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Species Boundaries in Gregarine Apicomplexan Parasites: A Case Study—Comparison of Morphometric and Molecular Variability in *Lecudina* cf. *tuzetae* (Eugregarinorida, Lecudinidae)

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ABSTRACT. Trophozoites of gregarine apicomplexans are large feeding cells with diverse morphologies that have played a prominent role in gregarine systematics. The range of variability in trophozoite shapes and sizes can be very high even within a single species depending on developmental stages and host environmental conditions; this makes the delimitation of different species of gregarines based on morphological criteria alone very difficult. Accordingly, comparisons of morphological variability and molecular variability in gregarines are necessary to provide a pragmatic framework for establishing species boundaries within this diverse and poorly understood group of parasites. We investigated the morphological and molecular variability present in the gregarine *Lecudina* cf. *tuzetae* from the intestines of *Nereis vexillosa* (Polychaeta) collected in two different locations in Canada. Three distinct morphotypes of trophozoites were identified and the small subunit (SSU) rDNA was sequenced either from multicell isolates of the same morphotype or from single cells. The aim of this investigation was to determine whether the different morphotypes and localities reflected phylogenetic relatedness as inferred from the SSU rDNA sequence data. Phylogenetic analyses of the SSU rDNA demonstrated that the new sequences did not cluster according to morphotype or locality and instead were intermingled within a strongly supported clade. A comparison of 1,657 bp from 45 new sequences demonstrated divergences between 0% and 3.9%. These data suggest that it is necessary to acquire both morphological and molecular data in order to effectively delimit the ''clouds'' of variation associated with each gregarine species and to unambiguously reidentify these species in the future.

Key Words. Alveolata, Apicomplexa, DNA barcode, gregarine, *Lecudina tuzetae*, molecular phylogeny, morphology, species, SSU rDNA.

GREGARINE apicomplexans are a diverse and poorly understood group of unicellular parasites that inhabit the intestines, coeloms, and reproductive vesicles of marine, freshwater, and terrestrial invertebrates. Of ~ 6,000 described apicomplexan species, ~ 1,600 are gregarines and many more are estimated to exist (Adl et al. 2007; Hausmann, Hülsmann, and Radek 2003; Levine 1988; Morrison 2009; Perkins et al. 2000). The description of new gregarine species has historically been based on the morphological species concept that emphasized features like trophozoite morphology, epimerite morphology (mainly septate eugregarines), timing of gamont association, gametocyst morphology, and the method of gametocyst dehiscence (Clopton 2009; Léger 1892; Smith and Cook 2008). Host association has also been used as a taxonomic criterion because many gregarines are inferred to be stenoxenous, being able to mature in only a single host genus or species (Levine 1979; Perkins et al. 2000). Nonetheless, some gregarine species can infect different host species, and one host species can be infected with several different gregarine species (e.g. Åbro 1974; Rueckert and Leander 2009). Moreover, the shape, size, and ultrastructure of trophozoites are often highly variable depending on different developmental stages of the parasite and host environmental conditions (e.g. a starved host vs. a fully nourished host). Therefore, although trophozoites are the most readily encountered, morphologically diverse, and conspicuous stage in the gregarine life cycle, it is very difficult to delimit the boundaries of different species on morphological criteria alone.

Another concept used for the delimitation of species is the (molecular) phylogenetic species concept, which defines a species as an irreducible group of organisms whose members are descended from a common ancestor and within this group possesses a combination of derived traits (Cracraft 1989). Diagnostic characters that can be used range from the molecular level to the behavioural level. Over the last decade, a growing dataset of small subunit (SSU) rDNA sequences has been used as additional evidence for species recognition and phylogenetic relationships among gregarines (Carreno, Martin, and Barta 1999; Clopton 2009; Leander 2007, 2008; Leander, Clopton, and Keeling 2003a; Leander, Harper, and Keeling 2003b; Leander et al. 2006; Rueckert and Leander 2008, 2009, 2010). This molecular marker has been explored extensively in many different groups of eukaryotes because (1) the gene is found in all eukaryotes, (2) many near identical copies of the gene are present in the nuclear genome and maintained through concerted evolution, (3) highly conserved regions allow for robust polymerase chain reaction (PCR) primer design, (4) the gene is relatively long (1,800 bp), and (5) the degree of sequence variation among different species provides phylogenetic signal at relatively deep levels. Although there are only 65 gregarine species with SSU rDNA sequences available in GenBank, these data clearly indicate that this marker is fast evolving in gregarines, which is typical for parasitic lineages in general (Moreira and López-García 2002). Presumably, the most divergent regions of this marker are useful for species-level discrimination (i.e. DNA barcoding approaches, Chantangsi and Leander 2010; Heger et al. 2011) and the most conserved regions of this marker are useful for inferences about deeper phylogenetic relationships. The SSU rDNA sequences were further investigated in our case study because it is currently unclear how the variability in this marker relates to the morphological species concept that has been used in gregarine systematics since their discovery in the 19th century. An improved understanding of how variability in SSU rDNA sequences relates to variability in gregarine morphology will provide needed guidance and justification for establishing and identifying different species of gregarines in the future.

The eugregarine *Lecudia tuzetae* was originally described from *Hediste* (*Nereis*) *diversicolor* in Wimereux, France (Schrével 1963, 1969). These studies documented a variety of different morphotypes in the trophozoites and offered two explanations for the polymorphisms: (1) the variation reflects different development stages in trophozoite growth; and (2) the variation reflects deformations associated with either cellular motility or different states of health of the parasite. Another possibility is that different

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morphotypes represent different (and perhaps cryptic) species. The multitude of possible reasons for morphological variation in gregarines makes the traditional approach of justifying new species on the basis of detailed morphometric analysis alone problematic. However, this traditional approach is testable with molecular data, which have the potential to more unambiguously discriminate different species at the level of DNA sequences.

In order to evaluate how morphological variability relates to SSU rDNA variability, we performed a case study involving trophozoites reminiscent of *L. tuzetae* that were isolated from two very closely related northeastern Pacific polychaete hosts, *Nereis neoneanthes* and *Nereis vexillosa*, from two different localities in British Columbia (Bamfield and Vancouver). The fact that the Pacific gregarines were recognized as being similar to *L. tuzetae* reflects ambiguities in the morphological species concept and a de-emphasis on geographical location (i.e. France vs. British Columbia) and the precise host species (*H.* [*N.*] *diversicolor* vs. *N. neoneanthes*/*N. vexillosa*). As there are no SSU rDNA data available for the type material, we decided to use *L.* cf. *tuzetae* for our specimens from Pacific polychaetes. As in previous studies, we consistently found several different morphotypes of the trophozoites in the intestines of the Pacific hosts. We generated SSU rDNA sequences from three distinct morphotypes isolated from the two different geographical locations: morphotype 1, Vancouver; morphotype 1, Bamfield; morphotype 2, Vancouver; morphotype 2, Bamfield; morphotype 3, Bamfield. Using molecular phylogenetic analyses, we evaluated whether the SSU rDNA sequences from these six isolates clustered according to morphotype, geographical location, or both variables. The design of this particular case study not only provided the potential to discover cryptic species in the *L.* cf. *tuzetae* morphotypes but also provided the context needed to justifiably delimit the range (or ''clouds'') of morphological and molecular variation associated with gregarine species in general.

MATERIALS AND METHODS

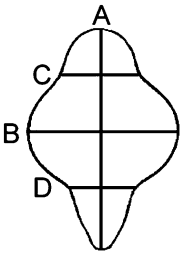
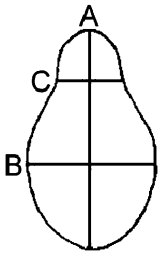
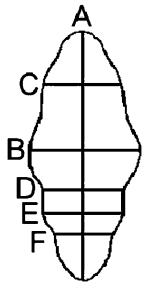
Study design and collection of organisms. Three different morphotypes of *L.* cf. *tuzetae* Schrevél, 1963 were collected from two polychaete hosts, namely *N. vexillosa* Grube, 1851 and *N. neoneanthes* Hartman, 1848 in 2007 and 2009 (Table 1 and Fig. 1–9). Morphotypes 1, 2, and 3 were isolated from hosts collected from Grappler Inlet, Bamfield, Vancouver Island, Canada (48°50′17″N, 125°08′02″W). Morphotypes 1 and 2 were also isolated from hosts collected in English Bay Beach, Downtown Vancouver (49°17′18″N, 123°08′37″W). Trophozoites were isolated in seawater by teasing apart the intestines of the respective host under a Leica MZ6 dissecting microscope (Wetzlar, Germany). The gut material was examined under a Zeiss Axiovert 200 inverted microscope (Göttingen, Germany) and parasites were removed by micromanipulation and washed 3 times in seawater in preparation for digital microphotography and DNA extraction.

Microscopy. Differential interference contrast light micrographs were produced by securing parasites under a cover slip with vaseline and viewing them with either a Zeiss Axioplan 2 microscope connected to a Leica DC500 colour digital camera and/or a Zeiss Axiovert 200 inverted microscope connected to a PixeLink Megapixel colour digital camera.

Individual trophozoites of *L.* cf. *tuzetae* morphotype 2 ($n = 55$) were prepared for scanning electron microscopy (SEM) using the OsO_4 vapour protocol. Isolated cells were deposited directly into the threaded hole of a Swinnex filter holder, containing a 5-μm polycarbonate membrane filter (Millipore Corp., Billerica, MA) that was submerged in 10 ml of seawater within a small canister (i.e. 2 cm diam., and 3.5 cm tall). A piece of Whatman No. 1 filter paper was mounted on the inside base of a 20-ml beaker that was slightly larger than the canister. The Whatman filter paper was saturated with 4% (w/v) OsO_4 and the beaker was turned over the canister. The parasites were fixed by OsO_4 vapours for 30 min. Ten drops of 4% (w/v) OsO_4 were added directly to the seawater and the parasites were fixed for an additional 30 min on ice. A 10-ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with CO_2. Filters were mounted on stubs, sputter coated with 5-nm gold particles, and viewed under a Hitachi S4700 SEM (Pleasanton, CA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

DNA isolation, PCR, cloning, and sequencing. Trophozoites representing the three morphotypes were separated from each other, washed 3 times in filtered seawater, and deposited into 1.5-ml microfuge tubes. Some of the isolates representing a

Table 1. Cell-shape and measurements of length, widths, and nucleus diameter in micrometres of morphotypes 1–3 from *Lecudina* cf. *tuzetae* isolated from *Nereis vexillosa* in Vancouver and Bamfield.

	Length (μm)	Width (μm)					Diameter (μm)
	A	B	C	D	E	F	Nucleus
Morphotype 1	105	48	30	30	_	_	16
	90	54	30	30	_	_	15
	109	45	32	26	_	_	14
	105	55	33	30	_	_	15
	79	38	26	30	_	_	14
	80	50	25	25	_	_	13
	146	98	50	40	_	_	_
Morphotype 2							
	65	40	24	_	_	_	15
	47	29	14	_	_	_	11
	60	43	18	_	_	_	13
	72	50	25	_	_	_	16
	68	45	24	_	_	_	14
	52	35	18	_	_	_	15
Morphotype 3	108	50	35	35	38	28	19
	182	87	71	56	58	33	23
	112	50	38	34	36	24	18
	102	46	37	25	28	16	18
	91	50	33	39	40	23	16

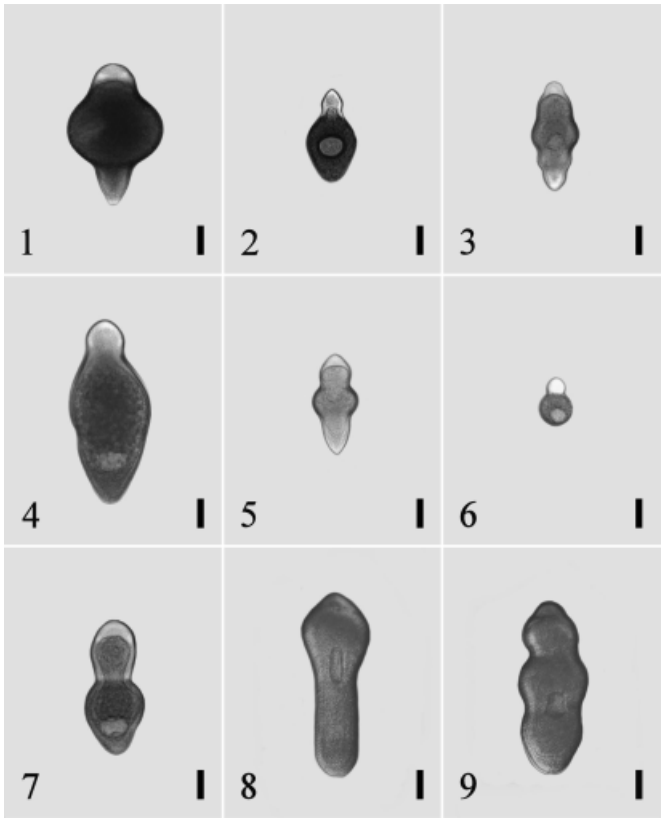


Fig. **1–9.** Differential interference contrast light micrographs showing trophozoites of the different morphotypes of *Lecudina* cf. *tuzetae* from *Nereis vexillosa*. **1.** Morphotype 1 showing a prominent widened part in the middle of the cell. This cell was used for DNA isolation and sequences were generated for two clones 1.2VANa and 1.2VANb. **2.** Morphotype 2 with the typical pear-like cell shape of *L. tuzetae*. **3.** Morphotype 3 trophozoite with cascading anterior and posterior ends starting at the widest part in the middle of the cell. **4.** A single cell of which sequences were generated for five clones 4BAMa–4BAMe. **5.** Another example for a morphotype 1 trophozoite. **6.** A smaller trophozoite of morphotype 2. **7–9.** Examples of different morphotypes that were isolated from *N. vexillosa* during this study. Cell shapes of those morphotypes varied greatly. Some cells had a constriction in the middle of the cell and a broader posterior half (7); some cells were elongated with their widest part located in the anterior half (8); some cells had two constrictions forming anterior, middle, and posterior bulges (9). The nucleus in these cells was located in the middle of the cell or slightly shifted to the posterior end (scale bars = 10 μm).

morphotype were composed of a single trophozoite that was used for single-cell PCR; other isolates consisted of 50 or fewer trophozoites of the same morphotype. DNA was extracted from each single-cell or multicell isolate using the total nucleic acid purification protocol as specified by EPICENTRE (Madison, WI). The SSU rDNA sequences were PCR amplified from each extraction using puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec City, QC, Canada) and the following primers: F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCT GCAGGTTCACCTAC-3′ (Leander et al. 2003a); F2 5′-GTCAT AYGCTTGTCTYGCAG-3′ and R2 5′-TGACTTTATCTGTTTC TGGG-3′.

Polymerase chain reaction products corresponding to the expected size were gel isolated and cloned into the PCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Ten cloned plasmids were digested with EcoR1 and screened for size. Up to nine clones from each PCR product were sequenced with ABI big dye reaction mix using vector primers and internal primers oriented in both directions. The SSU rDNA sequences were initially identified by BLAST analysis, verified with phylogenetic analysis, and then submitted to GenBank.

Sequence name format. The sequences were named in accordance with the following scheme: morphotype designation, isolate designation, location designation (Vancouver or Bamfield), and clone designation. For instance, ''1.1BAMa'' means morphotype ''1,'' isolate ''1,'' Bamfield (BAM), clone ''a.''

Molecular phylogenetic analysis. Thirty-nine new SSU rDNA sequences of *L.* cf. *tuzetae* were aligned with 38 other SSU rDNA sequences, representing marine and terrestrial gregarines with cryptospridians designated as the outgroup, using MacClade 4 (Maddison and Maddison 2000) and visual fine-tuning; gaps and ambiguously aligned bases were excluded from the 77-taxon alignment resulting in 1,169 unambiguously aligned sites. PhyML (Guindon and Gascuel 2003; Guindon et al. 2005) was used to analyse the dataset (one heuristic search) with maximum likelihood (ML) using a general-time reversible (GTR) model of base substitutions (Posada and Crandall 1998) that incorporated invariable sites and a discrete gamma distribution with eight rate categories (GTR+I+gamma model). The GTR model was selected using the program MrAIC 1.4.3 with PhyML (http://www.abc.se/ ~ nylander/mraic/mraic.html), and model parameters were estimated from the original dataset ($\alpha = 0.437$, proportion of invariable sites = 0.000). Maximum likelihood bootstrap analyses were conducted with the same settings described above (100 pseudoreplicates; one heuristic search per pseudoreplicate). Bayesian analysis of the 77-sequence dataset was performed using the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001). The program was set to operate with GTR, a gamma-distribution, and four Monte Carlo Markov chains (default temperature = 0.2). A total of 2,000,000 generations were calculated with trees sampled every 50 generations and with a prior burn-in of 100,000 generations with 2,000 sampled trees discarded (burn-in was checked manually). A majority rule consensus tree was constructed from 38,001 post burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post burn-in trees. Independent Bayesian runs on each alignment yielded the same results.

The 45 new SSU rDNA sequences were also analysed without outgroups and aligned with MacClade 4 (Maddison and Maddison 2000) and visual fine tuning. The ML method was performed under a GTR substitution model on the 45-taxon alignment containing 1,668 unambiguous sites. All gaps were excluded from the alignments before phylogenetic analysis. The α-shape parameter was estimated from the data using the GTR model for base substitutions (Posada and Crandall 1998) and a gamma-distribution with invariable sites ($-\ln L = 4{,}176.31592$, eight rate categories, $\alpha = 0.605$, fraction of invariable sites = 0.322).

RESULTS

All trophozoites had a brownish appearance under the light microscope (LM) suggesting an accumulation of amylopectin granules within the cytoplasm. There was a huge variety of cell shapes (Fig. 1–9), but three constantly reoccurring morphotypes were selected for further investigation (Table 1).

Morphotype 1 (Fig. 1, 5). These trophozoites were found in the intestines of *N. vexillosa* collected from Vancouver and Bamfield. They were 79–146 μm long and 38–98 μm wide at their widest part ($n = 7$), which was located in the centre of the cell. The anterior end of the cell, bearing the mucron, was rounded and free of amylopectin granules, while the posterior end of the cell

was more pointed. The nucleus was located in the middle of the cell (Fig. 5). Trophozoites of this morphotype were rigid and capable of gliding.

Morphotype 2 (Fig. 2, 6). The trophozoites of this morphotype represent the typical pear-like cell shape of *L. tuzetae*. They were isolated from the intestines of *N. vexillosa* from Vancouver and Bamfield as well as from *N. neoneanthes* from Bamfield. Trophozoites were 47–72 μm long and 29–50 μm wide at their widest part located in the posterior half of the cell ($n = 6$). Both ends were rounded, the mucron was free of amylopectin granules, and the nucleus was located in the posterior half of the cell. Fifty-five cells were examined with SEM, which consistently showed a cell cortex inscribed with longitudinal epicytic folds with a density of 3 folds/μm (Fig. 10–12). In some of the examined trophozoites one undulating fold was framed by one or two straight folds (Fig. 11); in other trophozoites, there were no undulating folds (Fig. 12). The trophozoites of this morphotype were rigid and capable of gliding.

Morphotype 3 (Fig. 3). Trophozoites of this morphotype were isolated from the intestines of *N. vexillosa* in Bamfield. They were usually 91–182 μm long and 46–87 μm wide at their widest part located in the middle of the cell ($n = 5$). The cell shape was reminiscent of cascades becoming wider in steps from the rounded anterior tip to the widest part in the middle of the cell and narrowing down in two steps to the more pointed posterior end. The nucleus was located in the middle of the cell or slightly shifted to the posterior end. Trophozoites of this morphotype were rigid and capable of gliding.

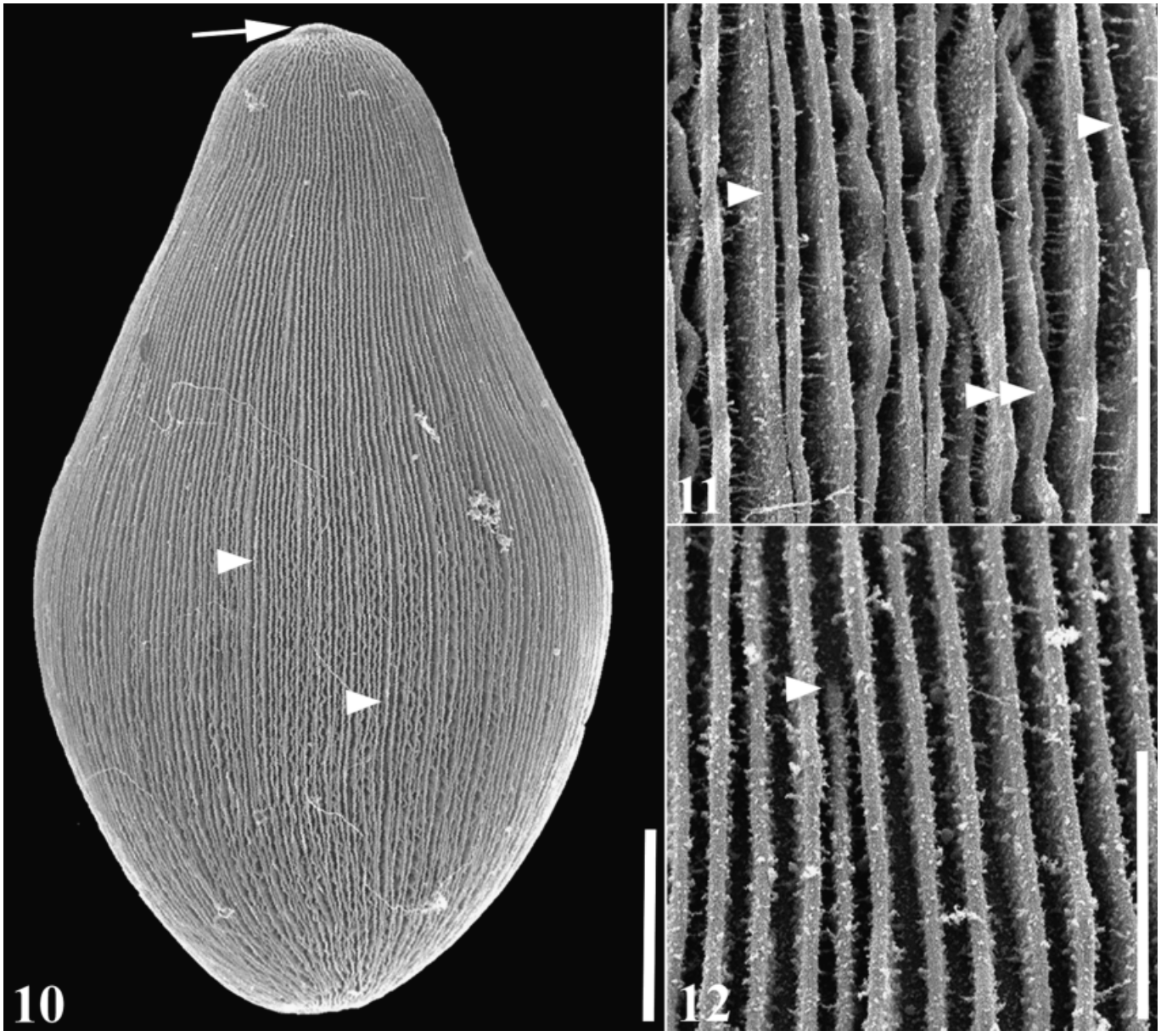


Fig. **10–12.** Scanning electron micrographs of *Lecudina* cf. *tuzetae* morphotype 2 showing the general morphology and surface ultrastructure. **10.** A trophozoite with a rounded mucron (arrow). Arrowheads indicate epicytic folds on the cell surface (scale bar = 12 μm). **11.** Higher magnification view of the cell surface showing epicytic folds. The displayed cell showed a mixture of undulating (double arrowhead) and non-undulating (arrowhead) folds. In most cases one undulating fold was framed by one or two straight folds (scale bar = 2.2 μm). **12.** Higher magnification view of the surface of another cell, again showing epicytic folds. This trophozoite showed a lack of undulating epicytic folds. The arrowhead indicates a terminating fold (scale bar = 3 μm).

Other morphotypes (Fig. 4, 7–9). Trophozoites with morphotypes that differed from the three described above were also isolated from the intestines of *N. vexillosa* from Bamfield and Vancouver. Five sequences were generated from the exact trophozoite morphotype 4 shown in Fig. 4. Other morphotypes encountered during this study differed by having a constriction in the middle of the cell and/or a broader posterior half containing the nucleus (Fig. 7). Some trophozoites were elongated being widest near the anterior half (Fig. 8). Other trophozoites had two constrictions forming anterior, middle, and posterior bulges (Fig. 9).

Molecular phylogeny. Phylogenetic analyses of the 77-taxon dataset resulted in a moderately supported clade of cryptosporidians (outgroup), a strongly supported terrestrial gregarine clade I and a poorly supported backbone for the marine gregarine clade (Fig. 13). The sequences from marine archigregarines *Selenidium*, *Platyproteum*, and *Filipodium* formed several different lineages that branched at the base of the marine gregarine clade. Marine eugregarines formed two sister clades that were moderately supported: (1) a strongly supported clade consisting of the urosporids *Pterospora* and *Lithocystis* and lecudinids *Difficilina*, *Lankesteria*, and *Lecudina*; and (2) a strongly supported clade consisting of two morphotypes of *Lecudina polymorpha* (Fig. 13). The sequences from the different *L.* cf. *tuzetae* morphotypes formed a strongly supported subclade within the strongly supported marine eugregarine clade (Fig. 13).

We also analysed separately a total of 45 new SSU rDNA sequences from the three different morphotypes of *L.* cf. *tuzetae* (Fig. 1–3), and five sequences were derived from one single cell representing a fourth morphotype, morphotype 4. There was no obvious pattern in the unrooted tree (Fig. 14). Sequences from the distinct morphotypes did not cluster together, and sequences from specimens collected at the same sampling site (i.e. Bamfield or Vancouver) did not cluster together. However, two clusters were recognized: Cluster 1 included sequences from all three morphotypes and both sampling sites and Cluster 2 included sequences from two morphotypes and clones from the single cell of morphotype 4 isolated from Bamfield (Fig. 14).

Moreover, a total of 1,657 base pairs were compared between all 45 sequences. A pair-wise distance calculation based on the Kimura 2-parameter model (Kimura 1980) of 1,657 nucleotides (excluding the indels) resulted in a 0.0–3.9% sequence divergence between all sequences, including different clones derived from single cells (Table 2, see Supporting Information Table S1). Variation between sequences from morphotype 1 was 0.0–3.0%; variation between sequences from morphotype 2 was 0.0–3.3% and variation between sequences from morphotype 3 was 1.4%. There was also 1.0–1.8% divergence between five clones from the single trophozoite representing morphotype 4 (i.e. 4BAMa–e, Fig. 4) and 0.9–1.6% divergence between four clones from a single trophozoite representing morphotype 2 (i.e. 2.2VANa–d). Two clones, 1.2VANa, b from a single trophozoite representing morphotype 1, were identical. There were also three identical sequences from morphotype 2, two sequences were from Vancouver (i.e. 2.1VANd, h) and one was from Bamfield (i.e. 2.1BAMb).

DISCUSSION

Many gregarines within the Lecudinidae (Levine 1976) look similar under the LM and have been separated on the basis of different host organisms, because most gregarines are thought to be host specific to some degree (Perkins et al. 2000). Ultrastructural and molecular phylogenetic data are especially helpful to confirm formerly described species based only on LM and to establish the boundaries of new species. The data presented here from *L.* cf. *tuzetae* demonstrate that a ''cloud'' of variation exists within the SSU rDNA sequences derived from different trophozoite morphotypes, and this range of molecular variation does not correspond to geographical locality, host associations, and specific morphotypes.

Lecudina cf. *tuzetae* was isolated from two different hosts and two localities. Morphotypes 1 and 2 were isolated from *N. vexillosa* from Vancouver and Bamfield; morphotype 2 was also isolated from *N. neoneanthes* from Bamfield; morphotype 3 was isolated from *N. vexillosa* in Bamfield. Morphotype 2 best matched the typical pear-like cell shape featured in the original species description of *L. tuzetae* from *H.* (*N.*) *diversicolor* collected in France (Schrével 1963). Therefore, *L. tuzetae* can be interpreted as a stenoxenous gregarine with a host specificity that is restricted to the host genus *Hediste*/*Nereis* rather than any particular species within it (Perkins et al. 2000).

Clopton (2004) published a standardized nomenclature and a metric set for 278 plane shapes in 23 shape series to be used in the morphological description of species within the Eugregarinorida. However, taking replicate measurements of multiple dimensions of multiple trophozoites is potentially uninformative due to trophozoite plasticity. For instance, the detailed measurements of cell shapes, geographies, and host associations we observed allowed for the possibility that different morphotypes of *L.* cf. *tuzetae* could represent different gregarine species. Molecular phylogenetic data were needed to test this possible inference.

Clouds of variation in molecular data. There were no distinct clades formed in the *L.* cf. *tuzetae* sequences that represented any particular morphotype, host, or locality. In our study, the range of divergence in the *L.* cf. *tuzetae* sequences was inconsistent between different morphotypes, within the morphotypes, and even between clones derived from the same cell. There are several possible explanations for these differences: (1) this variation might reflect slightly different SSU rDNA genes encoded in the same nucleus (e.g. paralogy), (2) it is known, for example, that there are five slightly different copies of the SSU rDNA gene in the *Cryptosporidium* genome (Fayer and Santín 2009; Le Blancq et al. 1997; Xiao et al. 1999), (3) it is also possible that some sequence heterogeneity is the result of PCR amplification errors associated with the Taq polymerase used in the puReTaq Ready-to-go PCR beads (GE Healthcare); however, this Taq polymerase is highly purified and has an excellent batch-to-batch reproducibility in PCR results.

A comparison of 1,657 bp from five different clones from a single cell isolation (i.e. 4BAMa–e) resulted in divergences of 1.0–1.8% (17–30 different bp); similarly, a comparison of 1,657 bp from four different clones of another single cell isolate (i.e. 2.2VANa–d) resulted in divergences of 0.9–1.6% (14–26 different bp). By contrast, there was no sequence divergence whatsoever in some clones derived from different cells collected in two different localities (i.e. Bamfield—2.1BAMb and Vancouver—2.1VANd, h). These data taken together show that, like morphology, gregarine species can be characterized with a range of variation present in the SSU rDNA marker.

The highest degree of sequence divergence found in this study was 3.9% of 1,657 bp or 62 different bp between a clone derived from a single cell isolated from Bamfield (i.e. 4BAMa, e) and a clone derived from a single cell isolated from Vancouver (i.e. 2.2VANa). By comparison, two new species of *Lankesteria* were established recently and were only 2.1–3.1% different in their SSU rDNA. However, these species were also separated because (1) the SSU rDNA clones from each species clustered into separate clades, (2) each *Lankesteria* species inhabits different host species, and (3) the trophozoites of each species were consistently different in size by an order of magnitude (Rueckert and Leander 2008). Moreover, the interspecies variation in the SSU rDNA was much higher in these two species than the intraspecies variation, namely 0.3–0.5% intraspecific variation in *Lecudina chelyosomae*

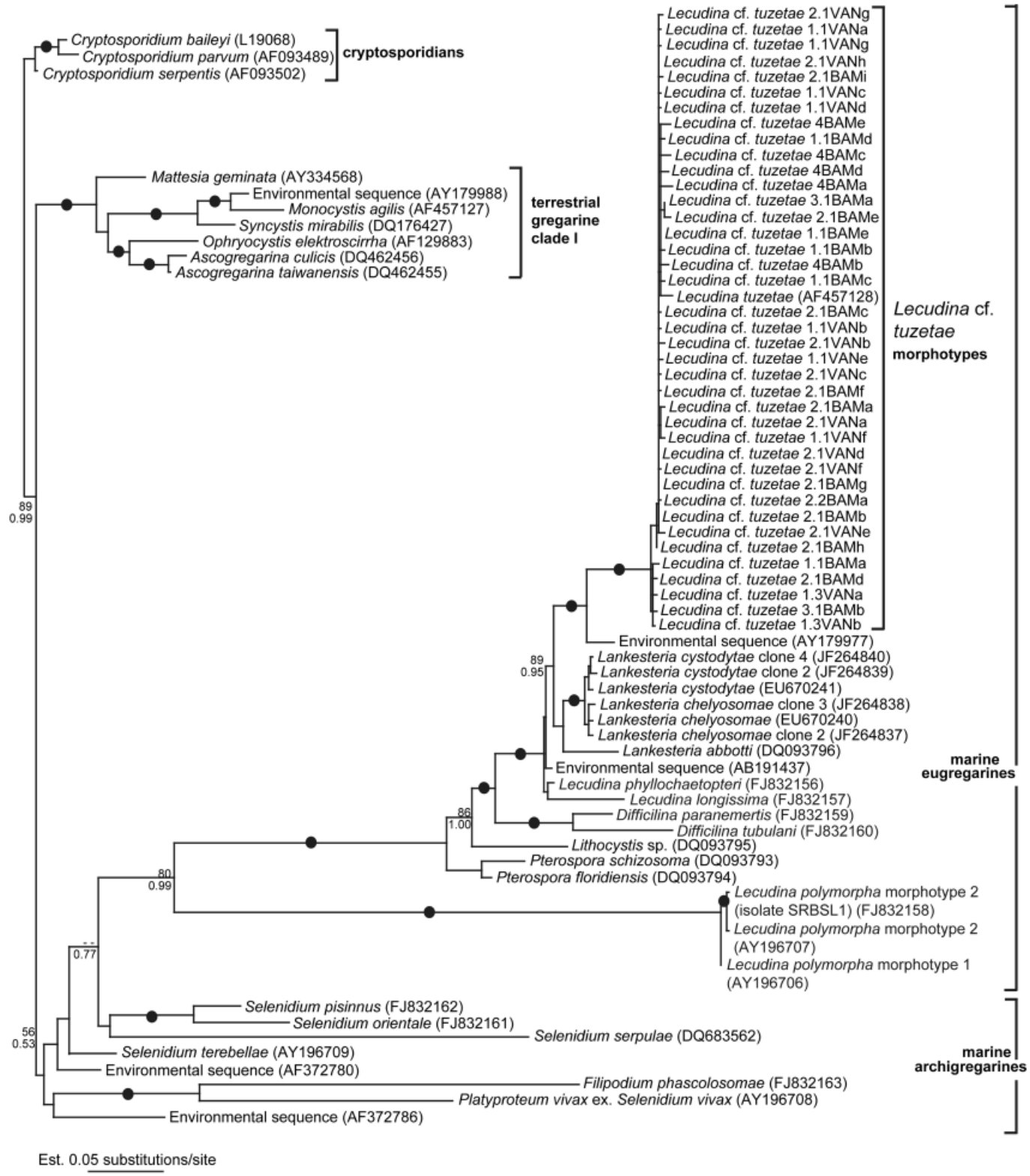


Fig. **13.** Phylogenetic tree of gregarine species using cryptosporidians as an outgroup. This gamma-corrected maximum likelihood tree ($-\ln L = 11{,}903.41485$, $\alpha = 0.437$, fraction of invariable sites = 0.000, eight rate categories) inferred using the general-time reversible model of substitution on an alignment of 77 small subunit rDNA sequences and 1,169 unambiguously aligned sites. Numbers at the branches denote bootstrap percentage (top) and Bayesian posterior probabilities (bottom). Black dots on branches denote Bayesian posterior probabilities and bootstrap percentages of 90% or higher.

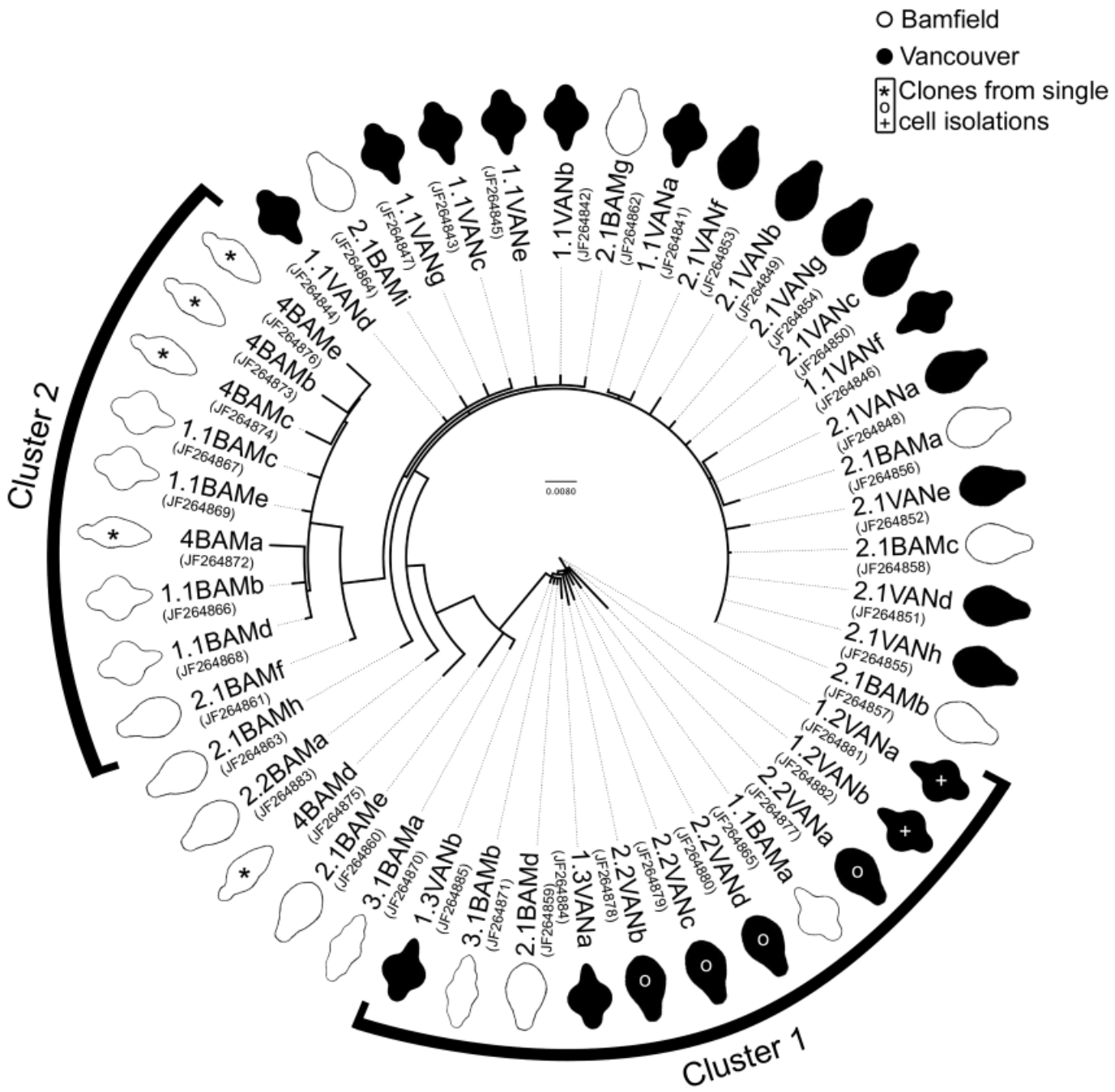


Fig. **14.** Unrooted gamma-corrected maximum likelihood tree ($-\ln L = 4{,}176.31529$, $\alpha = 0.605$, fraction of invariable sites = 0.322, eight rate categories) inferred using the general-time reversible model of substitution on an alignment of 45 small subunit rDNA sequences and 1,668 unambiguously aligned sites. There were no clusters that corresponded to distinct morphotypes or specimens collected at the same locality (i.e. Bamfield or Vancouver). Nonetheless, Cluster 1 including sequences from all three morphotypes and both sampling sites, and Cluster 2 including sequences from two morphotypes and clones from the single cell of morphotype 4 isolated from Bamfield. However, neither of these clusters corresponded to specific morphotypes or sampling sites. The icons and labels stand for the different morphotypes and clones (see text for details). White icons are cells isolated from hosts in Bamfield, black icons are cells isolated from hosts in English Bay. *, °, and + highlight different clones from single cell isolations.

and 1.3–1.5% intraspecific variation in *Lecudina cystodytae* (Rueckert and Leander 2008).

In another study, two new species of *Difficilina* that are morphologically similar but inhabit different species of nemerteans were described, in part, on the basis of 14.1% divergence in the SSU rDNA sequences (i.e. 221 different bp out of 1,732 bp) (Rueckert and Leander 2010). By contrast, three sequences from two very different morphotypes of *L. polymorpha* have SSU rDNA divergences of 0.6–0.8% (i.e. 10–13 different bp out of 1,704) (Rueckert and Leander 2010). A comparison of our observations with these previous studies demonstrates the difficulties in pinpointing a consistent and pragmatic degree of SSU rDNA sequence divergence for species discrimination. This shows that the level of sequence divergence in gregarine apicomplexans is in and

Table **2.** Summary of sequence divergences in (%) and differences in nucleotides of several isolates and clones of morphotypes 1–3 from *Lecudina* cf. *tuzetae* collected in Vancouver and Bamfield based on 1,657 nucleotides.

	Morphotype 1		Morphotype 2		Morphotype 3
	Vancouver 1.1a–g; 1.2a,b; 1.3a,b	Bamfield 1.1a–e	Vancouver 2.1a–h; 2.2a–d	Bamfield 2.1a–g; 2.2a	Bamfield 3.1a,b
Morphotype 1					
Vancouver					
1.1a–g; 1.2a,b; 1.3a,b	0.0–2.3% 0–37	0.7–2.8	0.1–3.0	0.1–2.6	0.2–2.2
Bamfield					
1.1a–e	11–46	0.4–3.3% 6–49	0.7–3.5	0.7–2.8	0.7–2.8
Morphotype 2					
Vancouver					
2.1a–h; 2.2a–d	2–48	12–56	0.0–3.2% 0–51	0.0–3.3	0.7–2.4
Bamfield					
2.1a–g; 2.2a	2–43	11–46	0–53	0.1–2.7% 1–43	0.2–2.6
Morphotype 3					
Bamfield					
3.1a,b	4–36	11–46	9–39	4–43	1.4% 23

Upper matrix, sequence divergence in (%) calculated with the Kimura 2-parameter model; lower matrix differences in nucleotides between the sequences.

of itself not that important for species discrimination, because the level of variation differs from species to species. Nonetheless, gregarine species can be recognized based on branching patterns within the framework of a molecular phylogenetic tree (i.e. the phylogenetic species concept). Evaluating the degree of SSU rDNA sequence variability within any particular species of gregarines is, therefore, wholly dependent on additional analyses and data, such as consistent patterns in (1) how the sequences cluster within molecular phylogenetic analyses, (2) morphological variability, (3) host associations, and (4) geographic distributions.

This case study shows that a more comprehensive understanding of congruent morphological and molecular variation is necessary to confidently identify different species of gregarines. Species descriptions and identifications based on either morphological data alone, or molecular data alone, are limited by the absence of reciprocal reinforcement in the observed variation at both levels of organization. Millions of new gregarine species remain hidden in the multitude of marine and terrestrial animal lineages that have yet to be explored (Perkins et al. 2000). An approach to gregarine systematics that accounts for the observed levels of morphological and molecular variation in each species should greatly improve the ease and accuracy of future species identifications. For instance, once the range of molecular variation in SSU rDNA has been characterized from the *L tuzetae* that was originally described from *H.* (*N.*) *diversicolor* collected in France (Schrével 1963, 1969), we will be in a position to test whether or not the northeastern Pacific gregarines identified as *L.* cf. *tuzetae* in this study are indeed the same species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequence divergences (upper matrix) and differences in nucleotides (lower matrix) of all isolates and clones of morphotype 1–3 of the marine eugregarine *Lecudina* cf. *tuzetae* collected in Vancouver and Bamfield based on 1,657 nucleotides. The sequence divergence was calculated based on the Kimura 2-parameter model. *Indicates clones from single cell isolates.

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