Research Article

**Initiating laboratory culturing of the invasive ascidian *Didemnum vexillum***

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**Abstract**

Over the past few decades, the invasive colonial ascidian *Didemnum vexillum* Kott, 2002 has established in many temperate regions, sometimes bringing detrimental perturbations to native marine ecosystems and local economies. A reliable supply of healthy, genetically defined *D. vexillum* stocks/strains would greatly assist research into the traits of this species relevant to its invasive success. To this end, here we describe basic methodologies for the growth, maintenance, formation of chimeric colonies and their follow-up observations in *ex situ* cultures of *D. vexillum*. *Didemnum* colonies were collected during the austral late winter/early spring from a marina (Nelson, New Zealand) and were maintained in the laboratory using re-circulated, unfiltered seawater. Ramets (1 – 10 cm\(^2\); >70 fragments) from a variety of colony locations (peripheral/central, flat/lobe/tendril-shaped); were isolated by razor blade and secured to glass slides using cotton thread. The ramets attached to the glass slides within 24 hours, displayed 100% survivorship and could be successfully further sub-cloned for up to 10 weeks, when the study was terminated. Two distinct processes for tunic-substrate attachment were observed: (i) the secretion of growing marginal fronts made of tunic matrix containing spicules but without zooids and (ii) the secretion of thin tunic extensions with clusters of tunic cells at their tips. Allogeneic paired colony fusion assays, using naturally growing edges of colony fragments (i.e. mimicking natural fusion scenarios) resulted in fusions and chimeric colony formation. Zooids from both partners within a chimera appeared to intermingle and the resulting chimeric entities grew vigorously. This brief report provides an outline of simple, low-tech laboratory culture conditions for the maintenance, asexual propagation and chimeric colony formation of *D. vexillum*. The techniques described present a foundation for the development of genetically defined *D. vexillum* strains which will facilitate research into the traits of this species that are relevant to its invasive success.

**Key words:** allorecognition, culture, chimeras, management, ramet, tunic cells, tunicate

**Introduction**

Marine bioinvasions involving a wide range of marine species introduced into regions beyond their natural or historic ranges, and the associated perturbations such invasive species impose on marine ecosystems and human welfare, are recognized as a major, and growing, environmental concern (Mooney and Cleland 2001; Occhipinti-Ambrogi 2007; Molnar et al. 2008; Simkanin et al. 2012). One of the more prominent invasive marine taxonomic groups is class Ascidiae (subphylum Tunicata, phylum Chordata; Lambert 2001) whose members include both solitary and colonial forms that inhabit many benthic marine communities. Because of their ability to thrive in eutrophic environments and being strong competitors, some species of ascidians have easily invaded new localities, spread rapidly, and integrate with local biofouling communities (Lambert 2001, 2007; Dijkstra et al. 2007; Carman et al. 2010; Adams et al. 2011). Introductions of non-indigenous ascidians into new harbors, aquaculture facilities, infrastructure constructions and natural environments worldwide, in tropical, subtropical, temperate and subarctic waters alike, are increasing in frequency, and the damage they may inflict on existing ecosystems, and the associated human economies, is well documented (Molnar et al. 2008; Simkanin et al. 2012).
Clearly there is a need for research tools to assist in scientific analysis of ascidians’ environmental tolerances, biological traits (e.g., growth rates, interspecific competitive proficiencies), diseases and interactions with native species. A central requirement is a constant supply of healthy, genetically defined strains/lines suitable for laboratory conditions, thereby circumventing seasonal and unpredictable variations in colony availability along with the impact of unknown underlying genetic variability. Such strains would allow the initiation of long-term and controlled experiments evaluating biological aspects of invasiveness under defined and controlled conditions.

Over the past few decades, the colonial ascidian *Didemnum vexillum* Kott, 2002 has been expanding globally impacting aquaculture facilities and natural habitats (Lambert 2009; Smith et al. 2012; Stefaniak et al. 2012). The rapid expansion of *D. vexillum*, along with other invasive ascidians, has raised questions about the biological traits that underlie such impressive adaptive success - especially in the context of anticipated changes in oceanic temperatures and chemistry over coming years (McCarthy et al. 2007; Bullard and Whitlatch 2009; Lenz et al. 2011; Simkanin et al. 2012; Smith et al. 2012). Addressing such questions is currently hampered by experimental and procedural deficiencies such as there being no well-established, disease free, genetically-defined strains of *D. vexillum* available nor have robust procedures for the long-term culturing of *D. vexillum* in the laboratory been described. To help initiate addressing these deficiencies here we describe basic methodologies for laboratory culturing of *D. vexillum* colonies and show that such colonies are easily experimentally manipulated.

**Materials and methods**

Small to medium sized *Didemnum vexillum* colonies, growing >5 meters apart, were collected from ropes and other submerged artificial objects from depths of 0.3–1.5 meters in the Nelson marina (Nelson, New Zealand; 41°15.4′S, 173°16.6′E) and transported ~2 km to the Cawthron Institute (Nelson) in containers filled with seawater. Each colony was cleaned of fouling organisms and cut using a razor blade into several ramets (n = 2–6; depending on the sizes of colonies). Ramets were secured by cotton threads to 5.0×7.5 cm glass slides, one ramet/slide. On some slides, pairs of isogeneic or allogeneic colony fragments were placed with their natural growing edges juxtaposed. All slides were placed vertically in the slots of glass staining racks (following Rinkevich and Shapira 1998) in unfiltered, recirculated seawater. Such vertical positioning of cultured colonies reduces debris, food and fecal accumulation around the colonies.

The colonies were grown in 16 litre flow-through glass tanks (~1 litre/min; unfiltered, recirculated sea water), under a 12:12 hour light-dark regimen. Salinity (34.5±0.1), pH (8.07±0.01) and temperature (17±1°C; ambient 15 °C) were monitored and kept constant along with vigorous aeration by air stones. The colonies were fed 4–5 times / week by adding ~50 ml of an algal mix (~1:1 ratio of *Isochrysis galbana* Parke [8–9 × 10⁶ cells / ml], *Pavlova lutheri* Green [10–12 ×10⁶ cells/ml]). This protocol follows the recommendations of Rinkevich and Shapira (1998) for botryllid ascidians inland mariculture which demonstrated that a mixture of diet types is superior to a monotype diet.

Colonies were cleaned during observations, at least once a week, using a soft, small paintbrush to remove debris and fouling organisms. The substrate around the colonies was cleaned using a small brush and razor blade. All cleaning protocols were performed under a binocular dissecting microscope and the colonies were submerged in seawater during all procedures. The status of the colonies was monitored every 1–2 days and photographs were taken to document colonial attachment to the substrate, growth patterns, outcomes of isogeneic and allogeneic contacts, and any changes in distribution/morphology.

**Results**

To collect colonies having different genotypes (i.e., to avoid colonies that are clonally related), seven *D. vexillum* colonies, denoted A–G, were sampled from different pontoons >5 meters apart in the Nelson marina. Colonies were peeled off by hand, or using single-edge razor blades, together with a very thin substrate layer and brought to the laboratory in seawater within an hour. Upon arrival at the laboratory, each colony was carefully rinsed in seawater, to remove sedimentation, and attached macro-epibionts were removed using forceps.

As *D. vexillum* colonies fragment easily (Bullard et al. 2007a,b; Morris and Carman 2012; Reinhardt et al. 2012) we first attempted to secure colonial fragments to the glass slides using the
Figure 1. Tunic–substrate attachment processes displayed by *D. vexillum* colonies: production of growing marginal fronts with spicules but without zooids and production of thin tunic extensions with masses of tunic cells at their tips (a) Marginal front attachment of a fragment sampled from the central part of a colony three days after securing the fragment with cotton threads which subsequently became embedded within the tunic matrix; (b) Two days thereafter, formation of a flat, fast growing colony on the substratum with wider growing marginal fronts; (c) A marginal front of a colony showing the embedded cotton threads, and the zooid-free tunic area, with many spicules; (d, e) Development of tunic extensions from growing marginal fronts with the apex of each extension containing tunic cells but no spicules (arrowheads); (f) Newly developed spicule-free tunic extensions, positioned above the substrate, with clear masses of tunic cells (arrowheads).

Abbreviations: te = tunic extensions; sc = spicules condensed area.

methodology successful for botryllid ascidians (Rinkevich and Weissman 1987). Peripheral, central and lobe/tendril shaped colony fragments (sizes 1 – 10 cm²) were cut from each colony, and transferred to settlement glass slides, one ramet/slide. The ramets were allowed to adhere to the slides by placing them in a moisture chamber at ambient temperature for 35–55 minutes before transferring them to still water aquaria. However, most of the fragments detached from the slides upon placing into the aquaria. We therefore used the alternate methodology of securing the fragments to the slides using thin cotton threads. The cotton threads were removed, after a colony’s complete adherence to the substrate, by cutting with a razor blade the free cotton threads above and below each colonial fragment and pulling out the embedded section using fine forceps. Parts of the four colonies denoted A–D were cut into smaller ramets, and distributed onto several glass slides (3–10 slides / colony).

In total >70 small ramets and larger fragments, of sizes ranging 1–25 cm² were produced and all (with the exception of a single case where the cotton covered areas degenerated) attached to the glass slides, irrespectively of their origin within the colony (i.e., peripheral, central, lobe/tendril-shaped fragments). The attached colonies were observed for up to 10 weeks, when this study was terminated. Under our basic culturing conditions, all attached fragments grew rapidly.
**Figure 2.** *D. vexillum* chimeric colony formation. (a, b) Fusion of tunics between two allogeneic pairs through tunic spreading and secretion of new tunic matrix with spicules but without zooids; (c) Development of chimerism between two allogeneic pairs through thin tunic extensions; (d - f) A condensed chimera, at the center of the glass slide, two days after first tunic to tunic contacts. The zooids area of each of the two partners is still morphologically distinguishable due to colony color differences (d). Five days thereafter (e), the whole intermingled chimeric entity has moved upward and almost half of the chimera is overgrowing the opposite side of the glass slide (f). The deteriorating right tunic area in ‘e’ (marked by a star) corresponds to the low right side of the right partner in the chimera (d, marked by a star). **Abbreviations:** te = tunic extensions; sc = condensed zooid-free spicules area.

on the slides (forming only flat, encrusting colonial forms), and had 100% survivorship. When all available space on the slides was covered (indeed in many cases fragments rapidly grew onto the back of the slides; Figure 2 e, f), the colonies were further subcloned and transferred to additional slides, a protocol with the potential to provide large stocks of ramets of identical genotype.

Within 24 hours after transfer to a glass slide, the *D. vexillum* colony fragments started an active attachment process, apparently initiated from the periphery, which led to their firm connection to their substrate (Figure 1). We observed two distinct processes associated with such attachment used separately or concurrently at each fragment’s edge. The first process involved the fast (within 24 hours after transfer) spreading of thin peripheral tunic matrix with many spicules but with a paucity/absence of zooids, located either all around the fragment’s outlying zones or as a growing front (Figure 1a-d). In common with other aplousobranch ascidians, *Didemnum* spp. colonies lack a common vascular system and their tunics are made of a fibrous, semi-transparent gelatinous matrix fringed, and supported, by a thin layer of dense fibers covering the whole tunic matrix (Hirose et al. 1997). Thus, as didemnid spicules are produced intracellularly within the zooids’ lateral organs of the thorax (Lafargue and Kniprath 1978; Kniprath and Lafargue 1980) and this method of attachment is rapid, producing, what appears to be new thin tunic with many spicules but without zooids, all these conditions point to spreading, not new tunic secretion. In the second attachment process colonies attach to substrates through thin extensions originating from the basal tunic area.
Table 1. Attachment rates to substrate by peripheral and central *D. vexillum* colony fragments.

<table>
<thead>
<tr>
<th>Fragment origin</th>
<th>Partly or fully attached to the substrate (days)</th>
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<tbody>
<tr>
<td>Peripheral</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6/6</td>
</tr>
<tr>
<td>Central</td>
<td>1/6</td>
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*Areas of cotton strings holding a single fragment start to deteriorate before this fragment fully attached to substrate. This ramet was detached from substrate one day thereafter, and when laid on the aquarium bottom, had started developing new attachment fronts.

(Figure 1d, e) or extensions emerging from upper parts of the tunic (Figure 1f), but directing towards the substrate. In contrast to the whole front attachment process described above, the tips of these small extensions which contain many cells but no spicules, formed distinct aggregates as more opaque areas at specific zones (Figure 1d–f, arrows). Some of these tunic cells (at least four different tunic cell types were recently identified in *D. vexillum*: denoted phagocytic, morula, filopodial and bladder; Sellers et al. 2013) are possibly the source of the newly secreted tunic matrix material at the growing edges of *D. vexillum* colonies. While these attachment processes were seemingly developed irrespective of the existence of zooids in peripheral zones, the whole course of attachment appears to be a highly coordinated activity at the ramet level.

Interestingly, while all the isolated fragments used in this study eventually attached to their substrates, fragments taken from colony peripheral zones attached faster than those taken from central regions (Table 1). Twelve fragments (ca. 1.0 cm$^2$) were isolated from colonies E–G (four samples/colony, two peripheral, two central). Two days following their securement to the glass slides, 6/6 peripheral fragments had attached to their substrates (some with very distinct growing fronts) as compared to 1/6 central region fragments being attached ($p$<0.05; G test). However within three days following securement, 4/6 of the central colony derived fragments attached while and all fragments attached within a week (Table 1).

The differential growth patterns of fragments on the glass slide substrates were so rapid that it was possible to document daily changes (Figure 2d–f), including examples of directional growth, growth with no clear trajectory, and colonies growing in more than one direction. Two of the characteristic features of fast-growing colonies were the less compact distribution of zooids within the tunic matrix and the appearance of wider peripheral expansion zones from which zooids were essentially absent. In faster growing colonies, an area of tunic devoid of zooids usually developed in older parts of the growing area. In some cases this older tunic area rapidly degenerated, whereas in other cases it remained intact through the full observational period. As the *D. vexillum* colonies were collected during austral late-winter/early-spring (August-October) no larvae were seen in the tunic matrix (Fletcher and Forrest 2011). Some areas, varying in locations and numbers between observations, containing faecal pellets were observed indicating the zooids were feeding.

We also performed both iso- and allogeneic paired-colony fusion assays between the naturally growing edges of colony fragments. This work was undertaken to establish protocols to follow the fusion outcomes when *D. vexillum* colonies of differing genotypes come into tunic–to–tunic contact. All six pair-wise combinations of colonies A–D, along with the four isogeneic combinations used as positive controls, were tested for fusion. Paired colony fragments adjacent to each other but without direct tunic-to-tunic contact were attached to glass sides. Growth of the paired colony fragments toward each other was rapid and, interestingly, it appeared that the tunic front of one of the pair generally advanced more rapidly than the other. Following tunic-to-tunic contact, the outer cortical layers of the tunic dissolved, resulting in fusion and the formation of chimeric colonies (example shown in Figure 2a, b). Fusion also occurred following the formation of tunic extensions (Figure 2c). The fusion process, from first tunic-to-tunic contact to complete amalgamation, took 1–6 days and was observed in all four isogeneic and six allogeneic pairings. Many of the chimeric colonies were morphologically highly active and zooids mixed within the new entity so that within 1–2 days their colony of origin was hardly obvious. Like their progenitors the chimeric colonies grew directionally and after a few days some even grew onto the back surfaces of the glass slides (Figure 2d–f).
Discussion

Didemnum vexillum Kott, 2002, is one of the well-known marine invasive species that has invaded temperate water regions across the globe (Lambert 2009; Stefaniak et al. 2009; http://woodshole.er.usgs.gov/project-pages/stellwagen/didemnum/index.htm). Atlantic Europe (Gittenberger 2007) including the UK (Minchin 2007; Griffith et al. 2009), New Zealand (Kott 2002; Smith et al. 2012), both coasts of North America (Kott 2004; Bullard et al. 2007b; Cohen et al. 2011) and, more recently, the Mediterranean (Tagliapietra et al. 2012). Population genetic studies strongly indicate the north-west Pacific Ocean as being the probable native region of D. vexillum (Smith et al. 2012; Stefaniak et al. 2012). Outside its native range, D. vexillum covers submerged manmade substrates as well as natural habitats from the very low intertidal down to 80 meters of depth (Bullard et al. 2007b; Valentine et al. 2007; Lambert 2009; http://woodshole.er.usgs.gov/project-pages/stellwagen/didemnum/index.htm).

Colonies grow as flat, encrusting mats or produce irregular lobes, depending on the type of available substratum and colony size/age. Aside from larval dispersal which is very limited due to the short-lived nature of the tadpoles, both natural and anthropogenic processes that fragment D. vexillum colonies are probably a major accelerator of the spread of this species (Bullard et al. 2007a,b; Morris and Carman 2012; Reinhardt et al. 2012). Indeed reattachment success and fragment viability, as reported in this and earlier (Morris and Carman 2012; Reinhardt et al. 2012) studies, are considered two important ecological traits responsible for D. vexillum’s invasive success.

This study provides the tools to follow colony survivorship, growth and patterns of substrate acquisition under controlled laboratory conditions, using various ramets of the same genotype and differing genotypes from a given population to reveal genetic components for key biological attributes. We documented that whole colonies, or fragments of larger colonies, can be gently and easily peeled off the substratum and transported to the laboratory, where they are cultured and propagated in line with the research needs.

The colonies of Didemnum vexillum cultured ex situ with the methodology described here were very suitable for a variety of scientific investigations and almost unlimited number of ramets can be established from any single genotype. While we used a short culturing period (up to 10 weeks), it is perceived that such cultures can be extended to the maximal lifespan of this species (unknown under either field or relaxed ex situ conditions). We successfully attached colonial fragments to substrates, observed the way colonies acquire new substrates, documented allorecognition assays with ramets of various genets, and monitored the outcomes.

This study shows that different Didemnum vexillum genotypes, upon tunic-to-tunic contacts, can form stable (at least on the morphological level) chimeras that displayed fast and differential growth patterns on the available substrates. It has long been considered that establishing intraspecific chimerism in sessile, sedentary colonial marine organisms may confer various ecological advantages, culminating in the formation of a ‘novel entity’ with a greater store of genetic variability and hence with a wider range of physiological qualities, characterized by reduced onset of reproduction, increased competitive capabilities, enhanced growth rates, reduced whole-colony mortality rates, synergistic complementation, and assurance of mate location when needed (Buss 1981; Grosberg 1988; Bishop and Sommerfeldt 1999; Rinkevich 2002; Rinkevich and Yankelevich 2004). This state of chimerism may therefore present adjustable genotypic combinations of organismal traits to alterable and capricious natural selection operations (Pineda-Krch and Lehtila 2004; Rinkevich 2004; Rinkevich and Yankelevich 2004), thus better withstanding harsh and/or fluctuating environmental conditions (Rinkevich 2004). It seems likely that chimerism is an important trait in the life history of D. vexillum, allowing this species to spread worldwide into different environmental regimes and be successful under various biological/physical conditions (McCarthy et al. 2007; Lenz et al. 2011; Smith et al. 2012). Chimerism is the norm for all aplousobranchs, especially didemnids (Bishop and Sommerfeldt 1999). Allorejection may or may not ensue depending on relatedness of the fused colonies or fragments (Ishii et al. 2008).

In order to improve our understanding of the traits controlling invasiveness of D. vexillum (Bullard and Whitlatch 2009), and to help predict its future invasive potentialities, there is a need to develop culture methodologies amenable for detailed and controlled ex situ studies. The ability to obtain D. vexillum ramets/genets on demand is crucial for elucidating relevant life history parameters and the natural variability of associated biological traits. In this context this study describes simple, low-tech inland culture conditions to which the animals responded well.
ex situ cultures of *Didemnum vexillum*

(by survived, attached to substrates and expended/ grew on artificial substrates) and presents the first steps in the development of *D. vexillum* genetically defined lines amenable for various research needs. It should be noted that colonies collected in the wild for division into fragments for laboratory cultures may be chimerae, made up of genetically diverse zooids. Thus, it may be also practical to use sexually produced zooids. Thus, combined with recently described methods for collecting and culturing *D. vexillum* larvae (Fletcher and Forrest 2011), we are now well placed to develop mass cultures of genetically defined lines of *D. vexillum* thereby opening up exciting new experimental possibilities. This will allow the establishment of focused management responses and efficient resource allocation for this pest species.

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