Morphological assessment of the *Octopus vulgaris* species complex evaluated in light of molecular-based phylogenetic inferences


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Cryptic species are common in the ocean, particularly among marine invertebrates such as octopuses. Delineating cryptic species is particularly problematic in octopus taxonomy where the plasticity recorded among taxonomic characters often results in low resolution at the species level. This study investigated the morphological relationships among seven phylogenetic clades (identified using cytochrome c oxidase subunit I) of the broadly distributed *Octopus vulgaris* species complex and close relatives. Morphological analyses in this study were successful in delimiting *O. sinensis*, Brazilian *O. vulgaris* and *O. vulgaris sensu stricto*, which was congruent with the molecular findings of this study. Analyses based on male morphology were successful in distinguishing 14 of 15 total pairwise comparisons and proved to be a more reliable indicator of species-level relationships in comparison with female morphology. The majority of characters with the greatest discriminatory power were male sexual traits. Significant morphological differences were also recorded among sampling localities of conspecifics, with phenotype showing correlation with local environmental data. The findings of this study support the hypothesis that multiple *O. vulgaris*-like species are currently being incorrectly treated under a single species name, *O. vulgaris*. Octopuses being exported globally under the name *O. vulgaris* are of extremely high fisheries market value and profile. Our findings have potentially significant implications for the naming and conservation of commercially harvested members of this species complex throughout their ranges.

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Introduction

The marine environment has traditionally been thought of as a large continuous system with relatively few barriers to dispersal. Organisms with an effective dispersal capability may therefore have the potential to maintain global genetic homogeneity (Waples 1987). However, dispersal distances of pelagic larvae are influenced by several physiological and biological factors (Hohenlohe 2004) and are often unknown (Knowlton 1993). Several examples exist where organisms once thought to be cosmopolitan in distribution, are now understood to represent morphologically similar yet genetically distinct cryptic species with relatively restricted distributions (Knowlton 1993; Klautau et al. 1999; Bickford et al. 2007). Cryptic species are common among marine invertebrates (Knowlton 1993), many of which lack identifiable delineating morphological traits (Klautau et al. 1999). This results in cryptic taxa being ‘lumped’ into single morphospecies, despite being genetically distinguishable. Cryptic diversity is often missed due to an inability to recognise distinguishing morphological traits, distortion of specimens through preservation and/or an inability to quantify the chemical recognition/communication systems that delineate species.

One marine group where cryptic species are common are the cephalopods, including squids and octopuses (Norman et al. 2014a,b). Taxonomy (Norman & Hochberg 2005; Norman et al. 2014b) and phylogenetic relationships (Carlini et al. 2001; Guzik et al. 2005; Strugnell et al. 2008a,b, 2014; Kaneko et al. 2011; Acosta-Jofré et al. 2012) within the benthic octopuses have received greater attention in recent years, with a number of cryptic species being identified (Pickford & McConaughy 1949; Söller et al. 2000; Alcock 2005; Leite et al. 2008; Alcock et al. 2011; Amor et al. 2014; Reid & Wilson 2015). The difficulties in identifying octopuses and understanding their evolutionary relationships are well illustrated by the current uncertainty and confusion surrounding the phylogeny and taxonomy of genus Octopus Cuvier, 1797 (type genus of the family Octopodidae d’Orbigny, 1839). Octopus has long been considered a ‘catch all’ genus (e.g. Nesis 1998), with few morphological characters available for distinguishing among closely related taxa. More recently, the genus Octopus was characterised by a muscular mantle and arms, sacculary mantle with a wide opening, two rows of suckers on each arm, hectocotylised third right arm, terminal organ with diverticulum, functional ink sac, well-developed anal flaps, the absence of water pouches on the oral surface of webs and a benthic adult life history (Norman & Sweeney 1997; Sweeney & Roper, 1998).

Species-level taxonomy of octopuses has been hindered due to morphological plasticity (Robson 1929; Pickford 1945; Voight 1994; O’Shea 1999) because their characteristic soft body has few hard structures (Bookstein et al. 1985) and is subject to distortion upon preservation (Pickford 1964; Burgess 1966; Voight 2001). This means that using morphological characters to distinguish closely related species is particularly difficult (e.g. Norman & Kabodera 2006); however, recent morphology-based studies suggest that benthic octopuses can be delineated based on discrete phenotypic differences (Gleadall et al. 2010; Gleadall 2013, 2016; Amor et al. 2014). Recent taxonomic revisions (O’Shea 1999; Norman et al. 2014a) and molecular-based phylogenetic studies (Guzik et al. 2005; Kaneko et al. 2011; Acosta-Jofré et al. 2012; Lü et al. 2013) have confirmed that the genus Octopus is polyphyletic, containing a large assemblage of species groups comprising a number of different genera. The species group most similar in morphology and behaviour to the type species of the genus (Octopus vulgaris Cuvier, 1797) has been identified as the ‘O. vulgaris species group’, based on general similarities in overall size, mantle shape, arm length and skin sculpture (Robson 1929). Species in this group are now considered to comprise the genus Octopus sensu stricto (O’Shea 1999).
Historically, *O. vulgaris* was considered to be a cosmopolitan species. First reported from the Mediterranean Sea and eastern North Atlantic, *O. vulgaris* has been reported from the subtropical waters of Australasia, Europe, Africa, Asia and the Americas. However, recent analyses (Söller et al. 2000; Leite et al. 2008; Amor et al. 2014, 2015; Gleadall 2016) suggest that populations previously treated as *O. vulgaris* comprise a complex of morphologically similar but genetically distinct *vulgaris*-like species (the ‘*O. vulgaris* species complex’). *Octopus vulgaris sensu stricto* (s. s.) occurs in the Mediterranean and eastern North Atlantic. Other members of this species complex include several species ‘Types’, which have been recognised based on geographic isolation and lack of plausible gene flow mechanisms (Norman et al. 2014a; Fig. 1). Type I occurs in the Caribbean and Gulf of Mexico, Type II in the western South Atlantic along the coast of Brazil, and Type III occurs in the eastern South Atlantic and the Indian Ocean, along the coast of South Africa. Type IV occurs in subtropical to temperate eastern Asia. *Octopus jollyorum* Reid & Wilson, 2015 was described based on a phylogenetic analysis that included ‘*O. vulgaris*’ individuals sampled throughout its known range and the discovery of a member of the group in the Kermadec Islands. *Octopus jollyorum* was used to recognise a clade that included *O. vulgaris* individuals sampled throughout its known range and the discovery of a member of the group in the Kermadec Islands. *Octopus jollyorum* was used to recognise a clade that included specimens from Japan and Taiwan, as no type specimen existed for a potential available name, *O. sinensis*. Subsequently, Gleadall (2016) designated a neotype for *O. sinensis*. While not stated in Gleadall (2016), we now recognise *O. jollyorum* as a junior synonym of *O. sinensis* and the latter name is used for representatives of this clade in the current study. Recent molecular-based analyses support the hypothesis that *O. vulgaris* s. s., *O. sinensis* and *O. vulgaris* Type II represent distinct species within the *O. vulgaris* species complex (Amor et al. 2015). However, the only recent morphological comparisons undertaken to investigate the taxonomic relationships among members of the *O. vulgaris* species complex are those between *O. vulgaris* s. s. and *O. insularis* Leite et al. 2008 (in Leite et al. 2008) and *O. sinensis* (Gleadall 2016). This study employs the first ever global-scale sampling strategy to investigate morphological variation and determine the validity of morphological based identifications among members and close relatives of the *O. vulgaris* species complex. We combine analyses conducted using conventional morphological traits and a more extensive data set. Phylogenetic analyses based on the mitochondrial ‘barcode of life’ gene cytochrome *c* oxidase subunit I (*COI*) are also used to provide insights into taxonomic resolution among taxa currently being treated as *O. vulgaris*.

### Materials and methods

#### Sampling

Whole specimens and tissue samples of *O. vulgaris* species group individuals were sourced from museums, university collections and fish markets from the Atlantic, Indian and Pacific oceans and the Mediterranean Sea (Fig. 1, Table 1). Tissue samples were stored in 70–90% ethanol. After tissue samples were taken, whole specimens were fixed in 10% formalin and later preserved in 70% ethanol following methods outlined in (Roper & Sweeney 1983).

![Fig. 1 Sampling localities (triangles) for whole animals/tissue samples of members of the Octopus vulgaris species group and close relatives. Distributions of Octopus vulgaris sensu stricto and species ‘Types’ are shaded in dark grey (Norman et al. 2014a). Distributions of non-vulgaris species are represented by dashed lines. Externally sourced data (Banyuls-sur-Mer, France; Table 1) are represented by a circle.](image)
### Molecular analyses

**Sequencing.** Genomic DNA was extracted from mantle or arm tissue samples of 1–2 mm³ (avoiding skin where possible) using a QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer’s instructions. Partial COI sequences were amplified via PCR using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR solutions (25 μL) were composed of 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 12.5 μL MyTaq Red Mix (Bioline), 9.5 μL H₂O and 2 μL DNA (5–10 ng total concentration). PCR cycle conditions were as follows: a single initial denaturing step (two minutes at 95°C); 35 cycles of denaturing (30 seconds at 95°C); annealing (30 seconds at 48°C); and extension (30 seconds at 72°C); and a single final extension step (five minutes at 72°C). PCR products were sequenced by Macrogen Inc (Seoul, Korea). COI sequences generated in this study were deposited in GenBank under accession numbers KU525758–KU525769. Additional sequences from previously published work were obtained from GenBank (Table S1). *Octopus cyanea* was selected as the out-group to root the phylogenetic tree (Amor et al. 2015), as it is the closest known relative of the in-group (Acosta-Jofré et al. 2012). Multiple sequence alignment of the 482 base pair partial COI fragments was performed using the ‘MUSCLE Alignment’ feature (Edgar 2004) within Geneious v7.1.3 (created by Biomatters; available from http://www.geneious.com/).

**Molecular-based phylogenetic analyses.** jModelTest v0.1.1 (Posada 2008) was used to carry out statistical selection of best-fit models of nucleotide substitution of the COI alignment. The appropriate model (GTR+G) was chosen based on ‘goodness of fit’ via the Akaiake information criterion (AIC; Akaiake 1974). Maximum-likelihood (ML) topologies were constructed using RAxML v8.0.19 (Stamatakis 2014). Strength of support for internal nodes of ML construction was measured using 1000 rapid bootstrap replicates. Bayesian inference (BI) marginal posterior probabilities were calculated using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). Model parameter values were treated as unknown and were estimated. Random starting trees were used and the analysis was run for fifteen million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of <0.01 was used as a guide to ensure the two independent analyses had converged. The program Tracer v1.3 (Rambaut et al. 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct ‘burn-in’ for the analysis.

### Morphological analyses

Standard morphological characters were measured using digital callipers following Roper & Voss (1983) and Norman & Sweeney (1997): dorsal mantle length (MLd), ventral mantle length (MLv), mantle width (MW), head width (HW), funnel length (FL), free funnel length (FFL), gill length (GL), enlarged sucker diameter (SDe), non-enlarged sucker diameter (SDn), specialisations at the tip of the males hectocotylised (third right) arm (ligula length, LL; calamus length, CL), terminal organ length (TOL) and arm width (AW) were all recorded to the nearest 0.1 mm. Web depth (WD) was measured from the beak opening to the mid-point of the web sector; the length of the arms on the left (ALL1-4) and right (ALR1-4) side from the beak opening to the arm tip was measured to the nearest 1 mm using stretch-resistant cord. The number of suckers on the left third arm (SCL) and the right third arm (SCR; which

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### Table 1 Sample data for Octopus species analysed in this study

<table>
<thead>
<tr>
<th>Species/Type</th>
<th>Location</th>
<th>Institution</th>
<th>Sample type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. vulgaris s. s.</td>
<td>Banyuls-sur-Mer, France</td>
<td>Santa Barbara Museum of Natural History</td>
<td>Data</td>
<td></td>
</tr>
<tr>
<td>O. vulgaris s. s.</td>
<td>Galicia, Spain</td>
<td>Consejo Superior de Investigaciones Científicas (CSIC), Vigo</td>
<td>Whole/Tissue</td>
<td></td>
</tr>
<tr>
<td>O. vulgaris s. s.</td>
<td>Mauritania</td>
<td>Instituto Español de Oceanografía (IEO), Tenerife</td>
<td>Whole/Tissue</td>
<td></td>
</tr>
<tr>
<td>O. sinensis</td>
<td>China</td>
<td>Fisheries College, Ocean University of China, Qingdao</td>
<td>Whole/Tissue</td>
<td>Reid &amp; Wilson (2015)</td>
</tr>
<tr>
<td>O. sinensis</td>
<td>Keelung/Da si, Taiwan</td>
<td>National Taiwan Ocean University, Keelung</td>
<td>Whole/Tissue</td>
<td>Reid &amp; Wilson (2015)</td>
</tr>
<tr>
<td>O. sinensis</td>
<td>Kyushu/Sendai, Japan</td>
<td>Tohoku University, Sendai</td>
<td>Whole/Tissue</td>
<td></td>
</tr>
<tr>
<td>O. vulgaris Type II</td>
<td>Pontal do Paraná, Brazil</td>
<td>Universidade Federal do Paraná (UFPR)</td>
<td>Whole</td>
<td></td>
</tr>
<tr>
<td>O. insularis</td>
<td>Rio Grande do Norte/Brazil</td>
<td>Universidade Federal do Rio Grande do Norte (UFRN)</td>
<td>Whole</td>
<td></td>
</tr>
<tr>
<td>O. insularis</td>
<td>Saint Peter and Saint Paul Archipelago, Brazil</td>
<td>Universidade Federal do Rio Grande do Norte (UFRN)</td>
<td>Whole</td>
<td></td>
</tr>
<tr>
<td>O. insularis</td>
<td>Trinidad Island, Brazil</td>
<td>Universidade Federal do Rio Grande do Norte (UFRN)</td>
<td>Whole</td>
<td></td>
</tr>
<tr>
<td>O. mimus</td>
<td>Tocopilla/Pisagua, Chile</td>
<td>Consejo Superior de Investigaciones Científicas (CSIC), Vigo</td>
<td>Data</td>
<td>Guerra et al. (1999)</td>
</tr>
<tr>
<td>O. tetricus</td>
<td>New South Wales, Australia</td>
<td>Museum Victoria</td>
<td>Whole/Tissue</td>
<td></td>
</tr>
<tr>
<td>O. tetricus</td>
<td>Tasmania, Australia</td>
<td>Museum Victoria</td>
<td>Tissue</td>
<td></td>
</tr>
<tr>
<td>O. cf. tetricus</td>
<td>Western Australia, Australia</td>
<td>Fisheries and Marine Research Laboratories, Western Australia and Museum Victoria</td>
<td>Whole/Tissue</td>
<td></td>
</tr>
</tbody>
</table>
for males is the sucker count of the hectocotylised arm, HASC) was counted with the aid of a dissecting microscope. Arm lengths and sucker counts were excluded where damage to an arm was perceived to inhibit growth, suckers appeared damaged and no scars/remnants were visible, or arm regeneration was evident (Tables S2 and S3). All missing data due to these exclusions were replaced with the ‘local’ mean of that trait across the geographic location as missing data were not permitted in analyses.

Morphological data sets were recorded only for mature males and females. To account for differences attributed to variation in overall size, and to allow for investigation of size free trait variation, all morphometric and meristic traits (with the exception of SC, FFL, LL and DL) were transformed to indices, dividing each trait by the dorsal mantle length (a proxy for body size) of the respective specimen. The remaining indices were obtained as follows: sucker counts of each arm were divided by the respective arm length, FFL was divided by FL, LL was divided by CL, and DL was divided by TOL. Morphological relationships were investigated using the complete set of traits recorded during this study (25 traits for males; 20 traits for females; Tables S2 and S3, respectively). For comparison with published data, a reduced number of traits were also analysed independently (12 traits for males; 8 traits for females; see traits marked with *+ in Tables S2 and S3, respectively). The reduced set of traits were MLd, MW, HW, FL, FFL, WD, ALL3/R3, SDn, SCL3/R3 (HASC, males only), LL (males only) and CL (males only). Analyses of reduced and complete trait data sets were performed on males and females separately to enable the inclusion of male-specific reproductive characters in morphological analyses.

Morphological indices of both males and females were mean scale-transformed (Berner 2011) and normalised using the ‘normalise variables’ function in PRIMER E+ v6 and PERMANOVA+ (Anderson et al. 2008) to allow for comparisons of traits despite differing scales of measurement. All morphological analyses were performed using PRIMER E+ v6 (Clarke & Gorley 2006) and PERMANOVA+ (Anderson et al. 2008). Collinearity and redundancy of morphological traits was investigated via principal component analysis (PCA) vector plots, Draftsman plots and Spearman correlation matrices as detailed in the user manual (Anderson et al. 2008). Highly correlated variables (R² ≥ 85%) were considered redundant. The effect of within-clade multivariate dispersion (i.e. the significance of within-clade variation contributing to between-clade differences) was investigated via permutation-based distance-based tests for homogeneity of multivariate dispersions (PERMDISP). Differences in morphological traits among sampled individuals were analysed via permutational multivariate ANOVA (PERMANOVA). A resemblance matrix based on Euclidean distance was calculated. To visualise the relationships among locations, PCA was performed using the COI-based phylogenetic clade as an independent factor to group individuals into taxonomically informative entities. Variable contributions to variation were investigated via similarity percentages (SIMPER) analysis (Clarke 1993). To evaluate the discriminative power of the morphological traits used, estimates of group assignment were performed using canonical analysis of principal components (CAP).

Comparative analyses

Environmental data were incorporated to estimate correlations between morphological variation and each environmental predictor variable. Mean annual (1900–1997) sea surface temperature (SST), sea bottom temperature (SBT) and salinity were obtained from NOAA (2014). A distance-based linear model (Anderson et al. 2008) was used to perform a marginal test on each environmental variable to determine the overall morphological variation explained.

To quantify the variability in the morphological resemblance matrix that was explained by environmental variables, a stepwise sequential test was performed using the AIC to select the model of best fit.

Results

Phylogenetic relationships

Topologies resulting from molecular-based ML and BI analyses showed a highly supported monophyletic clade containing O. insularis, O. mimus Gould, 1852, O. bimaculoides Pickford & McConnaughey, 1949 and O. maya Voss and Solis Ramírez, 1966 (bootstrap value [BS] = 95, posterior probability [PP] = 1; Fig. 2). This clade was sister taxon to (1) a clade containing O. hummelincki Adam, 1936 and (2) a clade containing the O. vulgaris species complex O. tetricus Gould, 1852 and O. cf. tetricus of Australasia (BS = 64, PP = 0.66). All members of the O. vulgaris species complex formed a highly supported monophyletic clade that also included O. tetricus and O. cf. tetricus (BS = 95, PP = 1; O. vulgaris group). The O. vulgaris species complex formed three distinct monophyletic clades, which corresponded to three of the O. vulgaris ‘Types’ described in Norman et al. (2014a): Clade 9, O. simonis (Asia and Kermedec Is; BS = 75, PP = 1); Clade 10, O. vulgaris Type II (southern Brazil: BS = 69, PP = 0.83); and Clade 11, O. vulgaris s. s. and O. vulgaris Type III (South Africa: BS = 88, PP = 1), which also included a single individual from southern Brazil.

Morphological relationships

Comparison of complete and reduced trait data sets. PERMANOVA comparisons and assignment of individuals to their a priori molecular-based phylogenetic clade via CAP were more...
successful using male and female complete trait data sets (Tables 2–3 and S8-S11). Analyses based on the reduced trait data sets are presented in online supplementary data associated with this manuscript. Analyses based on the complete trait data sets are presented below.

**Analyses of male specimens.** Male arm lengths (L2, L3, L4 and R2) displayed ≥85% correlation with each other. Arm length data were most complete for arm L3; therefore, ALL3 was retained while the remaining correlated arm lengths were considered redundant and excluded from analyses. Within-clade variation had no significant impact on among clade analyses (P = >0.05). A significant difference was recorded among the six molecular-based phylogenetic clades investigated (Pseudo-F = 5.2805, df = 5, P = 0.001). Pairwise comparisons among these six phylogenetic clades showed 14 of 15 (93%) significant differences (Table 2). All members of the *O. vulgaris* species complex were distinguished based on morphological analyses (P = <0.02). *Octopus vulgaris* s. s. and *O. sinensis* were distinguished primarily by differences in GL and ALR4. *Octopus vulgaris* s. s. was distinguished from *O. vulgaris* Type II primarily by SDe. *Octopus sinensis* was distinguished from *O. vulgaris* Type II by significantly longer gills (GL). *Octopus insularis* specimens were found to be morphologically distinct from all other taxa in the *O. vulgaris* species complex (P = <0.002). The greatest sources of variation between *O. vulgaris* s. s. and *O. insularis* were attributed to differences in ALR3 and HASC. *Octopus vulgaris* Type II and *O. insularis* were primarily distinguished by DL and HASC. *Octopus sinensis* and *O. insularis* were distinguished by variations in GL and TOL. *Octopus tetricus* and *O. cf. tetricus* differed significantly from each other (P = 0.012), particularly through differences in SCL3 and DL. No morphological differences were found between *Octopus vulgaris* s. s. and *O. cf. tetricus* (P = 0.095).

Visualisation of the principal component biplot for males (Fig. 3A) showed *O. vulgaris* s. s. and *O. vulgaris* Type II males to have greater levels of morphological variability in comparison with other taxa, which was demonstrated by
Although Octopus vulgaris Type II individuals had relatively fewer suckers on the third arm than Octopus vulgaris s. s. and Octopus sinensis, Octopus tetricus, Octopus cf. tetricus and Octopus insularis demonstrated negative PC2 loadings attributed to high sucker numbers. Octopus tetricus and Octopus insularis showed the least overlap with other taxa included in the analysis, but Octopus cf. tetricus overlapped with all members of the Octopus vulgaris species complex.

Of the 68 male individuals analysed, 54 (79%) were correctly assigned to their a priori group via CAP (Table 2). For Octopus vulgaris s. s., 16 individuals (84%) were correctly classified: the remainder were misclassified as Octopus sinensis (n = 1). Twelve Octopus sinensis individuals (75%) were correctly assigned to their a priori group, with the remaining individuals being misclassified as Octopus vulgaris s. s. (n = 1), Brazilian Octopus tetricus Type II (n = 1), Octopus insularis (n = 1) or Octopus cf. tetricus (n = 1). Nine Octopus vulgaris Type II individuals (82%) were correctly classified, while the remaining individuals were misclassified as Octopus vulgaris s. s. (n = 1) and Octopus insularis (n = 1). Eight Octopus insularis individuals were correctly assigned (67%), with the remaining individuals misclassified as Octopus vulgaris s. s. (n = 1), Octopus tetricus (n = 1) or Octopus cf. tetricus (n = 2). Four Octopus tetricus individuals (80%) were correctly assigned, with the remaining individual being misclassified as Octopus sinensis. All Octopus cf. tetricus individuals (n = 5) were correctly assigned to their respective a priori group.

Analysis of female specimens. Significant within-clade variation was recorded for Octopus vulgaris s. s. and Octopus insularis females (P = 0.03). The main-effects model showed significant morphological differences among the six molecular-based phylogenetic clades of female individuals (Pseudo-F = 3.8184, df = 5, P = 0.001). Pairwise comparisons showed that 10 of 15 (67%) comparisons had significant morphology-based differences (Table 3). All members of the Octopus vulgaris species complex (Octopus vulgaris s. s., Octopus sinensis and Octopus vulgaris Type II) were successfully distinguished based on multivariate morphological analyses (P = ≤0.01). Octopus vulgaris s. s. and Octopus sinensis were distinguished primarily by arm length (L3) and sucker diameter. Arm width was the primary source of variation between Octopus vulgaris s. s. and Octopus vulgaris Type II. Octopus sinensis and Octopus vulgaris Type II were found to differ in gill length and arm width. All members of the Octopus vulgaris species complex were distinguished from Octopus insularis (p = ≤0.005). Variation between Octopus vulgaris s. s. and Octopus insularis was primarily attributed to

Table 2 Pairwise comparisons of male Octopus vulgaris species group and O. insularis individuals based on 25 morphological traits

<table>
<thead>
<tr>
<th></th>
<th>O. vulgaris s. s.</th>
<th>O. sinensis</th>
<th>O. vulgaris Type II</th>
<th>O. insularis</th>
<th>O. tetricus</th>
<th>O. cf. tetricus</th>
<th>Correct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. vulgaris s. s.</td>
<td>–</td>
<td>ALL3/SN</td>
<td>AW</td>
<td>SCR3/SCR3*</td>
<td>SCR3/HW</td>
<td>SCLR3</td>
<td>76.2</td>
</tr>
<tr>
<td>O. sinensis</td>
<td>0.004</td>
<td>–</td>
<td>GLL/AW</td>
<td>SCL3</td>
<td>SCR3/F</td>
<td>HW</td>
<td>50</td>
</tr>
<tr>
<td>O. vulgaris Type II</td>
<td>0.01</td>
<td>0.001</td>
<td>–</td>
<td>SCR3</td>
<td>SCR3/AW</td>
<td>AW</td>
<td>71.4</td>
</tr>
<tr>
<td>O. insularis</td>
<td>0.001*</td>
<td>0.001</td>
<td>0.003</td>
<td>–</td>
<td>SCL3/F</td>
<td>ALL1/3</td>
<td>100</td>
</tr>
<tr>
<td>O. tetricus</td>
<td>0.093</td>
<td>0.119</td>
<td>0.039</td>
<td>0.004</td>
<td>–</td>
<td>HW</td>
<td>75</td>
</tr>
<tr>
<td>O. cf. tetricus</td>
<td>0.181</td>
<td>0.05</td>
<td>0.041</td>
<td>0.012</td>
<td>0.114</td>
<td>–</td>
<td>50</td>
</tr>
</tbody>
</table>

Lower left diagonal represents PERMANOVA results with significant differences (P < 0.05) highlighted in bold. Upper right diagonal represents results of SIMPER analyses showing traits that contribute most to variation between groups. SIMPER results are also shown in bold if corresponding PERMANOVA showed a significant difference. Asterisks represent pairwise comparisons effected by significant within-clade variation. Far right column represents the percentage of individuals assigned to their a priori group via CAP analysis (see Table S4 for full CAP analysis table).
differences in the number of suckers on the third arm pair, which was also the greatest source of variation between *O. vulgaris* Type II and *O. insularis*. *Octopus sinensis* and *O. insularis* were best delineated by the variation in sucker numbers on the third left arm. *Octopus tetricus* and *O. cf. tetricus* were unable to be distinguished based on morphology (*P* = 0.3).

Visualisation of the principal component biplot for females (Fig. 3B) showed that *O. vulgaris* s. s. and *O. sinensis* had the most morphological variability, with highly positive and negative PC1 and PC2 loadings. *Octopus vulgaris* Type II was characterised by positive PC2 loadings (low SCL/R3). *Octopus insularis* individuals formed a distinct group characterised by positive PC1 and negative PC2 loadings (low arm lengths and high sucker counts).

Overall, 41 of the 62 analysed female individuals (66%) were correctly assigned via CAP (Table 3). Sixteen *O. vulgaris* s. s. individuals (76%) were correctly classified, while four individuals were misclassified as *O. sinensis* and a single individual as *O. tetricus*. Ten *O. sinensis* individuals (50%) were correctly assigned to their a priori group, with the remaining individuals being misclassified as *O. vulgaris* s. s. (*n* = 5), *O. insularis* (*n* = 1), *O. tetricus* (*n* = 2) and *O. cf. tetricus* (*n* = 2). Five *O. vulgaris* Type II individuals (71%) were correctly assigned, with a single individual misclassified as *O. vulgaris* s. s., *O. sinensis* and *O. tetricus*. All *O. insularis* individuals (*n* = 6) were correctly assigned, while 75% of *O. tetricus* and 50% of *O. cf. tetricus* individuals were assigned correctly.

**Reduced trait analyses of male *O. vulgaris* s. s.** Significant differences were recorded among Galician, Mediterranean and Mauritanian males (*P* = 0.001), with the pairwise multivariate model showing a significant difference among the three localities (*P* = ≤0.004; Table 4).

A PC biplot (Fig. 4A) showed that each sampling locality for *O. vulgaris* s. s. males could be distinguished, although there was some overlap. Individuals from the Mediterranean were found to have greater sucker numbers (L3, R3) in comparison with Galician and Mauritanian (eastern North Atlantic) individuals. Galician and Mauritanian individuals were able to be distinguished along PC1, as Galican males had longer arms (L3, R3).

Based on the CAP, 24 of the 27 *O. vulgaris* s. s. males (89%) were correctly assigned (Table 4). All individuals from Mauritania (*n* = 8) were successfully assigned to their correct sampling locality: eight of the nine Mediterranean individuals (89%) were correctly assigned, with a single individual being misclassified as Galician, and eight of the ten Galician individuals (80%) were correctly assigned,

### Table 4: Pairwise comparisons of *O. vulgaris* s. s. individuals based on 12 morphological traits

<table>
<thead>
<tr>
<th></th>
<th>Galicia</th>
<th>Mediterranean</th>
<th>Mauritania</th>
<th>Correct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediterranean</td>
<td>P = 0.004</td>
<td>--</td>
<td>ALR3/SCL3</td>
<td>80</td>
</tr>
<tr>
<td>Mauritania</td>
<td>P = 0.003</td>
<td>ALR3</td>
<td>FFL/LL</td>
<td>88.9</td>
</tr>
</tbody>
</table>

Lower left diagonal represents *PERMANOVA* results with significant differences (*P* = 0.05) highlighted in bold. Upper right diagonal represents results of SIMPER analyses showing traits that contribute most to variation between groups. SIMPER results are also shown in bold if corresponding *PERMANOVA* showed a significant difference. Far right column represents the percentage of individuals assigned to their a priori group via CAP analysis (see Table S5 for full CAP analysis table).
with the remaining two individuals misclassified as Mauritanian.

Variation attributable to environmental data explained 31.4% of the variation in male morphology ($R^2 = 0.31354$). Investigating each trait independently via marginal tests, SST explained 21.3% ($P = 0.001$) and SBT 21.2% ($P = 0.001$) of the variation. Sequential tests revealed that SST accounted for 21.3% of the variation seen in the morphological data ($P = 0.002$). Once SST was accounted for, SBT explained a further 10% of the variation ($P = 0.002$). Latitude, longitude and depth did not explain any further variation, although each was found to explain a significant amount of the variation in morphology when analysed independently ($P = 0.001$, $P = 0.005$ and $P = 0.023$, respectively).

Reduced trait analyses of female *O. vulgaris* s. s. A significant difference was recorded among Galician, Mediterranean and Mauritanian females ($P = 0.001$), with the pairwise multivariate model showing a significant difference among the three localities ($P = 0.001$; Table 5).

A PC biplot (Fig. 4B) distinguished *O. vulgaris* s. s. females by locality. Individuals from the eastern North Atlantic (Galicia and Mauritania) were more similar to each other than they were to Mediterranean females, which have longer funnels (FL). Individuals from the eastern North Atlantic differed, with Galician males possessing more suckers (SCL/SCR) and a larger head (HW).

Of 27 female *O. vulgaris* s. s. individuals, 26 (96%) were correctly assigned to their *a priori* group (Table 5). Individuals from Mauritania and the Mediterranean (France) were all assigned with 100% accuracy, and nine of the ten Galician individuals were assigned correctly (90%), with the remaining individual misclassified as Mediterranean.

Of the overall variation in female morphology, 33.9% was correlated with variation in environmental data ($R^2 = 0.33854$). Investigating each trait independently via marginal tests showed that latitude explained 20.8% ($P = 0.001$) and SST 18.8% ($P = 0.001$) of the variation. In sequential tests, latitude accounted for 20.8% of the morphological variation ($P = 0.001$). With latitude accounted for, SST explained a further 13% of the variation ($P = 0.002$); once both latitude and SST were accounted for, SBT, longitude and depth explained no further variation (although a significant amount of variation in morphology was explained when these parameters were analysed independently: $P = 0.002$, $P = 0.001$ and $P = 0.001$, respectively).

Table 5 Pairwise comparisons of female *Octopus vulgaris* sensu stricto individuals based on eight morphological traits

<table>
<thead>
<tr>
<th></th>
<th>Galicia</th>
<th>Mauritania</th>
<th>Mediterranean</th>
<th>Correct (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galicia</td>
<td>–</td>
<td>SCL3/HW</td>
<td>FFL/FL</td>
<td>90</td>
</tr>
<tr>
<td>Mauritania</td>
<td>$P = 0.001$</td>
<td>–</td>
<td>FFL/SCL3</td>
<td>100</td>
</tr>
<tr>
<td>Mediterranean</td>
<td>$P = 0.002$</td>
<td>$P = 0.001$</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>

Lower left diagonal represents PERMANOVA results with significant differences ($P < 0.05$) highlighted in bold. Upper right diagonal represents results of SIMPER analyses showing traits that contribute most to variation between groups. SIMPER results are also shown in bold if corresponding PERMANOVA showed a significant difference. Far right column represents the percentage of individuals assigned to their *a priori* group via CAP analysis (see Table S7 for full CAP analysis table).
Discussion

Molecular-based phylogenetic analyses of *O. vulgaris* species group individuals support the presence of six distinct clades, which were used as a discriminant factor in morphological analyses. Multivariate morphological analyses using conventional morphological traits were successful in distinguishing the majority of these clades and support the hypothesis of greater species-level diversity within the *O. vulgaris* species complex (*O. vulgaris* s. s., *O. vulgaris* Type II and *O. sinensis*). Although each of these species was successfully distinguished, further distinctions were detected among the sampling localities of *O. vulgaris* s. s., suggesting a requirement of broad sampling across the known distribution to ensure robust future morphological analyses of this group.

Previous molecular-based phylogenetic analyses using five mitochondrial genes placed Chinese and Japanese *O. vulgaris* into a well-supported monophyletic clade, distinct from all other members of the *O. vulgaris* species complex (Amor et al. 2014). Reid & Wilson (2015) considered mitochondrial-based differences to warrant the distinction of Kermadec Island individuals from *O. vulgaris* s. s., establishing the name *O. jollyorum* for this clade, which also encompassed Asian Type IV *O. vulgaris* individuals. The recent designation of a neotype for *O. sinensis* effectively renames the clade member taxa and places *O. jollyorum* in synonymy with *O. sinensis*. We formally synonymise the two species here. The latter species was redescribed by Gleadall (2016) and can be distinguished from *O. vulgaris* with the former species having shorter arms and fewer suckers. Although individuals from Asia and the Kermadec Islands are currently understood to comprise a single species, the substantial geographic distance between these two geographic regions warrants further investigation into their species-level diversity.

Vidal et al. (2010) compared the morphology and chromatophore patterns of *O. vulgaris* paralarvae from the eastern North Atlantic (Galicia, Spain; *O. vulgaris* s. s.) and the western South Atlantic (southern Brazil; *O. vulgaris* Type II), noting considerable differences in chromatophore numbers. These differences support the hypothesis that *O. vulgaris* Type II is distinct from *O. vulgaris* s. s. The present study reports differences in adult morphology and places individuals from southern Brazil into a monophyletic clade, distinct from *O. vulgaris* s. s. and *O. sinensis*. We therefore recognise *O. vulgaris* Type II as a distinct species within the *O. vulgaris* species complex.

Superficial morphological similarity among species in the *O. vulgaris* species complex had resulted in the assumption that *O. vulgaris* is a single cosmopolitan species. Despite estimates of 3–15 million years divergence between Australasian and Asian taxa (Amor et al. 2014) and 19–41 million years divergence between *O. insularis* and other members of the *O. vulgaris* species group (Amor et al. 2015), principal component plots show that the morphology of these taxa is relatively conservative. The distinct molecular-based clades within the *O. vulgaris* species complex have allopatric distributions; therefore, the selective pressures to adapt their phenotype due to interspecific competition may be reduced. Differentiation in morphological traits is often most extreme where closely related species occur in sympathy (Brown & Wilson 1956), which is thought to limit resource overlap and interspecific competition and allow otherwise directly competing taxa to coexist. Such ‘ecological character displacement’ appears to be a common strategy among closely related taxa and has been documented in a number of plant, reptile, mammal, bird, fish and snail taxa (Dayan & Simberloff 2005). One exception within the *O. vulgaris* species group is the parapatric distribution of *O. vulgaris* Type II (subtropical southern Brazil) and *O. insularis* (mid-Atlantic islands and tropical northern Brazil). Although these two taxa are relatively distantly related, they are very similar in morphology, which may represent a unique opportunity to investigate the extent of this phenomenon within the *O. vulgaris* group.

The sexual traits of male individuals were found to be important characters for morphology-based species discrimination in the *O. vulgaris* species complex, confirming the utility of male sexual traits in cephalopod systematics. Similar findings associated with other animal groups also show that sexual traits are more variable than non-sexual traits (Pomiankowski & Moller 1995), and are often the only reliable delimiting characters among species (Arnqvist 1998). Amor et al. (2014) used 17 morphological characters (five of which were sexual traits) to distinguish *O. tetricus* (from New Zealand and the eastern coast of Australia) and *O. cf. tetricus* (western Australia). HASC was found to be the primary source of variation between the two species, with significantly greater values for *O. cf. tetricus*. However, a study of the genus Pareledone found that morphological traits (including three sexual traits) were unsuccessful in resolving species-level relationships; however, clear genus level resolution was achieved (Allcock et al. 2008).

The utility of HASC has previously been demonstrated in species-level resolution of octopuses (Toll 1988). Among 12 species, Toll (1988) reported HASC values to be relatively fixed among conspecifics. In contrast, the present study found HASC values for *O. vulgaris* s. s. differed significantly among sampling localities. Individuals from the Mediterranean (France) and the eastern North Atlantic (Spain) had overlapping but significantly differing HASC.
values (144–168 and 156–183, respectively). Mauritanian specimens were found to have significantly lower HASC values (114–150) than those for both France and Spain. The significant differences in HASC values reported within *O. vulgaris* s. s. are considered to represent population-level differences. Alternatively, as specimens from Mauritania display minimal overlap in this character compared with those from France and Spain, this may indicate the presence of further species-level diversity within *O. vulgaris* s. s. Such a wide range in HASC values within *O. vulgaris* s. s. therefore suggests the need for caution in basing species within this group on discrimination between HASC values. Voight (2012) questioned the validity of using HASC as a species delimiting trait, also citing wide variation in HASC as a potential problem for species-level inferences, concluding that variation in sucker numbers of ≤15% between potential species should be interpreted with caution. Although variation in HASC values among Australasian members of the *O. vulgaris* species group showed western Australian *O. cf. tetricus* have ~40% greater sucker numbers than those for eastern Australian *O. tetricus*, which was determined to reflect species-level differences (Amor et al. 2014).

The discriminatory power of female-based morphological analyses was weaker than that for males. In the complete and reduced trait data sets, more morphological traits were available for males (male-specific reproductive characters) and these traits were found to be important in distinguishing among the molecular-based phylogenetic clades. In contrast, the female traits found to have the greatest delimiting power among species were non-sexual. Sexual traits, particularly the hectocotylus, are also important distinguishing taxonomic characters for many cephalopods (Bello 1995; Brakoniecki 1996; Norman & Lu 1997; O’Dor & Lipinski 1998; von Byern & Klepal 2010). In comparison with body size and shape traits (which are likely to be less phenotypically and genetically variable between species), sexual traits are often exaggerated and diverse among close relatives (Pomiankowski & Moller 1995), making them ideal candidates for distinguishing among species. While sexual traits were the primary source of morphological variation in the present study, non-sexual traits for both male and female morphology were successful in distinguishing among sampling localities of *O. vulgaris* s. s. (Galicia, France and Mauritania).

The need for greater taxonomic resolution within the family Octopodidae is particularly important in the light of the growing global exploitation of octopuses as a commercial fisheries resource (Norman & Finn 2014). Global production of octopuses exceeds 350 000 tonnes with a total export value of US$1.07 billion, surpassing many valuable finfish fisheries (FAO, 2012). A major limitation of the global catch statistics reported by the FAO is the poor state of octopus taxonomy, with only five (*O. vulgaris*, *O. maya*, *Eledone cirrhosa*, *Eledone moschata* and *Enteroctopus dofleini*) of the estimated 100 species of commercially harvested octopuses listed in global statistics (Norman & Finn 2014). As the majority of octopus fisheries worldwide are in decline (Norman & Finn 2014), this low taxonomic resolution highlights the requirement for more accurate species identification to develop more sustainable octopod fisheries practices. Octopuses being exported globally under the name *O. vulgaris* are of extremely high market value and profile (Norman et al. 2014a), particularly in north-western Africa, the largest single-species octopus fishery in the world (FAO, 2012). Aquaculture and captive growing of wild-caught juveniles are receiving increasing funding, particularly in China (Norman et al. 2014a). Differences among geographic areas in hatching features and paralarvae viability (Iglesias et al. 2007, 2014) may also be linked to taxonomic differences. The findings presented here support the hypothesis that multiple *O. vulgaris*-like species are currently being incorrectly treated under a single species name. Our findings therefore have significant implications for the naming, marketing, value, documentation and potentially conservation of commercially harvested members of this species complex throughout their ranges.

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consumables and sequencing. An Australian Biological Resources Study (ABRS) grant awarded to MDA assisted with the costs of travel to Brazil. MDA thanks Julian Finn and the staff of the Department of Marine Invertebrates at Museum Victoria for assistance in carrying out this project.

References


composition of *Octopus vulgaris* (Cuvier 1797) paralarvae. *Aquaculture International*, 22, 1041–1051.


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

Fig. S1. Principal Component biplot of a) male and b) female Octopus vulgaris species-group individuals and close relatives, grouped by COI based phylogenetic clade. Analysis is based upon 12 and 8 morphological traits respectively.

Table S1. List of COI sequences accessed from GenBank and corresponding species and locality information.

Table S2. Raw male morphological data for Octopus vulgaris species-group individuals and close relatives.

Table S3. Raw female morphological data for Octopus vulgaris species-group individuals and close relatives.

Table S4. Canonical Analysis of Principal Components (CAP), based on 25 morphological traits of male Octopus vulgaris species-group and O. insularis individuals.

Table S5. Canonical Analysis of Principal Components (CAP) results, based on 12 morphological traits among 27 male Octopus vulgaris sensu stricto individuals.

Table S6. Canonical Analysis of Principal Components (CAP), based on 20 morphological traits of female Octopus vulgaris species-group and O. insularis individuals.

Table S7. Canonical Analysis of Principal Components (CAP) results, based on ten morphological traits among 27 female Octopus vulgaris sensu stricto individuals.

Table S8. Pairwise comparisons of male members of the Octopus vulgaris species-group and close relatives, based on 12 morphological traits.

Table S9. Canonical Analysis of Principal Components (CAP), based on 12 morphological traits of male Octopus vulgaris species-group individuals.

Table S10. Pairwise comparisons of female Octopus vulgaris species-group individuals and close relatives, based on 10 morphological traits.

Table S11. Canonical Analysis of Principal Components (CAP), based on 10 morphological traits of female Octopus vulgaris species-group individuals and close relatives.