PHYLOGENETIC ANALYSIS OF *DENDRONOTUS* NUDIBRANCHS WITH EMPHASIS ON NORTHEASTERN PACIFIC SPECIES

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(Received 2 June 2009; accepted 17 May 2010)

ABSTRACT

Molecular and morphological evidence suggests that the taxonomic status of several eastern Pacific species of *Dendronotus* needs a reassessment. *Dendronotus diversicolor* and *D. albus* are synonymized due to lack of genetic variation in the 16S rRNA gene and of any significant morphological differences. *Dendronotus nanus* and *D. iris* are also regarded as synonyms based on a reevaluation of ecological and morphological data. Finally, the name *D. venustus* is resurrected for eastern Pacific populations previously considered to be *D. frondosus*. Pacific *D. venustus* display consistent morphological and molecular differences from Atlantic *D. frondosus*. The molecular phylogeny presented here is not robust enough to shed light on the evolution and biogeography of *Dendronotus*, but preliminary evidence indicates that the Pacific species of *Dendronotus* are not a monophyletic group, because Arctic and Atlantic species are nested within them. The 16S rRNA genetic diversity within *Dendronotus* is very small compared to that of other related groups. These data, along with the comparatively large diversity of *Dendronotus* in the Pacific, suggest the possibility that relatively recent Arctic migration and vicariance along Beringia may have been involved in the evolution of this group.

INTRODUCTION

Dendronotus Alder & Hancock, 1845 (Dendronotidae) contains mostly northern temperate species, with the greatest diversity occurring along the eastern Pacific coast and other species distributed in the northern Atlantic, Arctic and western Pacific Oceans (MacFarland, 1966; Marcus & Marcus, 1967; Robilliard, 1970; Thompson, 1976; Behrens, 1991). There are currently 15 recognized species in *Dendronotus* (Table 1), 11 of which were reviewed, described or redescribed by Robilliard (1970, 1972). Since Robilliard's (1970, 1972) publications, *D. lacteus* (Thompson, 1840) has been resurrected as a species distinct from *D. frondosus* (Ascanius, 1774) based on allozyme electrophoresis studies (Thollesson, 1998), and *D. comteti* Valdés & Bouchet, 1998 has been described from the hydrothermal vents of the mid-Atlantic ridge (Valdés & Bouchet, 1998). Two new species were recently described from the tropical Indo-Pacific, from which this genus had not previously been recorded (Pola & Stout, 2008).

Of the 15 currently recognized species, all but six are found in the eastern Pacific, from Alaska to Baja California (Table 1, Fig. 1). The exceptions are *D. gracilis* Baba, 1949 from New Zealand and northern Japan, *D. robustus* Verrill, 1870 from the Arctic and eastern Atlantic, *D. lacteus* from the eastern Atlantic, *D. comteti* from deep hydrothermal vents in the mid-Atlantic ridge, and *D. noahi* Pola & Stout, 2008 and *D. regius* Pola & Stout, 2008 from the tropical Indo-Pacific. Of the species that do occur in the eastern Pacific, *D. dalli* Bergh, 1879 also occurs in the northwestern Pacific along the coast of Russia and along the northeast Atlantic (M. Thollesson, personal communication), and *D. frondosus* is found on both sides of the Atlantic. There have also been reports of *D. frondosus* from the coast of Chile (Schrödl, 2003). Baba (1993) described some specimens of *D. frondosus* from Japan, but there is not enough anatomical information on these to include them in this study.

Questions on the validity of some Pacific species of *Dendronotus* have been raised in the last few decades. Robilliard (1970) described *D. diversicolor* as a species distinct from *D. albus*, but intermediate forms between these two very similar species have been found since then (Behrens, 2006, 2007). The validity of *D. nanus* Marcus & Marcus, 1967 has also been questioned because of its morphological similarity to *D. iris* Cooper, 1863 (Rudman, 2005; Velarde, 2005). Questions have also been raised about *D. frondosus*, the species with the most widespread range. It has been proposed that with further examination it may be shown to be a complex of several species instead of just one highly variable and widespread species (Robilliard, 1975; Sisson, 1998, 2002, 2005). None of these questions have yet been addressed using molecular data or phylogenetic analyses. The present paper attempts to provide molecular and morphological data to address these questions and to clarify the taxonomic status of the problematic Pacific species of *Dendronotus*.

MATERIAL AND METHODS

Morphological data

Specimens were obtained from the Natural History Museum of Los Angeles County (LACM), the California Academy of Sciences Department of Invertebrate Zoology and Geology in San Francisco (CASIZ) and the American Museum of Natural History in New York (AMNH). Fresh specimens were collected along the eastern Pacific from Alaska to San Diego. Specimens of *Dendronotus frondosus* and *D. lacteus* were also obtained from Scotland (Table 2). Specimens were dissected under a microscope and external and internal features were recorded. Reproductive structures were compared to drawings made by Robilliard (1970). The buccal mass of each specimen was extracted and soaked in a 10% sodium hydroxide solution for 1 week to dissolve the connective and muscle tissue, leaving only the radula and mandibles. The coated radula and mandibles of each species were examined and images were obtained using scanning electron

Table 1. Species and species distribution of currently recognized species of *Dendronotus* (prior to this study).

Species	Distribution
D. frondosus (Ascanius, 1774)	Arctic, northern Atlantic (east and west), northeastern Pacific
D. lacteus (Thompson, 1840)	Northeastern Atlantic
D. comteti Valdes & Bouchet, 1998	Mid-Atlantic Ridge
D. robustus Verrill, 1870	Arctic, northeastern Atlantic
D. gracilis Baba, 1949	Northern Japan, New Zealand
D. albopunctatus Robilliard, 1972	San Juan Archipelago, Washington
D. albus MacFarland, 1966	Alaska to Baja California
D. diversicolor (Robilliard, 1970)	Alaska to California
D. dalli Bergh, 1879	Bering Sea to Puget Sound, WA; NE Atlantic
D. rufus O'Donoghue, 1921	Alaska to Washington
D. subramosus MacFarland, 1966	British Columbia to Baja California
D. iris Cooper, 1863	Aleutian Island to Coronados Island, Mexico
D. nanus Marcus & Marcus, 1967	Sonora, Mexico
D. regius Pola & Stout, 2008	Tropical Indo-Pacific
D. noahi Pola & Stout, 2008	Tropical Indo-Pacific

microscopes (Leo 1450 VP, Hitachi S-3000). Characters and character states used in the phylogenetic analysis are shown in Table 3. All multistate characters were treated as unordered and unweighted. The outgroups were chosen primarily based on previous morphological and genetic studies that have shown the genera *Tritonia*, *Lomanotus* and *Melibe* to be phylogenetically closely related or sister to *Dendronotus* (Wägele & Willan, 2000; Wollscheid-Lengeling *et al.*, 2001; Pola, Rudman & Gosliner, 2009). These large-scale studies did not include all genera within Dendronotida (Bouchet & Rocroi, 2005) so representative species from other families within the same subclade were also included as outgroups. Species of *Doto* were also included for congruence between the morphological and molecular data sets and because of its traditional grouping with these taxa, despite analyses that have excluded the genus from this subclade (Wägele & Willan, 2000; Bouchet & Rocroi, 2005).

Molecular data

Because most museum specimens were fixed in formalin, only a limited amount of fresh material was available for genetic studies (Table 2) and DNA extraction success (based on DNA quantification readings), even with newly obtained and

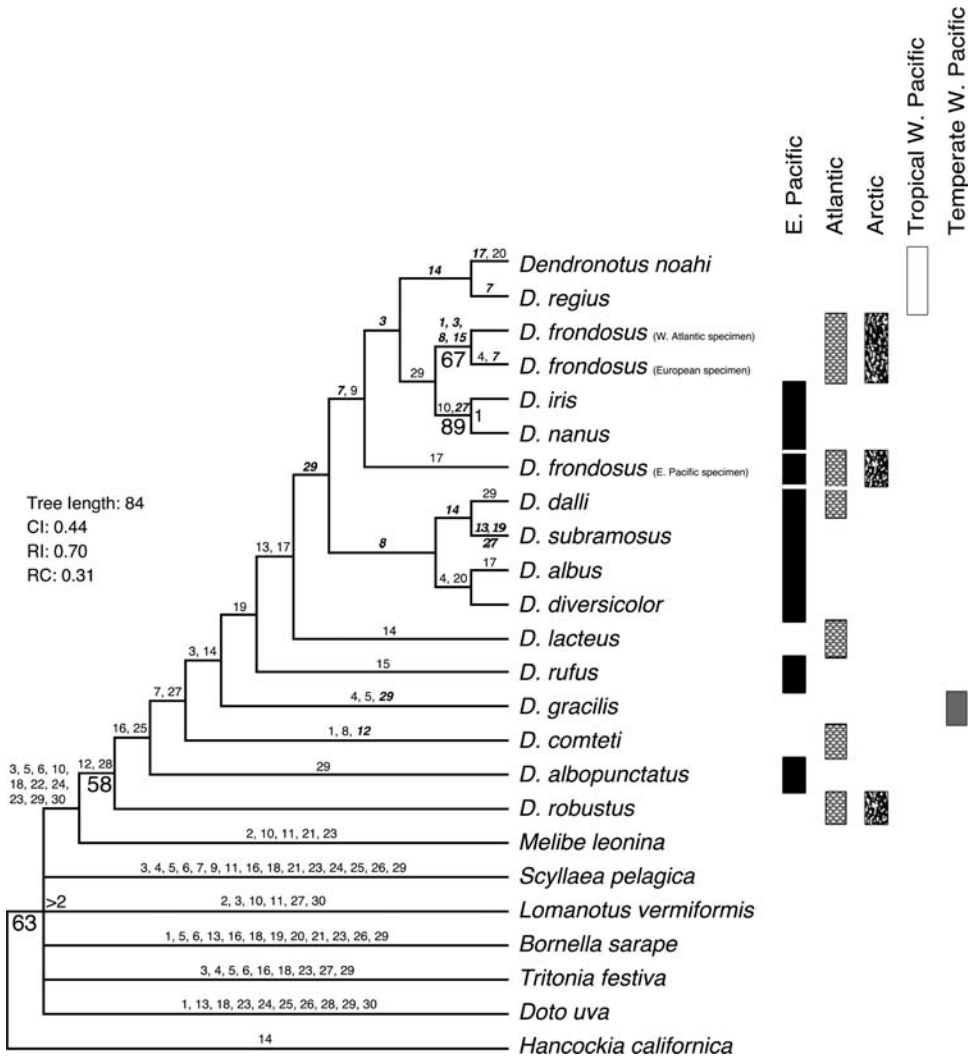

Figure 1. Phylogenetic tree for *Dendronotus* species based on analysis of 30 unordered and unweighted morphological characters. Distributions of species are coded on the right. Numbers along branches are character state changes with reversals indicated in italic bold. Larger numbers to the right of nodes represent Bremer support, and large numbers below nodes represent bootstrap support after 100 replicates.

Table 2. List of specimens of *Dendronotus* and outgroups used in this study.

Species (n = number of species if >1)	Locality	Voucher	GenBank accession no. for 16S sequences
D. albopunctatus	Vancouver I., British Columbia	LACM 172348	
D. albus ($n = 2$)	Alcala Pt., Vancouver I.	LACM 172347	
D. albus	Montaña de Oro, San Luis Obispo, California	LACM 174845	GU339185
D. albus	Santa Barbara, California	LACM 2004-2.2	
D. albus	Port Townsend, Washington	LACM 174853	
D. albus	Middle cove, Cape Arago, Oregon	LACM 174857	
D. dalli	Vancouver I., British Columbia	CASIZ 068459	
D. dalli	2 mi SW of Kodiak, St Paul Harbor, Alaska	LACM 73-33	
D. dalli	Victoria breakwater, Vancouver I., British Columbia	CASIZ 068449	
D. dalli ($n = 3$)	8 mi W of Point Barrow, Alaska	CASIZ 068459	
D. dalli	Trident Basin (152° 24.00′W 57° 46.50′N), Alaska	LACM 174862	
D. diversicolor	Montaña de Oro, San Luis Obispo, California	LACM 174846	GU339186
D. diversicolor	Cutter Rock, Ketchikan, Alaska	LACM 172350	
D. diversicolor	San Diego, California	LACM 174193	
D. frondosus	New Hampshire	CASIZ 079497	
D. frondosus	Walpole, Maine	AMNH 313488	
D. frondosus	Hancick Co., Maine	AMNH 3414	
D. frondosus ($n = 2$)	Wood's Hole, Massachusetts	AMNH 179130	
D. frondosus	Cape Neddick, Maine	AMNH 305758	
D. frondosus	Belmar, New Jersey	AMNH 313492	
D. frondosus	Nuchatlitz Inlet, Nootka Dist., Vancouver I., British Columbia	LACM 172352	
D. frondosus	Cutter Rock, Ketchikan, Alaska	LACM 172351	
D. frondosus	Coast guard breakwater, Monterey, California	CASIZ 170765	
D. frondosus	San Diego, California	LACM 174190	
D. frondosus	San Diego, California	LACM 174191	
D. frondosus	Newport, Oregon	CASIZ 174472	
D. frondosus	Redondo Canyon, California	LACM 174850	GU339198
D. frondosus ($n = 2$)	Bodega Bay (38.19.45.55 N, 123.03.27.52 W), California	LACM 174852	GU339199, GU339200
D. frondosus	Imperial Reef, Garvellach Is, Firth of Lorne, Scotland (56.15.1736N, 5.44.5624W)	LACM 174860	GU339187
D. frondosus	Cordova, Alaska	LACM 174869	
D. frondosus	Morro Bay docks, California	LACM 174870	
D. frondosus	Cape Arago, Oregon	LACM 174871	
D. frondosus	Montaña do Oro, San Luis Obispo, California	LACM 174872	
D. frondosus	Neah Bay, Washington	LACM 174873	
D. frondosus	Middle cove, Cape Arago, Oregon	LACM 174875	
D. iris	Galiano I., Cowichan Dist., Vancouver I., British Columbia	LACM 172353	
D. iris	Channel Is, California	CASIZ 098809	
D. iris ($n = 2$)	Point Reyes, Marin Co., California	CASIZ 068425	
D. iris	Galiano I., British Columbia	LACM 174847	
D. iris	San Diego, California	LACM 174194	GU339188
D. iris	Gig Harbor, Washington	CASIZ 174471	GU339189
D. iris	Gig Harbor, Washington	CASIZ 174469	
D. iris	La Jolla Canyon, San Diego, California	LACM 174858	GU339190
D. iris ($n = 3$)	Washington	LACM 174859	
D. lacteus	Imperial Reef, Garvellach Is, Firth of Lorne, Scotland (56.15.1736N, 5.44.5624W)	LACM 174877	
D. robustus	Massachusetts	CASIZ 024973	
D. robustus	New Hampshire	CASIZ 079513	
D. rufus	Mountain Point, Ketchikan, Alaska	LACM 172354	
D. rufus	King County, Washington	CASIZ 068450	
D. rufus ($n = 4$)	British Columbia	LACM 174851	
D. rufus	Washington	LACM 174876	
D. rufus	Buoy 16 (152°31.80′W, 57°43.00′N) Alaska	LACM 174861	GU339191
D. rufus	Cargo Pier (152° 30.87′W, 57°43.80′N), Alaska	LACM 174863	
D. rufus	Alaska	LACM 174864	

Continued

Table 2. *Continued*

Species (n = number of species if >1)	Locality	Voucher	GenBank accession no. for 16S sequences
D. rufus	Marginal Pier (152°31.47′W, 57°43.35′N), Alaska	LACM 174865	
D. subramosus ($n = 4$)	Puget Sound, Washington	CASIZ 068445	
D. subramosus	Bee Rock, Santa Rosa I., Channel Is, California	CASIZ 071370	
D. subramosus	Alcala Point, Greater Vancouver, British Columbia	LACM 172355	
D. subramosus	Marin Co., California	LACM 126379	
D. subramosus	Hudson's Point, Washington	LACM 174854	GU339195
D. subramosus ($n = 2$)	Port Townsend, Washington	LACM 174855	GU339196, GU339197
D. subramosus ($n = 3$)	San Diego, California	LACM 174192	GU339192 GU339193 GU339194
D. subramosus	Montaña de Oro, San Luis Obispo, California	LACM 174868	
D. subramosus	North cove, Cape Arago, Oregon	LACM 174874	
Lomanotus vermiformis	Panama	LACM 153371	
Mariona sp.	Panama	LACM 153497	GU339201
Melibe leonina	Cabrillo Beach docks, California	LACM 174849	GU339202
Melibe leonina	Cabrillo Beach docks, California	LACM 174856	
Scyllaea pelagica	Nova Scotia	LACM 124383	
Scyllaea pelagica	Costa Rica	LACM INB 0001497362	
Tritonia diomedea	South of Point Vicente, Los Angeles, California	LACM 2004-16.3	GU339203
Tritonia festiva	Catalina I., California	LACM 140812	
Hancockia californica	California	CASIZ 069133	
Bornella sarape	Gulf of California	LACM 36-71	
Bornella sarape	Jalisco, Mexico	LACM 14801	

properly preserved specimens, was limited. DNA was extracted using either the DNeasy extraction kit (Qiagen, Valencia, CA, USA) or Chelex (BIO-RAD, Hercules, CA, USA). For DNeasy extractions, the protocol suggested by the manufacturer was followed, adjusting only the time for incubation at 56°C to run overnight instead of 1–3 h. For Chelex extractions, 3 mm of finely cut tissue from the foot was placed in 200 µl of 10% Chelex in 1× TE solution, incubated at 56°C in a water bath for 20 min and vortexed for 10 s. Samples were incubated in a dry heat block at 100°C for 8 min and again vortexed for 10 s. After centrifugation for 3 min at 15,000 × g, the supernatant was ready for PCR.

Primers for the 16S ribosome subunit gene (16S ar-L 5′-CGCCTGTTTATCAAAAACAT-3′, 16S br-H 5′-CCGGTCT GAACTCAGATCACGT-3′) developed by Palumbi *et al.* (1991) were used to amplify this region of the mitochondrial genome. This gene has been used to build phylogenies at various taxonomic levels because of the variable rates of mutation in the stem *vs* loop regions of the gene (Wollscheid-Lengeling *et al.*, 2001; Lüter & Cohen, 2002; Valdés, 2003). Stem regions are highly conserved, but loop regions are subject to increased rates of mutation because of reduced selection pressure. Attempts were made to include COI sequences as well, but despite several attempts at designing new primers, very few specimens were successfully amplified for this gene.

For amplification of 16S rRNA gene, each 50 µl PCR consisted of 50 mM KCl, 10 mM Tris, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 mM of each primer, 0.25 µl *Taq* polymerase and 2 µl DNA template. The conditions were as follows: 2 min at 94°C initial denaturation; 30 cycles of 94°C for 30 s (denaturation), 50°C for 30 s (annealing), 1 min at 72°C (extension); and final extension at 72°C for 7 min. Purified products were sequenced at the City of Hope DNA sequencing lab in Duarte, CA, USA. Consensus sequences were obtained from assembled forward and reverse sequences in Geneious version 3.7 (Drummond *et al.*, 2007). The sequences for *Dendronotus dalli* and all three *Doto* species (*D. eireana*, *D. koenneckeri* and *D. pinnatifida*) were acquired from GenBank (accession numbers: AF249252.1, AF249248.1, AF249249.1 and AF249250.1, respectively). All consensus sequences were then aligned using CLC Free Workbench version 4.0.3 (CLC bio).

Tree building

The morphological data matrix was generated in MacClade v. 4.06 (Maddison & Maddison, 2003) and imported into *PAUP** v. 4.0b10 (Swofford, 2002). A heuristic search was done under parsimony using accelerated transformation (ACCTRAN) for character state optimization and by treating unknown character states as missing data. Stepwise addition with 100 replicates was applied and branch support was assessed with nonparametric bootstrapping (100 replicates) and Bremer support using decay analysis (Bremer, 1994).

For genetic analyses, aligned sequences were imported into MEGA4 (Tamura *et al.*, 2007) for maximum parsimony (MP) analysis and subjected to 2000 bootstrap replicates for statistical support. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000) using the complete deletion option where all positions containing gaps and missing data were eliminated from the data set. The initial trees were obtained with the random addition of sequences (10 replicates). For maximum likelihood (ML) trees, aligned sequences were tested against models of evolution in ModelTest v. 3.7 (Posada & Crandall, 1998) to find the model that best fit the data using Akaike Information Criterion (AIC), which was TVM + I + G. This model was implemented into *PAUP** and then subjected to 1,000 bootstrap replicates. A Bayesian approach was also used (MrBayes v. 3.1.2, Huelsenbeck &

Table 3. Characters and characters states for the morphological phylogenetic analysis of *Dendronotus* species.

Character	Character states
1. Jaw denticles on masticatory process	0: present; 1: absent
2. Median tooth	0: present; 1: absent
3. Median tooth morphology	0: elongate; 1: quadrangular; 2: wider than higher
4. Median tooth denticulation furrows	0: absent; 1: present
5. Outermost lateral tooth cusp	0: short and wide; 1: long and skinny
6. Inner lateral teeth morphology	0: broad and flat plate-like; 1: thin and delicate
7. Outer lateral teeth morphology	0: thin; 1: broad, plate-like
8. Outer lateral tooth cusp	0: absent; 1: present
9. Denticulation on lateral teeth	0: absent; 1: present
10. Prostate	0: smooth and thick-walled; 1: globular and concentric ring-shaped; 2: very globular and long
11. Secondary branching of dorsolateral processes	0: absent; 1: present
12. Tertiary branching of dorsolateral processes	0: absent; 1: present
13. Lengths of primary stalk when bifurcations are present	0: branches end approximately equidistant from the body; 1: branches end at all different lengths
14. Secondary branch lengths	0: short; 1: long
15. Tertiary branch lengths	0: short; 1: long
16. Rhinophoral sheath	0: campanulate with smooth border; 1: knobby border; 2: long crown papillae extending from the border
17. Crown papillae	0: unbranched; 1: branched
18. Rhinophore	0: perfoliate vertically; 1: perfoliate horizontally; 2: palmated; 3: smooth
19. Lateral processes on rhinophoral sheath stalk	0: absent; 1: present
20. Branching rhinophoral lateral papilla	0: absent; 1: present
21. Large posterior flap on rhinophore sheath	0: absent; 1: present
22. Dorsum profile	0: distinctly marked profile; 1: rounded profile
23. Digestive gland in rhinophore	0: absent; 1: present
24. Oral veil	0: bilobed; 1: rounded
25. Veil papillae	0: absent; 1: digitate papillae; 2: branching papillae
26. External gills	0: absent; 1: present
27. Foot morphology	0: flared; 1: reduced
28. Reproductive system	0: diaulic; 1: triaulic
29. Cardiac prominence	0: absent; 1: slightly raised; 2: very large
30. Gastric teeth	0: present; 1: absent

Ronquist, 2001) to construct a phylogeny with the sequence alignment. The tree was constructed using 1 million generations sampled every 100 generations with the first 2,500 samples discarded as burn-in. Support for the tree was inferred from the posterior probabilities generated in the analysis.

RESULTS

Cladistic analysis of morphological data

A total of 30 morphological characters were parsimony-informative for the 23 taxa (Tables 3, 4). Parsimony analysis using a heuristic search resulted in seven most parsimonious trees (consensus of the trees in Fig. 1) with 84 steps, a consistency index of 0.44 and a retention index of 0.70. Synapomorphies for *Dendronotus* as a monophyletic group include a triaulic reproductive system and tertiary branching of the dorsolateral processes. Only *D. noahi* and *D. regius* represent a distinct clade found in only one of the previously defined geographical regions (tropical western Pacific), but they do not form a monophyletic group with *D. gracilis* which is found in temperate western Pacific waters. These tropical western Pacific species are sister to each other and most closely related to *D. iris* and *D. nanus*, both of which occur along the eastern Pacific coast. All other regions have species that occur throughout the tree (Fig. 1). The most basal member of the genus is *D. robustus* from the Atlantic and Arctic regions. While *D. frondosus* that occur on both sides of the Atlantic do group together, they are not monophyletic with *D. frondosus* from the eastern Pacific. Of species of doubtful validity, *D. iris* and *D. nanus* showed no variation in morphological characters and *D. diversicolor* showed only one character state difference (character 17: branching of crown papillae) from *D. albus*.

Phylogenetic analysis of 16S rRNA sequences

The alignment of 23 specimens consisted of 395 single-nucleotide positions, of which 66 were parsimony-informative. Not all ingroup taxa were represented (Table 2). The 17 ingroup specimens included eight of the 12 currently recognized species, but neither of the two tropical species (*D. noahi* and *D. regius*), and *D. frondosus* was represented only from Europe and the Pacific (not western Atlantic).

The MP analysis shows strong support for the monophyly of *Dendronotus* (Fig. 2). In all trees (Fig. 3) conspecifics group together, except for *D. frondosus* in which the Pacific specimens form a clade separate from the European specimen. All analyses reject a Pacific clade; Pacific specimens of *D. frondosus* group together but are not most closely related to Atlantic specimens of *D. frondosus*. *Dendronotus albus* and *D. diversicolor* show no genetic divergence. The low genetic diversity within *Dendronotus* (Fig. 2) compared to the genera within the outgroup is remarkable. There is more genetic diversity among

Table 4. Data matrix of morphological character states (see Table 3) for 30 characters across 24 taxa of *Dendronotus* and outgroups.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
D. noahi	0	0	0	1	?	1	1	?	1	?	1	1	1	0	0	2	0	1	1	0	0	1	1	1	2	1	1	?	2	1
D. gracilis	0	0	1	0	0	1	0	1	0	?	1	1	0	1	–	2	0	1	0	–	0	1	?	1	2	1	1	?	1	?
D. regius	0	0	0	1	?	1	1	?	1	1	1	1	1	0	–	2	1	1	1	1	0	1	1	1	2	1	1	1	2	1
D. frondosus Pacific	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	2	0	1	1	1	0	1	1	1	2	1	1	1	2	1
D. frondosus W. Atl.	1	0	2	1	1	1	1	0	1	1	1	1	1	1	1	2	1	1	1	1	0	1	1	1	2	1	1	1	1	1
D. frondosus Europe	1	0	2	0	1	1	0	0	1	1	1	1	1	1	1	2	1	1	1	1	0	1	1	1	2	1	1	1	1	1
D. iris	0	0	0	1	1	1	1	1	1	2	1	1	1	1	0	2	1	1	1	1	0	1	1	1	2	1	0	1	1	1
D. nanus	0	0	0	1	1	1	1	1	1	2	1	1	1	1	0	2	1	1	1	1	0	1	1	1	2	1	0	1	1	1
D. robustus	0	0	0	1	1	1	1	1	0	1	1	1	0	0	–	2	0	1	0	–	0	1	1	1	1	1	0	1	0	1
D. dalli	0	0	1	–	1	1	0	0	0	1	1	1	1	0	–	2	1	1	1	1	0	1	1	1	2	1	1	1	1	1
D. rufus	0	0	1	1	1	1	0	1	0	1	1	1	0	1	1	2	1	1	1	1	0	1	1	1	2	1	1	1	0	1
D. subramosus	0	0	1	1	1	1	0	0	0	1	1	1	0	0	–	2	1	1	0	–	0	1	1	1	2	1	0	1	2	1
D. albus	0	0	1	0	1	1	0	0	0	1	1	1	1	1	0	2	0	1	1	0	0	1	1	1	2	1	1	1	2	1
D. diversicolor	0	0	1	0	1	1	0	0	0	1	1	1	1	1	0	2	1	1	1	0	0	1	1	1	2	1	1	1	2	1
D. albopunctatus	0	0	0	1	1	1	1	1	0	1	1	1	0	0	–	2	0	1	0	–	0	1	1	1	2	1	0	1	1	1
D. lacteus	1	0	1	0	1	1	0	1	0	1	1	1	1	1	0	2	1	1	1	1	0	1	1	1	2	1	1	1	0	1
D. comteti	1	0	0	1	1	1	0	0	0	?	1	0	0	0	–	2	0	1	0	–	0	1	1	1	2	1	1	1	0	1
Hancockia californica	0	0	2	1	0	0	–	1	0	0	1	0	0	1	–	1	–	0	0	–	0	0	1	0	1	1	1	0	2	0
Melibe leonina	–	1	–	–	–	–	–	–	–	2	0	0	–	–	–	1	–	1	0	–	1	1	0	1	1	1	0	0	0	1
Scyllaea pelagica	0	0	0	0	1	1	0	1	1	0	0	0	–	–	–	0	–	1	0	–	1	0	0	1	0	0	1	0	0	0
Lomanotus vermiformis	0	1	–	–	0	0	–	1	0	1	0	0	–	–	–	1	0	0	0	–	0	0	1	0	1	1	0	0	2	1
Bornella sarape	1	0	0	1	1	1	1	1	0	0	1	0	1	0	–	2	0	1	1	0	1	0	0	0	1	0	1	0	0	0
Tritonia festiva	0	0	1	0	1	1	1	1	0	0	1	0	0	0	–	0	–	2	0	–	0	0	0	0	1	1	0	0	0	0
Doto uva	1	0	2	1	0	–	–	–	–	0	1	0	1	0	–	1	–	3	0	–	0	0	0	1	0	0	1	1	1	1

0, plesiomorphic state; 1, 2, apomorphic states; –, not applicable; ?, missing data.

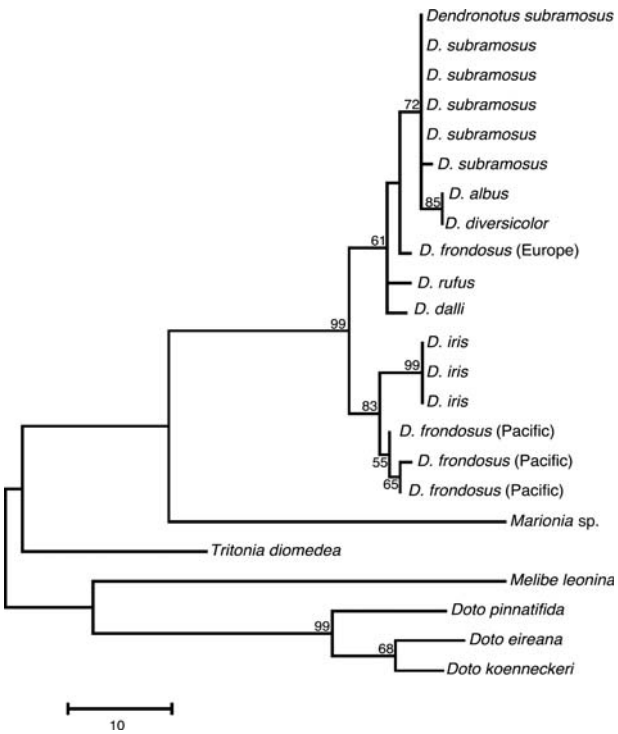

Figure 2. Phylogenetic tree for *Dendronotus* species based on MP analysis of 16S rRNA sequences. Values at nodes are percentage bootstrap support after 2,000 replicates. Scale bar represents 10 changes.

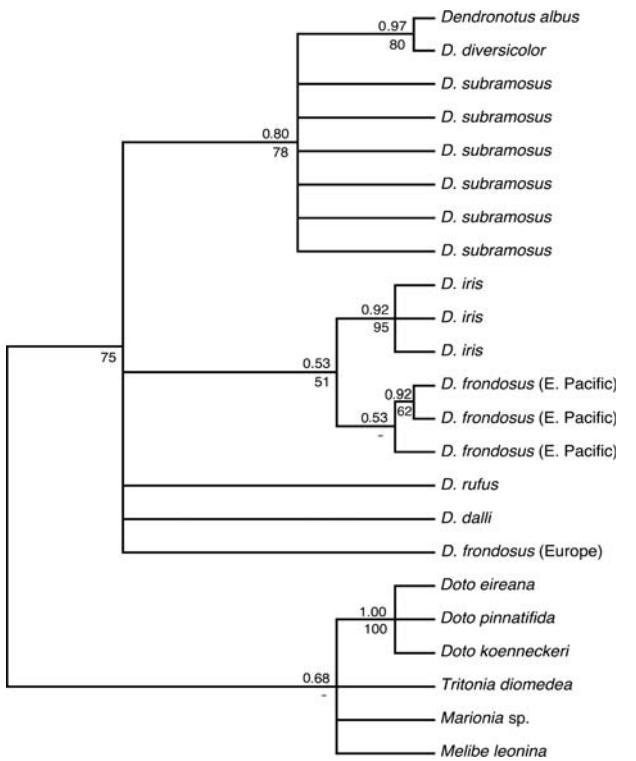

Figure 3. Phylogenetic tree for *Dendronotus* species based on Bayesian inference and ML analysis of16S rRNA sequences. Numbers above nodes are posterior probabilities, and below nodes ML bootstrap percentages after 1,000 replicates. Nodes with <0.50 posterior probability are collapsed.

three species of *Doto* than among the entire ingroup, measured both in terms of number of base substitutions per site and number of changes over the entire sequence (Table 5).

DISCUSSION

Tree congruence and stability

The morphological and molecular trees show a degree of congruence. For those species that were included in both analyses the phylogenetic relationships are often similar. For example, in all trees Atlantic and Pacific *Dendronotus frondosus* are consistently in separate clades. Also, *D. albus* and *D. diversicolor* are consistently grouped together, because they show no morphological or molecular differences. There is also a close relationship between *D. subramosus*, *D. albus* and *D. diversicolor* in both morphological and molecular analyses. However, in the molecular trees *D. iris* is closely related to Pacific *D. frondosus*, whereas morphologically it appears to be more closely related to Atlantic *D. frondosus*.

The validity of Dendronotus diversicolor *and* Dendronotus albus

Originally, *D. diversicolor* was thought to be a distinct species based mainly on colour differences from *D. albus*. Specimens of *D. albus* are generally white or lilac with white or orange pigment on the cerata, whereas specimens of *D. diversicolor* are white, sometimes cream or pink-brown (Robilliard, 1970). Other differences discussed by Robilliard (1970) included the body size at maturity, the number of pairs of cerata, the body texture, the number and location of hepatic diverticula, the denticulation of the radula, the overall shape of the jaws, and the proportions and shape of the reproductive organs; he also observed them showing disinterest in copulation with each other under captive conditions. Robilliard, who described *D. diversicolor* in 1970, has since expressed doubts of its validity based on many observations of animals with intermediate morphologies occurring together (D.W. Behrens, personal communication).

The evidence presented here indicates that *D. diversicolor* should be synonymized with *D. albus* due to the lack of either genetic or significant morphological differences. The only difference between these two taxa is the presence of long, unbranched crown papillae along the rhinophoral sheath in *D. albus*, whereas the specimens assigned to *D. diversicolor* have branched papillae (character 17, Table 3).

The validity of Dendronotus nanus *and* Dendronotus iris

Robilliard (1972) hypothesized that *D. nanus* appears to be simply a juvenile form of *D. iris*. He observed that some of the juveniles of *D. iris* collected in the state of Washington exhibited many of the same external features as *D. nanus*. They were grown in captivity and confirmed as *D. iris* when they reached lengths of at least 35 mm. This hypothesis would explain the shorter branches of dorsolateral processes seen in *D. nanus*. While genetic data were not available to resolve this question, morphological evidence suggests that *D. nanus* should be synonymized with *D. iris*. The only morphological differences lie in slight colour pattern variations and the length of dorsolateral processes (Robilliard, 1972), but the original description of *D. nanus* was based on only two specimens. The ranges of these two species have in the past been considered distinct, with *D. nanus* occurring further south on the mainland Mexican coast of the Gulf of California (type locality is Sonora, Mexico) and *D. iris* having a more northern distribution that extends only as far south as San Diego, CA, USA. Recently specimens with colour patterns resembling *D. nanus* have been recorded in San Diego, CA, USA (Chapman, 2005), suggesting that the range of these two colour forms do overlap.

The validity of Dendronotus venustus

Molecular and morphological evidence strongly support the suggestion that *D. frondosus* consists of at least two distinct species. European and eastern Pacific specimens display morphological differences (Table 4). The molecular and morphological trees place European and eastern Pacific specimens in different clades, with sequence divergences ranging from 1.8% to 2.1% between Pacific and European specimens compared to 0.3–0.5% within Pacific specimens (Table 5). In the 16S trees, eastern Pacific *D. frondosus* is sister to *D. iris*, whereas Atlantic *D. frondosus* is sister to a clade of *D. subramosus* and other species. The type locality of *D. frondosus* is Norway and therefore the European specimens should retain the species name. MacFarland (1966) described specimens from the eastern Pacific under the new name *D. venustus*. These specimens had white patches between the cerata and match the external and internal characteristics of the animals examined here. *Dendronotus venustus* was later synonymized with *D. frondosus* by Robilliard (1970), who found intermediate colour forms from the Pacific. In a later paper, Robilliard (1975) argued that at least four species previously identified as *D. frondosus* may co-exist on the Pacific coast of North America. These hypothetical four species display different colour patterns and have distinct food and habitat preferences. The specimens of Pacific *D. frondosus* examined in this study display substantial colour and morphological diversity, but have small genetic differences (0.4%; Table 5) in the 16S gene, which suggests that they may be conspecific. However, the fact that European and Pacific specimens of *D. frondosus* belong to different clades, in both molecular and morphological trees, supports the resurrection of the name *D. venustus* for eastern Pacific specimens currently recognized as *D. frondosus*. The range of *D. venustus* is unknown and more species from the Arctic need to be examined. *Dendronotus frondosus* appears to have the largest worldwide distribution of any *Dendronotus* species (including the Arctic seas and the North Atlantic, from the Bay of Biscay to Cape Cod) and varies greatly in external morphology (Rudman, 2006). Further examination of additional specimens from various locations may reveal a complex of species currently considered to be *D. frondosus*.

Evolution and diversity of Dendronotus

Dendronotus has an interesting pattern of distribution, with the largest number of species concentrated along the eastern Pacific coast. The present molecular phylogenetic analysis shows that the three species of *Doto* included in the analysis show greater genetic divergence than all the *Dendronotus* combined ($\zeta = 36.721$, $P \ll 0.001$). The low genetic divergence suggests that *Dendronotus* has either undergone rapid and recent speciation or that the rate of evolution of the 16S gene is unusually low. Divergence between this clade and the members of the outgroup is large (Fig. 2). Examination of additional loci and specimens are required to elucidate the evolution of *Dendronotus*. The fact that the Pacific species of *Dendronotus* do not appear to be monophyletic (Arctic and Atlantic species are nested within them) could suggest an Arctic origin for many of the Pacific species, perhaps a result of intermittent openings and closures of the Bering Strait. This idea needs further testing.

An explanation for the high diversity found along the eastern Pacific coast remains elusive, but it is possible that successive vicariant events along with the presence of ecologically

Table 5. Estimates of genetic divergence between 165 sequences.

Species	GenBank accession no.	Within species avg.	Within genus avg.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	GU339196																								
	GU339193			0%																					
Dendronotus subramosus	GU339197	0.001		0%	0%																				
	GU339192			0%	0%	0%																			
	GU339195			0.3%	0.3%	0.3%	0.3%																		
	GU339194			0%	0%	0%	0%	0.3%																	
D. albus	GU339185	N/A		0.5%	0.5%	0.5%	0.5%	0.8%	0.5%																
D. diversicolor	GU339186	N/A		0.5%	0.5%	0.5%	0.5%	0.8%	0.5%	0%															
	GU339189		0.019	3.5%	3.5%	3.5%	3.5%	3.8%	3.5%	3.5%	3.5%														
D. iris	GU339188	0.000		3.5%	3.5%	3.5%	3.5%	3.8%	3.5%	3.5%	3.5%	0%													
	GU339190			3.5%	3.5%	3.5%	3.5%	3.8%	3.5%	3.5%	3.5%	0%	0%												
D. frondosus (Europe)	GU339187	N/A		0.8%	0.8%	0.8%	0.8%	1.0%	0.8%	1.3%	1.3%	2.7%	2.7%	2.7%											
	GU339199			2.7%	2.7%	2.7%	2.7%	2.9%	2.7%	2.7%	2.7%	1.8%	1.8%	1.8%	1.8%										
D. frondosus (Pacific)	GU339200	0.004		2.9%	2.9%	2.9%	2.9%	3.2%	2.9%	2.9%	2.9%	1.6%	1.6%	1.6%	2.1%	0.3%									
	GU339198			2.7%	2.7%	2.7%	2.7%	2.9%	2.7%	2.7%	2.7%	1.3%	1.3%	1.3%	1.8%	0.5%	0.3%								
D. rufus	GU339191	N/A		1.3%	1.3%	1.3%	1.3%	1.6%	1.3%	1.8%	1.8%	3.2%	3.2%	3.2%	1.0%	2.9%	2.7%	2.4%							
D. dalli	AF249252.1	N/A		1.3%	1.3%	1.3%	1.3%	1.6%	1.3%	1.8%	1.8%	3.2%	3.2%	3.2%	1.0%	2.4%	2.1%	1.8%	1.0%						
Tritonia diomedea	GU339203	N/A	N/A	12.8%	12.8%	12.8%	12.8%	13.1%	12.8%	12.5%	12.5%	12.8%	12.8%	12.8%	13.1%	12.8%	12.8%	12.5%	13.1%	12.5%					
Doto eireana	AF249248.1	N/A		13.0%	13.0%	13.0%	13.0%	13.3%	13.0%	13.0%	13.0%	14.0%	14.0%	14.0%	12.4%	13.3%	13.7%	13.3%	13.3%	13.7%	14.6%				
Doto koenneckeri	AF249249.1	N/A	0.047	12.7%	12.7%	12.7%	12.7%	13.0%	12.7%	12.7%	12.7%	13.7%	13.7%	13.7%	12.7%	13.0%	13.4%	13.0%	13.0%	13.4%	13.3%	2.9%			
Doto pinnatifida	AF249250.1	N/A		14.9%	14.9%	14.9%	14.9%	15.3%	14.9%	14.9%	14.9%	15.6%	15.6%	15.6%	15.0%	15.3%	15.6%	15.3%	15.3%	15.3%	14.3%	6.0%	5.1%		
Melibe leonina	GU339202	N/A	N/A	17.8%	17.8%	17.8%	17.8%	18.2%	17.8%	18.2%	18.2%	17.2%	17.2%	17.2%	17.2%	17.2%	17.2%	16.8%	17.8%	1.7%	18.2%	20.2%	19.9%	20.5%	
Marionia sp.	GU339201	N/A	N/A	15.0%	15.0%	15.0%	15.0%	15.3%	15.0%	14.7%	14.7%	15.4%	15.4%	15.4%	15.0%	14.7%	14.7%	14.4%	15.0%	14.1%	16.6%	20.5%	18.8%	20.5%	20.7%

The percentage of base substitutions from analysis between sequences is shown. All results are based on the pair-wise analysis of 23 sequences. Analyses were conducted using the Kimura two-parameter method in MEGA4 (Kimura, 1980; Tamura *et al.*, 2007). All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 395 positions in the final data set.

distinct habitats in the Pacific may have contributed to the evolution and persistence of a relatively large number of species. Previous studies have shown that conditions during the late Neogene through the Pleistocene were favourable for radiation and diversification along the eastern Pacific in a variety of taxa (Jacobs, Haney & Louie, 2004; Ilves & Taylor, 2008).

ACKNOWLEDGEMENTS

We are very grateful to Lindsey Groves of the Natural History Museum of Los Angeles County, Elizabeth Kools and T.M. Gosliner from the California Academy of Sciences (San Francisco) and Marla Coppolino from the American Museum of Natural History (New York) for their loans of the material examined. The constructive comments of two anonymous reviewers are much appreciated. Funding for this project was provided through the US National Science Foundation PEET grant DEB-0329054 (to T.M. Gosliner and Á.V.) and the SEM work was supported by the US National Science Foundation MRI grant DBI-0216506 (to Á.V. *et al.*).

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