Global patterns of genetic diversity and geographical distribution in the marine protist morphospecies *Oxyrrhis marina*

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Abstract

Species' diversity and the related processes that drive it are fundamental to understanding patterns of biodiversity. There is a wealth of literature that quantifies the ranges of "large" organisms, and hence our understanding of the processes that determine macrospecies' distributions; by contrast, this is obviously a more challenging field for microorganisms, of which protists seem to be least studied. Despite this, there are two long debated views regarding protist distribution, proposing that protists display either ubiquity or moderate endemicity. *Oxyrrhis marina* is an ecologically important marine protist that is used widely as a model organism, and from the pattern of genetic divergence, likely consists of two species. However, patterns of diversity and the distribution of different species (or genetic lineages) are unknown on a global scale. In this thesis I use *Oxyrrhis* as a model protist to quantify the amount of genetic diversity that may exist between regions at a global scale and to determine whether there are any patterns between genetic diversity and geography. First, I assess current levels of diversity within the *O. marina* morphospecies. Morphological and molecular literature on *O. marina* was reviewed and the genus subsequently split into two species: *Oxyrrhis marina* and *Oxyrrhis maritima*. Second, genetic divergence between global samples of the *Oxyrrhis* genus was quantified using multiple gene phylogenies to determine levels of diversity and global patterns of distribution. The three genes COI (mitochondrial), 5.8S ITS and α-tubulin (nuclear) defined the two distinct lineages (*O. marina* and *O. maritima*); moreover, 5.8S ITS and α-tubulin uncovered further genetic diversity in strains that were predominantly from East Asian waters. The divergence between these strains and both *O. marina* and *O. maritima* is such that they may represent a new species, but further morphological and phylogenetic characterisation is required to support this. The lineages displayed contrasting patterns of distribution and abundance, one being broadly distributed and abundant and the other being geographically restricted and rare in comparison, seemingly supporting both sides of the protist distribution debate. These patterns were not exclusive (*i.e.* they overlapped) and require further sampling to draw more precise conclusions about the processes that led to their present distributions. This thesis has uncovered high levels of genetic diversity and contrasting distribution patterns displayed in a single genus; it is therefore clearly unrealistic to make generalisations about "protist biogeography" as they display a wide range of responses and distributions.

Chapter 1

Introduction

1.1 Diversity and distribution of organisms

Organisms' distributions vary in space and time, which gives rise to differences in community structure and genetic diversity. Biogeography is the study of the distribution of organisms in a spatial and temporal context, with the aim of revealing where they live, at what abundance and why (Avise, 1994; Martiny *et al*., 2006). The current distributions are as a result of both historical (past environment, vicariance) and present processes (dispersal, physiological/ecological tolerance). An organism's distribution is also closely linked to its diversity as mechanisms such as speciation, dispersal and extinction are often driven by similar processes affecting distribution.

For example, the Pleistocene climate changes, and in particular ice ages, considerably altered species ranges and for well studied European and North American fauna there are well defined centres of refugia and patterns of colonisation following the last glacial maxim (Avise, 1992; Hewitt, 1999; Taberlet *et al*., 1998). Such patterns become less clear for microorganisms as their small size and high abundance makes many aspects of their existence difficult to assess; species numbers are at best an estimate and much of the microbial world remains undescribed. Perhaps the least studied microorganisms are the protists (Caron, 2009).

1.2 Protist biogeography

Protist biogeography is an ongoing subject of heated debate, proposing two contrasting views, both with ostensibly credible arguments and supporting evidence; (1) the ubiquitous distribution model and (2) the moderate endemicity model. The ubiquitous distribution model states that microorganisms do not display biogeographies; their small size and abundance means they are not restricted by barriers that would affect larger organisms, giving them little risk of extinction and the potential to disperse eerywhere (Fenchel, 1993; Finlay, 2002; Finlay & Clarke, 1999; Finlay & Fenchel, 2004). This ubiquitous "seedbank" and the conditions of the local environment determine the presence of a species in a particular habitat. The model proposes that microorganisms have high levels of gene flow, which leads to a

low species number as well as a lack of distribution patterns. By contrast the "moderate endemicity model" (Foissner, 2006, 2008) proposes that the geographic patterns of protists and other microorganisms are intrinsically similar to those of macroorganisms. Despite their small size and abundance, they can still be subject to barriers that affect their dispersal.

Elements of both sides of the debate also tackle mechanisms about species divergence and speciation. In relation to species diversity, the ubiquity school argues that protists' abundance and the lack of barriers to dispersal provide little opportunity for allopatric speciation, giving rise to low species richness (Fenchel, 1993; Finlay, 2002). However, the moderate endemicity school asserts that it was not just small size and abundance but that many microorganisms have a high phylogenetic age, giving them much more time to disperse as well as to accumulate a high level of genetic diversity (Foissner, 2006). In light of the evidence for diversity and spatial distribution patterns, and the fact that much of the protist world remains undersampled and undescribed (Foissner, 2006; Foissner *et al.*, 2002), the claims of the ubiquity model seem very generalised for such a diverse group of organisms (Figure 1.1). It seems more reasonable to view this as one extreme of a continuum, along which dispersal rates and gene flow varies, leading to a situation at the opposite end where gene flow is very restricted and divergence occurs over a relatively small spatial scale.

One environment in particular that lacks obvious barriers to dispersal to pelagic species is the marine environment. Despite its potential for high connectivity, there is much increasing evidence of cryptic species and endemism in marine species (Boenigk *et al.*, 2006; Gentekaki & Lynn, 2009; Slapeta *et al.*, 2006; Stern *et al.*, 2010). There are many aspects of the marine environment that can act as barriers to microorganisms and limit their dispersal, *e.g.* currents, salinity, temperature, pH (Gachter & Weisse, 2006; Lowe *et al*., 2005; Montagnes & Weisse, 2000; Tatebe *et al*., 2010; Weisse & Montagnes, 1998; Weisse *et al*., 2007). Protist dispersal rates and gene flow likely vary in relation to these barriers, supporting the view that where these barriers limit gene flow, there is greater scope for species diversity and that they can indeed display biogeographies (Foissner, 2006).

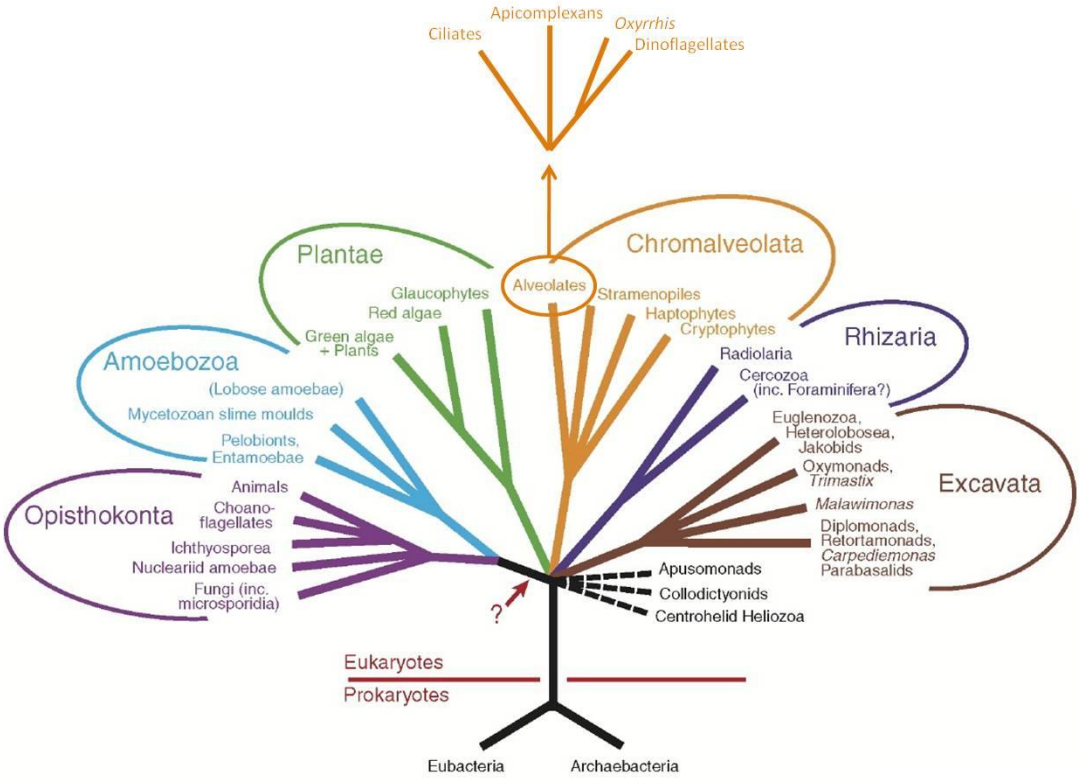

Figure 1.1. A diagrammatic tree representing the organisation of most eukaryotes into 6 major groups, which consists predominantly of protists. Figure is from Simpson & Roger (2004) and adapted to indicate groups within Alveolates including *Oxyrrhis*.

1.3 *Oxyrrhis marina*, a model organism

Oxyrrhis marina Dujardin (Figure 2.1) is an ecologically important marine protist that is widely abundant in coastal environments (Figure 3.1; Droop, 1959; Watts *et al*., 2011). It is easily cultured under laboratory conditions (Lowe *et al*., 2011b) and has been used as a model species in many studies on grazing rates and growth rates, synecology, autecology, phylogenetic analysis and also for alveolate evolution (Fast *et al.*, 2002; Jeong *et al.*, 2008; Kimmance *et al.*, 2006; Lowe *et al.*, 2005; Slamovits & Keeling, 2008; Slamovits *et al.*, 2007; Zhang *et al.*, 2007). *Oxyrrhis marina* possesses several unusual morphological and genomic characteristics for a dinoflagellate (see Chapter 2) and as a result there has been considerable debate about where to place *O. marina* within alveolates (see Figure 1.1). It has been classed as a dinoflagellate based on morphology (Dodge, 1982), yet others have excluded it from this group as it lacks features specific to dinoflagellates (Leander & Keeling, 2004; Zhang *et al*., 2007). The most exclusive studies and recent

classification places *O. marina* as an ancestral dinoflagellate lineage (Hoppenrath & Leander, 2010; Slamovits *et al*., 2007).

1.4 Species diversity and distribution in *Oxyrrhis* sp.

Although many of these studies have focussed on the taxonomic position of *O. marina* relative to other alveolates very few have considered intraspecific variation. The few studies that have reported such comparisons focus on COI and 5.8S ITS regions of *O. marina* and they reveal high levels of genetic divergence that separates the strains into two divergent lineages (Lowe *et al*., 2005; Lowe *et al*., 2010b). This poses a problem for the many studies working on different strains of *O. marina*, as they could potentially be working on different species, and responses detailed in one species may not be applicable to another. Therefore this highlights the need to quantify the amount of divergence found in *O. marina* and to determine whether this morphospecies is a species complex.

In a distribution sense, the ~60 strains from these two divergent lineages displayed some spatial genetic structure and different geographic distributions between divergent lineages (Lowe *et al*., 2010b). These strains included several from a culture collection from USA, and 2 from East Asian waters, but were predominantly from European waters. Therefore phylogenetic and distributional patterns of *Oxyrrhis* are still unknown at a world wide scale. Thus, the questions remain: how many more lineages will be revealed with adequate global sampling, and do they have distinct ranges?

1.5 Thesis aims and outline

The main aims of this thesis are twofold; (1) to assess whether historical literature provides any indications of morphological variation in *Oxyrrhis* that may correlate to the two divergent lineages and to determine whether *Oxyrrhis* represents several species; (2) to determine whether further sampling at a global scale reveals additional cryptic species and whether these species have similar distributions and levels of interlineage diversity to previous studies.

The next two chapters will address the above aims. Chapter 2 provides a brief overview of the historical literature on *Oxyrrhis marina* with particular reference to

morphological studies which suggest possible variation in *Oxyrrhis* (the content of Chapter 2 forms part of the study Lowe *et al.*, 2011a, published in Appendix C). In relation to this Chapter 2 also summarises recent studies on genetic and molecular data and finally provides a case for two current species within the genus *Oxyrrhis*. It is important to quantify genetic diversity and to resolve species number before tackling the issue of what patterns of global biogeography are present (Chapter 3). Unrecognised species would risk drawing flawed conclusions about distribution patterns, since separate species could be grouped and this would mask subtle distribution patterns. Chapter 3 focuses on the phylogeny and biogeography of a global sample of strains of *O. marina*. Using multiple genes this chapter examines the phylogenetic patterns of global strains of *Oxyrrhis* and also infers possible evolutionary histories from its geographic distribution. In light of the decision in the preceding chapter to describe two current species of *Oxyrrhis*, the strains used in this study are treated as two separate species or lineages based on the phylogenies presented in this chapter and by (Lowe *et al*., 2010b). Chapter 4 provides a summary of the main issues in the thesis with reference to the above aims and provides direction for further studies.

Chapter 2

Morphological and phylogenetic studies assessing diversity in *Oxyrrhis marina*

2.1. Introduction

Oxyrrhis marina is an extensively studied protist within the alveolate taxon, commonly employed as a 'model' to examine a broad range of ecological, physiological and behavioural responses (Boakes *et al.*, 2011; Buskey *et al.*, 1998; Jeong *et al.*, 2008; Lowe *et al.*, 2005; Montagnes *et al.*, 2011b; Roberts *et al.*, 2011). It is an easily recognisable (see Figure 2.1) and apparently monospecific genus. A few early morphological studies point towards phenotypic variation in *Oxyrrhis* but remain rather unclear. However, recent work indicates that this taxon harbours extensive cryptic diversity: levels of genetic and physiological variation are potentially sufficient to suggest that *O. marina* represents more than one species (Lowe *et al*., 2010b). Isolates form two distinct lineages and it has been suggested that these represent two separate species (Cavalier-Smith & Chao, 2004; Lowe *et al*., 2005). This is alarming, as researchers around the world continue to isolate strains and run experiments on this "species". Different research groups employing different isolates of the "model" *O. marina* are potentially working on highly divergent strains or even different species.

Indeed, a review of the literature (see Lowe *et al.*, 2011a) indicates that ~160 studies have examined various aspects of *O. marina* biology and reveals that: (1) most studies examine a single strain; (2) many isolates are reported only once in the literature; and (3) most laboratories (research groups) work on only a single strain. Of the 38 *O. marina* isolates reported in the literature (see Watts *et al*., 2011), most are poorly characterised beyond their gross morphology. Consequently, the bulk of studies are not interpretable in a comparative context, and there are limited molecular, morphological or ultrastructural data to corroborate such diversity or aid the delineation of potentially multiple species in the genus. There is increasing evidence of cryptic speciation masked by gross morphology in other species of protists (Gentekaki & Lynn, 2009; Slapeta *et al*., 2006; Stern *et al*., 2010). Because of the potential for high gene flow, such diversity is often unexpected in such small and highly abundant organisms (see Chapter 3), thus highlighting the importance of

quantifying levels of diversity and assessing species status in separate taxa, especially for widely used "models" as *Oxyrrhis*.

To place current and future work in a taxonomic context, it is essential to primarily explore our current understanding of who *O. marina* is and the likely extent of diversity in this taxon. Specifically, it is of significant importance that species diversity is firstly assessed to avoid confounding any comparisons made in the following chapter on *Oxyrrhis* distribution patterns. The present chapter provides a brief overview of the morphological and phylogenetic literature that has defined the genus *Oxyrrhis*, highlighting in particular the historical and contemporary evidence for multiple species. Ultimately, this chapter indicates that despite extensive study, *Oxyrrhis marina* remains poorly characterised and most critically that *O. marina* actually represents more than one species, for which justification for reclassifying is provided.

2.2 Morphological studies of *Oxyrrhis marina*

Gross morphology. *Oxyrrhis marina* Dujardin (Dujardin, 1841) (Figure 2.1a, Table 2.1) was originally described as oblong, oval bodied, with pointed anterior, obliquely notched anteriorly, possessing "several" flagella protruding sideways from the notch centre. Diagnostic features were: colourless, sub-cylindrical, rough bodied cell, with rounded posterior, 0.05 long (no units, but remarks on magnification of the original figure indicate 44 µm long). The type location was the Mediterranean (likely on the French coast), and as was typical of protistan studies of the time, no type material was deposited.

The first main revision by Saville-Kent (Saville-Kent, 1880), provided further details (Figure 2.1b, Table 2.1), based on the literature and observations of isolates from Jersey (UK). The revision provided information on: two flagella, one extending and the other coiled within the oral aperture; swimming and feeding behaviour (*e.g.* the longitudinal flagellum being responsible for trapping prey, while the transverse flagellum pushes it into the oral cavity); division by transverse fission; an anterior contractile vacuole; and, in illustrations, a posterior ventral bulge (or tentacular lobe) within the posterior ventral depression.

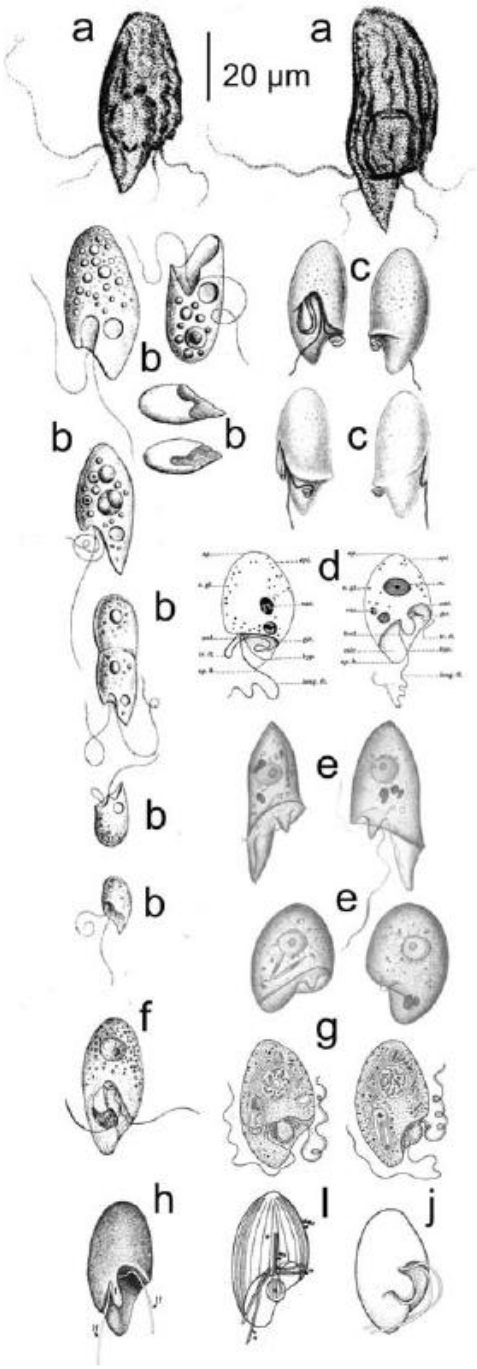

Figure 2.1. Illustrations of *Oxyrrhis marina* over the last 160 years: (a) the original description (Dujardin, 1841); (b) eight drawings by Saville Kent, including variation in size and division (1880); (c) four of many illustrations by Senn (Senn, 1911); (d) two illustrations from many provided by Hall (Hall, 1925); (e) four illustrations, indicating osmotic influence on cell size, by Diskus (Diskus, 1956); (f) an illustration from a guide to protozoa of Woods Hole (Calkins, 1902); (g) the two general illustrations presented in Dodge and Crawford (Dodge & Crawford, 1971a); (h) a simple schematic presented in Roberts (Roberts, 1985); (i) a schematic, indicating ultrastructure and microtubules (Brown *et al*., 1988); (j) a general illustration from Lowe *et al*. (Lowe *et al.*, 2011a). All illustrations presented to be associated with the scale bar.

Table 2.1. The designations, and naming authorities, for species in the genus *Oxyrrhis*.

Oxyrrhis	**Date**	**Length (µm)**	**Flagella**	**Shape**	**Location**
marina Dujardin	1841	44*	several	Oblong, oval bodied, rounded posteriorly	Mediterranean
marina Kent	1880	28-51	2	Body conical, subcylindrical, rounded posteriorly	St Helier, Jersey
phaeocysticola	1900	20	2	Rounded posterior, pointed anterior, excavated oral region with trunk-like projection.	Helgoland, Gemany
tentaculifera Conrad	1939	38	2	Twice as long as wide, has a tentacle	Belgium
maritima Van Meel	1969	16-24	2	More voluminous than *O. marina*	Belgium

* No units were provided in the original description – value is inferred by the author.

Several other older *O. marina* reviews exist. Senn (Senn, 1911) extensively reviewed the literature and provided new details (Figure 2.1c), indicating: no observable contractile vacuole; the flagella insert on either side of the ventral bulge; *O. marina* was a dinoflagellate, possibly related to *Gymnodinium*; and there is only one species of *Oxyrrhis*. Of the older literature Kofoid and Swezy (Kofoid & Swezy, 1921) seem to provide the best synthesis and most rigorous diagnosis of the genus and species, also supporting the notion that there is only one species of *Oxyrrhis*.

However, three other free-living *Oxyrrhis* species have been described: *O. phaeocysticola* (Scherffel, 1900) *O. tentaculifera* (Conrad, 1939) and *O. maritima* (Van Meel, 1969) (Figure 2.2, Table 2.1). *Oxyrrhis phaeocysticola* (Figure 2.2a) was distinguished as *Oxyrrhis*-shaped, including possessing a ventral bulge, but its swimming pattern was flagella first, in contrast to *O. marina* , which swims with the flagella in the posterior (Scherffel, 1900) *Oxyrrhis maritima* (Figure 2.2b) and *O. tentaculifera* (Figure 2.2c) were both isolated from Belgian coastal waters. *Oxyrrhis maritima* was ambiguously distinguished as larger and rounder than *O. marina*, while

O. tentaculifera was defined as possessing a long tentacle (probably a longer version of the ventral bulge indicated above), extending from the notch, but otherwise it was superficially similar to *O. marina*. *Oxyrrhis phaeocysticola* was moved to the genus *Hemistasia* (Elbrächter *et al*., 1996) thus creating the new combination *Hemistasia phaeocystidicola*. *Oxyrrhis tentaculifera* and *O. maritima* were synonymised with *O. marina* by Dodge (Dodge, 1982), whose reasoning was that as *O. marina* exhibits considerable morphological variation, these two species were insufficiently different from *O. marina*. The description by Conrad (Conrad, 1939) of *O. tenticulifera* seems sufficiently distinct (particularly the presence of a conspicuous, long tentacle) to stand as a distinct species, though the lack of corroborating observations of this morphotype limits further consideration. Regardless, the question of multiple *Oxyrrhis* species has not been revisited until relatively recently (see below); thus the literature suggests that the current genus *Oxyrrhis* contains only the original species, *O. marina*.

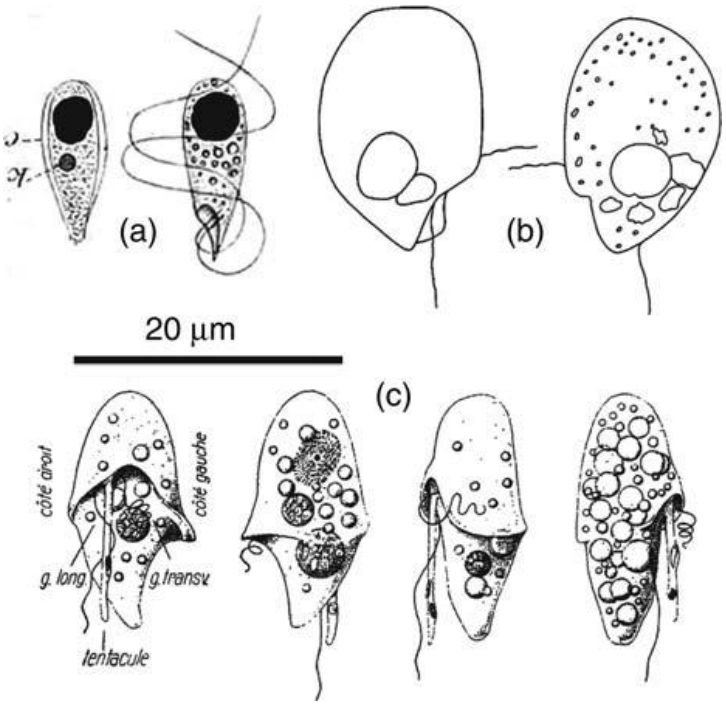

Figure 2.2. Illustrations of the other three species described in the genus *Oxyrrhis*: (a) *O. phaeocysticola* Scherffel (Scherffel, 1900), moved to *Hemistasia phaeocystidicola* (Scherffel) comb. nov. (Elbrächter *et al*., 1996); (b) *O. maritima* van Meel (Van Meel, 1969); (c) *O. tentaculifera* Conrad (Conrad, 1939). Scale bar applies to all illustrations.

Other observations of gross morphology are distributed throughout the literature (Figure 2.1). For example, isolates of *O. marina* collected from Venice were illustrated with a ventral bulge and were noted to vary extensively in cell size and shape in response to a range of osmotic conditions (Diskus, 1956; Figure 2.1e). Cell shape and size appear to be highly variable in *O. marina*: Triemer (Triemer, 1982) noted changes in shape following food ingestion; concurrently, in my own experience of culturing large numbers of *O. marina* isolates, variation in size occurs depending on food concentration and culture status (see also Kimmance *et al*., 2006). Furthermore, recent observations on clonal isolates collected across Europe suggest clone-specific variation in cell size (unpublished data), though whether these differences are systematic and correlated with phylogenetic identity is not yet clear.

Oxyrrhis *- an unusual dinoflagellate*

As noted above, early studies recognised *O. marina* to be a dinoflagellate, though a somewhat unusual one. Virtually all of the subsequent morphological and ultrastructural work has focused on providing data to characterise *O. marina* and to assess its affinity within the alveolates (*i.e*. the taxonomic group that contains the dinoflagellates, apicomplexans and ciliates; Hausmann *et al.*, 2003). In fact very few of these studies even consider making comparisons within *O. marina*.

Oxyrrhis marina possesses several morphological features that differ from those of dinoflagellates such as the structure and function of flagellar apparatus (Cachon *et al*., 1994; Cachon *et al*., 1988; Dodge & Crawford, 1971b; Godart *et al*., 1992; Roberts, 1985; Roberts & Roberts, 1991), flagellar and body scales (Clarke & Pennick, 1972, 1976), the microtubular cytoskeleton structure (Roberts *et al.*, 1993) and the nuclear structure (Cachon *et al.*, 1979; Gao & Li, 1986; Hall, 1925; Kato *et al.*, 1997; but see Slamovits & Keeling, 2011; Triemer, 1982). It is implied that these features are consistent across the taxon, but observations have not been made in a comparative context. Most of these studies use a single strain, or do not report which isolate was used. Only a small number of studies use multiple strains, and even then the most recorded was 6 (5 from UK, 1 unknown location) in Dodge and Crawford (Dodge & Crawford, 1971a, b).

The ventral bulge or tentacle seems to be the only morphological feature that varies within *Oxyrrhis*. This medial, ventral structure is well documented in the earlier literature and its length was used to diagnose *O. tentaculifera* (Figure 2.2c). In *O. marina* it is relatively small (~5 µm), is constricted proximally and is located below the horizontal ridge (Dodge & Crawford, 1971a, b). The microtubular structural differences between *Oxyrrhis* and dinoflagellates make it difficult to assess what this ventral bulge structure is. It has been suggested to be a reduced hyposome (Brown *et al*., 1988), but the lack of associated transverse microtubules in *Oxyrrhis* prevents comparison to the same structures in other dinoflagellates; if the microtubule structures were homologous across taxa, it would suggest that the ventral bulge is not a hyposome (Roberts *et al*., 1993).

2.3 Contemporary evidence for cryptic *Oxyrrhis* species

A striking conclusion that arises from the morphological literature is that despite early descriptions of multiple *Oxyrrhis* species, most studies accept the opinions of Kofoid and Sweezy (Kofoid & Swezy, 1921) and Dodge (Dodge, 1982) that only the single species of *O. marina* exists. More recently *O. marina* has been examined in various molecular phylogenetic studies, including taxonomic studies using multiple gene phylogenies (Leander & Keeling, 2004; Lenaers *et al*., 1991; Saldarriaga *et al*., 2003; Slamovits *et al*., 2007) and assessing mitochondrial genome structure (Slamovits *et al*., 2007; Zhang & Lin, 2008), but the focus of these, similar to that of most morphological studies are also to resolve the *Oxyrrhis* position in relation to alveolates and the dinoflagellates. As a result the taxonomic and phylogenetic affiliations of *O. marina* relative to alveolates are well described, in contrast to our understanding of genetic, physiological, and morphological variability within *O. marina* which remains limited. Indeed, while early morphological studies argue for multiple *Oxyrrhis* species, assessments of variability between different *O. marina* strains and isolates are rare. Given the increasing number of examples of cryptic diversity in a broad range of free-living protist taxa (*e.g.* Darling *et al.*, 2004; Slapeta *et al.*, 2005), this lack of study represents an important oversight. In fact, recent studies of *O. marina* suggest that high levels of genetic diversity occur within the current *O. marina* morphospecies (Cavalier-Smith & Chao, 2004; Lowe *et al*., 2005; Lowe *et al*., 2010b). The following section examines assessments of variability within *O. marina*, highlights that current observations of morphological and

cytological variation are scarce, and indicates that genetic studies reveal extensive diversity. Based on the strength of the molecular phylogenetic data, two *Oxyrrhis* species are proposed- *O. marina* and *O. maritima* - for which new diagnoses are provided (the existence of a third species, *O. tenticulifera*, is also discussed below). Ultimately, this re-designation reflects the extent of diversity within the genus and provides an important framework to direct future comparative morphological, physiological, and genetic studies.

2.4 Combining morphological and molecular data.

Six studies have examined variation between *O. marina* isolates; of the morphological and cytological studies, only Dodge and Crawford (Dodge & Crawford, 1971a, b), Clarke and Pennick (Clarke & Pennick, 1972, 1976) and Roberts (Roberts, 1985) compared *O. marina* isolates, based on cell structure, scales and flagellar structure, none of which noted variation. The most extensive assessments of diversity within *O. marina* are phylogenetic, though these too are limited. Three studies have quantified the level of genetic variation between *O. marina* isolates based on a single gene (rDNA) and a small number of isolates (n = 2, 3, 11, for Cavalier-Smith & Chao, 2004; Lowe *et al*., 2005 respectively; Saldarriaga *et al*., 2003). These studies indicate: (1) an exceptionally high level of divergence in the basal *O. marina* branch (Saldarriaga *et al*., 2003) and (2) two divergent lineages that have been proposed as separate species (Cavalier-Smith & Chao, 2004; Lowe *et al*., 2005; Lowe *et al*., 2010b). Following this, a recent assessment of diversity within *O. marina* examined 5.8S ITS rDNA and mitochondrial cytochrome c oxidase I COI) in 58 *O. marina* isolates; this work supported two highly divergent lineages, each composed of two distinct clades (Figure 2.3; Lowe *et al*., 2010b). Based on the COI gene, sequence divergence between lineages was 10.5 % (within lineage divergence was <1% in both cases). Mitochondrial COI sequences in particular are now commonly used to aid species delineations across a broad range of organisms including protists (Evans *et al.*, 2009; Evans *et al.*, 2005; Frezal & Leblois, 2008; Hebert *et al.*, 2003; Lin *et al.*, 2009; Sites & Marshall, 2003). For example, 3 - 11% divergence for the COI gene has been used to delineate species across a range of protist taxa (*e.g.* Chantangsi *et al*., 2007; Evans *et al*., 2005; Gentekaki & Lynn, 2009). Comparisons of these divergence estimates strongly support the occurrence of two *Oxyrrhis* species.

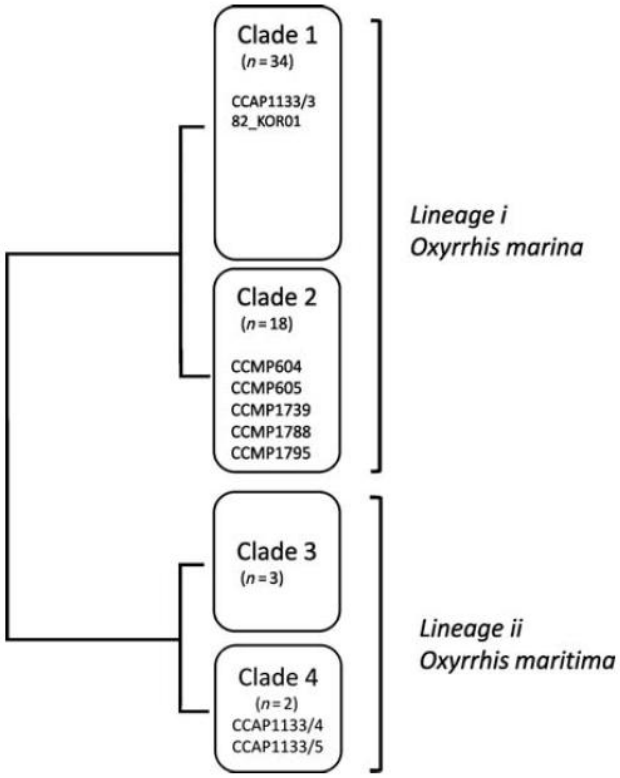

Figure 2.3. Cladogram (redrawn from Lowe *et al*., 2010b) of the four *Oxyrrhis* clades defined based on 5.8S ITS rDNA and mitochondrial COI sequence data. Representations of the four clades are scaled according to the number of isolates known to belong to each clade. Indicated are the proposed species names for the two *Oxyrrhis* lineages and the most commonly used *Oxyrrhis* strains for which affiliations are known (CCAP and CCMP indicate the source culture collection: CCAP—Culture Collection of Algae and Protozoa, Dunstaffnage, UK; CCMP—Provasoli—Guillard National Center for Culture of Marine Phytoplankton,West Boothbay Harbour, ME, USA).

Oxyrrhis marina *is more than one species.*

Based on the molecular evidence detailed above, two *Oxyrrhis* species are proposed: *O. marina* and *O. maritima* (see Lowe *et al.*, 2011a for full diagnoses). Following recommendations by Foissner *et al*. (Foissner *et al*., 2002), that previously employed species names be used, the synonymised specific epithet *O. maritima* is resurrected to denote the second *Oxyrrhis* species.

A third species, *O. tentaculifera*, may also occur. As noted, the description of *O. tentaculifera* (Conrad, 1939) seems sufficiently distinct to stand as a separate species – though contemporary observations and DNA sequence data for this species are

clearly required to support its existence and assess its precise relationship to the two other *Oxyrrhis* species.

2.5 Reasoning for diagnosis of three species of *Oxyrrhis*

High levels of cryptic diversity, as highlighted here for *Oxyrrhis*, are now documented for many free-living protist taxa. Such variation raises important questions – is extensive genetic variation paralleled by functional diversity, and does this need to be accounted for in evaluations of physiological responses and ecological interactions? Clearly, the use of experimentally tractable model organisms, such as *O. marina*, is an important strategy to address these questions; however, failure to recognise the sources and extent of cryptic variation in these organisms is plainly problematic.

For *Oxyrrhis*, the designation of two species highlights for future studies that: (1) a more cautious approach must to be taken in selecting and characterising *Oxyrrhis* isolates for experimental study (*i.e.* it is inappropriate to report assessments concerning poorly characterised isolates) and (2) comparative studies of multiple isolates are required to assess individual, population and species level variation in the *Oxyrrhis* genus. Such recommendations are obviously relevant to all protist species and it should now be exceptionally clear that new species designations should include morphological and genetic data, and where possible examination of multiple isolates to assess variability.

The reasoning for the designation of Lineage *i* and Lineage *ii* (Figure 2.3; Lowe *et al*., 2010b) as *O. marina*, and *O. maritima*, respectively, follows (summarised in Table 2.2). As indicated above, there are no morphological data to tie the proposed molecular-based species to the original description of *O. marina*, nor does type location (*i.e.* coastal Mediterranean, France) provide a criterion to assign species names: representatives from all clades occur in the French Mediterranean (see Chapter 3, Figure 3.1; Lowe *et al*., 2010b; Lowe, unpublished data). The original description of *O. maritima* as larger and rounder than *O. marina* offers a potential distinguishing morphological characteristic; however, observations to date (personal observation) do not suggest a difference in cell size between *Oxyrrhis* lineages. Furthermore, the small amount of work on recognising ecophysiological differences

between isolates (Lowe *et al*., 2005) offers no guidance on defining "ecotypes". Therefore, species are designated based on the least disruptive classification, using occupied names of junior synonyms. In this respect, there are a range of criteria that suggest that *O. marina* should be represented by Lineage *i* (*i.e.* it is the most prevalent, has the widest distribution, and has the highest number of confirmed isolates and therefore changing its name would be most disruptive; Table 2.2); the overriding reason, however, is simple: there are only two well-studied (genetically identical; Lowe *et al*., 2010b) isolates of *Oxyrhis* that are available from commercial culture collections in Lineage *ii*, while there are six genetically distinct, well-studied, commercially available isolates in Lineage *i*. Thus, by assigning the specific epithet *maritima* to Lineage *ii*, both the need to reassign names to past work and any future confusion are minimised.

Table 2.2. Criteria for the re-designation of species names in the genus *Oxyrrhis*. Bold text indicates the lineage from which the majority of isolates are used in each criterion.

Criteria for species assignment to *Oxyrrhis* lineages	**Lineage *i***	**Lineage *ii***	**Citation**
Environmental prevalence (*i.e.* occurrence in the ~150 samples that have been collected by us, to date	**83**	17	unpublished data
Global breadth of distribution	**broad**	narrow	Chapter 3 Watts *et al.* (2011)
History of study (years), based on isolation date of commercial cultures	<20	**>50**	Lowe *et al*. (2011)
Citations for the single most studied strain in each lineage	6	**17**	Lowe *et al*. (2011)
Citations for all stains within each lineage	**25**	21	Lowe *et al*. 2011)
Number of confirmed isolates within each lineage	**52**	5	Lowe *et al.* (2010, 2011)
Proposed species designation	*marina*	*maritima*	

2.6 Conclusions

These species designations are based on moleular data only, as despite extensive morphological and ultrastructural observations, such comparative studies of multiple *Oxyrrhis* isolates are virtually absent from the literature. Clearly then there is scope to re-visit, in a comparative context, many morphological studies conducted on single *O. marina* isolates to better define the extent of diversity within the genus. Based on the morphological literature mentioned above, studies of flagellar scales, tentacular structure and size, cyst formation and potentially flagellar rootlet structure may be fruitful directions for such work.

In a broader context, general understanding of the ecological and evolutionary processes that drive patterns of diversity and speciation in free-living protists as a whole remains poor. Model protists such as *O. marina* and *O. maritima* for which an extensive pool of variation is beginning to be recognised and characterised, present ideal opportunities to unravel these fundamental processes. As species diversity and the processes that drive it are closely linked to distribution patterns, which also remain poorly understood in protists, I continue this study in a geographic sense, assessing global diversity and distribution patterns in *O. marina* and *O. maritima* (Chapter 3).

Chapter 3

Contrasting patterns of genetic differentiation and biogeography in globally distributed samples of the *Oxyrrhis* genus.

3.1. Introduction

Protist dispersal and gene flow have proved to be a rather contentious issue over the past two decades, with opposing views debating whether small (<1 mm) organisms, in particular protists, either show ubiquitous (*i.e*. tending towards cosmopolitism) or limited geographical distributions (*i.e*. endemism). The ubiquity model states that protists exist everywhere as a "seedbank" and it is the local environment that determines their presence or absence in a particular location (Fenchel, 1993; Finlay, 2002; Finlay & Clarke, 1999; Finlay & Fenchel, 2004). One corollary of small size and high abundance is that protists are easily dispersed and less likely to become extinct, giving rise to the idea that they do not experience restrictions to dispersal, colonisation and population persistence in the way macroorganisms do. This leads to the idea that gene flow is high, which generally limits the amount of genetic differentiation among populations; hence rates of allopatric speciation are dampened and protist species tend to be cosmopolitan in distribution. In contrast the moderate endemicity model argues that despite their relatively high abundance, protists are affected by barriers to their dispersal, resulting in lower gene flow, and the potential for genetic divergence, within and between areas, and high species diversity (Foissner, 2006, 2008).

Of course, given the diversity of taxa encompassed by the term "protist" it is highly unlikely that either model can fully describe the distribution patterns of all protist species; they span such a huge range of sizes and include an array of morphologies, life histories, environmental tolerances and behaviours (Caron, 2009). Different protist species with different environmental tolerances will contribute to these varying distribution patterns. Nonetheless, there is increasing evidence of cryptic species and endemism in protists (Boenigk *et al*., 2006; Chen & Hare, 2008; Knowlton, 2000; Slapeta *et al*., 2006; Westheide & Schmidt, 2003) seem to support the moderate endemicity model. In fact the current distribution of protists is most likely determined by interplay of both present processes as above and historical processes, such as historical contingency and vicariance.

In freshwater environments, genetic differentiation among protist populations has been reported (*e.g.* Evans *et al*., 2009; Katz *et al*., 2005) together with evidence for high gene flow in some species (Gentekaki & Lynn, 2009; Katz *et al*., 2005). Somewhat surprisingly, in the marine environment where barriers to dispersal are less obvious for pelagic protists, there are also contrasting patterns of genetic divergence among populations of protists (*e.g.* Darling *et al.*, 2004; Lowe *et al.*, 2005; Lowe *et al.*, 2011a; Lowe *et al.*, 2010b) and a lack of differentiation (Katz *et al*., 2005; LaJeunesse, 2001; Pawlowski *et al*., 2007) can be identified. These contrasting patterns emphasise the role for climatic and ecological factors in driving genetic divergence (*e.g.* see Darling *et al*., 2004; Katz *et al*., 2005).

The *Oxyrrhis* genus is an ideal model protist as it is commonly found in coastal areas and displays an apparent global distribution. It contains several unusual characteristics (see Chapter 2) which make it a very interesting study organism and it has been used in a wide variety of studies including growth, grazing rates, ecology and ecophysiology studies (Fast *et al*., 2002; Jeong *et al*., 2008; Kimmance *et al*., 2006; Lowe *et al*., 2005; Slamovits & Keeling, 2008; Slamovits *et al*., 2007; Zhang *et al*., 2007). The *Oxyrrhis* morphospecies also contains high levels of genetic variation, with the two most genetically divergent lineages described as separate species, *O. marina* and *O. maritima* (Chapter 2; Lowe *et al.*, 2011a). These patterns of speciation and spatial genetic structure are based on samples predominantly from European waters and the level and pattern of genetic differentiation, both within and among these two species of *Oxyrrhis*, at a global scale is unknown. Therefore, this study aims to (1) determine whether increased sampling effort, both in terms of numbers and geographic coverage, will reveal further diversity within the *Oxyrrhis* morphospecies and (2) assess whether these species tend towards ubiquitous or endemic biogeographic patterns.

3.2. Materials and Methods

Sample collection and maintenance

A total of 180 samples were collected from 155 locations throughout this taxon's known distribution (Figure 3.1; see Watts *et al*., 2011; see Appendix D). The sample sites were spatially distributed across North and South America, the Atlantic, Africa, Asia and Australasia (Figure 3.1). Most isolates were obtained *de novo* after

environmental sampling by collaborators (see Appendix D), predominately from intertidal habitats (see Table 3.1 and Figure 3.1 for full details). Environmental samples consisted of 15-100 ml of seawater with their positions geolocated by GPS or latitude/longitude (Table 3.1, Figure. 3.1). As samples were considered to have originated from the same location if they were collected from within 100 m of each other, there are multiple isolates from some locations (Table 3.1). The maximum number of replicates per site was 10, although at the majority of sites only one or two samples were collected.

Upon receipt environmental samples were inoculated with 5-20 ml of *Dunaliella primolecta* (at ~$5x10^5$ cells ml^{-1}) and left for ~4 days in natural sunlight to grow. Next, the samples were visually inspected using inverted compound and dissection microscopes. For samples that contained *O. marina*, cells were serially transferred using fine-drawn Pasteur pipettes, and mono-clonal cultures were ensured by three serial single cell isolations. All cultures were grown in artificial seawater at 32 PSU (Ultramarine Synthetica Sea Salt, Waterlife Research Industries Ltd., Middlesex, UK). A single clone was analysed for each sample location.

Of the total number of samples collected, 28 were positive (Table 3.1). These isolates of *Oxyrrhis* were analysed in this study in addition to 22 representative samples that have been characterised previously (Lowe *et al*., 2005; Lowe *et al*., 2010b). Five of the isolates were obtained from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, Bigelow Laboratory (CCMP), and one from the Culture Collection of Algae at the University of Texas, Austin (UTEX), along with an established culture of "*O. marina"* isolated from Korea (kindly donated by Dr H. J. Jeong, Seoul National University, Korea).

Table 3.1. Sample locations, and strain ID codes for isolates of *Oxyrrhis* used in this study, and including representatives from Lowe *et al*. (Lowe *et al*., 2010b) for which Genbank accession numbers are also included. *n* is the number of samples per site.

Accession No. COI/ITS/α-tubulin	Strain Ids	*n*	Location	Date collected	Lat (N)	Long (W)
	N&S America					
	1_HAW05	1	Hilo, Hawaii, USA	Mar09	19.7296	-155.0641
	1_MAD02, 03	2	Madison, Connecticut, USA	Aug09	41.2700	-72.6089
	1_MAS01	1	Martha's Vineyard, Massachusettes, USA	Jun09	41.4077	-70.538
	1_NLN01, 03	2	New London, Connecticut, USA	Aug09	41.3153	-72.0650
	1_SDG01 - 03	3	La Jolla, San Diego, USA	Jan10	32.8510	-117.2732
	55_BZL02, 05	2	Rio de Janeiro, Brazil	Aug09	-22.7380	-41.8737
	E. Atlantic					
	354_ICE02	1	Hvalnes, Iceland	Sep09	64.4047	-14.5457
FJ853703/FJ853679	351_AZO01	1	Sao Roque, Azores	Sep08	37.7320	-25.5510
FJ853696/FJ853670	351_AZO02	1	Mosteiros, Azores	Sep08	37.8965	-25.8234
	Africa					
	20_SHA01	1	Sharm el Sheik, Egypt	Oct09	27.9528	34.3872
	27_SAF01	1	Bantry Bay, Cape Town, S. Africa	Apr09	-33.9279	18.3754
	Asia					
	60_BOR03, 04	2	N. Borneo, Malaysia	Aug09	7.0151	116.7373
	60_BOR05	1	Sarawak, Borneo, Malaysia	Oct09	1.7536	110.3150
FJ853699/FJ853675	81_JAP01	1	Ishigaki Island, Okinawa, Japan	Oct08	24.3383	124.1533
	81_JAP02	1	Sata, Japan	Apr09	31.3273	130.8016
FJ853692/FJ853666	82_KOR01	1	Keum Estuary, Kunsan, Korea	May09	35.9800	126.7000
	86_CHN01, 04	2	Daya Bay, Shen Zhen, China	Apr09	22.5882	114.6364
	86_CHN05, 07	2	Qing Dao, China	Jun09	36.0616	120.3184
	Oceania					
	61_AUS01, 02	2	Sydney, Australia	Apr09	-33.8534	151.1714
	61_AUS08	1	Sydney Harbour, Australia	May09	-33.8591	151.2220
	64_NZL05	1	Seatoun, Wellington, New Zealand	Oct09	-41.3186	174.8293
	UK & Ireland					
FJ853690/FJ853664	44_GLE01	1	Glenuig, Scotland, UK	Apr08	56.8370	-5.8337
FJ853700/FJ853676	44_PLY01	1	Plymouth, England, UK	Apr08	50.3632	-4.1391
FJ853685/FJ853659	353_GAL03	1	Carraroe, Galway, Ireland	Mar08	53.2504	-9.6240
	Europe					
FJ853684/FJ853658	45_BOG01	1	Bogense, Denmark	Aug08	55.5706	10.0841
FJ853688/FJ853662	351_FAR01	1	Faro, Algarve, Portugal	Jan08	37.0170	-7.9932
GQ487326/GQ487327	34_BAR01	1	Barcelona, Spain		41.2952	2.1241
FJ853697/FJ853671	39_NAP03	1	Rocce Verdi, Naples, Italy	Aug08	40.7972	14.1983
	39_NAP06	1	Rocce Verdi, Naples, Italy	Aug08	40.7972	14.1983
FJ853704/FJ853680	30_SUN01	1	Sounio, Greece	Oct08	37.6516	24.0285
FJ853698/FJ853674	30_NAX01	1	Agios Giorgio, Naxos, Greece	Nov08	37.0948	25.3738
FJ853694/FJ853668	356_MAL01	1	Bahar ic Caghaq, Malta	Oct08	35.9401	14.4565
FJ853701/FJ853677	30_POR01	1	Porto Rafti, Greece	Oct08	37.8731	24.0199
FJ853702/FJ853678	30_POS01	1	Posithonia, Greece	Oct08	37.6778	24.0524
	Culture Collection					
FJ853706/AY566416*	CCAP1133/5	1	Långskar, Tvärminne, Finland	1951	60.1705	21.2064
FJ853707/AY566413*	CCMP1739	1	Texas, USA	Jul93	27.8333	-97.1330
FJ853708/AY566412*	CCMP1788	1	St. Maarten, Caribbean, USA	May97	18.0280	-63.0530
FJ853709/AY566411*	CCMP1795	1	Groton, Connecticut, USA	Oct96	41.3100	-72.0716
FJ853710/AY566415*	CCMP604	1	San Juan Island, Washington, USA		48.5440	-123.0100
FJ853711/AY566414*	CCMP605	1	Fort Pierce, Florida, USA	Dec83	27.4323	-80.3100
	UTEX LB1974	1	La Jolla, California, USA		32.8675	-117.2588

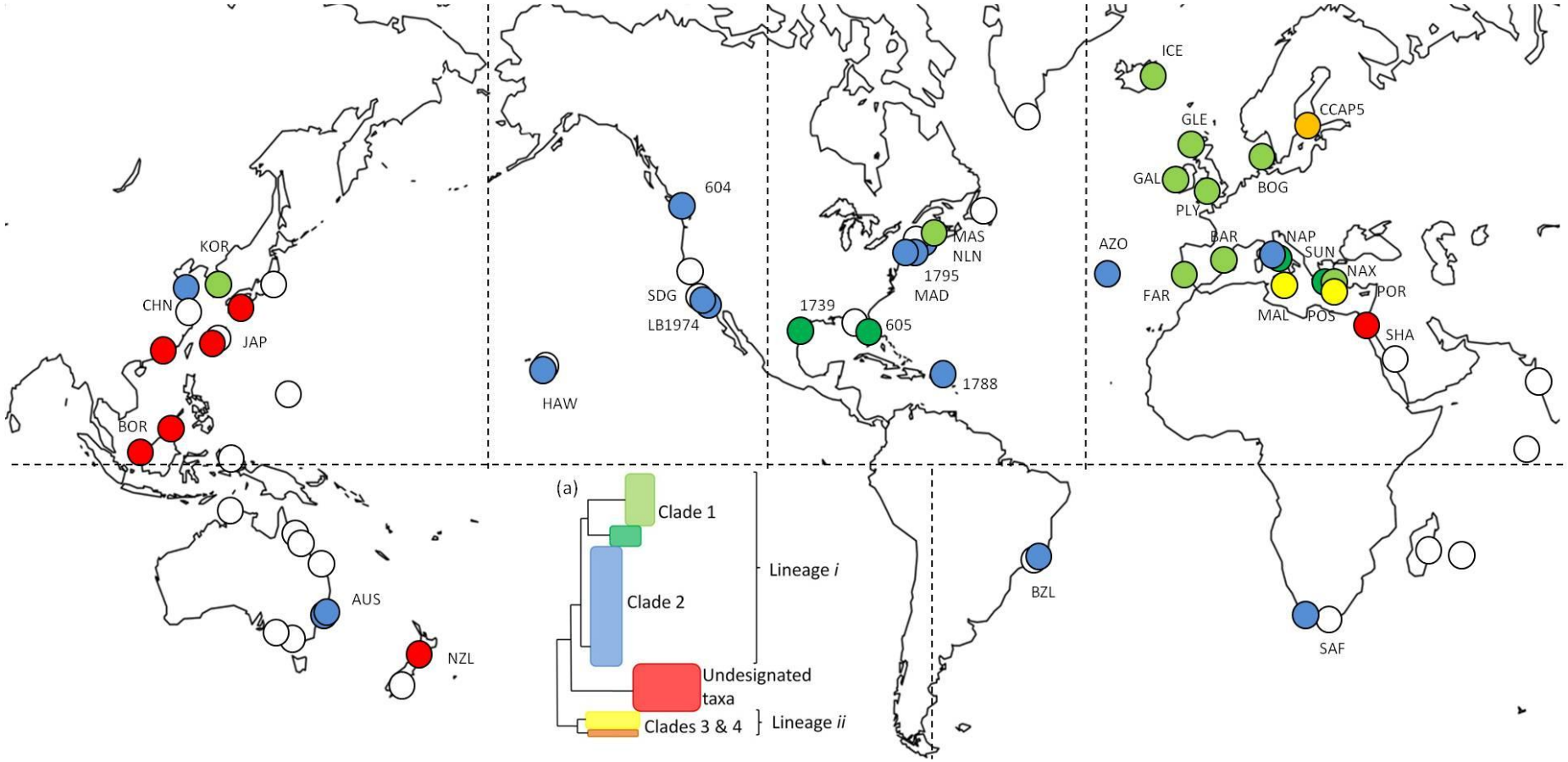

Figure 3.1. All global locations sampled for the presence of *Oxyrrhis*. Clear circles show sites that were sampled but which did not yield *Oxyrrhis*, Filled circles indicate the positive samples used in this study, and are coloured according to clade (see inset a).

DNA extraction, PCR and sequencing

DNA was extracted from samples by centrifuging 1.5 ml to 50 ml of cultures ($2x10^4$ – $2x10^5$ cells ml^{-1}) and treating the pellet with either Chelex-100 or a standard high-salt extraction method (Sambrook & Russell, 2001; Walsh *et al.*, 1991). Where the latter procedure was used, DNA concentration was estimated using a Nanodrop spectrophotometer (Nanodrop Technologies Inc).

Three gene regions were PCR-amplified and sequenced for phylogenetic analysis: (1) a 432 bp region of the 5.8S ITS rDNA region (5.8S ITS), (2) a 628 bp region of the mitochondrial cytochrome *c* oxidase I gene (COI), (3) a 510 bp region of α-tubulin. For PCR, ~1-20 ng of DNA (from high salt extractions), or 1-2 µl of Chelex prepared DNA was used; for all gene loci, each 10 µl PCR contained: 75 mM Tris-HCl pH 8.9, 20 mM $(NH_4)_2SO_4$, 0.01 % v/v Tween-20, 0.2 mM each dNTP, 1.5-3.0 mM $MgCl_2$, 2 pmol each primer and 0.25 U *Taq* polymerase (ABgene). Thermal cycling conditions on PTC-0221 Dyad thermocyclers (MJ research) for the 5.8S ITS region were: 95 °C for 3 min, 5 cycles of (95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s), 35 cycles of (92 °C for 30 s, 53 °C for 45 s, 72 °C for 55 s) and a final extension at 72 °C for 10 mins. Annealing temperatures for COI and α-tubulin were 53 °C and 57 °C respectively. To ensure consistency of the sequence data, a minimum of three independent PCR products for each gene locus were directly sequenced using BigDye™ v3.1 chemistry (Applied Biosystems), following the manufacturer's standard protocols, and capillary electrophoresis on an ABI3130*xl* genetic analyser.

Data analysis

Sequences were analysed using Sequence Analysis 5.2 (Applied Biosystems). Alignments were constructed using the DNA Star suite of software (DNAstar, Madison, WI, USA). 5.8S ITS fragments for CCAP and CCMP isolates had been sequenced previously (Lowe *et al.*, 2005) and are available in the NCBI database (Table 1). Following sequence quality trimming, 386 bp of the 5.8S ITS, 592 bp of the COI and 428 bp of the α-tubulin sequences were available for alignment (alignment lengths were 386, 592 and 428 bp, respectively). The α-tubulin and 5.8S ITS regions contained apparently heterozygous sequence, suggesting allelic variation or multiple gene copies. Generally most dinoflagellates are haploid, however the ploidy of *Oxyrrhis* is unknown (Montagnes *et al.*, 2011a; Sano & Kato, 2009). In

order to be conservative, ambiguous sites were removed from the data as they comprised a small percentage (3-4%) of the sequence.

Outgroups were included in the COI and α-tubulin alignments to root the phylogenetic trees: *Hetercapsa triquetra, H. rotundata, Amphidinium carterae,* and *A. operculatum* (Zhang *et al*., 2007) and *Perkinsus marinus, Amphidinium herdmanii, Heterocapsa triquetra,*and *H. rotundata* (Saldarriaga *et al*., 2003); (see Table 3.1 for accession numbers). The level of genetic variation among species in the 5.8S ITS region was too high to include these species as outgroups (and is therefore presented as an unrooted tree, Appendix A). An alignment of three genes was concatenated to analyse all sequence data simultaneously.

Bayesian inference was carried out using MrBayes v3.1.1 (Ronquist & Huelsenbeck, 2003). A generalised time reversible model and gamma was used for all gene alignments, where the burnin = 0.25 of the samples and the samplefreq = 100. Runs consisted of: $1.5x10^6$ generations for α-tubulin, $1x10^6$ generations for COI, $1x10^6$ for 5.8S ITS and $2.5x10^6$ for the concatenated alignment. MrBayes was allowed to optimise model parameters based on codon-based data partition.

Phylogeographic analyses

Phylogeographic analyses were carried out on Lineages *i* and *ii* separately as they have been defined as two species (Chapter 2; Lowe *et al.*, 2011a) using ARLEQUIN ver.3.5.1.2 (Excoffier & Lischer, 2010). Strain locations were grouped into 6 major geographic regions and 12 sub-regions, based on the location of known barriers to dispersal for other taxa, defined marine biogeographic realms and major ocean currents (see Figure 3.2 inset; data taken from Schwaninger, 2008; Spalding *et al*., 2007). Analysis of molecular variance (AMOVA) was used to characterise the population genetic structure for these groups in a geographical context. Standard molecular diversity indices such as haplotype diversity (*h*), number of polymorphic sites (*S*) and nucleotide diversity (π) were calculated for each of these regions (Table 3.2).

To detect any variation in population demography (*i.e*. expansion or decline) among areas, the following statistics were calculated (1) Tajima's *D* (Tajima, 1989) (2) Fu's

F_s (Fu & Li, 1993) and (3) performed a mismatch analysis of mtDNA sequences (Rogers & Harpending, 1992; Schneider & Excoffier, 1999). Tajima's *D* and Fu's F_s were developed to detect deviations from neutral expectations of diversity, however significantly negative values of *D* or F_s are often taken to be indicative of population expansion. A mismatch analysis (a frequency distribution of pairwise differences among haplotypes) is based on the distinctive pattern of DNA sequence variation in populations that have rapidly expanded compared with those that have maintained a stable size. Stable populations are expected to have a bimodal or multimodal (ragged) distribution, while populations that have undergone a rapid-expansion in size will have a clear unimodal distribution of pairwise differences between haplotypes (Rogers & Harpending, 1992). All statistics were calculated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010) with a randomisation procedure used to test the significance of *D* and F_s, while for the mismatch analysis a generalised least-square approach was used to estimate the parameters associated with sudden population expansion (see Excoffier *et al.*, 2005; Schneider & Excoffier, 1999). For each sample, the validity of a model of sudden expansion is determined from the sum of squared deviations (SSD) between the observed and the expected mismatch distributions, and also by the raggedness index (Harpending, 1994) which takes larger values for multimodal distributions (*i.e*. stationary populations) than for unimodal distributions (*i.e.* expanding populations).

Haplotypes were also defined using Arlequin ver.3.5.1.2, and a minimum spanning network was generated based on the number of pairwise differences. Haplotype networks were constructed using Hapstar (Teacher & Griffiths, 2011). The 6 major geographic regions were colour coded and applied to the haplotype networks to visualise any patterns between genetic structure and geographic region.

3.3. Results

Phylogenetic structure of the genus Oxyrrhis

A broadly similar tree topology was produced by all three genes – cytochrome c oxidase I (COI), 5.8S internal transcribed spacer 1 and 2 rDNA (5.8S ITS) and α-tubulin – and also when these genes were concatenated, with most of the isolates separating into the same clades (Figures 3.2, 3.3, 3.4; see Appendix A for 5.8S ITS which could not be rooted to a suitable outgroup). Note however, that in the single-

gene phylogenies *n* = 2, 3 and 8 isolates failed to PCR-amplify and they are thus missing for α-tubulin, 5.8S ITS and COI respectively; hence, there are some structural differences between phylogenies. Nonetheless, in all phylogenies, isolates of *Oxyrrhis* were partitioned into the two previously defined lineages (*i* and *ii*, see Lowe *et al*., 2010b; and also defined as *O. marina* and *O. maritima* in Chapter 2 and Lowe *et al*., 2011a). Lineages *i* and *ii* could be further subdivided into 2 clades in each lineage (defined by Lowe *et al.*, 2010b as Clades 1 & 2 [Lineage *i*] and Clades 3 & 4 [Lineage *ii*]). However, additional sampling revealed a number of new, divergent strains within *Oxyrrhis*, which did not fall within Lineages *i* and *ii*; these are the "undesignated taxa" and they form a third "lineage". Interestingly, the 8 isolates that could not be placed in the COI phylogeny (due to PCR failure) all were from the new, undesignated taxa.

In all analyses (single genes and concatenated data) the isolates of Lineage *i* (*O. marina*) were apparently derived and separated into two clades (1 and 2), with little variation between isolates (sequence identities for COI, 5.8S ITS and α-tubulin respectively were 99.3, 86 and 88.5% between Clades 1 and 2), and which is consistent with previous work on this genus (Lowe *et al*., 2010b). The only inconsistency was a sub-group of four strains (two from the Caribbean and two from the Mediterranean Sea), which changed position between Clade 1 in the 5.8S ITS and concatenated phylogenies and Clade 2 in the COI and α-tubulin phylogenies (*c.f.* Figures 3.2 and 3.3 with Appendix A). There is some indication of spatial genetic structure in Clade 1, which predominantly represents samples from the North Atlantic and the Mediterranean; however Clade 2 is comprised of samples from all major geographic locations (Figure 3.2, 3.3, 3.4).

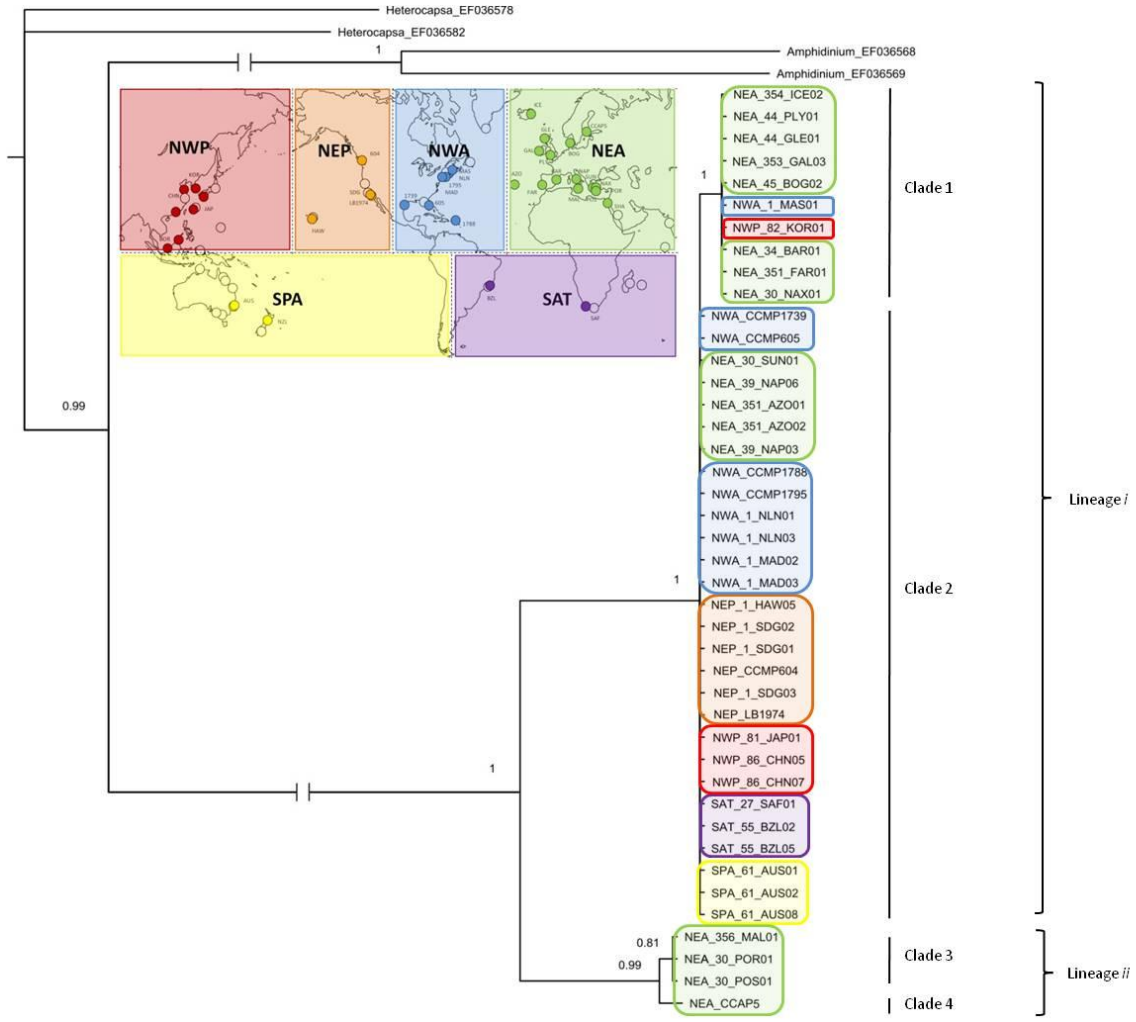

Figure 3.2. Maximum likelihood tree for 42 isolates of *Oxyrrhis* based on COI. Posterior probabilities are indicated at the nodes. Strains are coloured according to the 6 major geographic regions indicated in the inset: North West Pacific (NWP), North East Pacific (NEP), North West Atlantic (NWA), North East Atlantic (NEA), South Pacific (SPA) and South Atlantic (SAT).

Strains belonging to Lineage *ii* (*O. maritima*) and the undesignated taxa consistently separated into a position basal to *O. marina* and contained more genetic divergence between strains (sequence identities for COI, 5.8S ITS and α-tubulin respectively were 99.1, 77.6 and 87.6% between Clades 3 and 4; also for 5.8S ITS and α-tubulin between Clade 3 and undesignated taxa 64 and 88.6%, and between Clade 4 and undesignated taxa 65.1 and 89.5%). Clades 3 and 4 (Lineage *ii*) had limited distributions and were recorded only from the Baltic and Mediterranean Seas. The new undesignated group consists of a further 9 strains which were collected from the

North West Pacific, New Zealand coast and the Red Sea. The relationship between the undesignated taxa and Clades 3 and 4 (Lineage *ii*) is ambiguous (Figures 3.3, 3.4). Notably, both the α-tubulin and concatenated phylogenies place Lineage *ii* (*O. maritima*) and the undesignated taxa as basal to Lineage *i* (*O. marina*). However, there is disagreement about the branching structure within the basal group; the α-tubulin phylogeny infers the undesignated taxa are basal to Lineage *ii* strains, in contrast to the concatenated tree which suggests the opposite. The basal branches of the α-tubulin phylogeny are poorly resolved and posterior probabilities are weak (*i.e.* less than 0.9). Unfortunately, the concatenated phylogeny lacks COI information for the undesignated taxa, making it difficult to identify with any confidence the basal group. Therefore, for further spatial analysis the undesignated taxa and Lineage *ii* were analysed together as a group (see Figure 3.5).

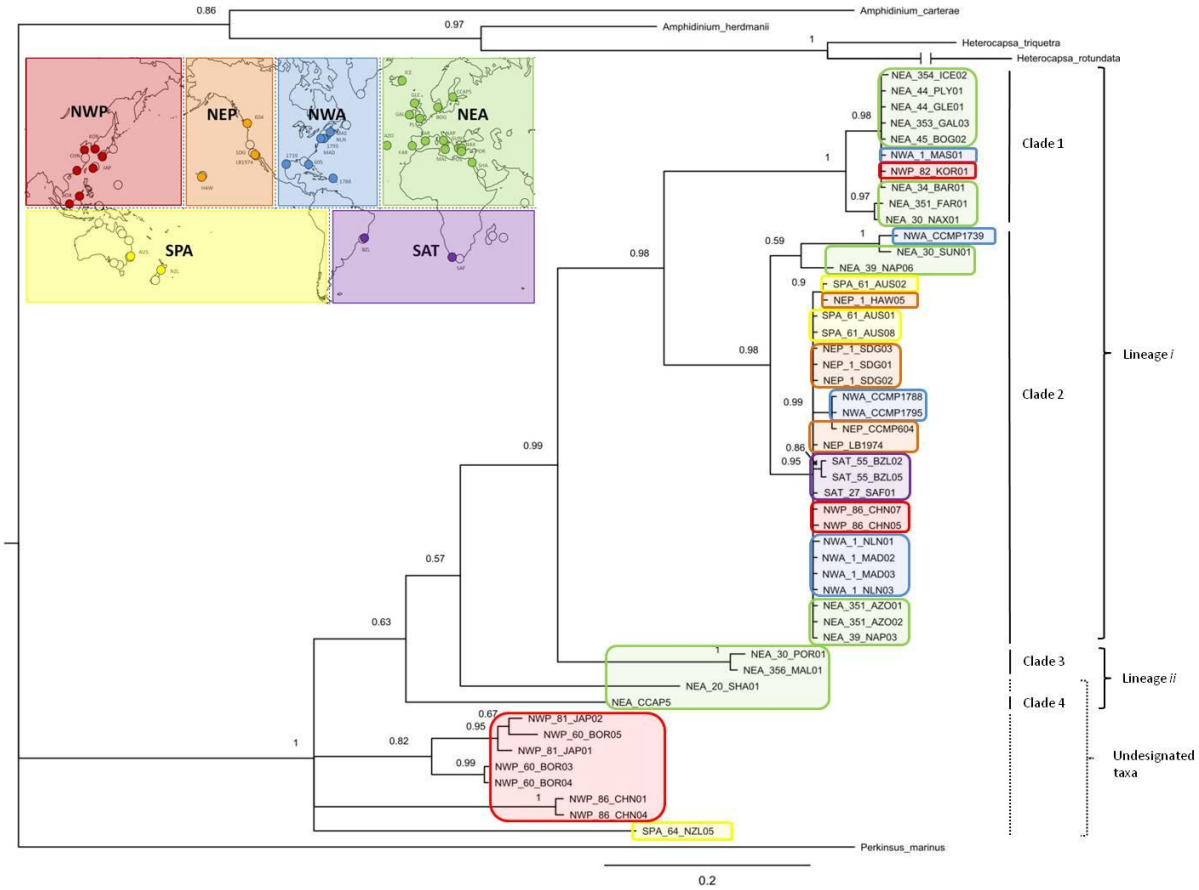

Figure 3.3. Maximum likelihood tree for 48 *Oxyrrhis* isolates, based on the α-tubulin gene.

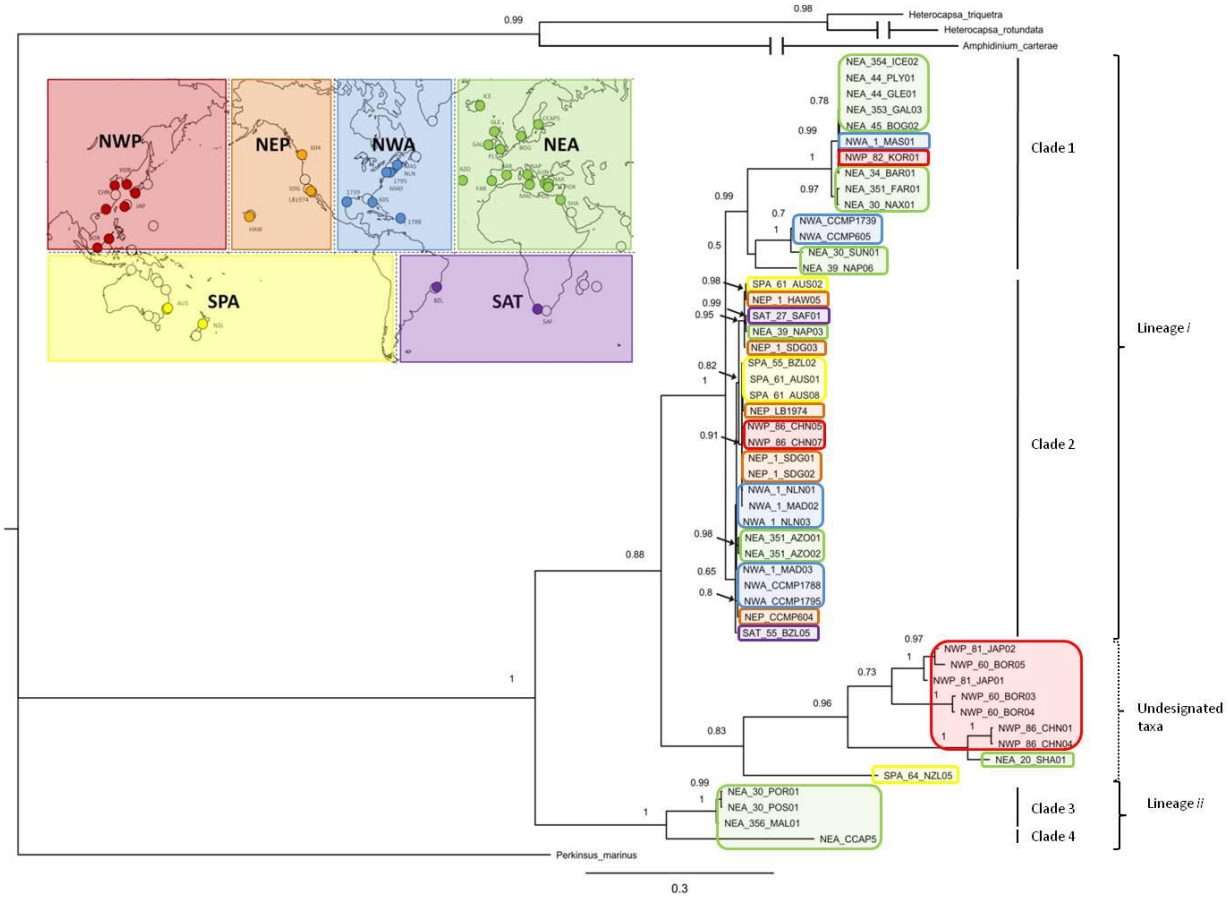

Figure 3.4. Phylogeny of the concatenated dataset for 3 gene regions, COI, 5.8S ITS and α-tubulin, for 50 isolates of *Oxyrrhis*.

Geographic distribution and spatial structure of lineages of Oxyrrhis

Oxyrrhis is widespread, and positive samples were found in most areas that were sampled, with most absences occurring on the east Australian coast and in the Indian Ocean (Figure 3.1). However, the distribution and abundance of the two lineages differed. No geographical clustering of haplotypes was evident in Lineage *i* (*O. marina*), which is abundant and geographically widespread, found throughout European waters and elsewhere in the Atlantic and Pacific Oceans (Figure 3.5). In contrast, Lineage *ii* (*O. maritima*) and the undesignated taxa are rare in comparison to Lineage *i* (only 8% of the total strains were Lineage *ii* and 18% are undesignated taxa) and also seem to be spatially restricted; Lineage *ii* was only isolated from the Mediterranean and the Baltic Seas, while the, as yet, undesignated taxa were present only in samples from the West Pacific and the Red Sea (Figures 3.3, 3.4, 3.5).

Table 3.2. Standard genetic diversity indicies for *Oxyrrhis marina* Lineages *i* and *ii* for three genes: α-tubulin, COI and 5.8S ITS. Number of strains (*n*), haplotype frequencies (*h*), number of polymorphic sites (*S*) and nucleotide diversity (π) at both regional and sub-regional levels. Sub-regional geographic groups were; UK and European waters (UKEU), Mediterranean (MED), East US coast (EUS), Caribbean (CAR), South American coast (SAM), South African coast (SAF), West US coast (WUS), North Asian waters (NAS), South Asian waters (SAS) and Australian waters (AUS). These were in turn grouped into the 6 major geographic regions (see Figure 3.2 for details)

α-tubulin Lineage *i*

Reg	*n*	*h*	*S*	π	Sub Reg	*n*	*h*	*S*	π
NEA	13	9	57	0.061 +/-0.032	**UKEU**	8	5	49	0.055 +/- 0.032
					MED	5	4	56	0.082 +/- 0.054
NWA	8	6	58	0.044 +/- 0.025	**EUS**	6	4	49	0.040 +/- 0.024
					CAR	2	2	27	0.063 +/- 0.064
SAT	3	3	1	0.002 +/- 0.003	**SAM**	2	2	0	0.000 +/- 0.000
					SAF	1	1	0	0.000 +/- 0.000
NEP	6	5	6	0.006 +/- 0.004	**WUS**	6	5	6	0.006 +/- 0.004
NWP	3	3	48	0.075 +/- 0.057	**NAS**	3	3	48	0.075 +/- 0.057
					SAS	0	-	-	-
SPA	3	3	3	0.005 +/- 0.004	**AUS**	3	3	3	0.005 +/- 0.004
α-tubulin Lineage *ii*									
NEA	4	4	71	0.104 +/-0.069	**UKEU**	1	1	0	0.000 +/- 0.000
					MED	3	3	56	0.087 +/- 0.066
NWA	0	-	-	-	**EUS**	0	-	-	-
					CAR	0	-	-	-
SAT	0	-	-	-	**SAM**	0	-	-	-
					SAF	0	-	-	-
NEP	0	-	-	-	**WUS**	0	-	-	-
NWP	7	6	62	0.074 +/- 0.042	**NAS**	4	4	56	0.087 +/- 0.058
					SAS	3	2	23	0.036 +/- 0.028
SPA	1	1	0	0.000 +/- 0.000	**AUS**	1	1	0	0.000 +/- 0.000

COI Lineage *i*

Reg	*n*	*h*	*S*	π	Sub Reg	*n*	*h*	*S*	π
NEA	13	4	4	0.004 +/- 0.002	**UKEU**	8	2	4	0.003 +/- 0.002
					MED	5	2	4	0.003 +/- 0.002
NWA	9	3	4	0.002 +/- 0.001	**EUS**	6	2	4	0.002 +/- 0.002
					CAR	3	1	0	0.000 +/- 0.000
SAT	3	2	0	0.000 +/- 0.000	**SAM**	2	1	0	0.000 +/- 0.000
					SAF	1	1	0	0.000 +/- 0.000
NEP	6	1	0	0.000 +/- 0.000	**WUS**	6	1	0	0.000 +/- 0.000
NWP	4	2	4	0.003 +/- 0.003	**NAS**	4	2	4	0.003 +/- 0.003
					SAS	0	-	-	-
SPA	3	1	0	0.000 +/- 0.000	**AUS**	3	1	0	0.000 +/- 0.000

Table 3.2. cont'd

COI Lineage *ii*									
NEA	4	2	6	0.005 +/- 0.004	**UKEU**	1	1	0	0.000 +/- 0.000
					MED	3	1	0	0.000 +/- 0.000
NWA	0	-	-	-	**EUS**	0	-	-	-
					CAR	0	-	-	-
SAT	0	-	-	-	**SAM**	0	-	-	-
					SAF	0	-	-	-
NEP	0	-	-	-	**WUS**	0	-	-	-
NWP	0	-	-	-	**NAS**	0	-	-	-
					SAS	0	-	-	-
SPA	0	-	-	-	**AUS**	0	-	-	-
ITS Lineage *i*									
Reg	***n***	***h***	***S***	π	**Sub Reg**	***n***	***h***	***S***	π
NEA	13	7	120	0.156 +/- 0.081	**UKEU**	8	3	95	0.111 +/- 0.062
					MED	5	4	119	0.181 +/- 0.111
NWA	9	7	123	0.127 +/- 0.069	**EUS**	6	5	100	0.095 +/- 0.056
					CAR	3	2	74	0.133 +/- 0.100
SAT	3	3	16	0.029 +/- 0.023	**SAM**	2	2	5	0.013 +/- 0.015
					SAF	1	1	0	0.000 +/- 0.000
NEP	6	5	19	0.018 +/- 0.011	**WUS**	6	5	19	0.018 +/- 0.011
NWP	3	2	97	0.176 +/- 0.132	**NAS**	3	2	97	0.176 +/- 0.132
					SAS	0	-	-	-
SPA	3	3	14	0.027 +/- 0.021	**AUS**	3	3	14	0.027 +/- 0.021
ITS Lineage *ii*									
NEA	5	4	195	0.304 +/- 0.186	**UKEU**	1	1	0	0.000 +/-0.000
					MED	4	3	168	0.265 +/- 0.174
NWA	0	-	-	-	**EUS**	0	-	-	-
					CAR	0	-	-	-
SAT	0	-	-	-	**SAM**	0	-	-	-
					SAF	0	-	-	-
NEP	0	-	-	-	**WUS**	0	-	-	-
NWP	5	5	167	0.302 +/- 0.184	**NAS**	3	3	131	0.264 +/- 0.198
					SAS	2	2	116	0.322 +/- 0.324
SPA	0	-	-	-	**AUS**	0	-	-	-

Table 3.3. Analysis of molecular variance (AMOVA) for *Oxyrrhis* Lineages *i* and *ii* at three genes: α-tubulin, COI and 5.8S ITS. Groups were defined at region level and populations were sub-region level (see Table 3.2 for details). Significant p-values are those in bold. Variance components (*V.C.*), F indices (*F.I.*) and percentage of variation (% var) are also indicated.

α -tubulin, Lineage *i*					
Var	***d.f.***	***V.C.***	***F.I.***	***p***	**% var**
Am grps	5	2.949 Va	FCT= 0.229	0.259	22.9
Am pops w/in grps	3	0.285 Vb	FSC= 0.029	0.186	2.21
W/in pops	27	9.641 Vc	FST= 0.251	**0.024**	74.89
Lineage *ii*					
Var					
Am grps	2	7.626 Va	FCT= 0.290	0.075	28.99
Am pops w/in grps	2	3.913 Vb	FSC= 0.210	0.243	14.88
W/in pops	7	14.762 Vc	FST= 0.439	**0**	56.13
ITS, Lineage *i*					
Var	***d.f.***	***V.C.***	***F.I.***	***p***	**% var**
Am grps	5	0.656 Va	FCT= 0.026	0.599	2.62
Am pops w/in grps	3	6.676 Vb	FSC= 0.274	**0.03**	26.67
W/in pops	28	17.704 Vc	FST= 0.293	**0.006**	70.71
Lineage *ii*					
Var					
Am grps	1	19.143 Va	FCT= 0.271	0.308	27.1
Am pops w/in grps	2	10.594 Vb	FSC= 0.206	0.253	15
W/in pops	6	40.903 Vc	FST= 0.421	**0.047**	57.9
COI, Lineage *i*					
Var	***d.f.***	***V.C.***	***F.I.***	***p***	**% var**
Am grps	5	0.034 Va	FCT= 0.043	0.547	4.34
Am pops w/in grps	3	0.204 Vb	FSC= 0.275	0.115	26.34
W/in pops	29	0.536 Vc	FST= 0.307	**0.036**	69.32
Lineage *ii*					
Var					
Am grps	-	-	-	-	-
Am pops w/in grps	1	3.000 Va			100
W/in pops	2	0.000 Vb	FST= 1	0.257	0

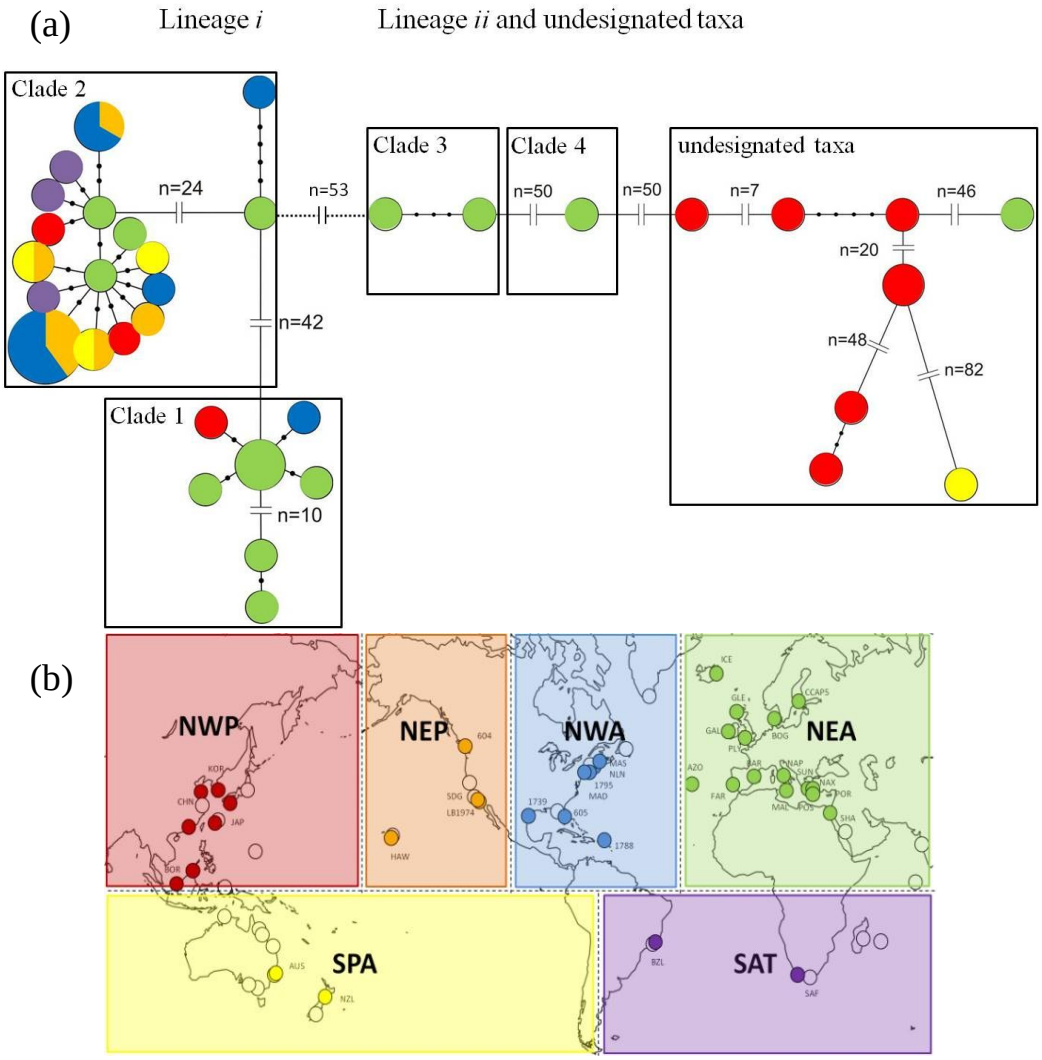

Figure 3.5. (a) Haplotype network for Lineages *i* and *ii* of the α-tubulin gene. Colours relate to those of the geographic locations specified in (b). Size of haplotype circle is proportional to the number of strains in the haplotype, dots and *n* indicate the number of nucleotide substitutions between haplotypes.

Highest levels of nucleotide diversity (π) for the α-tubulin and 5.8S ITS genes were observed in the Northwest Pacific and the Northeast Atlantic (predominantly from samples in the Mediterranean Sea) in both Lineages *i* and *ii*, and in the same geographic regions for Lineage *i* at COI (Table 3.2). Complicating a detailed appraisal of this apparent variance in the distribution of genetic diversity, however, is sampling bias in the North Atlantic and Mediterranean Sea.

Analysis of molecular variance (AMOVA) revealed that the greatest amount of genetic variation occurred within "populations" – that is, at the sub-regional level

(p<0.05; for all genes and both lineages); in just one instance, at the 5.8S ITS gene for Lineage *i*, was significant (p=0.03) variation among groups within regions detected (Table 3.3). Neither Tajima's *D*, Fu's F_s nor the mismatch analysis revealed a convincing pattern of population expansion in any of the geographic regions examined (see Appendix B).

Both α-tubulin (Figure 3.5) and 5.8S ITS gene regions produced similar haplotype topologies. As the α-tubulin region contained the least number of dropouts (*i.e*. strains that failed PCR amplification), I will focus on the haplotype network for this gene region. Lineage *i* (*O. marina*) contained 24 haplotypes, 19 of which were represented by single strains and 5 of which consisted of 2-5 strains. Haplotypes group into two larger clusters, within which there is little variation, which represent the two clades of Lineage *i*. However, there was no spatial genetic structure in Lineage *i*, as all haplotypes were found in all geographic regions. In contrast to the results presented above (see Appendix B), the large central cluster formation of Lineage *i* forms a star-like distribution of haplotypes that may indicate a population expansion may have occurred in the past; if this is the case, however, no obvious geographic region defines the expansion (Figure 3.5). Lineage *ii* (*O. maritima*) and the undesignated taxa consisted of fewer haplotypes separated by greater genetic distances. Lineage *ii* and the undesignated taxa contained fewer strains from the NW Pacific, New Zealand and the Mediterranean Sea (Figure 3.5).

3.4. Discussion

Phylogenetic diversity in Oxyrrhis

Global sampling of *Oxyrrhis* has revealed additional diversity of strains, and possibly uncovered new species, to that previously described in the genus (Lowe *et al*., 2010b). The global phylogeny of *Oxyrrhis* presented here, extends on previous studies by sampling a far broader geographic area and increases data confidence by sequencing an additional gene locus (alpha tubulin). To allow direct comparison between the current phylogeny and that of Lowe *et al*. (Lowe *et al*., 2010b), 22 strains of *Oxyrrhis* were common between the two studies. Two aspects of the new phylogeny are obvious: firstly, Lineage *i* (*O. marina*) appears to maintain comparatively low levels of divergence between strains and is robust to additional sampling. New strains in this lineage separate into the existing clade structure,

which is well supported by individual gene and concatenated phylogenies. Second, by contrast, the clade structure of Lineage *ii* (*O. maritima*) has become less clear. The basal portion of the phylogeny now includes a number of divergent strains in addition to Lineage *ii*, and the relationships between these are poorly resolved. Indeed, the structure of the basal part of the tree is inconsistent between phylogenies based on different genes. One reason which may contribute to the poorer resolution of the basal portion of the phylogeny is the occurrence of paralogs, however the α-tubulin topology is supported by the same groupings in the 5.8S ITS phylogeny. It is more likely that this poor resolution is as a result of (1) relatively modest sampling relative to the likely extent of diversity and (2) missing data (COI sequences).

Species status

The specific status of these new haplotypes is difficult to assess from the current study. The relatively large genetic distances between these basal operational taxonomic units (OTUs) and the lack of repeated haplotype sampling (*i.e.* none of the basal haplotypes are represented by multiple strains) suggests that poor resolution in the basal region of the phylogeny may account for the inconsistency between phylogenies derived from different genes. As a result, a rigorous assessment of the potential occurrence of additional species divisions within *Oxyrrhis* will require further sampling of basal strains within the current phylogeny.

The current dataset does, however, provide some insight into additional diversity within *Oxyrrhis*. COI is often used to delineate species boundaries and uncover cryptic species as it is relatively conserved and is informative about diversity at a species level (Evans *et al*., 2005; Frezal & Leblois, 2008; Hebert *et al*., 2003; Hebert *et al*., 2004; Lin *et al*., 2009; Sites & Marshall, 2003; Stern *et al*., 2010). Unfortunately, it was not possible to amplify the COI region in the new strains of undesignated taxa, likely because of polymorphisms in the COI primer sites; this suggests that these strains are genetically divergent from the strains of *Oxyrrhis* so far characterised (Lowe *et al*. 2005, 2010). The α-tubulin region is less conserved than COI and therefore may be less informative in terms of species delineation. Perhaps surprisingly therefore, I achieved PCR-amplification success for the α-tubulin and 5.8S ITS gene regions (Figure 3.2 and Appendix A), but as the 5.8S ITS phylogeny could not be rooted to a suitable outgroup, the α-tubulin is at present the

most informative for phylogeny reconstruction. The α-tubulin phylogeny shows 90% mean sequence identity between Lineages *i* and *ii* (already defined as separate species *O. marina* and *O. maritima*); by comparison the sequence identities between the undesignated taxa and Lineages *i* and *ii* are 88% and 89% respectively. In an effort to place level of genetic variation between lineages of *Oxyrrhis* in context comparisions with other dinoflagellate taxa are potentially informative. For example, these levels of genetic diversity are consistent with interspecific variation in *Amphidinium* sp. and *Heterocapsa* sp. (mean sequence identity is 84.8% and 88.8% respectively). However similar levels of diversity are also seen within lineages for the α-tubulin gene region (between Clades 1 & 2 and 3 & 4 sequence identities are 88.5 and 87.6%), making it difficult to confirm the status of the undesignated taxa. Levels of diversity in the 5.8S ITS gene, between the undesignated taxa and Lineages *i* and *ii* (~65% for both) are similar to diversity between the two lineages (71.1%) and within lineage diversity is consistently lower (sequence identity within Lineages *i* and *ii* are 86 and 87.6%). As such I tentatively suggest that these undesignated lineages of *Oxyrrhis* represent new species; researchers working on "*Oxyrrhis*" from East Asian waters, where these isolates originated, should be aware that these different strains may not be representative of patterns displayed by *O. marina* (see Chapter 2 for implications of working on divergent strains, also Lowe *et al.*, 2011a; Montagnes *et al.*, 2011b). However, additional sampling, and development of new genetic markers (particularly COI which separates Lineages *i* and *ii* – see Figure 3.2), is required to assess species boundaries and crucially to develop a more robust root to the phylogeny of *Oxyrrhis*.

***Global biogeography patterns in* Oxyrrhis**

It is interesting given the polarisation of the debate about protist distributions that the global biogeography of *Oxyrrhis* presents two contrasting patterns of geographic distribution and abundance; *Oxyrrhis* lends support to both sides of the protist distribution debate! Lineage *i* is broadly distributed and relatively abundant; commonly found in the environment and is prevalent throughout the Atlantic, and Pacific Oceans. On the other hand, the undesignated taxa and Lineage *ii* (basal lineages) are rare in comparison to those of Lineage *i*. They also seem to be more geographically restricted, the undesignated taxa are mainly found in the western

Pacific, with one strain present in the Red Sea, and Lineage *ii* is only found in the Mediterranean and Baltic Seas (see Figures 3.3, 3.4)

Irrespective of the wide generalisations made about protist distribution, it is striking that such contrasting patterns can be seen within *Oxyrrhis*; similar differences between taxa have been reported for other protists, but not within a single genus (*e.g.* Katz *et al*. 2005; Darling *et al*. 2008). Numerous factors can determine strain or species' distribution, but for most protists how these effects differ between species is not known. In the marine environment, for example, pelagic protist species will be dispersed passively via physical processes such as water currents or gyres; subtle behavioural differences between species could affect the efficacy of passive dispersal but this has not been studied. What is clear, however, is that protist species (and even strains within a nominal species) display different responses to physico-chemical factors, such as temperature, salinity and pH (Boenigk *et al*., 2007; Gachter & Weisse, 2006; Lowe *et al*., 2005; Montagnes & Weisse, 2000; Weisse & Montagnes, 1998) and this is likely a major influence on species distributions (Weisse, 2008); moreover, there is some evidence that biotic factors such as food availability and predation pressures may affect habitat suitability (Weisse, 2008). Where individual strains are adapted to local conditions then migrants are likely to be outcompeted by native individuals and therefore fail to establish after dispersal (De Meester *et al*., 2002; DeMeester, 1996; Okamura & Freeland, 2002). Thus, despite seemingly high capacity for widespread, passive dispersal successful gene flow among populations can be limited. The extent to which processes relating to adaptation and gene flow vary among closely-related taxa remains an interesting avenue for further study.

Current patterns of spatial distribution may also reflect a lasting signature of historical events. For example, patterns of divergence in Antarctic populations of the planktonic foraminferan *Neogloboquadrina pachyderma* are linked to glacial-interglacial climate dynamics during the Quaternary (Darling *et al*., 2004). In order to further understand the past environmental conditions and geological processes that may have contributed to the *Oxyrrhis* biogeography in particular, an estimate of the timing of divergence is required. Based on 10% divergence between the two *Oxyrrhis* lineages in COI (Lowe *et al*., 2010b) and applying a rate of 1.4-2.6%

divergence per Mya (for snapping shrimp *Alpheus* and similar rates in a range of crustacea *e.g.* see Chen & Hare, 2008; Knowlton & Weigt, 1998; Knowlton *et al*., 1993), divergence between these two lineages of *Oxyrrhis* occurred around 3.8-7.1 Mya. This places the timing of divergence towards the end of the Miocene to the Pliocene epochs. Several major climatic events occurred at this time, including the Messinian salinity crisis (5.98-5.33 Mya) and the closing of the Isthmus of Panama (*ca*. 3.5 Mya), which cut equatorial ocean currents and intensified the Gulf Stream current (Coates & Obando, 1996). In particular, the Mediterranean Sea appears to host numerous divergent lineages of *Oxyrrhis* which may have been facilitated by divergence in allopatric refugia during the Messinian salinity crisis. Northeast Atlantic strains of *Oxyrrhis* contain quite low levels of genetic diversity (given the high sampling effort) and strains that appear to be derived from other areas. Hence, the North Atlantic appears to have been recently colonised by *Oxyrrhis*, and the low diversity may suggest a recent expansion (although there is no statistical support for this). The last glacial period which lasted until approximately 10,000 years ago, caused changes in species geographic ranges, and for the North Atlantic and Europe there are patterns of refugia and post-glacial recolonisation (Consuegra *et al*., 2002; Darling *et al*., 2004; Gysels *et al*., 2004; Hewitt, 1999; Stefansson *et al*., 2009). This is consistent with the apparent recent colonisation in the Northeast Atlantic by *Oxyrrhis*, suggesting that this pattern is indicative of post glacial colonisation. This is at best conjecture about the processes that may have led to the divergent patterns of biogeography in *Oxyrrhis*, but indicates the major diversification between Lineages *i* and *ii* occurred prior to the well documented Pleistocene climatic fluctuations that apparently drove diversification in many marine (non-protist) taxa (*e.g.* Nikulina *et al.*, 2007; Stefansson *et al.*, 2009).

While the two species show different distributions, their distributions overlap extensively. Centres of diversity are East Asian waters (mainly arising from the new undesignated taxa) and the North East Atlantic, particularly the Mediterranean Sea. High levels of genetic diversity seems to be a typical feature of both of these areas (Boudouresque, 2004; Coll *et al*., 2010; Derycke *et al*., 2008; Nagai *et al*., 2009; Schwaninger, 2008) and may relate to presence of refugia during periods of climate change. Interestingly, there are suggestions that East Asian and North Pacific waters are a centre of origin for several species (Barber *et al.*, 2006; Drew & Barber, 2009;

Liao *et al.*, 2007; Schwaninger, 2008; Williams, 2007). It is possible that this may be the case for the basal taxa of *Oxyrrhis*, however the branches are too poorly resolved to determine this with any confidence: more sampling is required to advance this hypothesis beyond speculation.

Indeed, throughout its distribution, evidence for differences in population demography between areas and lineages was equivocal with no statistical support for an expansion. On the other hand, in the well-sampled and well-defined lineage *O. marina*, part of the haplotype network is consistent with a pattern of population expansion; however no spatial genetic structure could be detected and if it occurs, the "expansion" was not limited to a certain geographic region such as the North Atlantic. In part, detecting demographic signals may be affected by the resolution (Weisse, 2008); the regional groups may cover too wide an area, as there is evidence of spatial structure at smaller geographic scales (Lowe *et al.*, 2010a). Population demography is also potentially affected by life history strategies, of which little is known for *Oxyrrhis*. There are various detailed descriptions of the *Oxyrrhis* cell cycle, where cells divide by transverse fission (Gao & Li, 1986; Hall, 1925; Kato *et al.*, 1997; Montagnes *et al.*, 2011a; Sano & Kato, 2009; Senn, 1911; Triemer, 1982), and there is one known report of sex in *Oxyrrhis* (von Stosch, 1972a, b). Most dinoflagellates are haploid with some exceptions (*e.g. Noctiluca*), yet it is unclear whether *Oxyrrhis* cells are haploid, diploid or polyploid (Montagnes *et al.*, 2011a; Sano & Kato, 2009). Further research on *Oxyrrhis* life and cell cycles is required to understand the implications this has on population structure as well as its use as a model organism (see Lowe *et al.*, 2010a; Montagnes *et al.*, 2011a).

Conclusions

Increasing the sampling of *Oxyrrhis* to encompass its global distribution has revealed further genetic diversity within this cosmopolitan genus. The new undesignated taxa uncovered in this study were predominantly found from East Asian waters, and so I would recommend further sampling in this region. I expect that this would reveal additional diversity and also help to resolve the relationship between lineages. In particular, it would be interesting to determine the support for the idea that Asian Seas represent the centres of origin and sustain greatest diversity. These new undesignated taxa also require further genetic characterisation, particularly through

the use of COI which delineates the major lineages of *Oxyrrhis* characterised in Chapter 2. Moreover, such work must be combined with detailed morphological studies to determine whether these new strains represent a separate lineage or a distinct third species. Different lineages show contrasting distributions, but more sampling is required to better characterise the biogeographic patterns. Indeed, while a huge effort was involved in sampling on such a scale, and the study samples isolates from every major ocean/sea, in relation to the size and abundance of this taxon, it remains hugely undersampled.

Chapter 4

General Discussion

4.1 Synthesis

Cosmopolitan species potentially have interesting biologies: for example, what physiological and dispersal mechanisms allow a species to be widely distributed and tolerate such a wide range of ecological conditions? Indeed, given the many mechanisms that lead to genetic divergence, and ultimately speciation, among allopatric populations there is much scepticism about the validity of the majority of cosmopolitan species (*e.g.* Barroso *et al*., 2010; Klautau *et al*., 1999), even for protist species that have the potential for very wide dispersal (see *e.g.* Fenchel & Finlay, 2004). Here, I acquired samples of the apparently cosmopolitan genus *Oxyrrhis* that encompass much of its known geographic distribution. Sequence data at three gene loci (1) confirmed the validity of two previously-suspected species within *Oxyrrhis*, (2) identified the presence of additional, previously unrecognised cryptic species and (3) highlighted the potential for very different patterns of distribution and gene flow within a single genus.

Species status of Oxyrrhis

High levels of genetic diversity in *Oxyrrhis* is a consistent result, such that the two distinct lineages identified in past studies have been defined here as the two separate species: *Oxyrrhis marina* and *Oxyrrhis maritima*. A search of the historic literature uncovered several descriptions about species in the genus *Oxyrrhis*, *O. maritima* and *O. tentaculifera*, both of which have been synonymised with *O. marina*. This and the description of a supposed "tentacle" were the only instances where morphological variation within the genus *Oxyrrhis* was suggested in the historical and morphological literature, although very few of the studies were comparative. The lack of morphological characters combined with its distinctive general shape likely lead to the recognition of *Oxyrrhis* as a monospecific genus. This is clearly incorrect: isolates formed two distinct lineages between which consistently high levels of genetic differentiation at several genes, both nuclear and mitochondrial. This led to the epithet *maritima* being resurrected and redescribed for the lineage that, from a literature survey, should cause least disruption or confusion to past and future work.

***Global patterns of diversity and distribution in* Oxyrrhis**

Global sampling has confirmed the widespread distribution of *Oxyrrhis*. Many of the new isolates of *Oxyrrhis*, fell into the pre-existing clade structure that defined Lineage *i* (*O. marina*) and had little genetic variation among strains within this lineage. *O. marina* is thus a well defined species group. However, in areas where this taxon is less well-studied (*e.g*. Asian coastal waters), I have revealed further genetic divergence between the new strains and both *Oxyrrhis marina* and Lineage *ii Oxyrrhis maritima*. The new strains are basal to the well-defined *O. marina* lineage; they are also genetically different from *O. maritima*, indicative of a third lineage, but their relationship to Lineage *ii* (*O. maritima*) need further characterisation with more samples and additional gene loci.

Interestingly, the lineages of *Oxyrrhis* show contrasting patterns of geographic range and abundance. *O. marina* is abundant and broadly distributed, found throughout European waters, the Atlantic and the Pacific. Conversely *O. maritima* and new undesignated taxa are rare and geographically restricted in comparison; *O. maritima* is found in the Mediterranean and the Baltic (no new strains were found) and the undesignated taxa are mainly found in the West Pacific with one strain in the Red Sea. The mechanisms that promote this discrepancy – potential response to dispersal, environmental tolerance and capacity for genetic divergence/cohesion – are simply not understood. It is possible that these patterns represent the action of past climatic events; lineages in Asian waters and possibly the Mediterranean Sea may have diverged in response to climate cycles; by contrast, a recent range expansion in the North Atlantic could account for the low levels of diversity. However, there is a lack of support for such differences in population demography and the sample size in Asian Seas is small.

As evidence for cryptic species continues to increase, particularly in those that lack distinct morphological characters, (Boenigk *et al*., 2006; Gentekaki & Lynn, 2009; Klautau *et al*., 1999; Slapeta *et al*., 2006; Stern *et al*., 2010) there is an obvious need to assess other protist species in a similar way to this study. New species designations should include morphological and genetic data, and where possible an evaluation of multiple isolates to quantify the level of intra- and inter-specific variation.

In light of the findings of this study, it is clear that neither ubiquity nor endemism fully explain distributional patterns for all protists. In hindsight, this is perhaps obvious: the term protist includes a vast range of taxa and it is highly unlikely they all conform to a universal pattern (Caron, 2009), especially when contrasting patterns of distribution can be seen here within a single genus. Given the recognition of such contrasting patterns the challenge now is to determine the processes that generate unique biogeographic signatures. The current distribution pattern is a result of an interaction of historic contingency (climatic fluctuations that may drive vicariance) and contemporary process, such as dispersal and different ecophysiological responses to factors such as temperature and salinity.

A further issue that makes it difficult to draw conclusions about the level of diversity and patterns of distribution in *Oxyrrhis* is that of undersampling. Despite extensive sampling and having representatives from each continent, this remains a small snapshot of the true composition and this may represent the true challenge to protistologists in the future. For example, in recent papers of "global protist biogeography" as few as 25 sample sites were used to describe biogeographic patterns (Darling & Wade, 2008). Are such studies truly representative of global patterns? The challenge is perhaps illustrated by the contrast between the traditional view of three main European refugia (during the last glaciation) for terrestrial animals (see reviews Hewitt, 1999; Taberlet *et al*., 1998); more detailed sampling in the Iberian Peninsula has now uncovered the presence of distinct "refugia within refugia" (*e.g.* Gomez & Lunt, 2007). The marine environment clearly differs from the mountainous structure of the Iberian Peninsula, but further sampling is required to ensure that samples from a specific area are representative of the region as a whole.

4.2 Future directions

In relation to species diversity in *Oxyrrhis*, there is scope for revisiting morphological studies in a comparative context. In particular studies on the flagellar scales, tentacular structure and size, and potentially flagellar rootlet structure may be fruitful directions for such work (see Chapter 2 and Lowe *et al.*, 2011a). The new diversity in global samples also requires further characterisation, both in a morphological sense as well to determine the phylogenetic stability of lineages. The

new isolates were predominantly found in the East Asian waters, and it seems high levels of diversity are typical in this region. Therefore, I would recommend further sampling in West Pacific regions as it may yield further diversity and perhaps some further understanding of distribution patterns and processes that promote genetic divergence. In addition, there is a need to further develop methods of molecular dating to enable more accurate estimates of dates of genetic divergence; this is required to provide a better indication of the processes and climatic events that may have driven species' distributions.

Finally, *Oxyrrhis* contains cryptic diversity and so, almost 30 years on from the synonymisation of various species into a monomorphic genus, *Oxyrrhis* has now been split into two species, *O. marina* and *O. maritima*. Global samples reveal further diversity as well as contrasting patterns of distribution in the genus *Oxyrrhis*, which seem to support opposing sides of the protist distribution debate. This study provides a preliminary indication of the world wide diversity and distribution of *Oxyrrhis*, and highlights the importance of using specific models to assess protist distributions.

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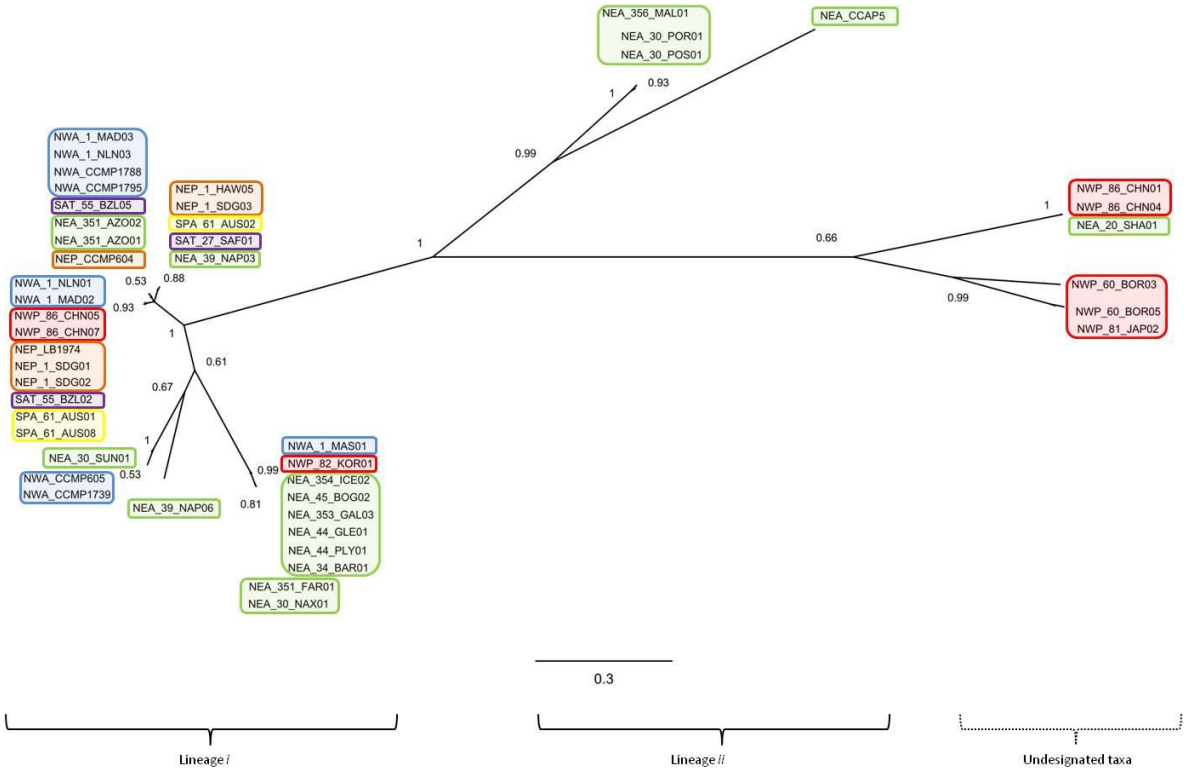
Appendices

Appendix A. 5.8S ITS phylogeny for 47 isolates of *Oxyrrhis*. Colours relate to geographic regions defined in Figure 3.2.

Appendix B. Tajima's *D*, Fu's *Fs* and Mismatch indices for strains geographically grouped into ocean, region and subregions. Ocean groups are North Atlantic (NA), South Atlantic (SA), North Pacific (NP) and South Pacific (SP).

Appendix C. Lowe *et al*., 2010b, which also in part forms Chapter 2.

Appendix D. List of global sample locations and those who kindly collected them and contributed to this project.



Appendix A. 5.8S ITS phylogeny for 47 isolates of *Oxyrrhis*. Colours relate to geographic regions defined in Figure 3.2.

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α-tubulin Lineage *i*

Ocean	n	D	P (D)	Fs	P (Fs)	SSD	P(SSD)	Rag	P(Rag)
NA	21	2.377	1.000	11.478	1.000	0.057	0.000	0.040	0.065
SA	3	0.000	0.985	0.693	0.455	0.014	0.815	0.111	0.998
NP	9	-1.828	0.007	2.067	0.819	0.454	0.001	0.036	1.000
SP	3	0.000	0.897	-0.693	0.124	0.042	0.928	0.222	0.995
Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
NEA	13	1.570	0.964	8.661	1.000	0.082	0.002	0.073	0.185
NWA	8	-0.873	0.215	4.111	0.955	0.100	0.300	0.151	0.311
SAT	3	0.000	0.988	0.693	0.438	0.014	0.838	0.111	0.996
NEP	6	-0.496	0.366	-0.168	0.374	0.041	0.576	0.129	0.779
NWP	3	0.000	0.666	2.347	0.576	0.277	0.131	0.444	0.880
SPA	3	0.000	0.888	-0.693	0.176	0.042	0.921	0.222	0.993
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	8	0.991	0.874	9.100	1.000	0.181	0.182	0.279	0.229
MED	5	1.520	0.898	1.712	0.514	0.113	0.415	0.167	0.942
EUS	6	-1.351	0.043	3.781	0.953	0.130	0.408	0.253	0.382
CAR	2	0.000	1.000	3.296	0.599	0.000	0.000	0.000	0.000
SAM	2	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
SAF	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
WUS	6	-0.496	0.327	-0.168	0.374	0.041	0.591	0.129	0.801
NAS	3	0.000	0.635	2.347	0.561	0.277	0.141	0.444	0.866
SAS	0	-	-	-	-				
AUS	3	0.000	0.879	-0.693	0.141	0.042	0.903	0.222	0.990

Appendix B. cont'd

α-tubulin
Lineage *ii*

Reg	n	D	P (D)	Fs	P (Fs)	SSD	P(SSD)	Rag	P(Rag)
NEA	4	1.027	0.854	1.959	0.533	0.104	0.523	0.167	0.963
NWA	0	-	-	-	-	-	-	-	-
SAT	0	-	-	-	-	-	-	-	-
NEP	0	-	-	-	-	-	-	-	-
NWP	7	1.437	0.96	2.116	0.779	0.088	0.182	0.161	0.433
SPA	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
MED	3	0.000	0.915	2.504	0.574	0.321	0.112	0.444	0.964
EUS	0	-	-	-	-	-	-	-	-
CAR	0	-	-	-	-	-	-	-	-
SAM	0	-	-	-	-	-	-	-	-
SAF	0	-	-	-	-	-	-	-	-
WUS	0	-	-	-	-	-	-	-	-
NAS	4	2.315	1	1.780	0.521	0.265	0.091	0.333	0.754
SAS	3	0.000	0.667	4.9456	0.953	0.474	0.004	1.000	0.492
AUS	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000

Appendix B. cont'd

COI, Lineage *i*

Ocean	n	D	P (D)	Fs	P (Fs)	SSD	P(SSD)	Rag	P(Rag)
NA	22	2.155	0.992	5.393	0.975	0.470	0.000	0.736	0.919
SA	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
NP	10	-1.667	0.021	1.744	0.789	0.058	0.040	0.720	0.699
SP	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
NEA	13	2.279	0.995	4.768	0.974	0.315	0.051	0.793	0.005
NWA	9	-1.610	0.035	1.844	0.782	0.071	0.022	0.704	0.695
SAT	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
NEP	6	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
NWP	4	-0.780	0.195	2.197	0.832	0.500	0.000	0.750	0.953
SPA	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	8	0.485	0.709	3.149	0.922	0.367	0.000	0.694	0.939
MED	5	-1.094	0.105	2.202	0.824	0.213	0.064	0.680	0.502
EUS	6	-1.295	0.084	2.139	0.837	0.153	0.041	0.667	0.574
CAR	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
SAM	2	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
SAF	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
WUS	6	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
NAS	4	-0.780	0.191	2.197	0.839	0.500	0.000	0.750	0.957
SAS	0	-	-	-	-	-	-	-	-
AUS	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000

Appendix B. cont'd

COI, Lineage *ii*									
Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
NEA	4	-0.809	0.174	2.944	0.898	0.500	0.000	0.750	0.950
NWA	0	-	-	-	-	-	-	-	-
SAT	0	-	-	-	-	-	-	-	-
NEP	0	-	-	-	-	-	-	-	-
NWP	0	-	-	-	-	-	-	-	-
SPA	0	-	-	-	-	-	-	-	-
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
MED	3	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
EUS	0	-	-	-	-	-	-	-	-
CAR	0	-	-	-	-	-	-	-	-
SAM	0	-	-	-	-	-	-	-	-
SAF	0	-	-	-	-	-	-	-	-
WUS	0	-	-	-	-	-	-	-	-
NAS	0	-	-	-	-	-	-	-	-
SAS	0	-	-	-	-	-	-	-	-
AUS	0	-	-	-	-	-	-	-	-

Appendix B. cont'd

ITS, Lineage *i*

Ocean	n	D	P (D)	Fs	P (Fs)	SSD	P(SSD)	Rag	P(Rag)
NA	22	2.845	1.000	19.687	1.000	0.085	0.000	0.141	0.000
SA	3	9.3E+06	1.000	1.240	0.468	0.452	0.004	1.111	0.362
NP	9	-1.613	0.031	9.051	0.999	0.102	0.313	0.155	0.252
SP	3	2.2E+07	1.000	1.139	0.482	0.239	0.157	0.444	0.800
Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
NEA	13	2.149	0.995	13.266	1.000	0.156	0.000	0.187	0.000
NWA	9	0.145	0.604	12.071	1.000	0.130	0.115	0.221	0.079
NEP	6	-1.289	0.081	3.664	0.956	0.122	0.189	0.267	0.307
NWP	3	0.000	0.975	7.691	0.990	0.532	0.012	1.000	0.487
SPA	3	2.2E+07	1.000	1.139	0.452	0.239	0.141	0.444	0.804
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	8	0.642	0.768	13.434	1.000	0.533	0.001	0.432	0.930
MED	5	1.183	0.890	4.943	0.937	0.137	0.059	0.230	0.413
EUS	6	-1.308	0.070	8.997	0.997	0.150	0.210	0.240	0.341
CAR	3	0.000	0.744	7.163	0.983	0.532	0.010	1.000	0.462
SAM	2	0.000	1.000	1.609	0.507	0.000	0.000	0.000	0.000
SAF	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
WUS	6	-1.289	0.069	3.664	0.945	0.122	0.208	0.267	0.308
NAS	3	0.000	0.962	7.691	0.982	0.532	0.009	1.000	0.480
SAS	0	-	-	-	-	-	-	-	-
AUS	3	2.2E+07	1.000	1.139	0.461	0.239	0.153	0.444	0.811

Appendix B. cont'd

ITS, Lineage *ii*

Reg	n	D	P (D)	Fs	P (Fs)	SSD	P(SSD)	Rag	P(Rag)
NEA	5	0.261	0.594	5.674	0.970	0.146	0.037	0.270	0.138
NWA	0	-	-	-	-	-	-	-	-
SAT	0	-	-	-	-	-	-	-	-
NEP	0	-	-	-	-	-	-	-	-
NWP	5	1.867	0.993	5.732	0.964	0.136	0.104	0.310	0.074
SPA	0	-	-	-	-	-	-	-	-
Sub Reg	**n**	**D**	**P (D)**	**Fs**	**P (Fs)**	**SSD**	**P(SSD)**	**Rag**	**P(Rag)**
UKEU	1	0.000	1.000	0.000	N.A.	0.000	0.000	0.000	0.000
MED	4	-0.702	0.250	6.561	0.975	0.263	0.126	0.417	0.324
EUS	0	-	-	-	-	-	-	-	-
CAR	0	-	-	-	-	-	-	-	-
SAM	0	-	-	-	-	-	-	-	-
SAF	0	-	-	-	-	-	-	-	-
WUS	0	-	-	-	-	-	-	-	-
NAS	3	0.000	0.172	8.280	0.992	0.532	0.014	1.000	0.501
SAS	2	0.000	1.000	4.754	0.609	0.000	0.000	0.000	0.000
AUS	0	-	-	-	-	-	-	-	-

Appendix C

This text box is where the unabridged thesis included the following third party copyrighted material;

Lowe, C. D., P. J. Keeling, et al. (2011). "Who is Oxyrrhis marina? Morphological and phylogenetic studies on an unusual dinoflagellate." Journal of Plankton Research **33**(4): 555-567.
doi: http://dx.doi.org/*10.1093/plankt/fbq110*

Appendix D. List of global sample locations and those who kindly collected them and contributed to this project.

New sample name	Region 1	Region 3	Country	Country Code	Collected by	Lat	Long	date collected
1_DLW06	Lewes (outer estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_DLW07	Lewes (outer estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_DLW08	Lewes (outer estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_MAS02	Marthas Vineyard	USA (E)	USA	1	Hugh McAllister	41.4077	-70.538	01/06/2009
1_MAS03	Marthas Vineyard	USA (E)	USA	1	Hugh McAllister	41.4077	-70.538	01/06/2009
1_NLN02	Avery Pt	Connecticut	USA	1	George McManus	41.3153	-72.0650	21/08/2009
55_BZL04	Brazil	S. America	Brazil	55	David Montagnes	22.7380	-41.8737	26/08/2009
60_BOR04	Borneo (North)		Malaysia	60	Adam Caris	7.0151	116.7373	01/07/2009
86_CHN06	Qing Dao	China	China	86		36.0616	120.3184	13/06/2008
1_HAW05	Naniloa Resort	Hawaii	USA	1	Gary & Caroline Davis	19.7296	-155.0641	16/03/2009
1_MAS01	Marthas Vineyard	USA (E)	USA	1	Hugh McAllister	41.4077	-70.538	01/06/2009
1_NLN01	Avery Pt	Connecticut	USA	1	George McManus	41.3153	-72.0650	21/08/2009
1_SDG01	La Jolla	San Diego	USA	1	Harry Noyes	32.8510	-117.2732	11/01/2010
1_SDG02	La Jolla	San Diego	USA	1	Harry Noyes	32.8510	-117.2732	11/01/2010
1_SDG03	La Jolla	San Diego	USA	1	Harry Noyes	32.8510	-117.2732	11/01/2010
27_SAF01	Bantry Bay	South Africa	South Africa	27	Tom Heyes	33.9279	18.3754	01/04/2009
354_ICE02	Hvalnes	Iceland (S)	Iceland	354	David Wilson	64.4047	-14.5457	08/09/2009
55_BZL02	Brazil	S. America	Brazil	55	David Montagnes	22.7380	-41.8737	26/08/2009
55_BZL05	Brazil	S. America	Brazil	55	David Montagnes	22.7380	-41.8737	26/08/2009
60_BOR03	Borneo (North)		Malaysia	60	Adam Caris	7.0151	116.7373	01/07/2009
60_BOR05	Damai	Borneo	Malaysia	60		1.7536	110.3150	28/10/2009
61_AUS01	Balmain	New South Wales	Australia	61	David Montagnes	33.8534	151.1714	26/04/2009
61_AUS02	Balmain	New South Wales	Australia	61	David Montagnes	33.8534	151.1714	26/04/2009
81_JAP01	Urasoko Bay	Okinawa	Japan	81	Kazuhiko Koike	24.3383	124.1533	12/10/2008
81_JAP02	Sata	Japan	Japan	81	David Montagnes	31.3273	130.8016	25/04/2009
86_CHN01	Daya Bay	China	China	86	David Montagnes	22.5882	114.6364	01/04/2009
86_CHN04	Daya Bay	China	China	86	Montagnes	22.5882	114.6364	01/04/2009
86_CHN05	Qing Dao	China	China	86		36.0616	120.3184	13/06/2008
CCMP1739	Texas	USA (E)	USA	1	CCMP	27.8333	-97.1330	08/07/1993
CCMP1788	St. Martin	Caribbean	USA	1	CCMP	18.0280	-63.0530	01/05/1997
CCMP1795	Groton, Connecticut, North Atlantic	USA (E)	USA	1	CCMP	41.3100	-72.0716	01/10/1996
CCMP604	San Juan Island, Strait of Georgia	USA (W)	USA	1	CCMP	48.5440	-123.0100	missing
CCMP605	Fort Pierce	USA (E)	USA	1	CCMP	27.4323	-80.3100	02/12/1983
LB1974	SIO Pier	California	USA	1	R A Lewin	32.8675	-117.2588	missing

82_KOR01	Keum Estuary	Korea	Korea	82	Hae Jin Jeong	35.9800	126.7000	01/05/2001
1_CAL01	California	California	USA	1	Mark Caddick	34.0208	-118.5068	01/03/2009
1_CAL02	Half moon bay	California (N)	USA	1	Karen Evans	37.4986	-122.4958	11/04/2009
1_CAL03	Venice Beach	California	USA	1	Neil Hall	33.9850	-118.4743	11/01/2010
1_CAL04	Venice Beach	California	USA	1	Neil Hall	33.9850	-118.4743	11/01/2010
1_CHS01	Serc Dock, Rhode River	Maryland	USA	1	Wayne Coats	38.8856	-76.5147	13/08/2009
1_CHS02	Carr's Wharf, Rhode River	Maryland	USA	1	Wayne Coats	38.8878	-76.5236	13/08/2009
1_CHS03	St Mary's City	Maryland	USA	1	Wayne Coats	38.1892	-76.4339	13/08/2009
1_CHS04	Pt Lookout	Maryland	USA	1	Wayne Coats	38.0400	-76.3208	13/08/2009
1_CHS05	Cambridge	Maryland	USA	1	Wayne Coats	38.5731	-76.0694	13/08/2009
1_CHS06	Hooper Island (lower)	Maryland	USA	1	Wayne Coats	38.3031	-76.2122	13/08/2009
1_CHS07	Hooper Island (upper)	Maryland	USA	1	Wayne Coats	38.3511	-76.2292	13/08/2009
1_CHS08	Crisfield	Maryland	USA	1	Wayne Coats	37.9772	-75.8642	13/08/2009
1_DLW01	Lewes (outer estuary)	Delaware	USA	1	Catherine House	38.7891	-75.1626	missing
1_DLW02	Lewes (outer estuary)	Delaware	USA	1	Catherine House	38.7891	-75.1626	missing
1_DLW03	Lewes (outer estuary)	Delaware	USA	1	Catherine House	38.7891	-75.1626	missing
1_DLW04	Lewes (outer estuary)	Delaware	USA	1	Catherine House	38.7891	-75.1626	missing
1_DLW05	Lewes (outer estuary)	Delaware	USA	1	Catherine House	38.7891	-75.1626	missing
1_DLW10	Woodland Beach (inner estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_DLW11	Woodland Beach (inner estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_DLW12	Woodland Beach (inner estuary)	Delaware	USA	1	Catherine House	39.3312	-75.4835	missing
1_FLA01	Tampa Bay	Florida	USA	1	Courtney Kagan	27.9712	-82.6980	29/04/2009
1_FLA02	Clearwater Beach	Florida	USA	1	Courtney Kagan	27.9730	-82.8306	29/04/2009
1_HAW01	Coconut Island	Hawaii	USA	1	Gary & Caroline Davis	19.7284	-155.0681	16/03/2009
1_HAW02	Richardson Ocean Park	Hawaii	USA	1	Gary & Caroline Davis	19.7371	-155.0135	16/03/2009
1_HAW03	Carlsmith Beach Park	Hawaii	USA	1	Gary & Caroline Davis	19.7351	-155.0269	16/03/2009
1_HAW04	Hilo Yacht Club	Hawaii	USA	1	Gary & Caroline Davis	19.7370	-155.0327	16/03/2009
1_HAW06	Onekahakaha Beach Park	Hawaii	USA	1	Caroline Davis	19.7379	-155.0407	18/03/2009
1_HAW07	Keauhou beach resort	Hawaii	USA	1	Caroline Davis	19.5806	-155.9681	01/10/2009
1_HAW08	Pu'uhonua O Honaunau	Hawaii	USA	1	Caroline Davis	19.4228	-155.9108	01/10/2009
1_HAW09	Kahulu'u beach	Hawaii	USA	1	Caroline Davis	19.5831	-155.9678	01/10/2009
1_HAW10	Kailua-Kona	Hawaii	USA	1	Caroline Davis	19.6358	-155.9917	02/10/2009
1_HAW11	Kailua-Kona	Hawaii	USA	1	Caroline Davis	19.6286	-155.9881	02/10/2009
1_HAW12	Kailua-Kona	Hawaii	USA	1	Caroline	19.6056	-155.9758	02/10/2009

					Davis			
1_MAD01	Madison	Connecticut	USA	1	George McManus	41.2700	-72.6089	19/08/2009
1_NEW01	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.867	-73.6452	11/05/2009
1_NEW02	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.867	-73.6452	11/05/2009
1_NEW03	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.867	-73.6452	11/05/2009
1_NEW04	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.867	-73.6452	11/05/2009
1_NEW05	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.8675	-73.4716	11/05/2009
1_NEW06	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.8675	-73.4716	11/05/2009
1_NEW07	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.8675	-73.4716	11/05/2009
1_NEW08	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.87099	-73.4757	11/05/2009
1_NEW09	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.87099	-73.4757	11/05/2009
1_NEW10	Cold Spring Harbor	New York	USA	1	Harry Noyes	40.87099	-73.4757	11/05/2009
1_NFL01	St. Pierre	Newfoundland	Canada	1		46.7933	-56.1617	04/10/2009
1_NFL02	St. Pierre	Newfoundland	Canada	1		46.7933	-56.1617	04/10/2009
1_VAN01	Vancover	Canada (W)	Canada	1	C. Brauner	49.3404	-123.2478	29/06/2009
1_VAN02	Vancover	Canada (W)	Canada	1	C. Brauner	49.3404	-123.2478	29/06/2009
230_MAU01	Mauritius	Mauritius	Mauritius	230	Dawn Cranmer	-20.0009	57.6579	01/06/2009
230_MAU02	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU03	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU04	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU05	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU06	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU07	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU08	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU09	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU10	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
230_MAU11	La Pirogue		Mauritius	230	Ravi Gopaul	-20.2998	57.3635	27/10/2009
254_KEN01	Takaungu		Kenya	254	Steve Kemp	-3.6371	39.8449	01/08/2009
254_KEN02	Takaungu		Kenya	254	Steve Kemp	-3.6388	39.8445	01/08/2009
261_MAD01	Madagascar		Madagascar	261	Sandra Telfer	-20.3343	48.6475	01/06/2009
261_MAD02	Madagascar		Madagascar	261	Sandra Telfer	-20.3343	48.6475	01/06/2009
27_SAF02	Plettenberg Bay	South Africa	South Africa	27	Tom Heyes	-34.0545	23.3793	19/04/2009
299_GLD01	Narsarsuaq	Greenland	Greenland	299		61.1517	-45.4350	30/09/2009
299_GLD02	Narsarsuaq	Greenland	Greenland	299		61.1517	-45.4350	30/09/2009
299_GLD03	Narsarsuaq	Greenland	Greenland	299		61.1517	-45.4350	30/09/2009
354_ICE01	Hvalnes	Iceland (S)	Iceland	354	David Wilson	64.4047	-14.5457	08/09/2009
354_ICE03	Jokulsarlon	Iceland (S)	Iceland	354	David Wilson	64.0426	-16.1794	10/09/2009
354_ICE04	Hoen	Iceland (S)	Iceland	354	David Wilson	64.2457	-15.1944	10/09/2009

354_ICE05	Hoen	Iceland (S)	Iceland	354	David Wilson	64.2504	-15.2172	10/09/2009
354_ICE06	Kollafjordur	Iceland (W)	Iceland	354	David Wilson	64.2031	-21.7125	13/09/2009
354_ICE07	Hralfjordur	Iceland (W)	Iceland	354	David Wilson	64.3943	-21.4421	13/09/2009
55_BZL01	Brazil	S. America	Brazil	55	David Montagnes	22.9739	-43.2029	26/08/2009
55_BZL03	Brazil	S. America	Brazil	55	David Montagnes	22.7380	-41.8737	26/08/2009
60_BOR01	Borneo (North)		Malaysia	60	Adam Caris	7.0373	116.7417	01/07/2009
60_BOR02	Borneo (North)		Malaysia	60	Adam Caris	7.0373	116.7417	01/07/2009
60_BOR06	Damai	Borneo	Malaysia	60		1.7536	110.3150	28/10/2009
60_BOR07	Damai	Borneo	Malaysia	60		1.7536	110.3150	28/10/2009
61_AUS03	Balmain	New South Wales	Australia	61	David Montagnes	33.8534	151.1714	26/04/2009
61_AUS04	Cairns marina, Australia	Cairns	Australia	61	Lucy Hopcroft	16.9182	145.7813	15/04/2009
61_AUS05	Picnic Bay, Magnetic Island	Australia	Australia	61	Lucy Hopcroft	19.1763	146.8452	11/04/2009
61_AUS06	Yeppoon1	Queensland (S)	Australia	61	Lucy Hopcroft	23.1198	150.7501	09/04/2009
61_AUS07	Yeppoon2	Queensland (S)	Australia	61	Lucy Hopcroft	23.1198	150.7501	09/04/2009
61_AUS09	Sydney harbour	New South Wales	Australia	61	Fiona Hobden	33.8591	151.2220	26/05/2009
61_AUS10	Sydney harbour	New South Wales	Australia	61	Fiona Hobden	33.8591	151.2220	26/05/2009
61_AUS11	Point Lonsdale	Victoria	Australia	61		38.2833	144.6000	25/11/2009
61_AUS12	Point Lonsdale	Victoria	Australia	61		38.2833	144.6000	25/11/2009
61_AUS13	Point Lonsdale	Victoria	Australia	61		38.2833	144.6000	25/11/2009
61_AUS14	Point Lonsdale	Victoria	Australia	61		38.2833	144.6000	25/11/2009
61_AUS15	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS16	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS17	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS18	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS19	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS20	Buffalo Creek	Darwin	Australia	61	Per Juel Hansen	12.3376	130.9081	03/01/2010
61_AUS21	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS22	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS23	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS24	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS25	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS26	Nightcliff	Darwin	Australia	61	Per Juel Hansen	12.3864	130.8411	04/01/2010
61_AUS27	Woolley Lake	South Australia	Australia	61	Jan Strugnell	37.4678	140.0346	missing
61_AUS28	Salmon Hole	South Australia	Australia	61	Jan Strugnell	37.4864	139.9994	missing
62_IND01	Pulau Wayag		Indonesia	62	Adam Caris	0.1604	130.0617	01/07/2009
62_IND02	Pulau Wayag		Indonesia	62	Adam Caris	0.1604	130.0617	01/07/2009
62_IND03	Pulau Wayag		Indonesia	62	Adam Caris	0.1767	130.0150	01/07/2009

62_IND04	Pulau Wayag		Indonesia	62	Adam Caris	0.1767	130.0150	01/07/2009
64_NZL01	Butterfly Bay	Karitane	New Zealand	64	Tracy Farr	45.6424 -	170.6635	19/10/2009
64_NZL02	Butterfly Bay	Karitane	New Zealand	64	Tracy Farr	45.6424 -	170.6635	19/10/2009
64_NZL03	Lyall Bay	Wellington	New Zealand	64	Tracy Farr	41.3354 -	174.8045	21/10/2009
64_NZL04	Lyall Bay	Wellington	New Zealand	64	Tracy Farr	41.3354 -	174.8045	21/10/2009
64_NZL06	Seatoun	Wellington	New Zealand	64	Tracy Farr	41.3186 -	174.8293	21/10/2009
64_NZL07	Scorching Bay	Wellington	New Zealand	64	Tracy Farr	41.2982 -	174.8326	21/10/2009
64_NZL08	Scorching Bay	Wellington	New Zealand	64	Tracy Farr	41.2982 -	174.8326	21/10/2009
64_NZL09	Evans Bay	Wellington	New Zealand	64	Tracy Farr	41.3046 -	174.8021	21/10/2009
64_NZL10	Evans Bay	Wellington	New Zealand	64	Tracy Farr	41.3046	174.8021	21/10/2009
671_GUA01	Tagachang Reef	Guam	Guam	671	Chris Lobban	13.4203	144.7845	missing
81_JAP03	Tenjin	Japan	Japan	81	David Montagnes	31.4677	131.1429	25/04/2009
81_JAP04	Tokyo Bay		Japan	81	Teppei Nakamoto	35.6260	139.7510	13/05/2009
81_JAP05	Miyako Islands	Shakishima Islands	Japan	81	Teppei Nakamoto	24.8120	125.1800	08/05/2009
81_JAP06	Miyako Islands	Shakishima Islands	Japan	81	Teppei Nakamoto	24.8130	125.1450	08/05/2009
81_JAP07	Miyako Islands	Shakishima Islands	Japan	81	Teppei Nakamoto	24.7430	125.2600	10/05/2009
852_HON01	Coffee Bay	Hong Kong	Hong Kong	852	David Montagnes	22.3218	114.1531	25/04/2009
852_HON02	Golden Bay	Hong Kong	Hong Kong	852	David Montagnes	22.3161	114.1926	25/04/2009
852_HON03	Tsing Yi	Hong Kong	Hong Kong	852	David Montagnes	22.3598	114.1090	25/04/2009
852_HON04	Yung shue wan	Hong Kong	Hong Kong	852	Lucy Hopcroft	22.2214	114.1051	18/04/2009
86_CHN02	China	China	China	86	Yang Zhou	31.9522	120.8716	Jan-09
86_CHN03	Shen Zhen, China (QD?)	China	China	86	David Montagnes	22.5205	114.0240	18/04/2009
86_CHN08	Qing Dao	China	China	86		36.0616	120.3184	13/06/2008
86_CHN09	Qing Dao	China	China	86		36.0616	120.3184	13/06/2008
86_CHN10	Qing Dao	China	China	86		36.0616	120.3184	13/06/2008
86_CHN13	Techeng Island		China	86		21.1502	110.4407	12/04/2010
86_CHN14	Techeng Island		China	86		21.1502	110.4407	12/04/2010
91_IND01	Agronda	India	India	91	Phill Watts	15.1782	73.9407	missing
91_IND02	Cave	India	India	91	Phill Watts	15.1782	73.9407	missing
94_SLK01	Weligama		Sri Lanka	94		5.9596	80.4220	01/08/2009
94_SLK02	Weligama		Sri Lanka	94		5.9596	80.4220	01/08/2009
94_SLK03	Weligama		Sri Lanka	94		5.9596	80.4220	01/08/2009
94_SLK04	Weligama		Sri Lanka	94		5.9596	80.4220	01/08/2009
960_MLD01	Male	Maldives	Maldives	960	Laura Martin	4.1902	73.5267	12/09/2008
966_ALN01	Alanbar	Saudi Arabia	Saudi Arabia	966	Naseem Radi	21.6330	39.1044	05/10/2008
972_EIL01	Eilat	Red Sea	Israel	972	Dror Angel	29.4665	34.9161	01/07/2009
972_EIL02	Eilat	Red Sea	Israel	972	Dror Angel	29.4665	34.9161	01/07/2009
972_EIL03	Eilat	Red Sea	Israel	972	Dror Angel	29.4665	34.9161	01/07/2009
972_EIL04	Eilat	Red Sea	Israel	972	Dror Angel	29.4665	34.9161	01/07/2009
64_NZL05	Seatoun	Wellington	New Zealand	64	Tracy Farr	41.3186 -	174.8293	21/10/2009