Molecular and anatomical analyses reveal that *Peronia verruculata* (Gastropoda: Onchidiidae) is a cryptic species complex

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Abstract

Recent years have seen an increase in the number of studies that use DNA sequence information in addition to morphological methods, as the latter alone can be inadequate for morphologically similar, cryptic species. Marine onchidiid slugs (Mollusca: Gastropoda: Onchidiidae), which commonly inhabit intertidal environments and have a near-global distribution, comprise 11 genera and 86 described species. Singapore has 19 recorded species of onchidiids with *Peronia verruculata* (Cuvier, 1830) being the most abundant. Here we present mitochondrial DNA sequence data from 63 *P. verruculata*-like specimens to show that they constitute two closely-related clades with distinct sequence signatures and a clear barcode gap. Intraclade distances are 0–1.8% while interclade distances range from 4.7 to 6.0%. Scanning electron microscopy of internal copulation organs reveals that the two clades have distinct penial and accessory gland spine structures, which also differ in size. Using an integrative taxonomic approach, we propose that what was initially thought to be *P. verruculata* in Singapore, actually are two different species of *Peronia*.

Introduction

Species delimitation is the *sine qua non* for biology. After all, what is defined as species often has material implications on areas such as biodiversity conservation or invasive species management (Mace, 2004; Armstrong and Ball, 2005; Bickford *et al.*, 2007; Hendrixon *et al.*, 2015). Molecular evidence is becoming more important for taxonomic work (Hajibabaei *et al.*, 2007; Kress *et al.*, 2015; Meier *et al.*, 2016). The technique of DNA barcoding, which is predicated on the use of short, standardised DNA sequences (i.e. barcodes), has facilitated species identification in many taxonomic groups (Hebert *et al.*, 2003, 2004; Witt *et al.*, 2006; Moura *et al.*, 2008). Species are also increasingly being delimited based on sequence data, a pursuit known as DNA taxonomy (Pons *et al.* 2006; Vogler and Monaghan, 2007). Notwithstanding the potential pitfalls associated with these DNA-based approaches (Will *et al.*, 2005; Meier *et al.*, 2006), the integration of molecular and morphological methods has led to numerous discoveries of cryptic species complexes (Hebert and Gregory, 2005; Bickford *et al.*, 2007; Tan *et al.*, 2010). In particular, DNA sequences have helped clarify the taxonomy of many gastropod species, which are notorious for being either phenotypically plastic or static; examples include *Lepetodrilus* (Johnson *et al.*, 2008), Carychiidae (Weigand *et al.*, 2011), Plakobranchus (Meyers-Muñoz *et al.*, 2016), Phyllidiidae (Stoffels *et al.*, 2016), *Rhagada* (Johnson *et al.*, 2016), Conidae (Lawler and Duda Jr., 2017), *Dendronotus* (Lundin *et al.*, 2017), and Chromodorididae (Layton *et al.*, 2018).

DNA sequences also have the potential to help with many taxonomic problems associated with gastropods

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of the family Onchidiidae. Onchidiids are shell-less, air-breathing gastropods, comprising at least 11 genera and 86 valid species (MolluscaBase, 2018). Apart from the Arctic and Antarctic, onchidiids have a near-global distribution, and typically inhabit the upper intertidal zone in sandy, rocky and muddy habitats; only three species are known to be terrestrial (Dayrat, 2009). Yet, until Dayrat's (2009) seminal review on Onchidiidae systematics, they have not been subject to much taxonomic research since the beginning of the 20th century (e.g. Labbé, 1934, Starobogatov, 1976, in Dayrat, 2009). This is due to the family's complex taxonomic history; almost all species descriptions in the past were made from preserved specimens rather than live animals, thus lacking in important information that Dayrat *et al.* (2016, 2017) have shown to be crucial for species identification. Moreover, many of the characters chosen for taxonomic work either displayed high intraspecific variability or were based on reproductive structures that were difficult to access (Dayrat *et al.*, 2016, 2017). In addition, the type specimens of many species were often lost or poorly preserved, thus rendering them of little help for clarifying species boundaries (Dayrat *et al.*, 2016, 2017). As such, onchidiid classification remains in disarray and the nomenclatural status of most species remains unresolved, making new species descriptions nearly impossible.

These problems notwithstanding, the last decade has seen a growing body of work focused on clarifying the status of various onchidiids, such as *Semperoncis montana* Plate, 1893 (Dayrat, 2010a), *Onchidium vaigiense* Quoy and Gaimard, 1824 (Dayrat, 2010b), *Hoffmannola* Strand, 1932 and *Onchidella* Gray, 1850 in the Tropical Eastern Pacific (Dayrat *et al.*, 2011b), *Onchidium* Buchannan, 1800 (Dayrat *et al.*, 2016), *Melayonchis* Dayrat and Goulding, 2017 (Dayrat *et al.*, 2017) and *Platevindex mortoni* Britton, 1984 (Zhang *et al.*, 2017). The study on *Onchidium* by Dayrat *et al.* (2016) was noteworthy for being the first to apply an integrative taxonomic approach (see Dayrat, 2005) to show that distinct morphological differences observed are well-supported by molecular data in three species of *Onchidium*. The same approach was once again successfully applied in the delimitation of four new species in the novel genus *Melayonchis* (Dayrat *et al.*, 2017). It is thus likely that such an integrative framework could also help with resolving taxonomic problems within onchidiid genera such as *Peronia* Fleming, 1822.

In the present study, we focus on a common intertidal onchidiid, *Peronia verruculata* (Cuvier,

1830). The species was initially described as *Onchidium verruculatum* based on specimens likely to have come from the Red Sea (Dayrat, 2009). While Cuvier (1830: 46) did not accompany that name with a description, the name is considered valid (Dayrat, 2009). It was later transferred to the genus *Peronia* by Labbé (1934) to distinguish species bearing dorsal gills from other onchidiids (Liow, 1997; Dayrat, 2009). Geographically, *P. verruculata* has been recorded from the Indo-Pacific region (Awati and Karandikar, 1948; Liow, 1997; Sun *et al.*, 2014). Liow's (1997) study of onchidiids in Singapore ranked *P. verruculata* as the most abundant of 19 onchidiid species recorded, and it was also the only known species in *Peronia* (Tan and Woo, 2010). It primarily inhabits the high intertidal, preferring rocky shores and artificial seawalls. During low tides, these onchidiids emerge from their holes to graze on algae-covered rocks, producing characteristic faecal homing trails which contain directional information for navigating back to their crevices before the tide washes in (McFarlane, 1980, 1981). During high tides, they remain dormant in their inundated crevices, relying on their dorsal gills for gaseous exchange. Despite its abundance in Singapore's intertidal environment, little is known about *P. verruculata*, and it is thus chosen as the subject for molecular and morphological characterisation.

Here, we provide a detailed analysis of the cytochrome c oxidase subunit I (COI) gene and internal morphology to reveal unexpected division of *P. verruculata*-like slugs in Singapore into two closely-related clades. These results support the presence of a previously unrecognised *Peronia* species in Singapore, which is confirmed here based on DNA sequence and scanning electron microscopy (SEM) data.

Material and methods

Specimen collection and preservation

A total of 63 *Peronia* specimens were collected from the seawalls and rocky shores of nine intertidal localities across Singapore, during low spring tides between July 2015 and January 2017 (Figure 1). Seawalls are a predominant feature of Singapore's reclaimed coastline (Lai *et al.*, 2015). They are constructed out of granite boulders and fitted to form flat, sloping surfaces facing the sea (Tan *et al.*, 2016; fig. 2a). Conversely, rocky shores have now become a rarity, as most have been reclaimed and replaced with seawalls (Todd and

Chou, 2005). These rocky shore environments are characterised by scattered sedimentary rock, housing communities of algae, molluscs and crustaceans (Tan *et al.*, 2016; fig. 2b).

Specimens were identified based on Britton’s (1984) and Liow’s (1997) descriptions, namely: (i) opening of the male genital pore on the left of the right eye stalk (Figure 3b), (ii) presence of knobbly papillae on the dorsum bearing eyes (Figure 3c), (iii) presence of clearly differentiated gill tubercles on the posterior dorsum (Figure 3d), and (iv) green-coloured sole and hyponotum.

A piece of the hyponotum was excised from each specimen and preserved in 100% molecular grade ethanol for molecular analyses. Voucher specimens were fixed in 10% formalin, transferred for preservation in 70% ethanol, and deposited at the Lee Kong Chian

Natural History Museum (LKCNHM, Singapore) under catalogue numbers ZRC.MOL.10094 to ZRC.MOL.10125.

The Onchidiidae collection at the LKCNHM was also examined. While the catalogue amounted to some 200 onchidiids, the vast majority of specimens were identified only as “Onchidiidae sp.” and were collected from mangroves, outside the known habitat of *Peronia* (Liow, 1997; Dayrat *et al.*, 2017). The rest of the collection were labelled “*Onchidium verruculatum*” and stored in unknown fixative that may prohibit DNA analysis, but specimens were inspected visually.

DNA extraction and genotyping

DNA was extracted from ~7mm² of tissue material taken from the interior of the hyponotum subsample to

Figure 1. Sampling localities of *Peronia* sp. in Singapore. Localities are abbreviated as follows: Punggol Jetty (PGJ), Changi Beach (CHG), Chek Jawa Wetlands (CJW), Tanah Merah Beach (TMB), St. John’s Island (SJI), Sentosa Island (SEN), Labrador Beach (LAB), Pulau Hantu (HAN) and Pulau Satumu (SAT). (Adapted from http://commons.wikimedia.org/wiki/File:Singapore_Outline.svg)

Figure 2. Sampling environments of *Peronia* onchidiids in Singapore: (a) seawall at Tanah Merah Beach (TMB), representative of sampling sites at St. John's Island (SJI), Labrador Beach (LAB) and Pulau Hantu (HAN); (b) rocky shore at Changi Beach (CHG), representative of sampling sites at Punggol Jetty (PGJ), Chek Jawa Wetlands (CJW), Sentosa Island (SEN) and Pulau Satumu (SAT). Photographs taken by JJM Chang.

reduce external contamination. Tissues were digested overnight using CTAB (hexadecyltrimethylammonium bromide) buffer with 0.4mg proteinase K, followed by phenol: chloroform: isoamyl-alcohol (25:24:1) for phase separation. Purified DNA pellets were eluted in 75–160µl of molecular grade water.

A partial segment of the cytochrome c oxidase subunit I (COI) gene was amplified via polymerase chain reaction (PCR) using LCO1490 and HCO2198 primers (Folmer *et al.*, 1994). PCRs were performed in 25µl reaction mixes comprising 2µl of template DNA, 1× BioReady rTaq buffer, 0.2µM of each dNTP, 0.4µM of each primer, 1U BioReady rTaq (cat. BSA12M1, Bioflux), and 0.02µg of bovine serum albumin (BSA). The thermal cycling profile was 94°C for 60s; 35

cycles of 94°C for 45s, 48°C for 45s, 72°C for 90s; and 72°C for 3 mins. Gene amplification was verified on 1% agarose gels. Successful amplicons were purified with SureClean Plus (Bioline) and sequenced using the BigDye Terminator v3.1 (Applied Biosystems) in a 3730xl DNA Analyzer (Thermo Fisher Scientific), following manufacturers' instructions.

Chromatograms were verified using Geneious v.10.1.3 (Kearse *et al.*, 2012). Sequences were translated to check for stop codons and deposited with GenBank under the accession numbers MH002545–MH002607.

Genetic analyses

For genetic distance analysis, we added *Peronia* spp. 1–6 sequences from Dayrat *et al.* (2011a) to our 63 sequences, for a total of 69 sequences. Following alignment using MAFFT v.7.311 under the default 'auto' parameters (Katoh *et al.*, 2002; Katoh and Toh, 2008; Katoh and Standley, 2013), MEGA 7 (Kumar *et al.*, 2016) was used to compute uncorrected p-distances amongst the 69 COI sequences. Uncorrected p-distance was chosen as opposed to other model-based distance methods like Kimura-2-parameter (K2P) because the latter have been shown to artificially inflate interspecific distances (Srivathsan and Meier, 2012). These 69 sequences were then consolidated together with 207 publicly available onchidiid sequences from GenBank (Dayrat *et al.*, 2011a, 2016; White *et al.*, 2011; Cumming, 2013; Layton *et al.*, 2014; Liu *et al.*, 2014; Sun *et al.*, 2014; Zhou *et al.*, 2016) in Mesquite 3.20 (Maddison and Maddison, 2017), for a total of 276 sequences. Alignments were performed again using MAFFT, yielding a 998-bp data matrix (without gaps and stop codons) for phylogenetic analysis.

We applied maximum likelihood (ML), Bayesian, as well as maximum parsimony (MP) tree reconstructions, with *Sarasinula linguaeformis* (HQ660055.1) and *Vaginulus taunaisii* (HQ660056.1) as outgroups (Dayrat *et al.*, 2011a). First, ML trees were inferred using RAxML 8.0.9 (Stamatakis, 2006, 2014) under the default GTRGAMMA model and 50 random starting trees. Node supports were quantified using 1,000 replicates of standard bootstrapping (Stamatakis *et al.*, 2008). Second, for Bayesian analyses, a model of molecular evolution was chosen from a list of 24 using Akaike Information Criterion (AIC) as implemented in jModelTest 2.1.10 (Darriba *et al.*, 2012). Bayesian inferences were performed

using the GTR + I + G model in MrBayes 3.2.2 (Ronquist *et al.*, 2012). Four Markov chains Monte Carlo (MCMC) of 9,000,000 generations were implemented in two runs, logging one tree per 100 generations. We employed Tracer 1.6 (Rambaut *et al.*, 2014) to evaluate MCMC convergence between the runs, and discarded the first 10,001 trees as burn-in. Lastly, MP tree searches were performed in TNT 1.1 (Goloboff *et al.*, 2008), using 1,000 replications comprising 100 cycles of tree fusing, drifting and ratcheting, followed by 1,000 bootstrap replicates (gaps were treated as missing).

Morphological observations

All specimen observations and excisions were made under Leica S8 APO or M205C stereo microscopes. Measurements for each specimen comprised number of dorsal eyes, and Labbé’s (1934) ratio of hyponotum (H) to sole (S) width.

Following precedents set by contemporary onchidiid studies (Dayrat 2010a, 2010b; Dayrat *et al.*, 2011b, 2016, 2017; Zhang *et al.*, 2017), the radula and male reproductive organs were targeted for SEM. These structures were prioritised as they have been shown to be the most informative traits for onchidiid systematics (Britton, 1984). Radulae were cleaned in 10% NaOH for 3–4 days, rinsed in distilled water for 3–4 days, and finally sputter-coated with gold before examination under a JSM-6510LV SEM (JEOL

Ltd.). The penis and the accessory gland spines were dehydrated in 100% ethanol before sputter coating.

Results

Genetic analyses

Our analyses of 276 onchidiid COI sequences recover two separate clades nested within *Peronia* that contain all 63 *Peronia* sequences from Singapore. Each of the clades are strongly supported by Bayesian and MP, whereas ML bootstrap support is fairly low (Figure 4). Of these sequences from Singapore, 30 form a monophyletic group together with the sequence labelled “*Peronia* sp. 2” in GenBank (Accession number: HQ660044.1; Figure 4). The remaining 33 form a separate distinct clade on its own, with no match above 95% to any GenBank sequence, but this clade is nevertheless closely related to *P. verruculata* (Figure 4). Henceforth, we refer to the former clade as *Peronia* sp.2 *sensu* Dayrat *et al.* (2011a), while the latter clade is referred to as the Singapore clade. Overall, relationships between the *Peronia* clades mirror those in Dayrat *et al.* (2011a), except that *Peronia* sp. 2 is sister group to sp. 6. Relationships between *Peronia* spp. 1–6 are poorly supported and thus remain uncertain.

Pairwise genetic distances for the 69 *Peronia* sequences corroborate results from our COI tree reconstructions, with support for a clear distinction

Table 1. Intra- and interclade mean (and range of, in parentheses) pairwise genetic distances (uncorrected p-distances, expressed in percentage) calculated using COI sequences from our dataset and *Peronia* sp. 1–6 *sensu* Dayrat *et al.* (2011a) via MEGA 7. Only a single sequence for each of the *Peronia* sp. 1–6 was made available on GenBank. Hence, where only one sequence was used in the calculation, the uncorrected p-distance is presented as a single value. 30 sequences from our study are grouped with *Peronia* sp. 2, while the 33 remaining sequences form a separate clade referred here as Singapore Clade (see Figure 4).

	Sp. 1	Sp. 2	Sp. 3	Sp. 4	Sp. 5	Sp. 6	Singapore Clade
Sp. 1	-	-	-	-	-	-	-
Sp. 2	14.03 (13.87 – 14.38)	0.18 (0 – 1.80)	-	-	-	-	-
Sp. 3	14.90	10.79 (10.62 – 10.96)	-	-	-	-	-
Sp. 4	15.41	6.03 (5.99 – 6.34)	10.10	-	-	-	-
Sp. 5	15.92	6.19 (6.16 – 6.34)	9.76	0.86	-	-	-
Sp. 6	14.04	1.57 (1.37 – 1.88)	10.10	6.16	6.34	-	-
SC	15.05 (14.73 – 15.24)	5.19 (4.65 – 6.00)	9.93 (9.59 – 10.27)	5.36 (5.14 – 5.65)	5.53 (5.31 – 5.82)	4.52 (4.11 – 4.79)	0.22 (0 – 0.75)

Figure 3. External morphological features present in both *Peronia* species: (a) *ex-situ* image of live specimen PV159 (ZRC. MOL.010111), cleaned, dorsal view; white arrow indicates black banding commonly seen on *Peronia* specimens; (b) anterior view of live specimen, black arrow indicates position of the male genital pore vis-à-vis the ocular tentacles; (c) dorsal eyes (black arrows) on the dorsum of the live specimen; (d) dorsal gills (white arrow), visible when specimen is immersed in seawater. Images (b)–(d) were observed and imaged by mounting Canon Powershot G10 on a Leica S8 APO Stereo Microscope. Images (b)–(d) are not tied to particular voucher numbers but are still included in this paper nonetheless to provide readers a clearer impression of the morphological features of *Peronia* species.

between Singapore clade and *Peronia* sp. 2. Interclade distances ranged between 4.7% and 6.0%, while intraclade genetic distances are fairly low in both Singapore clade and *Peronia* sp. 2 (maximum of 1.8%; Table 1). In other words, there exists a clear barcoding gap (*sensu* Meyer and Paulay, 2005) between the intra- and interclade genetic distances for the two clades (Figure 5).

External morphology

The external appearance does not vary consistently amongst individuals and between the two clades containing the *P. verruculata*-like samples (*Peronia* sp.

2 and Singapore clade) from Singapore. Dorsal notum colour is highly variable amongst individuals and is difficult to observe in the field as the specimens are often coated with mud or sand grains. Upon cleaning, specimens are either beige mottled with brown-black colouration, or vice versa. Some individuals also have black bands along the notum edges; these bands manifest in a radial fashion like spokes on a wheel from a dorsal perspective (Figure 3a).

The dorsal notum is dotted with tuberculate papillae, which sometimes bear dorsal eyes (in groups of one to four in *Peronia* sp. 2, and groups of two to nine in the Singapore clade; Figure 3c, Table 2). Dorsal eyes are typically distributed evenly across the anterior and

Table 2. Comparative external morphology of our specimens from *Peronia* sp. 2 (30 specimens examined) and Singapore Clade (33 specimens examined). Readers are directed to Figure 3 for images of the morphological features (i.e. male genital pore, dorsal eyes and dorsal gills) used in this comparison. H and S refer to hyponotum and sole respectively.

	<i>Peronia</i> sp. 2	Singapore Clade
Position of male genital pore	Left of the right ocular tentacle	Left of the right ocular tentacle
Dorsal eyes	Present, in groups of 1–4	Present, in groups of 2–9
Dorsal gills	Present, on posterior dorsum	Present, on posterior dorsum
Hyponotum (H) and Sole (S) colour	Green	Green
H:S ratio	H>S, H=S, H<S	H>S, H=S, H<S

middle regions of the dorsal notum. Posteriorly, the dorsal notum is covered with an even distribution of branched papillae, elsewhere referred to as dorsal gills. The gills are typically collapsed when specimens are exposed to the atmosphere but spread out and become erect when immersed (Figure 3d).

Ventrally, the ratio of the hyponotum (H) to pedal sole (S) widths varies across individuals as well as between genetic clusters, with no clear systematic pattern attributable to each clade (Table 2). Both the pedal sole and the hyponotum are green in all specimens.

Anteriorly, the ocular tentacles are superior to the left and right oral lobes as well as the mouth. The ocular tentacles are stubby (as opposed to long and thin in *Onchidium typhae*), and each bear an eye at the tip of the stalk. In preserved specimens, the eye stalks and oral lobes are retracted. The male genital pore is always located to the left of the right ocular tentacle (Figure 3b).

Morphology of male genitalia

The *Peronia* male reproductive organ comprises (i) the penial complex, consisting of the penis, penial sheath, deferent duct and retractor muscle, and (ii) the accessory penial gland. Both the penis and accessory gland spine open into the same male genital pore.

(i) Penial anatomy. The penial sheath of both *Peronia* sp. 2 and Singapore clade is a long straight stalk that protects the penis for its entire length. An insertion of the retractor muscle marks the separation between the penial sheath (and its constituent penis) and the deferent duct (Dayrat, 2010; Dayrat *et al.*, 2016). Deferent ducts across all specimens are highly convoluted. The penis is typically elongated and tube-like, and always possesses a distal portion

covered internally with conical hooks. Penile length is consistent between specimens from either clade, measuring 0.9–1.5 cm.

Noticeable differences in penial morphology can be observed between clades, nonetheless. The penis of *Peronia* sp. 2 (n=6) is narrower in diameter vis-à-vis Singapore clade (n=7; Figures 6a-b, Table 3). Moreover, the length of the distal portion of the penis with hooks (D) also differs markedly between clades: 0.4–0.5 cm for *Peronia* sp. 2 and 0.2–0.3 cm for Singapore clade. Taken together, the length of the proximal portion of the penis without hooks (P), when expressed as a ratio against D, is approximately about 1–2× in *Peronia* sp. 2 as compared to 3–4× in Singapore clade. Hooks cover the distal portion of the penis in both clades, but with approximately 50–90 hooks per 200×200 µm area for *Peronia* sp. 2, and only 30–50 hooks per same area for the Singapore clade (Figures 6c-d). Both clades are similar in terms of hook diameter and length (Figures 6e-f, Table 3).

(ii) Accessory penial gland. The accessory penial gland comprises regions of varying thickness; the thinner portion tends to be heavily coiled and is often entangled with the deferent duct. Position of the thicker portion of the accessory gland varies; it is typically embedded underneath the gut for *Peronia* sp. 2, but easily visible on the surface and posterior to the buccal mass for Singapore clade specimens. The accessory gland ends as a stiff, hollow spine that shares the same vestibule as the penis and is protected by a sheath as well. Gland spine length varies across specimens, but both clades (n=7 each) share the same range of 1.5–2.2 mm (Table 4). Singapore clade individuals tend to possess a noticeably wider diameter at the conical base of the spine compared to *Peronia* sp. 2 (Figures 7a-b, Table 4). SEM observations reveal that the distal ends of the gland spine are similar in both clades;

Table 3. Comparative penial anatomy of *Peronia* sp. 2 (6 specimens examined) and Singapore Clade (7 specimens examined).

	<i>Peronia</i> sp. 2	Singapore Clade
Sample size	6	7
Penial width (mm)	0.06–0.15	0.16–0.35
Penial length (cm)	0.9–1.5	0.9–1.4
Proximal area without hooks (P; cm)	0.5–1.0	0.7–1.1
Distal area with hooks (D; cm)	0.4–0.5	0.2–0.3
P/D	1–2×	3–4×
No. of hooks (per 200×200μm)	56–94	32–54
Hook diameter (μm)	13–43	17–33
Hook length (μm)	23–89	37–66

Table 4. Comparative accessory gland spine anatomy of our specimens from *Peronia* sp. 2 and Singapore Clade (7 specimens from each clade were examined).

	<i>Peronia</i> sp. 2	Singapore Clade
Spine length (mm)	1.54–2.16	1.50–2.06
Conical base diameter (C; μm)	113–181	187–267
Aperture diameter (A; μm)	22–28	58–79
C/A	5–7×	3–4×

the aperture is slightly apical, centered, with a small triangular lip at the opening (Figures 7c-d). While similar in appearance, diameter of the spine aperture in the Singapore clade is much larger (61–79 μm) than in *Peronia* sp. 2 (22–28 μm).

Radula morphology

The radula is located within two large postero-lateral muscular masses at the anterior portion of the visceral cavity. Due to similarities between the two clades, the description hereon applies to both clades (n=10 each clade). Complete radulae have an inverted heart-shaped appearance (Figures 8a-b). Each radula row consists of a rachidian tooth with two half rows of lateral teeth that are similar in size and shape (Figures 8c-d). Radula formulae are variable across specimens (Table 5). The rachidian tooth is always tricuspid; the median cusp is discernible, while the two lateral cusps are rounded and generally inconspicuous (Figures

8c-d). Its base, anchored to the radula membrane, is either flat or mildly triangular. The lateral sides of the rachidian tooth always extend from the membrane at an angle, and they are either straight, or mildly convex. Rachidian teeth tend to be smaller than lateral teeth.

Lateral teeth are arranged in two half rows angled at 45° from the rachidian axis (Figures 8e-f). They are hook-like; the base rises perpendicular to the radula membrane and the hook curves at almost 90° relative to the base. The shape of the hook is capsule-like with a rounded ending. There is also a pointed cusp on the lateral expansion of the base, which is usually obscured by the hook of the adjacent, outer lateral tooth and only visible when the teeth are further apart. Although the half-rows are angled at 45°, the hooks themselves are straight and run near-parallel to the rachidian axis. Apart from the first two innermost lateral teeth, the lateral teeth tend to be similar in size and shape.

Table 5. Comparative radula morphology between specimens from *Peronia* sp. 2 and Singapore Clade (10 specimens from each clade were examined).

Specimen No.	Radula	Rachidian Tooth	Lateral Teeth			
	Rows	Formula	Base length (μm)	Median cusp length (μm)	Length (μm)	Width (μm)
<i>Peronia</i> sp. 2						
PV042	33	67-1-67	52	45	100	29
PV047	31	51-1-51	52	40	74	31
PV052	35	69-1-69	64	45	100	29
PV057	25	71-1-71	57	37	106	34
PV072	28	65-1-65	67	52	102	36
PV103	35	56-1-56	45	40	90	31
PV108	41	53-1-53	48	33	79	29
PV124	42	56-1-56	55	43	69	26
PV166	40	74-1-74	67	52	102	33
PV263	34	54-1-54	53	53	79	24
Singapore Clade						
PV031	26	53-1-53	50	36	71	24
PV078	40	66-1-66	obscured	obscured	78	24
PV091	32	62-1-62	56	44	79	31
PV115	33	55-1-55	60	44	85	27
PV139	29	64-1-64	obscured	obscured	76	24
PV142	37	70-1-70	50	33	79	24
PV148	34	73-1-73	56	37	87	29
PV151	36	68-1-68	55	33	112	26
PV200	31	67-1-67	54	41	80	26
PV245	41	82-1-82	52	36	76	26

Discussion

COI sequencing of *Peronia verruculata*-like pulmonates in Singapore has revealed two distinct clades separated by a barcoding gap without overlap between intra- and interclade divergences (Figure 5, Table 1). COI tree reconstructions employing three different optimality criteria concur that these two clades are distinct (Figure 4). It is recognised that COI is not directly involved in speciation and is merely indicative of divergence times (Kwong *et al.*, 2012). Nevertheless, the molecular data are consistent with morphological observations revealing that the two clades exhibit anatomical differences in their male genitalia (specifically in P:D and C:A ratios; Tables

3-4). Such noted variation in the male genitalia hints at possible mechanical reproductive isolation between the two clades. The fact that they co-occur in six out of our nine study sites, and yet remain anatomically and genetically distinct (Figs. 4-7, Table 1), suggests that these two clusters are already on different evolutionary trajectories. It thus appears that there is more than one species of *P. verruculata* in Singapore (*contra* Liow, 1997; Tan and Woo, 2010). As these two clades are phenetically distinct, diagnosable and reciprocally monophyletic, this conclusion is supported by all commonly used species concepts (see Wheeler and Meier, 2000). Furthermore, *Peronia* is likely to represent yet another case where distinct genitalia combined with genetic data have successfully detected

Figure 4. Phylogenetic reconstruction of Onchidiidae based on COI sequences, with *Sarasinula linguaeformis* and *Vaginulus taunaisii* as outgroups. Sequences from our own dataset separated into either the Singapore Clade or *Peronia* sp. 2 (clades are indicated in bold; number of our sequences that separated into each clade are indicated in parentheses). Numbers below branches indicate bootstrap values for maximum likelihood (ML), Bayesian, and maximum parsimony (MP) respectively; only ML bootstrap > 50, Bayesian posterior probabilities > 0.9, MP bootstrap > 50 are reflected on the tree.

Figure 5. Histogram showing the distribution of pairwise uncorrected p-distances of 63 *Peronia* sequences from our dataset, obtained via MEGA 7. All intraclade genetic distances (light grey) are below 2.0%, and all interclade distances (dark grey) are minimally 4.5% and as high as 6.0%. A clear and unambiguous barcode gap (*sensu* Meyer and Paulay, 2005) between 2.0 and 4.5% is present.

and helped resolve a gastropod species complex (see also *Satsuma* (Kameda *et al.*, 2007), *Bullidae* (Malaquias and Reid, 2008), *Mandarina* (Chiba and Davison, 2008), *Leptaxinae* (Jordaens *et al.*, 2009), *Pseudamnicola* (Delicado and Ramos, 2012), *Aiteng* (Neusser *et al.*, 2011), and *Anteaeolidiella* (Carmona *et al.*, 2014)).

However, several challenges hamper identification of these *Peronia* onchidiid clades to species. Cuvier (1830: 46) named *P. verruculata* based on illustrations in Savigny's (1817) *Description de l'Egypte* (the latter did not provide any species names in his publication; Dayrat, 2009). Unfortunately, Cuvier (1830) did not furnish a description to accompany the name (Dayrat, 2009). As we have also shown, traits proposed by Britton (1984) are of little value as they overlap between both clades and are more appropriate for narrowing down onchidiids to genus rather than species level. The status of the type specimen remains unclear as well (Dayrat, 2009). We were also unable to sample from the type locality of *P. verruculata*

in the Red Sea (Dayrat, 2005, 2009), nor the waters surrounding Singapore. While no taxonomic decisions are made here, it is worth noting that the name *Onchidium griseo-fuscum* (Tapperone-Canefri, 1874), *nomen dubium*, was established with Singapore as the type locality. Dayrat *et al.* (2016) suggested the name might belong to *Peronia*, and thus could be re-established in future revisions for these Singapore clade onchidiids. Furthermore, the status of *Peronia* sp. 2 remains unclear as well. Apart from sequence data and locality (Oman), no other information was provided by Dayrat *et al.* (2011a), curbing further investigative work on this species. It thus remains difficult to assign precise names to these onchidiids as it is impossible to ascertain if they are new to science.

Identification problems remain a pertinent stumbling block in natural history research (Tewksbury *et al.*, 2014), and is especially severe in the tropics where species diversity is high. Yet most species remain undescribed because old descriptions are so superficial that they cannot be used for comparisons (Dayrat *et*

Figure 6. Comparative morphology of penial anatomy, visualised under Leica M205C stereo microscope and SEM. (a), (c), (e) are from our specimens that grouped with *Peronia* sp. 2 *sensu* Dayrat *et al.* (2011a); (b), (d), (f) are from the Singapore Clade. (a) Penis, general view, PV108 (ZRC.MOL.010103). (b) Penis, general view, PV105 (ZRC.MOL.010102). (c) Penial hooks, PV108. (d) Penial hooks, PV068 (ZRC.MOL.010101). (e) Penial hook, PV166 (ZRC.MOL.010111). (f) Penial hook, PV068.

al., 2016, 2017; Meier, 2017). The way forward would undoubtedly be better quality (re)descriptions with high-resolution imaging techniques like SEM (Dayrat, 2010a, 2010b; Dayrat *et al.*, 2011b), complemented with DNA barcodes (Hebert *et al.*, 2003; Dayrat *et al.*, 2016, 2017). The latter is becoming more cost-efficient (Meier *et al.*, 2016), and will undoubtedly be more prevalent in taxonomic studies. Furthermore, the availability of more genome-wide molecular markers could help reduce uncertainty of phylogenetic relationships amongst *Peronia* species and advance our understanding of Onchidiidae in relation to other higher pulmonate taxa (Dayrat *et al.*, 2001, 2011a; Vonnemann *et al.*, 2005; Wägele *et al.*, 2014).

The present study has tested a suite of anatomical characters amongst clades of closely related onchidiids. Specifically, we evaluated the potential of the male genitalia (consisting of the penis and the accessory penial gland) and radula in distinguishing onchidiid species. The reproductive system, especially

Figure 7. Comparative morphology of the accessory spine gland under SEM. (a) and (c) are from our specimens that grouped with *Peronia* sp. 2 *sensu* Dayrat *et al.* (2011a); (b) and (d) are from the Singapore Clade. (a) Accessory gland spine, general view, PV042 (ZRC.MOL.010099). (b) Accessory gland spine, general view, PV148 (ZRC.MOL.010108). (c) Spine aperture, PV042. (d) Spine aperture, PV148.

Figure 8. Comparative morphology of radula under SEM. (a), (c), (e) are from our specimens that grouped *Peronia* sp. 2 *sensu* Dayrat *et al.* (2011a); (b), (d), (f) are from the Singapore Clade. (a) Radula, complete, PV072 (ZRC.MOL.010103). (b) Radula, complete, PV200 (ZRC.MOL.010115). (c) Rachidian teeth and innermost lateral teeth, PV166 (ZRC.MOL.010111). (d) Rachidian teeth and innermost lateral teeth, PV151 (ZRC.MOL.010108). (e) Rachidian teeth and lateral teeth, PV103 (ZRC.MOL.010103). (f) Rachidian teeth and lateral teeth, PV142 (ZRC.MOL.010106).

the anterior male copulatory complex, is of great importance in onchidiid systematics and has long been applied by onchidiid taxonomists (e.g. Labbé, 1934, Starobogatov, 1976, in Dayrat, 2009). Traits that have been studied include the position of the male reproductive aperture vis-à-vis the ocular tentacles, the presence of a full-fledged penis that may or may not be covered with internal hooks, and morphology of the hooks themselves (Dayrat, 2010a; Dayrat, 2010b, 2011b, 2016, 2017). While useful for identifying higher taxa, these traits are insufficient in distinguishing the two *Peronia* clades present in Singapore.

Instead, our detailed measurements on the dimensions of the penis and accessory gland spine detected other informative characters (Tables 3–4). For several onchidiid genera, male copulatory complexes are characterised by accessory penial glands. Like the penis, presence of the penial gland and morphology of the spine aperture vary between genera (Dayrat, 2010a, 2010b; Dayrat *et al.*, 2011b, 2016, 2017). Observations here reveal that the overall morphology of the spine gland is invariable amongst *Peronia* species (Figure 7), but there are differences in dimensions and proportions of the structure between *Peronia* sp. 2 and Singapore clade (Table 4). We suggest that a morphometric approach applied to genitalia anatomy has potential for detecting morphological differences between closely-related onchidiid species.

The radula formula was not useful in distinguishing the two *Peronia* clades, largely due to its high variability. Our results not only mirror findings from Bertsch (1976), who noted intraspecific radula plasticity within gastropods, but also lend support to claims that radula morphology might be similar amongst closely related onchidiid species (Dayrat, 2010a, 2010b; Dayrat *et al.*, 2017). The radula formula is thus ineffective for species delimitation. Nevertheless, radula morphology and formula might be useful for comparisons between genera as more SEM data become available. For example, the lateral teeth of *Peronia* specimens are straight and near-parallel to the rachidian axis, as opposed to those in *Semperoncis montana* and *Platevindex mortoni*, which are spatulate and tend to curve inward (Dayrat, 2010a: figs. 15–20; Zhang *et al.*, 2017: fig. 3); or even *Melayonchis* onchidiids, which have a distinctive protuberance on the inner lateral margin of each lateral tooth that is insofar specific to that genus (Dayrat *et al.*, 2017: figs. 6, 13, 18, 24).

Evidently, external morphology has not been useful in distinguishing members of the *Peronia* cryptic species complex found in Singapore, unlike that of

Onchidium (Dayrat *et al.*, 2016) or *Melayonchis* (Dayrat *et al.*, 2017) onchidiids, which do not have overlapping morphologies between species. For instance, foot and dorsal colouration of the live animals is specific to each species of *Onchidium* and *Melayonchis*, respectively (Dayrat *et al.*, 2016, 2017), making it easy to identify them. We have demonstrated that distinctive traits of *Peronia* are present in both members of the genus, and that variations in these traits are not clade-specific, a point echoed by Dayrat *et al.* (2017).

Given the relatively short branch lengths on the COI tree (Figure 4), *Peronia* spp. 2–6 *sensu* Dayrat *et al.* (2011a) and Singapore clade may constitute a case of adaptive radiation, in which species are only beginning to diverge into separate lineages. Such a radiation would certainly support the prevailing hypothesis that onchidiids originated out of warm, tropical waters (Dayrat *et al.*, 2011a), from which *Peronia* spp. 1–6 and Singapore clade specimens were obtained. If these species are part of an adaptive radiation, the more interesting task would be to uncover the drivers behind this evolutionary process, which could involve niche differentiation in the form of habitat or resource partitioning (Rundell and Price, 2009). However, more ecological and biogeographical data for onchidiids are needed to test these hypotheses. For instance, future work could explore diets of the two onchidiids in Singapore via faecal metagenomic sequencing (e.g. Srivathsan *et al.*, 2016) to discern if this observed sympatry may be due to resource partitioning or associated with competitive exclusion.

Interestingly, Liow (1997) observed that *Onchidium aberrans* Semper, 1885 (designated *nomen dubium*, Dayrat *et al.*, 2016) was not only similar to *P. verruculata* in terms of abundance, but also found to co-occur with *P. verruculata*. This is strikingly similar to what was observed for Singapore clade and *Peronia* sp. 2. However, Liow's (1997) detailed morphological analysis of *O. aberrans* showed that it did not possess dorsal gills and was thus unlikely to be the Singapore clade onchidiid found in this study. These patterns hint at a possible shift in onchidiid community composition over the years. The LKCNHM Onchidiidae collection was examined, but unfortunately, historical comparisons here with the museum specimens were unfruitful for several reasons. First, most of the specimens were recorded as “Onchidiidae sp.” making it difficult to establish past species composition in the absence of DNA barcoding as we were unable to identify them to species level. Second, most specimens were sampled from mangrove habitats which lie

outside known ranges of *Peronia* (Liow, 1997; Dayrat et al., 2017); they are unlikely to be *Peronia* and thus discussion pertaining to the collection as a whole is beyond the scope of this paper. Finally, most of the “*Onchidium verruculatum*” specimens were sampled from a single site (Punggol). Without DNA barcoding to identify these onchidiids and more localities for comparison, we remain unable to quantify community shifts over time. Clearly, more work is needed to better understand the ecology of these onchidiids.

Conclusion

In this paper, we complemented DNA sequence information of *P. verruculata*-like specimens from Singapore with SEM data of internal organs to show that they form two closely-related clades with distinct mitochondrial COI signatures and male genitalia measurements. Using this integrative approach, we present evidence that what was initially thought to be *P. verruculata* might be two different members of the genus in Singapore. Owing to the family’s complex taxonomic history, and the lack of samples from the type locality, we have opted for a tempered interpretation of our results and chosen not to propose formal taxonomic changes. Nonetheless, the provision here of detailed SEM images and data, a first for *Peronia* to our knowledge, contributes to a better understanding of morphological evolution and reproductive compatibility amongst onchidiid species.

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Online supplementary information

SI. List of *Peronia* specimens in this study.