *et al.*, 2014), and the retained reads were assembled with IDBA-UD v1.1.3 (Zhu *et al.*, 2010) using default parameters. To identify the mitochondrial genome contig of *E. thurstoni*, within the IDBA-UD assembly, which included both nuclear and mitochondrial contigs, a BLASTn search was conducted using a COI sequence of *E. thurstoni* from India (GenBank accession KX138509) as a query against the assembled contigs. A single contig showing >90% sequence identity to the Indian sequence was recovered. Reads were mapped to these contigs, and after few rounds of read mapping this mitochondrial contig, could be circularized to form a 13,894 bp sequence confirming that the complete mitochondrial genome was obtained for *E. thurstoni.* Coverage analysis in Geneious 2025.1.2 (Biomatters Ltd., Auckland, New Zealand) indicated a mean read depth exceeding 200X (Mean: 212.3±14.0, Minimum: 165, Maximum: 244), supporting the accuracy of the assembled COI sequence. A preliminary annotation of the mitochondrial sequence was done using MITOS2 (Donath *et al.*, 2019) followed by manual curation of protein-coding gene boundaries according to Rubinstein *et al.* (2013). Specifically, annotation was optimized to maximize similarity with published tunicate mitochondrial genomes while minimizing gene overlaps and intergenic regions. A detailed description of the mitochondrial genome will be presented elsewhere. For the present study, the complete COI sequence (1,527 bp) has been deposited in GenBank under accession number PV849434.

Phylogenetic reconstructions

To investigate the phylogenetic relationships of the Cypriot and Israeli sequences among other known ascidian sequences, a dataset was assembled by combining the newly-generated COI sequences with publicly available sequences from GenBank. Given that the genus *Ecteinascidia* has been shown to be non-monophyletic, with members of *Perophora* and *Ecteinascidia* placed within

a single clade (Montesanto *et al.*, 2024), all sequences belonging to these two genera available in the NCBI as of March 23, 2025, were downloaded.

Sequences containing premature stop codons, frameshifts, or excessive gaps were excluded from the dataset. Specifically, the following sequences were removed: AY116604, KU220956–KU220957, KU667276, KU667278, KU667280, and KX138509. Notably, KX138509 (*Ecteinascidia thurstoni* voucher DBTIC134), the only available *E. thurstoni* sequence in public databases, was excluded due to the presence of deletions inconsistent with the high conservation typically observed in COI sequences. The sequence MW278674 (*Ecteinascidia* sp. HAW01) was also excluded, as it clustered with the stolidobranch genus *Eusynstyela* rather than within Phlebobranchia. Six *Ascidiella* sequences were selected as outgroup taxa, based on the phylogenetic inference of Montesanto *et al.* (2024), which identified *Ascidiella* as a closely related to the *Perophora-Ecteinascidia* clade.

Multiple sequence alignment was performed using the MAFFT v1.5.0 plug-in (Katoh *et al.*, 2002) in Geneious Prime v2025.1.2 under the L-INS-i algorithm. The alignment was first trimmed to the length of the longest Cypriot sequence and then positions with more than 50% missing data were excluded. Specifically, the start and end of the Israeli *E. thurstoni* COI sequence were trimmed at this stage. The final alignment began at a first codon position and ended at a third, maintaining reading-frame integrity for codon-based analyses. A total of 71 sequences and 486 nucleotide positions were retained in the final alignment.

Phylogenetic reconstructions were conducted under both Maximum likelihood (ML) and Bayesian inference (BI) frameworks, using a codon-partitioned model. ML analysis was carried out with IQ-TREE v3.0.0 (Wong *et al.*, 2025) using the edge-linked proportional partition model, allowing separate substitution models and evolutionary rates for each codon position. The best-fitting substitution model was selected for each partition, using ModelFinder Plus (-m MFP) (Kalyaanamoorthy *et al.*, 2017), and branch support was assessed with 1,000 non-parametric bootstrap replicates (-b 1000). The best-fit model based on the Bayesian Information Criterion (BIC) was TN+F+I for codon position 1, F81+F+G4 for position 2, and HKY+F+G4 for position 3.

Bayesian phylogenetic inference was performed using MrBayes v3.2.7 (Ronquist *et al.*, 2012), employing a similar codon-partitioned approach. A General Time Reversible (GTR) model with six substitution types (nst = 6) was applied to each codon position. In agreement with the IQtree model selection, among-site rate variation was modeled using an invariant+gamma distribution for codon position 1, and a gamma distribution for positions 2 and 3. Two independent Markov Chain Monte Carlo (MCMC) runs were executed, each with four chains and 10,000,000 generations, sampling every 100 generations. A burn-in of 25% was applied. Convergence was assessed by confirming that the average standard deviation of split frequencies had dropped below 0.01 prior to the burn-in threshold and that all potential scale reduction factor (PSRF) values approached 1.0 at the end of the run.

Results

Employing a combination of classical taxonomic identification and genetic analyses, the specimens collected from Cyprus were assigned to *E. turbinata* and the Israeli specimen to *E. thurstoni*. Cypriot zooids were relatively large and rounded, ranging from 15 to 22 mm in length, with 17-20 rows of stigmata, connected by stolons arising from the postero-ventral region of the body (Fig. 3). The tunic of the zooid was thin and transparent, and the oral and atrial siphons lay close together, encircled by a conspicuous bright-orange ring. The intestinal tract formed a broad, slight C-shaped loop with almost no secondary bend, in which the ascending intestinal limb and the rectum diverged rather than running in parallel, and the rectum extended 9-10 stigmatal rows toward the atrial siphon (Fig. 3).

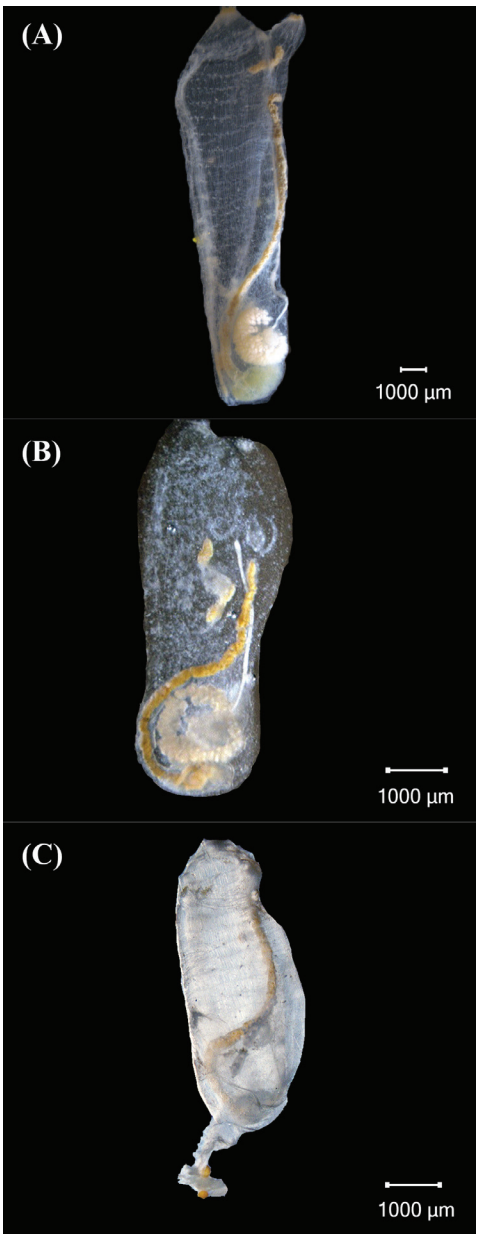

Fig. 3: Zooids of (A) *Ecteinascidia turbinata* from Cyprus (CY–AS180724) and *Ecteinascidia thurstoni* from (B) Israel and (C) Suez Canal (#AS8362).

Retrospective screening of prior surveys and photographic archives recovered additional records of *E. turbinata* from Zygi Marina (34.727317°N, 33.339599°E) in October 2019, where colonies were observed attached to submerged ropes. More recently, in November 2024, new photographic evidence and colonies (CY–AS071124) were recorded and collected from the same site. Additionally, larvae were observed in *E. turbinata* specimens collected from Limassol Marina in July 2024, and from Zygi Marina in early November 2024 available on FigShare. During subsequent visits to Zygi Marina in January and February 2025 the species was not observed, despite its documented occurrence in the preceding November. Environmental measurements indicated that salinity in Limassol Marina ranged from 39.1 to 39.6 PSU (Table S2) and temperature from 20.8 to 26.5°C between April and October 2024, while data-logger records showed peaks up to 33°C in early October 2024 (Table S3; Fig. S1).

Morphological examinations of the Israeli specimens showed that, in contrast to *E. turbinata*, *E. thurstoni* has small, rounded zooids. Similarly to the congeneric, zooids were connected by stolons arising from the postero-ventral region of the body, featuring a thin and transparent tunic with distinct yellow-orange ring around the aperture rims, similar to *E. turbinata* (Fig. 3). Zooid length ranged between 4-7 mm and each exhibited approximately 12-14 rows of stigmata. The gut loop formed a narrow first curve, followed by a second right-angled curvature, more pronounced than in *E. turbinata*. Distal to this bend, the ascending tract and the rectum run approximately parallel to each other and to the posterior body wall. In *E. thurstoni*, the rectum is shorter, extending upright toward the atrial siphon, spanning 6-8 rows of stigmata (Fig. 3). The gut-loop shape, together with the smaller zooid size

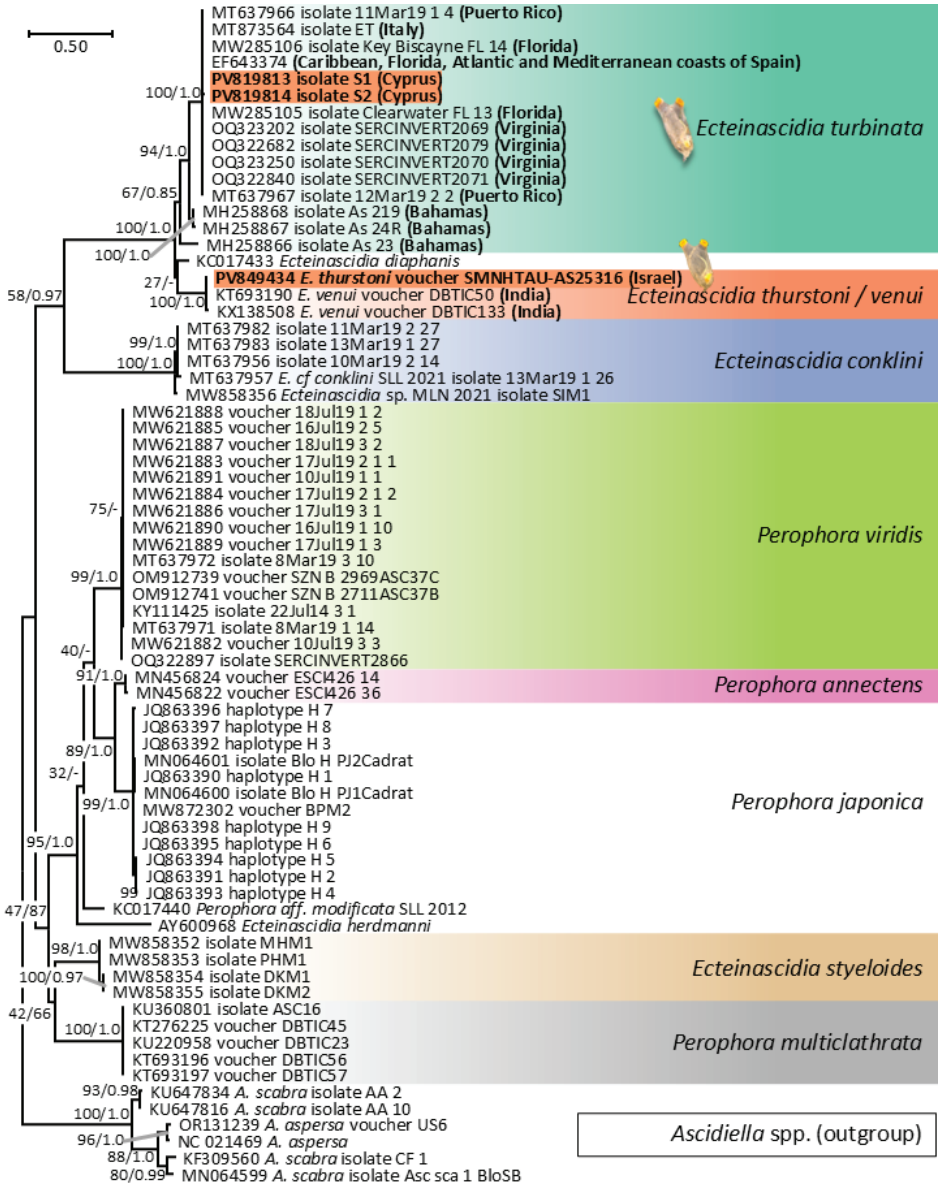

Fig. 4: Maximum likelihood (ML) phylogeny of the genera *Ecteinascidia* and *Perophora* based on COI sequences available in public databases, inferred using a codon-partitioned model. Newly-generated *Ecteinascidia* sequences from Cyprus and the Mediterranean coast of Israel are highlighted in bold against an orange background. Branch supports (i.e., ML bootstrap percentages [BP] / posterior probabilities [PP]) are indicated near the corresponding nodes. A dash indicates that the corresponding node was not recovered in the Bayesian consensus tree PP < 0.5.

and lower stigmatal count, are key diagnostic characteristics separating *E. thurstoni* from *E. turbinata*. Additional photographs of the examined specimens are available on FigShare.

ML and BI phylogenetic reconstructions were congruent, differing only in the placement of poorly supported branches (Fig. 4). Species-level monophyly was recovered for most taxa included in the analysis, with sequences assigned to specific species considered in this analysis forming well-supported, distinct clades (ML bootstrap percentage BP > 90, BI posterior probabilities PP = 1.0). The Cypriot sequences clustered among *Ecteinascidia turbinata* sequences and were found to be identical to sequences from the Mediterranean Sea (e.g., EF643374 and MT873564), the eastern and western Atlantic coast (e.g., OQ323202, and MW285106), the Gulf of Mexico (e.g., MW285105), and the Caribbean (e.g., MT637966) (López-Legentil & Turon, 2007; Nydam *et al.*, 2022; Salonna *et al.*, 2021; Streit *et al.,* 2021), supporting their morphological identification. The *E. turbinata* clade also included few divergent sequences from the Bahamas (i.e., MH258866-MH258868) which formed two distinct subclades separate from the one including the Cypriot sequences. Overall, the monophyly of *E. turbinata* was only weakly supported (BP = 67, PP = 0.85). Two other exceptions without high support for species monophyly were observed: *Ascidiella scabra*, which appeared polyphyletic; and the Israeli *E. thurstoni* sequence, which clustered with two *E. venui* sequences (BP=100; PP=1) from India (Akram *et al.*, 2018). Specifically, the Israeli *E. thurstoni* sequence was identical to KT693190, differing by only five base pairs from KX138508 (after trimming the final 20 bp of the latter, which showed poor alignment with other COI sequences).

In line with the morphological similarity observed between Israeli *E. thurstoni* and Cypriot *E. turbinata*, these two taxa clustered together in a maximally supported clade (BP = 100; PP = 1.0) that also included *E. diaphanis*, another species characterized by red bands around the siphon base and apertures (Kott, 1985). At the genus level, phylogenetic reconstruction was consistent with previous findings (Montesanto *et al.*, 2024), indicating that the genera *Ecteinascidia* and *Perophora* were not recovered as monophyletic, and that relationships among species of both genera are not yet resolved.

Discussion

In the Eastern Mediterranean, and particularly around Cyprus, limited sampling and taxonomic research on ascidians has resulted in significant knowledge gaps regarding their current diversity and distribution. Providing morphological and molecular tools for accurate identification of ascidian fauna in the region is essential to facilitate the detection of non-indigenous species. Where present, both *E. turbinata* and *E. thurstoni* can be common fouling organisms that colonize artificial substrates and tolerate a wide range of environmental conditions, particularly salinity (Goodbody & Cole, 2006). *E. turbinata* is found within a physiological salinity range of 22-40 PSU and at seawater temperatures reaching up to 32ºC (Carballo, 2000; Vázquez & Young, 2000). *E. thurstoni* occurs under hypersaline conditions up to 46 PSU and temperatures of ~27-29°C in the Suez Canal and adjacent Red Sea sites (Gab-Alla, 2008). In this study, *E. turbinata* in Cyprus was recorded at temperatures up to 33°C and salinity up to 39.6 PSU (October 2024; Table S2; Table S3; Fig. S1). Reproductive activity was confirmed in summer and autumn, with larvae observed in July and November 2024, but no colonies were observed at the study site in January and February 2025. Although environmental data are unavailable for those winter months, the species' disappearance suggests that seawater temperatures likely fell below its physiological tolerance. This seasonal pattern aligns with previous findings in the literature from other Mediterranean locations (Maciver *et al.*, 2017) and parallels observations for *E. thurstoni* in the Suez Canal, where the species shows temperature-linked seasonality with winter regression to stolons (Gab-Alla, 2008), as well as along the Mediterranean coast of Israel, where colonies occur only in spring-autumn when seawater temperatures are >~22 °C (Shenkar & Loya, 2009), suggesting that low temperatures may act as a key environmental constraint.

The two congeneric ascidians exhibit contrasting biogeographic histories and status in the Mediterranean. *E. thurstoni*, is a Lessepsian species (i.e., species of Red Sea/Indo-Pacific origin introduced into the Mediterranean through the Suez Canal) (López-Legentil & Turon, 2007; Shenkar & Loya, 2009). In contrast, the status of *E. turbinata* is ambiguous. The species is absent from several Mediterranean non-indigenous species checklists (e.g., Galanidi *et al.*, 2023; Zenetos *et al.*, 2010, 2017, 2022; Zenetos & Galanidi, 2020), likely reflecting its circumtropical distribution and long-standing records in the basin, first recorded in the early decades of the 20th century in the Balearic Islands, Spain (Rodríguez, 1922). Along the Mediterranean coasts of Tunisia, *E. turbinata* has been described as "probably native" (Chebbi *et al.*, 2010) and later as native (Amor *et al.*, 2016), being common in the Gulf of Gabès and exploited for pharmaceutical purposes. The European Alien Species Information Network (EASIN) currently treats *E. turbinata* as cryptogenic, a designation also retained by Maciver *et al.* (2017). *Sensu* Carlton (1996), cryptogenic taxa are those for which neither native nor introduced status can be demonstrated, often because any putative introduction was unobserved in historical times and the available evidence is insufficient to assign origin. Given this evidence, in the present study *E. turbinata* is best treated as cryptogenic.

Despite their contrasting status, both species occupy similar habitat types and tolerate comparable environmental conditions, making their future co-occurrence in the same locations possible. In Israel, *E. thurstoni* has already established widespread populations along the Mediterranean coast, occurring on both artificial substrates and natural rocky habitats. It has been recorded from the northern marine protected area of Achziv to southern sites such as Ashqelon and Ashdod (N. Shenkar pers.

obs.; Table S1). Similarly, *E. turbinata* has been reported on both artificial and natural substrates in various parts of the Mediterranean (Table S1; Fig. 1), although in Cyprus it has thus far been observed only in marinas. The geographic proximity of Cyprus to both the Israeli coastline and the Suez Canal, combined with heavy maritime traffic in the region, increases the likelihood of future introductions or the secondary spread of *E. thurstoni* to Cyprus and *E. turbinata* to the Israeli coast. Interestingly, the co-existence of the two species has already been documented in the Gulf of Gabès in Tunisia (Amor *et al.*, 2016).

Externally, *E. turbinata* and *E. thurstoni* initially appear similar. Both colonies form bouquets of translucent zooids connected by stolons and display a bright yellow-orange band surrounding the closely apposed siphons. As a result, rapid field surveys that rely on external morphology alone often fail to separate the two species. Discriminating diagnostic characteristics, such as the broader open gut loop and consistently higher stigmata row count in *E. turbinata* versus the more compact C-shaped loop and lower stigmata rows in *E. thurstoni*, are complicated and only evident under dissection or high-magnification imaging. Consequently, this resemblance has generated a long history of taxonomic confusion: Herdman's (1880) *E. moorei*, once considered as a valid species in several studies, is now considered as a junior synonym of *E. turbinata*; while the material that Sluiter (1905) called *E. moorei* was later considered *E. thurstoni* by Monniot & Monniot (1997) (see Maciver *et al.*, 2017 and references therein).

In the present study, in addition to the morphological evidence, our molecular analyses provide strong support for the identification of the Cypriot specimens as *E. turbinata*. The newly-generated sequences were found to be identical to those from individuals collected in the Mediterranean, the Gulf of Mexico, the Caribbean, and along the Atlantic coast of the United States, indicating minimal genetic divergence across a wide geographic range. This lack of variation may be explained by two non-mutually exclusive hypotheses: a recent colonization of Cyprus by *E. turbinata*, or a low rate of mitochondrial evolution in this species. The latter explanation was previously offered by López-Legentil & Turon (2007), who reported no COI sequence variation within or between populations across much of the species' distribution, suggesting a slowly evolving mitochondrial genome. The presence of the same haplotype in the Cypriot specimens as that identified by López-Legentil & Turon (2007) is consistent with both scenarios. Given increasing evidence that ascidians can rapidly expand their ranges (Montesanto *et al.*, 2024), a recent introduction or range expansion of *E. turbinata* into the Eastern Mediterranean cannot be eliminated, although additional molecular data and long-term monitoring will be necessary to confirm this scenario.

In contrast, the Israeli specimen clustered with two sequences currently deposited as *Ecteinascidia venui* from India (KT693190/voucher DBTIC50 and KX138508/voucher DBTIC133). This raises questions regarding the accuracy of taxonomic assignments in public databases and the validity of species boundaries between *E. venui* and *E. thurstoni*. The species *E. venui* was described by Meenakshi (2000) from the southeast coast of India and reported to differ from *E. thurstoni* in the presence of yellowish-orange pigment spots on the siphons and the absence of a complete layer of circular musculature interrupted only ventrally. However, these traits are problematic: pigmentation can disappear in preserved specimens (Kott, 1985; Meenakshi, 2000), and muscular arrangement may be difficult to assess due to muscle contraction and distortion during fixation. The weak diagnostic value of these features, combined with the absence of additional distinguishing characters, raises the possibility that *E. venui* may be a junior synonym of *E. thurstoni*. Upon closer examination of the COI sequence KX138509 (voucher DBTIC134), originally deposited as *E. thurstoni* from India and excluded from our phylogenetic reconstruction, we found that – after trimming a spurious region at the 3′ end – this sequence was identical to that of the Israeli *E. thurstoni* specimen. This finding provides additional evidence of the presence of *E. thurstoni* in India, and suggests that the two Indian sequences labeled as *E. venui* may in fact represent misidentified *E. thurstoni* or contaminations. The latter interpretation is further supported by known difficulties in amplifying COI from certain tunicates using standard primers, due to their high mitochondrial sequence divergence (Salonna *et al.*, 2021). In the present study, the amplification of *E. thurstoni* with universal Folmer primers, as well as with the tunicate-specific primers developed by Salonna *et al.* (2021), repeatedly failed and instead retrieved contaminant (amoebozoan) sequences (see methods). This technical challenge could potentially explain erroneous or contaminated sequence submissions from previous studies. Based on the available morphological and molecular data, the Israeli specimen is best identified as *E. thurstoni*, and the Indian sequences currently labelled as *E. venui* likely represent either the same taxon or contaminations. Further sampling and detailed morphological assessments of specimens from India are needed to clarify whether *E. venui* is a valid species or should be synonymized with *E. thurstoni*.

The present study highlights the ongoing challenges in ascidian systematics, where cryptic morphology, overlapping traits, rapid range expansions, and accelerated evolutionary rates complicate species-level identification (Shenkar & Swalla, 2011; Gulnick, 2024). Our findings underscore the importance of integrative taxonomic approaches that combine detailed morphological assessments with molecular data to disentangle species identities, trace invasion routes, and inform management in an increasingly ascidian-rich seascape.

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Data Availability Statement: All information regarding the collected data is included in this publication. Generated genetic data is publicly available through NCBI Genbank under PV819813, PV819814, PV849434 accession numbers. The samples examined have been deposited at the Marine and Environmental Research (MER) Lab (samples from Cyprus: CY–AS180724, CY–AS071124, CY–AS081024_1, CY–AS081024_2) and the Steinhardt Museum of Natural History and National Research Center (samples from Israel: SMNHTAU-AS25316, SMNHTAU-AS25263, SMNHTAU-AS25265, SMNHTAU-AS25266, samples from the Suez Canal: AS8362).
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Web links

European Alien Species Information Network (EASIN) https://easin.jrc.ec.europa.eu/easin
FigShare https://doi.org/10.6084/m9.figshare.29386667 Codes refer to: CY-Cyprus, IS-Israel, RS- Red Sea.

Supplementary Data

The following supplementary information is available online for the article:

Fig. S1. Seawater temperature (°C) in Limassol Marina, Cyprus, recorded by a HOBO data logger deployed at 1 m depth from April to October 2024. The solid red line shows the daily mean temperature (t_mean), and the shaded band shows the daily range (t_min–t_max) derived from sub-daily logger records. Vertical dashed lines mark the sampling dates of *Ecteinascidia turbinata* (18 July and 08 October 2024).

Table S1. Mediterranean records of *Ecteinascidia turbinata* and *Ecteinascidia thurstoni*, including year of record, substrate type, and depth (where available). Red denotes approximate coordinates.

Table S2. Sampling sites in Cyprus where *Ecteinascidia turbinata* colonies were observed, including substrate type, depth, environmental parameters (temperature, conductivity, salinity, oxygen levels, and pH), and the methods used for specimen collection and preservation, where applicable.

Table S3. Temperature data collected in Limassol Marina using a HOBO data logger between April and October 2024 deployed at 1 m depth.