Further morphological and molecular studies of driftwood hoppers (Crustacea: Amphipoda: Talitridae) from Mediterranean/north-east Atlantic coastlines

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An unknown talitrid was discovered in 2011 in the Swale, UK, living in driftwood. Sequencing of multiple mitochondrial and nuclear genes (cytochrome oxidase I, 18S rDNA and 28S rDNA) confirms that the unknown taxon was close to *Orchestia mediterranea* A. Costa 1857. The driftwood in which it was found was of Douglas fir (*Pseudotsuga menziesii*), probably imported from North America. Relative growth methods allow us to reject the null hypothesis that the unknown taxon was a juvenile *Orchestia mediterranea*. This is because the unknown taxon initiates sexual maturation at an earlier moult number (neoteny) and is predicted to reach terminal moult stages at a much smaller size (dwarfism) than its closest relative *O. mediterranea*. Both molecular and morphological data confirm that the unknown taxon from the Swale, UK, is a new driftwood specialist taxon, distantly related to the driftwood specialist genus *Macarorchestia*. Additional data for body length and sex ratios are presented for *Macarorchestia*. In addition, relative growth methods have shown that the driftwood species *Orchestia microphthalma* Amanieu and Salvat 1964 is closer to the genus *Macarorchestia* rather than to the wrack generalist one, *Orchestia*. This evidence is in line with the molecular results that do not cluster morphologically defined species of *Orchestia* monophyletically.

Keywords: *Orchestia microphthalma*; neotenic dwarfism; driftwood habitat; COI; *Macarorchestia*; *O. mediterranea*

Introduction

In an earlier publication (Wildish et al. 2012) an initial study of some of the known species of driftwood specialist talitrids found in the north-east Atlantic/Mediterranean region (inclusive of the Azores, Madeiras and Canaries archipelagos) was presented. In that paper, morphological-based, relative growth methods in combination with modern molecular analyses were employed to determine: (1) a close morphological and genetic similarity between *Macarorchestia remyi* from Italy, *M. roffensis* from the UK, and *M. martini* from the island of Terceira, the Azores; (2) that *Macarorchestia* was a monophyletic genus; and (3) that a putative new driftwood specialist taxon from the Swale, UK, was closely related to *Orchestia mediterranea*, rather than to *Macarorchestia*.

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In a continuation of this work using similar methods and analyses with the addition of nuclear genes and additional taxa, we tested the following specific null hypotheses:

Hypothesis 1 that *Orchestia microphthalmia* belonged to *Orchestia* rather than *Macarorchestia*.

Hypothesis 2 that the putative new taxon from the Swale, UK, was a juvenile of *Orchestia mediterranea* A. Costa 1857.

The results of this study, as well as the earlier one (Wildish et al. 2012), will be used in preparing formal taxonomic descriptions of two new taxa of driftwood talitrid specialists (Wildish 2014).

Materials and methods

Archived material

The Natural History Museum in London, UK (NHM) made available 71 paratype individuals of *Orchestia microphthalmia* for non-destructive morphological analysis, plus an additional five individuals for destructive sampling for molecular analyses. All specimens were collected from the type locality at Cap Ferret, near Arcachon, France (Amanieu and Salvat 1963). They were collected on 11 September 1967 by D.J. Wildish and deposited at the NHM with the following registration number: NHM 1967.10.6.1–75.

Data matrices containing morphological measurements and counts against age and moult number in cultured juveniles of *O. mediterranea* from the Medway estuary, UK, originally made in 1968 and archived at the NHM, were also used in this study. The data for the juvenile phase of the life history was part of a comparative growth study of talitrids from the Medway estuary, UK (Wildish 1972). A sample ($n = 30$) of *O. mediterranea* from the Ria Formosa, Faro, Portugal collected by Ms Natalia Dias in 1998–1999 was also analysed here.

The sample from the Swale, Kent, UK (Wildish et al. 2012) consisted of 16 individuals (one immature male, two immature females and 13 juveniles) collected during the summer of 2011 by K.J. Wildish from a single driftwood log. A total of eight individuals, including all immature stages, were used in the relative growth study.

Relative growth

As far as possible we used a non-destructive method to make measurements and counts on each individual. Observational methods were essentially the same as used previously (Wildish et al. 2012). The following antennal flagellum counts/measurements in mm were made for each individual examined:

- total body length from anterior cephalon to tip of telson (TBL) measured from photographs of each animal made with a Canon 990 digital camera attached to a binocular microscope. Using suitable calibration photographs TBL was

measured with Image Pro Plus software, which allows curved bodies to be measured accurately.

- second antenna article count (A2 FA)
- A2 peduncle length, measured dorsally on the two most distal segments (A2 PL)
- eye diameter (ED)
- third peraeopod propodus length measured on the anterior side (P3 PL)
- seventh peraeopod propodus length measured on the anterior side (P7 PL)
- third pleopod exopod length (pl3 ExL)
- third pleopod basis length (pl3 BL)
- first uropod exopod length (Up1 ExL)

All four population samples were treated separately and measurements/counts made on them as previously (Wildish et al. 2012). Data matrices were prepared for each population sample on separate worksheets in Microsoft Excel (2003). The model I linear regression equation used was: $y = ax + b$, to analyse each population sample, with $x = \text{TBL}$ and $y =$ each of the counts/measurements in turn.

Because the archived data for *O. mediterranea* juveniles from the Medway estuary lacked some measurements in the 1968 data, back calculation was used to provide it for TBL, P3 PL and P7 PL. The measurements were back calculated from the *O. mediterranea* sample from Faro, Portugal. The equations used were: $\text{TBL} = 12.06(\text{pl3 BL}) + 1.54$, $R^2 = 0.88$, $N = 32$; $\text{P3 PL} = 0.49(\text{pl3 BL}) + 0.12$, $R^2 = 0.83$, $N = 32$; $\text{P7 PL} = 1.39(\text{pl3 BL}) + 0.08$, $R^2 = 0.93$, $N = 32$.

Sex ratio

The sex ratio was calculated as the percentage of males within discrete population samples for all immature and adult specimens as follows: ‘ $\% \text{♂} = \text{total of immature and mature males} \times 100 / \text{total of immature and mature females plus intersexes}$ ’.

Each animal was examined with the aid of a binocular microscope to determine the presence of penes at the base of P7 (= male) and oostegites at bases of P2 to P5 (= female). Where secondary sexual characters were mixed we have designated them as intersex individuals.

Determining the provenance of driftwood occupied by the unknown taxon

Because it might indicate the origin of the driftwood and thus help identify the place of origin of the unknown taxon, we sought to identify the driftwood tree species. This was achieved by boiling a piece of driftwood from the Swale, UK, in which the unknown taxon was found; it was then sectioned transversely, tangential longitudinally and radial longitudinally at 20 μm thickness. Slide preparations mounted in glycerol were examined with a light microscope.

Statistics of morphology based measures/counts

Mean total body lengths (TBL) was calculated for each population sampled and compared by analysis of variance.

Two types of error are common in sex ratio data analysis: insufficient sample size and sampling bias (Wildish 1977). Assuming an equal sex ratio ($\% \text{♂} = 50$) we used

the normal approximation method to determine whether the expected and observed sex ratio were significantly different with normal deviate (c) tables of Mather (1964). The probabilities of significant difference from an equal sex ratio are given as: **** (> 99.9%); *** (99%); ** (95%); * (90%); ND (< 90%).

Discrimination of relative growth difference between counts and measurements was made by model I regression analysis with TBL as the independent variable. The fitted regression lines were compared by Reeve's (1940) method, from the goodness of fit with single, separate and parallel lines. Mean square differences, or residual variance, from all fittings were compared with the F statistic: F = larger mean square/smaller mean square.

We used Statgraphics XV software, which uses Reeve's method, to compare population sample slopes and intercepts, and determined the probabilities of significant difference by reference to F-ratios. The same probabilities of significant difference of p -values as indicated for the sex ratio analyses were adopted in this analysis. Significance levels test the null hypothesis that population #1 $\neq$ #2 for slope coefficient. If the slopes were not significantly different (ND) a further test of the intercept coefficient was performed.

Additional specimens for molecular study

The dataset of Wildish et al. (2012) was re-analysed and additional talitrid species and molecular markers (see next paragraph) were included in this study. In total, we analysed nine genera and 19 species (inclusive of two unrelated taxa as outgroups). For the genus *Orchestia*, we analysed seven species from 18 locations (Table 1), including an attempt to retrieve DNA from the historical, formalin preserved specimens of *O. microphtalma*. A complete list of the populations, species and molecular markers used is shown in Table 1.

Molecular methods

Laboratory procedure

DNA extractions and polymerase chain reaction (PCR) conditions for the historical formalin preserved specimens of *O. microphtalma* were performed as described in Wildish et al. (2012).

For contemporary samples DNA extractions, PCR reactions and sequencing were carried out according to Ketmaier et al. (2008), Pavesi et al. (2011, 2013) for the COI gene; primers and conditions detailed in Spears et al. (2005) and Jarman et al. (2000) were used for the 18S gene and two fragments of the 28S gene (primer pairs 28Sdd/28Sff and 28Sv/28Sx), respectively.

Sequencing was outsourced to LGC Genomics (<http://www.lgcgenomics.com>) and performed in both directions for each gene fragment. Sequences were edited with SEQUENCER v. 4.1 (Gene Code Corporation, Ann Arbor, MI, USA). Aligning the COI sequences was straightforward and was done in SEQUENCER. For the 18S and 28S fragments, we used CLUSTALX (Thompson et al. 1997) using the default settings (pair-wise alignment parameters: slow-accurate pairwise alignment method, gap opening penalty = 15.00, gap extension penalty = 6.66, IUB DNA weight matrix; and multiple alignment parameters: gap opening penalty = 15.00, gap

Table 1. Genera and species list of Mediterranean/north-east Atlantic Talitridae employed in molecular analyses.

Genus	Species	n	Sample code	Country	Sampling locality	COI	18s	28s dd/ff	28s v/x
<i>Arcitalitrus</i>	<i>dorrieri</i>	1	ADA	Australia/UK*	UK	✓	✓	n/a	✓
<i>Deshayesorchestia</i>	<i>deshayesii</i>	1	DDI	Italy	Sardinia	✓	✓	✓	✓
<i>Macarorchestia</i>	<i>renyi</i>	1	MRL	Italy	Apulia	✓	✓	✓	✓
		1	MRV	Italy	Apulia	✓	✓	✓	✓
	<i>roffensis</i>	1	MRU	Italy	Tuscany	✓	✓	✓	✓
	<i>martinii</i>	1	MRW	UK	Medway	✓ ^o	n/a	n/a	n/a
<i>Cryptorchestia</i>	<i>cavimana</i>	1	MMA	Portugal	Azores	✓ ^o	n/a	n/a	n/a
		1	OCC	Republic of Cyprus	Cyprus	✓	✓	✓	✓
	<i>garbinii</i>	2	OCI1, OCI2	Cyprus					
<i>Orchestia</i>	<i>aestuarensis</i>	4	H ‡	Italy	Lazio	✓	✓	✓	✓
	<i>guancha</i>	1	OGT	UK	Medway estuary	✓	✓	✓	✓
	<i>gammarellus</i>	5	A ‡	Spain	Canary Islands	✓	✓	✓	✓
		5	B ‡	UK	Lower Stoke	✓	✓	✓	✓
		5		UK	Roman War memorial	✓	✓	✓	✓
		5	C ‡	UK	Bedlams bottom	✓	✓	✓	✓
		5	E ‡	UK	Burham Court	✓	✓	✓	✓
		5	F ‡	Canada	Chittick Beach	✓	✓	✓	✓
<i>mediterranea</i>		1	OMA	Italy	Lazio	✓	✓	✓	✓
		5	D ‡	UK	Medway estuary	✓	✓	✓	✓
<i>montagui</i>		1	OMN	Italy	Sardinia	✓	✓	✓	✓
		1	OMS	Italy	Sardinia	✓	✓	✓	✓
		1	OMG	Malta	Gozo	✓	✓	✓	✓
		1	OMI	Italy	Apulia	✓	✓	✓	✓
		1	OML	Italy	Apulia	✓	✓	✓	✓

(Continued)

Table 1. (Continued).

Genus	Species	<i>n</i>	Sample code	Country	Sampling locality	COI	18s	28s dd/ff	28s v/x
<i>Palmorchestia</i> <i>Platorchestia</i>	<i>stephenseni</i> <i>epigea</i> <i>platensis</i>	1	OMM	Italy	Sardinia	✓	✓	✓	✓
		1	OSI	Italy	Tuscany	✓	n/a	✓	✓
		1	PEC	Spain	Canary Islands	✓	✓	✓	✓
		3	PP11, PP12, PP13	Italy	Lazio	✓	n/a	✓	✓
<i>Talitrus</i>	<i>saltator</i>	2	TST1, TST2	Italy	Lazio	✓	✓	✓	✓
		3	TSA1, TSA2, TSA3	Italy	Apulia	✓	✓	✓	✓
<i>Africorchestia</i> <i>ugolini</i>	<i>spinifera</i> <i>ugolini</i>	2	TSP1, TSP2	Morocco	Mehdia beach	✓	✓	✓	✓
		1	TUC	France	Cateraggio di Aleria	✓	✓	n/a	✓
Putative New Taxon	under descrip. (Wildish)	1	NTW	UK	Kent	✓	✓	✓	✓
		3	G ‡	UK	The Swale	✓	✓	✓	✓

Note: ° short fragments (see Wildish et al. 2012 for details); * Australian population established in the Royal Botanic Gardens, Kew, UK; ‡ newly analysed species; *n* = number of individuals examined.

extension penalty = 6.66, delay divergent sequences = 30%, DNA transition weight = 0.50), followed by limited manual editing to improve inferences of positional homology. No large gaps or poorly alignable regions were present in the 18S and 28S alignments.

Statistical analyses

For the COI dataset, Kimura two-parameter ($K2P_{COI}$) genetic distances were calculated with PAUP□ Portable version 4.0b10 (Swofford 2002). In order to assess levels of genetic divergence at the ‘among’ and ‘within’ genus levels, minimum, maximum and mean $K2P$ distances were calculated within *Macarorchestia* and *Orchestia* spp. For comparative purposes, $K2P_{all_genes}$ distances were also calculated on the 18S and 28S gene partitions with the two 28S gene fragments combined together.

The automatic barcode gap detection (ABGD) method (Puillandre, Lambert et al. 2012), which implements an automatic procedure of barcode gap discovery, was used to define species assignment based on COI haplotype groups within the genus *Orchestia*. The analysis was performed online (<http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>) with both Kimura 2P (K89) and Jukes-Cantor (JC69) distances and with default parameters. Given the limited number of sequences per species at our disposal, we additionally tested model combinations with P (prior limit to intraspecific diversity) ranging between 0.001 and 0.1, and X (proxy for minimum gap width) from 1.5 to 10. This was done to both evaluate the effect on our dataset, and to avoid the capture of smaller gaps as a result of weak sampling (Castelin et al. 2012; Puillandre, Modica, et al. 2012; Marin et al. 2013).

Phylogenetic searches were conducted on each gene separately, on COI plus the 28S fragment obtained with the 28Sv/x primer pair and all genes combined together. Phylogenetic trees were rooted using the homologous sequences of the hyperiid amphipod *Themisto gaudichaudii* retrieved from GenBank as outgroup (accession nos. HM053513, AY743940, AF244091 and AF244092 for the COI, 18S and 28S fragments, respectively). We used JMODELTEST 2 (Darriba et al. 2012) to determine the best model of sequence evolution for each data partition analysed. JMODELTEST 2 returned the GTR + G model for the 18S and 28Sdd/ff partitions and the GTR + G + I for all the others. These models were then implemented in the Bayesian searches carried out in MrBAYES (Ronquist et al. 2012). We allowed site-specific rate variation partitioned by gene and, for COI, by codon position in the Bayesian analysis. MrBAYES was run for 2,000,000 generations with a sampling frequency of 100 generations. We ran one cold and three heated Markov chains and two independent runs. To establish if the Markov chains had become stationary, we plotted the likelihood scores of the sampled trees against generation time. Trees generated before stationarity were discarded as burn-in (first 10% of the sampled trees) and posterior probability values for each node were calculated on the basis of the remaining 90% of sampled trees. The output of each independent Bayesian run was visualized using TRACER v1.4 (Drummond and Rambaut 2007) to check whether a reasonable effective sampling size (ESS, number of independent samples of the posterior distribution) had been reached during each search. Convergence of the Markov chains was further evaluated with AWTY (Wilgenbusch et al. 2004). TRACER and AWTY both consistently suggested that the Markov chains in both independent runs have converged and reached stationarity after the 10% burn-in. We

tentatively dated the main splits in the COI phylogeny using $K2P_{COI}$ values and the mutation rate adopted for *O. montagui* in Pavesi et al. (2013).

Results

Relative growth

Hypothesis 1

A scatterplot comparing *O. microphthalmalma* and *O. mediterranea* from Faro, Portugal for p13 ExL on TBL is shown in Figure 1A and suggests that the regression lines are far apart. All dependant variables tested are shown in Table 2. Because scatterplots of ED, A2 FA and A2 PL for *O. microphthalmalma* and *M. remyi* showed an inflection between juvenile and immature data, the juveniles were excluded in calculating the regression constants (indicated by brackets in Table 2). Of the eight dependent variables of Table 2 significant differences in slope values between *O. microphthalmalma* and *O. mediterranea* were found in: ED*, A2 FA***, A2 PL****, and P13 ExL****. For bivariate lacking significant difference in slope (Table 2) we tested the intercept differences with the following results: P3 PL (ND), P7 PL**, Up1 ExL**** and P13 BL****. The preponderance of allometric slope differences (four of eight) between the two taxa allow us to reject the null hypothesis that *O. microphthalmalma* belongs to *Orchestia*.

We examined an alternative hypothesis that *O. microphthalmalma* belonged to *Macarorchestia* by comparing the allometric slope differences between *O. microphthalmalma* and *M. remyi* from Principina Mare, Italy (data from Wildish et al. 2012). We found no slope coefficient differences (Figure 1B) between the two species, consistent with *O. microphthalmalma* belonging to *Macarorchestia*. Only intercept differences were present between the two sampled populations as follows: A2 Fa*** (Figure 1B), A2 PL**, P13 ExL** and Up1 ExL**** (not shown).

Hypothesis 2

A scatterplot comparing the unknown taxon and *O. mediterranea* juveniles from the Medway estuary, UK, suggested that the fitted regression lines for A2 PL on TBL (Figure 2A) and ED on TBL (Figure 2B) was linear and parallel for both taxa, with no evidence of an inflection (although this does occur in *O. mediterranea* between the juvenile stage and at the onset of sexual maturity). There appears to be no slope difference, but a significant intercept difference in the regression lines compared in Figure 2. Other points to note from Figure 2 are the few data points for the unknown taxon (maximum of eight) which renders their use in relative growth as questionable, although $R^2 = 0.89$ in Figure 2A and $R^2 = 0.88$ in Figure 2B for this dataset. The three largest individuals for the unknown taxon shown in Figure 2 consist of immature male and two immature females, as recognized independently by observing the presence of penes and oostegite buds at the base of P7 and P2 to P5, respectively. The regression data are shown in Table 3 and confirm the impression of no slope, but large intercept difference between the unknown taxon and juvenile *O. mediterranea*. Thus for all six measures/counts (P13 measures are excluded because only two measurements were available for the unknown taxon) none of the slope coefficients (except one), but all of the intercept differences were significantly different. Thus:

Figure 1. Scatterplots and fitted linear regressions for: (A) P13 ExL on TBL in *O. mediterranea* from Ria Formosa, Faro, Portugal, for all life history stages (◆); and *O. microphthalmus* from Cap Ferret, France (■). (B) A2 FA on TBL in *O. microphthalmus* from Cap Ferret, France (□) and *M. remyi* from Principina a Mare, Italy (◆).

ED***, A2 FA****, A2 PI****, P7 PL****, and Up1 ExL****. The one exception was for P7 PL where the slope coefficient was significantly different at the 95% level (Table 3). In general the regression lines for this pair of populations were similar in slope but far apart positionally, suggestive of a close relationship, but differing in relative growth characteristics which include the initial and final size reached by each population.

Table 2. Regression data for morphological measurements or counts (y) in: *Orchestia microphthalma* from Cap Ferret, France, *O. mediterranea* immatures and adults from Ria Formosa, Faro, Portugal. The equation is: $y = ax + b$, where $\times$ = total body length in mm (TBL). Brackets indicate juvenile data excluded.

Measure/count (y)	<i>Orchestia microphthalma</i>				<i>O. mediterranea</i> immatures and adults			
	A	b	R ²	n	a	B	R ²	n
ED	(0.01	+0.14)	0.49	22	0.02	+0.12	0.47	21
A2 FA	(0.41	+7.79)	0.46	22	0.83	+6.28	0.73	21
A2 PL	(0.06	+0.23)	0.62	21	0.14	−0.24	0.75	30
Up1 ExL	0.06	+0.01	0.90	39	0.06	−0.03	0.91	27
P3 PL	0.04	+0.03	0.90	34	0.04	+0.05	0.86	27
P7 PL	0.10	−0.05	0.88	37	0.11	+0.01	0.93	25
PI3 ExL	0.03	−0.03	0.86	30	0.07	−0.06	0.88	27
PI3 BL	0.06	+0.03	0.88	30	0.06	+0.17	0.81	27

We examined one possible alternative hypothesis, that the unknown taxon belonged to *Macarorchestia*, by comparing the slope differences between it and *M. roffensis* (data from Wildish et al. 2012). For the latter species an inflection was present for ED, A2 FA and A2 PL at the onset of sexual maturity and so the juvenile data were excluded in calculating the regression coefficients for these characters. Of the six available dependent variables, significant allometric slope differences were found in one of them (ED****), and intercept differences in A2 FA***. Of two measurements we were able to make of the PI3 characters in an immature female and immature male of the unknown taxon, the PI3 ExL was 0.500 and 0.600 mm. For *M. roffensis* at the same TBL as these specimens the PI3 ExL as predicted by the regression line would be 0.250 and 0.300 mm respectively. These large differences suggest that the unknown taxon was not directly related to *Macarorchestia*.

Total body length (TBL)

If we combined body size data from our earlier study (Wildish et al. 2012) with the additional taxa of this study, there was a considerable range in body size among species, as shown in Table 4. Whereas the smaller species, such as *M. roffensis*, had females > males, the larger species, such as *M. remyi*, had males > females, as measured by TBL. Insufficient population sample numbers were available for *M. martini* from the Azores and *M. remyi* from Lesina, Italy to decide if they too followed this trend.

Sex ratio

Although insufficient sample numbers were available to determine sex ratios for *M. martini* from the Azores and *M. remyi* from Lesina, Italy, the general trend was for the sex ratio (Table 5) to be positively related to size, such that the smaller the final body size, the more biased was the sex ratio to an excess of females.

Figure 2. Scatterplots and fitted regression lines for: (A) A2 PL on TBL for the unknown taxon from the Swale, UK ($\square$) and juvenile *O. mediterranea* from the Medway estuary, UK ($\triangle$). (B) ED on TBL for the unknown taxon from the Swale, UK ($\square$) and juvenile *O. mediterranea* from the Medway estuary, UK ($\triangle$).

Table 3. Regression data for morphological measurements or counts (y) in: unknown taxon from the Swale, Kent, UK, *O. mediterranea* juveniles from the Medway estuary, UK. The equation is: $y = ax + b$, where x = total body length in mm (TBL). Back calculated data indicated where R^2 values > 0.97 .

Measure/count (y)	Unknown taxon				<i>O. mediterranea</i> juveniles			
	A	b	R^2	<i>n</i>	a	B	R^2	<i>n</i>
ED	0.04	+0.04	0.88	7	0.04	−0.06	0.90	18
A2 FA	1.67	−0.09	0.92	7	1.77	−3.76	0.97	29
A2 PL	0.13	−0.07	0.89	7	0.12	−0.23	0.98	29
Up1 ExL	0.06	+0.04	0.83	7	0.07	−0.10	0.97	23
P3 PL	0.04	+0.07	0.67	8	0.04	+0.06	1.00	31
P7 PL	0.10	+0.08	0.93	6	0.12	−0.10	1.00	31
P13 ExL				1	0.10	−0.20	0.96	29
P13 BL				1	0.08	−0.13	1.00	31

Driftwood provenance

Growth rings in the Swale driftwood sample were narrow with 18 complete growth rings observable, which were 586–970 μm in width (mean = 762 μm). Axial resin canals were present mainly in the latewood. In tangential section some of the rays had radial canals. In both tangential and radial view the tracheids possessed shallow helical thickenings. This combination of axial and radial resin canals plus helical thickening is diagnostic of *Pseudotsuga* sp. (Douglas fir). Of the two known North American species of *Pseudotsuga* sp (Farjon 2001) the likely one is *P. menziesii* as it is the only one used in commercial lumbering. *Pseudotsuga menziesii* was introduced into the UK in 1827 (Edlin 1970) and today is widely grown, e.g. by the UK Forestry Commission. Since the early twentieth century large quantities of Douglas fir have been imported to the UK from North America. Thus the provenance of the Swale driftwood is uncertain (imported or locally grown Douglas fir?). The limited evidence suggests that the driftwood originated as imported Douglas fir, because growth rings indicate slow growth. Typically, North American growth conditions result in slow growth, versus faster growth in the UK. The large dimensions of the sawn driftwood sample found ($\sim 3 \times 0.5 \times 0.25\text{m}$) in the Swale driftline suggests that its original human use was a wharf marine piling in the Thames or Medway estuaries.

Molecular data

Hypothesis 1

In spite of the considerable effort and use of the specific protocols adopted successfully for the recovery of short fragments of the historical, formalin preserved specimens of *M. roffensis* and *M. martini* in Wildish et al. (2012), it was not possible to obtain any PCR product for *O. microptalma*. Formalin preservation for more than 40 years had probably caused high degradation of the DNA of these specimens.

Similarly, we were not able to amplify fragments of the 18S and 28S genes for *M. roffensis* and *M. martini*. All markers worked well for most species if contemporary samples were available, with only a few exceptions (Table 1). All sequences obtained

Table 4. Total body lengths (TBL in mm) of immature and adult *Macarorchestia* measured electronically by Image Pro Plus from photomicrographs of whole animals.

Taxon	Sampling location	Date	Sex	Maximum (mm)	Mean (mm)	SD	<i>n</i>
<i>Orchestia microphthalmia</i>	Cap Ferret, France	11.09.1967	M	14.10	11.21	1.98	13
			F	13.94	10.71	2.12	9
<i>M. remyi</i>	Principina-a-Mare, Italy	24.04.2011	M	10.38	8.46	0.97	10
			F	11.47	8.24	1.29	11
<i>M. remyi</i>	Lesina, Italy	31.10.2006 + 11.02.2006	M	10.11	7.67	1.20	10
			F	9.36	7.48	1.31	9
<i>M. roffensis</i>	Chatham Ness, Medway, UK	9.08.1968	M	8.11	5.98	1.01	9
			F	8.30	6.64	0.80	21
<i>M. martini</i>	Terceira, Azores	2.08.1987	M	6.06	–	–	1
			F	6.22	5.88	0.42	8

Table 5. Sex ratio of immature and adult *Macarororchestia* spp. including data from Stock (1989). Probability levels for significant difference from ♂% = 50 are shown.

Taxon	Sampling location	Date	Juveniles	M	F	Intersex	♂%	n
<i>Orchestia microphthalmia</i>	Cap Ferret, France	11.09.1967	6	22	23	?	48.9 N.D.	71
<i>M. remyi</i>	Principina a Mare, Italy	May2006 + April2007	705	729	1,728	268	26.8 ****	3430
<i>M. remyi</i>	Lesina, Italy	01.11.2006	0	3	3	8	21.4 N.D.	14
<i>M. roffensis</i>	Chatham Ness, Medway, UK	Summer 1967	307	88	331	?	21.0 ****	726
<i>M. martini</i>	Terceira, Azores	2.08.1987	0	1	9	?	10.0 *	10

M = males; F = females.

Table 6. Pairwise population and species comparisons of COI divergences based on Kimura two-parameter (K2P_{COI}) distances. K2P values based on the nuclear genes (18S and 28S) are also shown for further comparisons. Finally, COI-based divergence times (million years, myr) ± standard deviation (SD) are provided for those comparisons involving a sister taxon relationship in the COI phylogeny of Figure 4.

Population/species 1	Population/species 2	K2P% COI	T _{Myr} ± SD COI	K2P% 18S	K2P% 28S
Putative new taxon from the Swale, UK	<i>O. mediterranea</i> , euhaline, Medway, UK	2	0.83 ± 1.50	0	0
<i>O. gammarellus</i> , euhaline, Medway, UK	<i>O. gammarellus</i> , mesohaline, Medway, UK	4.3	1.8 ± 0.53	0	0.08
<i>O. gammarellus</i> , euhaline, Medway, UK	<i>O. guancha</i> , Tenerife, Canaries	22	9.2 ± 7.7	0.2	0.2
<i>O. aestuarensis</i> , mesohaline, Medway, UK	<i>O. mediterranea</i> , euhaline, Medway, estuary, UK	11	N/A	0.33	0.15
<i>O. gammarellus</i> , euhaline, Medway, UK	<i>O. mediterranea</i> , euhaline, Medway, estuary, UK	20	N/A	1.3	0.5
<i>O. gammarellus</i> , euhaline, Medway, UK	<i>O. montagui</i> , all populations	24	N/A	3.6	4
<i>O. gammarellus</i> , euhaline, Medway, UK	<i>C. cavimana</i> Republic of Cyprus	28	N/A	2.9	3.3
Average among 17 <i>Orchestia</i> populations and species		22	N/A	2.1	2.3
<i>Macarorchestia remyi</i> , Italy	<i>Palmorchestia</i> <i>epigaea</i> , La Palma, Canaries	25	N/A	2.5	3.1
Average within three <i>Macarorchestia remyi</i> populations	4	4	N/A	0	0

from COI (497 base pair, bp), 18S (639 bp) and 28S (821 bp and 558 bp for the primer pairs 28Sdd/ff and 28Sv/x, respectively) were submitted to GenBank (accession nos. COI: JX094864-85; JX104468-69, 18S: KP010833-65, 28S: KP010899-933).

Hypothesis 2

Pertinent genetic distances based on K2P of the COI gene are shown in Table 6 along with those based on the two nuclear genes. For the unknown taxon from the Swale, UK, and an *O. mediterranea* population from the euhaline part of the Medway estuary, UK, K2P_{COI} = 2%, which shows the closeness between the two species.

18S and 28S genes showed no differences between the unknown taxon and *O. mediterranea* (Table 6). Our results are consistent with the null hypothesis that the unknown taxon is an intraspecific form within *O. mediterranea*.

Other K2P differences

Other differences of interest (Table 6) show that between *O. mediterranea* and *O. aestuarensis* $K2P_{COI} = 11\%$, which is consistent with this pair being closely related sister species, although able to interbreed both in the laboratory and the field (Wildish 1970). The possibility that a mesohaline population of *O. gammarellus* from the Medway estuary was a new species was discounted because of an insufficient $K2P_{COI}$ difference of 4.3%. The genetic structuring (Ketmaier and Pavesi 2013) in the mesohaline population may result from its isolated up-estuary position where it received few immigrants. The genetic difference between *O. gammarellus* and *O. guancha* from Tenerife, the Canaries archipelago, with a $K2P_{COI} = 22\%$, is equivalent to the average for all *Orchestia* studied. Phylogenetic searches consistently retrieved *O. gammarellus* and *O. guancha* as sister species as first proposed by Villacorta et al. (2008). For these reason (and according to results from this study, see further down), we have decided to keep *O. guancha* within the genus *Orchestia* and not to transfer it to the genus *Cryptorchestia*, as recently proposed by Lowry and Fanini (2013). Villacorta et al. (2008) found both *O. gammarellus* and *O. guancha* close to *O. gomeri* from the island of La Gomera in the Canaries, a species that unfortunately we were not able to include in the study. The genetic difference between *Macarorchestia remyi* from Italy and *Palmorchestia epigaea* from the Canaries is $K2P_{COI} = 25\%$. Both are basal to the large clade which includes many *Orchestia* spp. (Figure 3; all genes). Multiple comparisons within the genus *Orchestia* returned $K2P_{COI}$ values comparable to that between the genera *Macarorchestia* and *Palmorchestia*, supporting the lack of monophyly for the genus *Orchestia* as apparent in the phylogenetic trees (Figures 3 and 4). Divergence times of supported cladogenetic events in the COI phylogeny ranges from about 0.83 million years (myr) for the split involving the putative new taxon and *O. mediterranea* to 9.2 myr between *O. gammarellus* and *O. guancha* (Table 6). We emphasize here that these time estimates should be considered with extreme caution given that (i) they are based on the assumption of a linear relationship between genetic divergence and evolutionary times; and (ii) our phylogeny is not exhaustive taxon-wise and this prevented us adopting more complex approaches (i.e. Bayesian approaches) that, being tree-based, require taxon-sampling denser than ours to avoid inflating divergence time estimates due to the presence of large gaps (unsampled taxa) in the branching pattern.

Talitrid phylogeny of the north-east Atlantic/Mediterranean region

Included in this region are the north-east Atlantic islands of the Azores, Madeira and Canary archipelagos. All of the known talitrid species found there by conventional, morphological methods are shown in Table 7. Species are categorized by the primary habitat (where this is known) that they normally occur in. The list in Table 7 includes nine genera and 26 morphologically defined species, for which we provide molecular data for seven of the nine genera, but only 13 of the 26 species. This limitation should be borne in mind when considering the phylogenetic analysis (Figures 3 and 4).

Figure 3. Bayesian estimate of the evolutionary relationships among the talitrid species included in the study based on all genes combined together (COI, 18S and two 28S fragments). Numbers at nodes are posterior probabilities.

Based on the COI alone (Figure 4) the evidence suggests that there are three groups, consisting of *C. cavimana* only and two subgroups of *O. montagui* + *O. stephenseni* and the rest. The latter consists of two subgroups made up of *Platorchestia* + *Orchestia* and *Deshayesorchestia* + *Talitrus* and a further subgroup consisting of *Macarorchestia* + *Africorchestia* + *Palmorchestia*. All genes combined yielded similar relationships (Figure 3) with the main discrepancies being the placement of *A. spinifera* and *P. epigea*; the two species cluster together in the COI phylogeny and separately in the tree based on all genes. Both phylogenies consistently rejected the genus *Orchestia* as a monophyletic.

Discussion

Growth data

Four of the eight dependent variables in the regression analysis shown in Table 2 are significantly different in slope between an *O. mediterranea* population from Portugal and the *O. microphthalmus* one from France. These variables included ED and P13 Ex1, which are reduced in *O. microphthalmus*. Both these features are included as diagnostic for *Macarorchestia* by Stock (1989) and allow us to reject the null hypothesis that

Figure 4. Bayesian estimate of the evolutionary relationships among the talitrid species included in the study based on the COI partition. Numbers at nodes are posterior probabilities; divergence time (myr) $\pm$ standard deviation (SD) is reported at each node. Taxa are labelled following the codes in Table 1. Species delimitations obtained with the ABGD method are shown next to the tree.

O. microphthalmus belongs to the genus *Orchestia*, where both characters are not reduced. Our results suggest that *O. microphthalmus* is more closely related to *Macarorchestia* and we examined this further by comparing *O. microphthalmus* and a population of *M. remyi* from Principina Mare, Italy. We found that none of the characters had a significant slope difference between *O. microphthalmus* and *M. remyi*, although there were four intercept significant differences which involved extensive overlapping, rendering them of little use in distinguishing the two species (Figure 1 B). The intercept differences are consistent with the hypothesis that *O. remyi* is neotenously derived from *O. microphthalmus* and that both species belong to the genus *Macarorchestia*.

The finding that the Swale unknown taxon included immatures at TBL sizes that in *O. mediterranea* would still be in the juvenile stage, supports a rejection of the null hypothesis that the unknown species is a juvenile *O. mediterranea*. The appearance of secondary sexual characters in the unknown taxon at a ‘juvenile’ TBL size for *O. mediterranea* suggests a neotenous origin for it. The explanation as to why in Figure 2 the parallel regression lines for the unknown species occurs above that of juvenile *O. mediterranea* is because the former first moult juvenile is hatched from the ova at a smaller TBL (~ 2.25 mm) size than the latter (3.7–4.2 mm). This is in line with the observed intercept differences in most of our data between the unknown

Table 7. Talitridae (Crustacea: Amphipoda) species list from the north-east Atlantic (inclusive of islands) and Mediterranean coastlines.

Primary habitat	<i>Africorchestia</i>	<i>Britorchestia</i>	<i>Cryptorchestia</i>	<i>Deshayesorchestia</i>	<i>Macarorchestia</i>	<i>Orchestia</i>	<i>Palmorchestia</i>	<i>Platorchestia</i>	<i>Sardorchestia</i>	<i>Talitrus</i>
Sand burrowing	<i>?spiniifera*</i>	<i>brito</i> <i>ugolini</i>		<i>deshayesi*</i>					<i>?pelecaniformis</i>	<i>saltator*</i>
Wrack generalist						<i>gammarellus*</i> <i>mediterranea*</i> <i>?aestuarensis*</i> <i>stephensi*</i> <i>montagui*</i> <i>?xylino</i>		<i>platensis*</i>		
Freshwater wrack			<i>cavimana*</i>			<i>?monticola</i> <i>?chevreuxi</i> <i>?guancha*</i> <i>?stocki</i> <i>?canariensis</i> <i>?goneri</i>	<i>epigea*</i>			
Rainforest leaf litter					<i>remyi*</i> <i>roffensis</i> <i>martini</i> (<i>microphthalma</i>)					
Driftwood										
Cave								<i>hypogea</i>		

Notes: ? indicates uncertain habitat status. * indicates molecular data available. Based on WoRMS (World Register of Marine Species; www.marinespecies.org) accessed in 2013.

taxon and *O. mediterranea* juveniles. One would normally only accept slope (allometric), and not intercept (isometric), differences between pairs of taxa as evidence of taxonomic difference. In the case between the unknown taxon and juvenile *O. mediterranea* from the Medway estuary the intercept differences occur for most of the dependent variables tested and demonstrate isometry, which supports the hypothesis of neotenus origin for the unknown species. On the other hand, the slope coefficient similarities also found support the view that the unknown taxon is closely related to *O. mediterranea*, perhaps only differing in that it is a neotenic form of it.

Having accepted the alternative hypothesis that the unknown taxon was a new form of driftwood hopper we tested the possibility that it belonged taxonomically to *Macarorchestia* by comparing it with *M. roffensis*, also from the Medway estuary. We found two slope differences (ED and PL3 ExL), which according to Stock's (1989) diagnosis of *Macarorchestia* discount it as belonging to that genus. The large, unreduced eyes and pleopod rami of the unknown taxon suggests that it did not belong to *Macarorchestia*.

Considerations based on relative growth measures, as above, hide the typically slow growth rates which are shown by all driftwood talitrids so far examined. Limited talitrid growth data are available (Wildish 1972) and we have used this to help construct hypothetical growth curves based on archived juvenile growth data available at the NHM for *O. mediterranea* and *M. roffensis*, from the Medway estuary, UK. Maximum adult TBLs for the former are 21 mm and 8 mm for the latter and these have been used to construct the growth curves of Figure 5. Shown for *M. roffensis* is a 12-month and for *O. mediterranea* an 18-month lifespan. The linear fitting for the unknown taxon (Figure 5A) is based on the assumptions of a first moult TBL = 2.5 mm and similar growth slope coefficient as in *O. mediterranea*. For a 12-month lifespan the maximum TBL reached by the unknown taxon would be 12 mm and for an 18-month lifespan it would be ~ 16 mm. A lognormal fitting for the same data is shown in Figure 5B.

The findings presented here suggest that neoteny, and the consequent small body size finally achieved (dwarfism), may be fundamentally important in the origin of all driftwood talitrids. If this is so the size, as determined by the TBL of driftwood hoppers, is important and so the accurate TBL of the data generated here and in Wildish et al. (2012) is included in Table 4. The range found is from ~ 14 mm to as low as 6 mm for *M. martini* from the Azores. A concomitant effect of reductions in body size, due to slower growth, is that the number of ova per brood is reduced (Wildish 1988). One way of offsetting small body size and consequent reductions in number of ova/brood is to increase the proportion of functional females in the brood. This is known to occur in talitrids commonly as a result of co-evolution of an 'endoparasite' and its talitrid host (Ginsburger-Vogel 1991) and the adaptive advantages are outlined (Wildish 1971). In our own data (Table 4) the tendency to female bias in the immature and adult populations does seem to increase as TBL decreases. However, our data are incomplete, particularly for *M. remyi* from Lesina, Italy and *M. martini* from the Azores and further sampling is required to firmly establish this point.

Our finding that the unknown driftwood talitrid found in the UK in 2011 was present in driftwood made up of Douglas fir confirms that driftwood talitrids can utilize both coniferous (Gymnospermae) and deciduous (Angiospermae) driftwood. This is consistent with earlier observations in the UK, of *M. roffensis* found in deciduous driftwood by Wildish (unpublished) and in the Mediterranean Sea region

Figure 5. Hypothetical growth curves for: (◆) *O. mediterranea* from the Medway estuary diamonds (18 months); (△) unknown taxon from the Swale, UK (12 months); (□) *M. roffensis* from the Medway estuary, UK (12 months). (A) Linear fitting; (B) lognormal fitting.

of other species of *Macarorchestia* in both deciduous (*Alnus* sp., *Betula* sp.) and coniferous (*Pinus* sp.) driftwood by Pavesi (2009). These findings are important because they establish that suitable driftwood (gymnosperms originated 300 mya and angiosperms 140 mya Atlantic Geoscience Society 2001) was available for driftwood colonization by talitrids prior to the supposed origin of talitrids from hyalid-like ancestors some 110 mya (Bousfield 1984).

Molecular data

The K2P_{COI} distances found in the current study confirmed the findings of Wildish et al. (2012) and are in line with the available literature on Crustacea (Adamowicz

et al. 2004; Costa et al. 2009). The driftwood hoppers (*Macarorchestia* spp.) are distinct from the non-driftwood talitrids, and the new taxon appears to be closer to *O. mediterranea* than to *M. remyi*.

Populations' ABGD was congruent with the current taxonomy for almost all species. In each analysis, when the prior maximal distance ($P_{\max}$) is small, the partition tends to find a higher number of groups, probably due to intra- rather than inter-specific genetic divergence. Indeed, many talitrid species show a high level of genetic structuring (see Ketmaier and Pavesi 2013 for a review on the issue). A threshold of 4% was the best to describe our data. The putative new taxon clustered, in each dataset and each parameter set-up, always together with *O. mediterranea*. For $P_{\max} > 4\%$ *O. aestuarensis* (H) would be grouped with *O. mediterranea*, and all the *O. gammarellus* populations would cluster together, while for $P_{\max} < 4\%$ the analysis would assign population B and all the other *O. gammarellus* populations to two distinct groups. The occurrence of probable divergent genetic lineages within amphipod species has sometimes been reported (Witt et al. 2006; Cabezas et al. 2013), including in some Mediterranean talitrids (Ketmaier and Pavesi 2013).

Although our phylogenetic tree includes only one half of the known species found in the Mediterranean/north-east Atlantic region we believe that it provides a preliminary view of what a more complete phylogeny might look like. The following are the salient findings:

- The divergence time for the splitting between the unknown taxon and *O. mediterranea* could be very recent (0.83 million years).
- *O. guancha* is phylogenetically close to *O. gammarellus*, supporting the earlier findings of Villacorta et al. (2008); this is circumstantial evidence in support of the hypothesis that the former species arose from a wrack generalist (Wildish 2012).
- *Palmorchestia epigea* is either close to *A. spinifera* (COI only) and both species form a clade sister to *M. remyi*; or close to *P. epigea* (all genes) and is basal to a large clade including various *Orchestia* species + the putative new taxon + *M. remyi*.
- The genus *Orchestia* is polyphyletic with at least four subgroups (*C. cavimana*, *O. stephensenii* + *O. montagui*, *O. aestuarensis* + *O. mediterranea* + unknown taxon, *O. gammarellus*).

This is supportive of the proposal of Lowry and Fanini (2013) that the former *Orchestia cavimana* belongs to a new genus *Cryptorchestia*, but not that the north-east Atlantic island terrestrial talitrids referable to *Orchestia* belong to the same new genus, represented in our sampling by the former *O. guancha*. The latter species is phylogenetically close to *O. gammarellus*.

Conclusions

The main findings of this study are that on morphological grounds *O. microphtalma* belongs to the genus *Macarorchestia*. Regarding the putative new taxon, morphological results indicate that the unknown taxon is not a juvenile *O. mediterranea*. Molecular analyses confirms that it does not belong to the well-established driftwood genus *Macarorchestia* and is very close genetically to the wrack generalist *O.*

mediterranea, having diverged from it *c.*0.83 myr. Based on allometric growth observations the unknown taxon is a neotenic dwarf form of *O. mediterranea*, differing only in that it is slower growing, begins sexualization at an earlier moult number and lacks dorsal pigment patterns.

Microscopic examination of the driftwood sample in which the unknown taxon was found established that it was from a Douglas fir tree.

The molecular results confirm the close relationship between the unknown taxon and *O. mediterranea*, with two additional nuclear genes supporting this. During this study approximately one half of the morphologically defined species present in the north-eastern Atlantic/Mediterranean region were analysed (for COI and 18S and 28S rDNA). The resulting phylogenetic tree provides a preliminary glimpse of the phylogenetic relationships of species from seven, of a total of nine, regional talitrid genera.

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