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

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Running title: Morphological variation in the *vulgaris* complex

Michael D Amor *et al.*

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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 This study investigated the morphological relationships among seven phylogenetic clades of the broadly distributed *Octopus vulgaris* species-complex and close relatives. Morphological analyses in the present study were successful in delimiting *Octopus sinensis* d'Orbigny, 1841, Brazilian *O. vulgaris* and *O. vulgaris* sensu stricto, which was congruent with the molecular findings of this study. Male morphology was successful in distinguishing 14 of 15 total pairwise comparisons, and proved to be a more reliable indicator of species species-level relationships in comparison to 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). A number of examples exist of organisms once thought to be cosmopolitan in distribution, that 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; Norman *et al.*, 2014b). In recent years, much attention has been focussed on the taxonomy (Norman & Hochberg, 2005; Norman *et al.*, 2014b) and phylogenetic relationships (Carlini *et al.*, 2001; Guzik *et al.*, 2005; Strugnell *et al.*, 2008a; Strugnell *et al.*, 2008b; Kaneko *et al.*, 2011; Acosta-Jofré *et al.*, 2012; Strugnell *et al.*, 2013) within the benthic octopuses and several cryptic species have been identified (Pickford & McConnaughey, 1949; Söller *et al.*, 2000; Allcock, 2005; Leite *et al.*, 2008; Allcock *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, but it has recently been characterised as octopuses with a with a well-defined 'patch-and-groove' skin topology, robust muscular arms with 200–350 prominent suckers in two columns down each arm, and arms two and three longer than arms one and four by a margin of around one mantle length (Gleadall, 2016).

Distinguishing octopus species is also hindered by their morphological plasticity (Robson, 1929; Pickford, 1945; Voight, 1994; O'Shea, 1999) since 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 & Kubodera, 2006) but recent morphology-based studies suggest that benthic octopuses can be distinguished based on discrete phenotypic differences (Gleadall *et al.*, 2010; Gleadall, 2013; Amor *et al.*, 2014; Gleadall, 2016). 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 genus *Octopus* as used previously was a polyphyletic assemblage of species groups comprising a number of different genera.. The species group closest 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 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).

Octopus vulgaris was long considered to be a cosmopolitan species. First reported from the Mediterranean Sea and eastern North Atlantic, *O. vulgaris* has been reported from around the world. However, recent analyses (Söller *et al.*, 2000; Leite *et al.*, 2008; Amor *et al.*, 2014; Amor *et al.*, 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). 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. *Octopus sinensis* d'Orbigny, 1841, occurs in the western North Pacific (Gleadall, 2016). Recent analyses based on molecular sequencing 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 comparison 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 & Haimovici, 2008 (in Leite *et al.*, 2008) and *O. sinensis* (Gleadall, 2016). The present study employs the first ever global scale sampling strategy to investigate morphological variation and determine the validity of morphologically based identifications among members and close relatives of the *O. vulgaris* species-complex. Analyses are combined for conventional morphological traits and a more extensive data set. Phylogenetic analyses based on the mitochondrial 'barcode of life' gene *COI* are also used to provide insights into taxonomic resolution among taxa currently included within the species *O. vulgaris*.

Materials and methods

Sampling

Whole specimens and tissue samples of *O. vulgaris* species-group individuals were obtained from museums, university collections and fish markets from the continental shelves of the Atlantic, Indian and Pacific oceans and the Mediterranean Sea (Fig. 1, Table 1). Tissue samples were removed from fresh or frozen specimens and stored in 70-90% ethanol. Specimens were then fixed in 10% formalin following methods outlined in Roper and Voss (1983), washed in tap water and later preserved in 70% ethanol.

[Insert Fig.1]

[Insert Table 1]

Molecular analyses

Sequencing: Genomic DNA was extracted from mantle or arm tissue samples of 1-2 mm^3 (after first trimming away skin where possible) using a QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer's instructions. Partial cytochrome *c* oxidase subunit I (*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 H_2O 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 outgroup to root the phylogenetic tree (Amor *et al.*, 2015). Multiple sequence alignment of the 482 base pair partial *COI* fragments was performed using *Geneious* 7.1.3 (created by Biomatters; available from http://www.geneious.com/) and the 'Muscle Alignment' feature (Larkin *et al.*, 2007).

Molecular-based phylogenetic analyses: *jModelTest* v0.1.1 (Posada, 2008) was used to select the best-fit models of nucleotide substitution of the *COI* alignment. The appropriate model (GTR+G) was chosen based on 'goodness of fit' via the Akaike Information Criterion (AIC; Akaike, 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 1,000 generations. A mean 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 & Drummond, 2003) 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 and Voss (1983) and Norman and 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) and length of the male hectocotylus (third right, R3). Enlarged sucker diameter (SDe), non-enlarged sucker diameter (SDn), specialisations at the tip of the hectocotylus (ligula length, LL; calamus length, CL), the length of the male reproductive tract 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; and the length of the arms on the left (ALL1-4) and right (ALR1-4) side from the beak opening to the arm tip, were 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 for males is the sucker count of the hectocotylised arm, HASC) were 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 was not permitted in analyses.

Morphological datasets 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 specimen's dorsal mantle length (a proxy for body size). 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 the present study (25 traits for males; 20 traits for females; Tables S2 and S3, respectively). For comparison with published data, a reduced number of traits was 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 enable 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 ($R^2 \geq 85\%$) 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 permutational 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). In order 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 step-wise 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 and McConnaughey, 1949, and *O. maya* Voss and Solís 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 which 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. sinensis* (Kermadec Is and Asia; 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.

[Insert Fig. 2]

Morphological relationships

Comparison of complete and reduced trait datasets: 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 datasets (Tables 2-3 and S8-S11). Analyses based on the reduced trait datasets 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 was most complete for arm L3, therefore ALL3 was retained whilst the remaining correlated arm lengths were considered redundant and excluded from analyses. Within-clade variation had no significant impact 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 14/15 (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* ($p = 0.095$).

[Insert Table 2]

Based on the principal component biplot for males (Fig. 3a), *O. vulgaris* s. s. and *O. vulgaris* Type II males showed the greatest morphological variability in comparison to other taxa, as demonstrated by their occupation of highly positive and highly negative PC1 and PC2 spaces. *Octopus vulgaris* s. s.*, O. sinensis and O. vulgaris* Type II

showed the least discrimination, although *O. vulgaris* s. s. and *O. vulgaris* Type II individuals had relatively longer arms than *O. sinensis* (PC1). *Octopus vulgaris* Type II individuals had relatively fewer suckers on the third arm pair than *O. vulgaris* s. s. and *O. sinensis* (PC2). *Octopus tetricus*, *O.* cf. *tetricus* and *O. insularis* demonstrated negative PC2 loadings attributed to high sucker numbers. *Octopus tetricus* and *O. insularis* showed the least overlap with other taxa included in the analysis but *O.* cf. *tetricus* overlapped with all members of the *O. vulgaris* species-complex.

[Insert Fig. 3]

Of the 68 male individuals analysed, 54 (79%) were correctly assigned to their *a priori* group via CAP (Table 2). For *O. vulgaris* s. s., 16 individuals (84%) were correctly classified: the remainder were misclassified as *O. sinensis* (n = 3). Twelve *O. sinensis* individuals (75%) were correctly assigned to their *a priori* group, with the remaining individuals being misclassified as *O. vulgaris* s. s. (n = 1), Brazilian Type II (n = 1), *O. insularis* (n = 1) or *O.* cf. *tetricus* (n = 1). Nine *O. vulgaris Type II* individuals (82%) were correctly classified whilst the remaining individuals were misclassified as *O. vulgaris* s. s. (n = 1) and *O. insularis* (n = 1). Eight *O. insularis* individuals were correctly assigned (67%), with the remaining individuals misclassified as *O. vulgaris* s. s. (n = 1), *O. tetricus* (n = 1) or *O.* cf. *tetricus* (n = 2). Four *O. tetricus* individuals (80%) were correctly assigned, with the remaining individual being misclassified as *O. sinensis*. All *O.* 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 *O. vulgaris* s. s. and *O. 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 10/15 (67%) comparisons had significant morphology-based differences (Table 3). All members of the *O. vulgaris* species-complex (*O. vulgaris* s. s., *O. sinensis* and south Brazilian Type II) were successfully distinguished based on multivariate morphological analyses (p = ≤0.01). *Octopus vulgaris* s. s. and *O. sinensis* were distinguished primarily by arm length (L3) and sucker diameter. Arm width was

the primary source of variation between *O. vulgaris* s. s. and *O. vulgaris* Type II. *Octopus sinensis* and *O. vulgaris Type II* were found to differ in gill length and arm width. All members of the *O. vulgaris* species-complex could be distinguished from *O. insularis* ($p = \leq 0.003$). Variation between *O. vulgaris* s. s. and *O. insularis* was primarily attributed to 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. No morphological distinctions were detected between *O. tetricus* and *O.* cf. *tetricus* ($p = 0.3$).

[Insert Table 3]

The principal component biplot for females (Fig. 3b) showed that *O. vulgaris* s. s. and *O. sinensis* have 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, whilst 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, whilst 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 ($p = \leq 0.004$; Table 4).

[Insert Table 4]

A PC biplot (Fig. 4a) showed that, basically, each sampling locality for *O. vulgaris* s. s. males could be distinguished, although a small number of individuals overlapped. Individuals from the Mediterranean were found to have more suckers (L3, R3) than Galician and Mauritanian (eastern North Atlantic) individuals. Galician males were distinct from Mauritanian males along PC1, Galician males having longer arms (L3, R3).

[Insert Fig. 4]

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, 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 ($p = \leq 0.002$; Table 5)

[Insert 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 explained by 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 ($p = 0.002$); and 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).

Discussion

Molecular-based phylogenetic analyses of *O. vulgaris* species-group individuals in the present study support the presence globally of six distinct clades, which were used as a discriminant factor in morphological analyses. Conventional morphological traits were successful in distinguishing the majority of these clades. Multivariate morphological analyses support the hypothesis of species distinctions 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 for broad sampling of species-level morphology across the known distribution for each species to ensure robust future morphological analyses of this group.

Previous molecular sequence-based phylogenetic analyses using five mitochondrial genes placed *O. sinensis* into a well-supported monophyletic clade, distinct from all other members of the *O. vulgaris* species-complex (Amor *et al.*, 2014). Reid and 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 *vulgaris* individuals. Subsequently, this clade was renamed *O. sinensis*, which was recently redescribed and distinguished morphologically from *O. vulgaris* s. s. (the former having shorter arms with fewer suckers; Gleadall, 2016). 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. Phylogenetic analyses and differences in adult morphology in the present study strongly support this hypothesis, showing that individuals from southern Brazil form a monophyletic clade, distinct from *O. vulgaris s. s*. and *O. sinensis*.

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/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. Within the *O. vulgaris* species-complex, the clades considered to be different at the species level have allopatric distributions, so the selective pressures for strong phenotypic adaptations through interspecific competition may have been low. Differentiation in morphological traits is often most extreme where closely related species occur in

sympatry (Brown & Wilson, 1956), as a means to reduce resource overlap and to limit interspecific competition, allowing otherwise directly competing taxa to co-exist. 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 (sub-tropical 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* species complex. The closely related sibling pair of ocellate species in southern California (*O. bimaculatus* Verrill, 1883, and *O. bimaculoides* Pickford & McConnaughey, 1949) have used a different strategy to maintain sympatry: *O. bimaculoides* undergoes direct benthic development, while *O. bimaculatus* undergoes the meroplanktonic paralarval development typical of this group of octopuses (Pickford & McConnaughey, 1949).

The sexual traits of male individuals were found to be important characters for morphology-based discrimination of species in the *O. vulgaris* species-complex, confirming the utility of male sexual traits in cephalopod systematicsin line with similar findings associated with other animal groups that sexual traits are more variable than non-sexual traits (Pomiankowski & Moller, 1995), and are often the only reliable way to identify closely related species (Arnqvist, 1998). However, a study of a different octopus genus, *Pareledone*, found that morphological traits (including three sexual traits) were unsuccessful in distinguishing among species, although they were well-defined traits characteristic of *Pareledone* at the generic level (Allcock *et al.*, 2008).

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*. The utility of HASC has been demonstrated previously for distinguishing among octopus species (Toll, 1988; Gleadall, 2016). Among 12 species, Toll (1988) reported intraspecific HASC values to be relatively fixed. In contrast, the present study found HASC values for *O. vulgaris* s.s. correlated significantly for sampling localities of similar latitude. 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, since specimens from Mauritania display minimal overlap in this character compared with those from France and Spain, this may indicate the development of further species-level diversity within *O. vulgaris* s.s. as currently recognized (cf. also the findings of Cabranes *et al*.,(2008)). Voight (2012) cited 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. Concerning HASC values among Australasian members of the *O. vulgaris* species-group, those of western Australian *O.* cf. *tetricus* are almost 40% greater than those for eastern Australian *O. tetricus*. 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.

The discriminatory power of female based morphological analyses was weaker than that for males. In the complete and reduced trait datasets, more morphological traits are available for male reproductive characters and these traits were found to be important in distinguishing among molecular sequence-based phylogenetic clades on the basis of morphology. The most significant female traits for distinguishing among species were non-sexual. Sexual traits, particularly the hectocotylus, are also important distinguishing taxonomic characters for decabrachian cephalopods, including the Idiosepiidae Appellöf, 1898 (Norman & Lu, 1997; von Byern & Klepal, 2010), Loliginidae Lesueur, 1821 (Brakoniecki, 1996), Ommastrephidae Steenstrup, 1857 (O'Dor & Lipinski, 1998) and Sepiolidae Leach, 1817 (Bello, 1995). In contrast to 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 highly successful in distinguishing among sampling localities of *O. vulgaris* s. s. (Galicia, France and Mauritania).

The need for better species resolution within the family Octopodidae is particularly important in view 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 the catch and value of many fisheries for finfish (FAO, 2012). A major limitation of the global catch statistics reported by the FAO is the poor resolution of octopus species, with only five (*O. vulgaris*, *O. maya*, *Eledone cirrhosa, Eledone moschata* and *Enteroctopus dofleini*) of the estimated 100 species of commercially harvested octopus listed in global statistics (Norman & Finn, 2014). As the majority of octopus fisheries world-wide are in decline (Norman & Finn, 2014), this low species resolution highlights the requirement for more accurate species identification in order to develop more sustainable octopod fisheries practices. Octopuses being exported globally under the name *O. vulgaris* are of high market value and profile (Norman *et al.*, 2014a), with those of northwestern Africa the largest single-species octopus fishery in the world (FAO, 2012). Aquaculture and captive growing of wild caught juveniles are receiving increasing profile and funding, particularly in China, Japan, Mexico and Spain. Differences among geographical areas in hatchling features and paralarvae viability (Iglesias *et al.*, 2007; Iglesias *et al.*, 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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Table 1: Sample data for octopus species analysed in the present study. Sample type refers to the type of data used: whole = whole animals, tissue = tissue samples or data = existing data from the literature.

Species/Type	Location	Institution	Sample Type	Reference
O. vulgaris s. s.	Banyuls-sur-Mer, France	Santa Barbara Museum of Natural History	Data	
O. vulgaris s. s.	Galicia, Spain	Consejo Superior de Investigaciones Científicas (CSIC), Vigo	Whole/Tissue	
O. vulgaris s. s.	Mauritania	Instituto Español de Oceanografía (IEO), Tenerife	Whole/Tissue	
O. sinensis	China	Fisheries College, Ocean University of China, Qingdao	Whole/Tissue	Reid and Wilson (2015)
O. sinensis	Keelung / Da si, Taiwan	National Taiwan Ocean University, Keelung	Whole/Tissue	Reid and Wilson (2015)
O. sinensis	Kermadec Islands, New Zealand	Australian Museum, Sydney	Whole/Tissue	Reid and Wilson (2015)
O. sinensis	Kyushu / Sendai, Japan	Tohoku University, Sendai	Whole/Tissue	
Type II (Brazil)	Pontal do Paraná, Brazil	Universidade Federal do Paraná (UFPR)	Whole	
O. insularis	Rio Grande do Norte/Brazil	Universidade Federal do Rio Grande do Norte (UFRN)	Whole	
O. insularis	Saint Peter and Saint Paul Archipelago, Brazil	Universidade Federal do Rio Grande do Norte (UFRN)	Whole	
O. insularis	Trindade Island, Brazil	Universidade Federal do Rio Grande do Norte (UFRN)	Whole	
O. mimus	Tocapilla / Pisagua, Chile	Consejo Superior de Investigaciones Científicas (CSIC), Vigo	Data	Guerra *et al.* (1999)
O. tetricus	New South Wales, Australia	Museum Victoria	Whole/Tissue	
O. tetricus	Tasmania, Australia	Museum Victoria	Tissue	
O. cf. *tetricus*	Western Australia, Australia	Fisheries and Marine Research Laboratories, Western Australia Museum Victoria	Whole/Tissue	

Table 2: Pairwise comparisons of male *Octopus vulgaris* species-group and *O. insularis* individuals based on 25 morphological traits. 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 Canonical Analysis of Principal Components (CAP) analysis (see Table S4 for full CAP analysis table).

	***O. vulgaris* s. s.**	***O. sinensis***	**Type II (Brazil)**	***O. insularis***	***O. tetricus***	***O.* cf. *tetricus***	**Correct (%)**
***O. vulgaris* s. s.**	-	**GLL/ALR4**	**SDe**	**SCR3/ALR 3**	**SCL3/SDn**	SCL3/DL	84.2
O. sinensis	**0.003**	-	**GLL/GLR**	**TOL/GLL**	**SDn**	**SCL3/TOL**	75.0
Type II (Brazil)	**0.011**	**0.001**	-	**DL/SCR3**	**SCL3/AW**	**SCL3/DL**	81.8
O. insularis	**0.002**	**0.001**	**0.001**	-	**SDe**	**ALR3**	66.7
O. tetricus	**0.009**	**0.001**	**0.001**	**0.001**	-	**SCL3/DL**	80.0
O.* cf. *tetricus	0.095	**0.001**	**0.01**	**0.001**	**0.012**	-	100

Table 3: Pairwise comparisons of female *Octopus vulgaris* species-group and *O. insularis* individuals based on 20 morphological traits. 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 S6 for full CAP analysis table).

	***O. vulgaris* s. s.**	***O. sinensis***	**Type II (Brazil)**	***O. insularis***	***O. tetricus***	***O.* cf. *tetricus***	**Correct (%)**
***O. vulgaris* s. s.**	-	**ALL3/SDn**	**AW**	**SCR/L3***	SCR3/HW	SCL/R3	76.2
O. sinensis	**0.004**	-	**GLL/AW**	**SCL3**	SCR3/FL	HW	50
Type II (Brazil)	**0.01**	**0.001**	-	**SCR/L3**	**SCR3/AW**	**AW**	71.4
O. insularis	**0.001***	**0.001**	**0.003**	-	**SCL3/FL**	**ALL1/3**	100
O. tetricus	0.053	0.119	**0.039**	**0.004**	-	HW	75
O.* cf. *tetricus	0.181	0.05	**0.041**	**0.012**	0.114	-	50

Table 4: Pairwise comparisons of male *Octopus vulgaris* sensu stricto individuals based on 12 morphological traits. 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).

	Galicia	Mediterranean	Mauritania	Correct (%)
Galicia	-	**ALR3/SCL3**	**ALR3**	80
Mediterranean	**p=0.004**	-	**FFL/LL**	88.9
Mauritania	**p=0.001**	**p=0.003**	-	100

Table 5: Pairwise comparisons of thee sampling locations of female *Octopus vulgaris* sensu stricto individuals based on eight morphological traits. 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).

	Galicia	Mauritania	Mediterranean	Correct (%)
Galicia	-	**SCL3/HW**	**FFL/FL**	90
Mauritania	**p=0.001**	-	**FFL/SCL3**	100
Mediterranean	**p=0.002**	**p=0.001**	-	100

Fig. 1: Sampling localities (triangles) for whole animals/tissue samples of members of the *Octopus vulgaris* species-group and close relatives. Distributions of *O. 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) is represented by a circle.

Fig. 2: Bayesian topology depicting the relationships among members of the *Octopus vulgaris* species-group and close relatives *O. mimus* and *O. insularis*. Analyses are based on partial sequence of the mitochondrial *COI* gene, showing BI posterior probabilities above and ML bootstrap values below major nodes. Outgroup is *O. cyanea*. Node labels represent geographic locations represented. Clade number is also shown (C1-11). *Octopus vulgaris* 'Types' refer to; Mediterranean/NE Atlantic (*O. vulgaris* s. s.), southern Brazil (Type II), South Africa (Type III) and *O. sinensis* (Norman *et al.*, 2014a). Haplotype characters in parentheses correspond to individuals in Table S1.

Fig. 3: Principal Component biplot of male (a) and female (b)*Octopus vulgaris* species-group and *O. insularis* individuals grouped by *COI* based phylogenetic clade. Analysis is based upon 25 and 20 morphological traits respectively.

Fig. 4: Principal Component biplot 27 *Octopus vulgaris* sensu stricto males (a) and females (b), grouped by locality. Analysis is based on 12 and 8 morphological traits respectively.