PHYLOGENY OF THE SEA HARES IN THE *APLYSIA* CLADE BASED ON MITOCHONDRIAL DNA SEQUENCE DATA

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RUNNING HEAD: *Aplysia* mitochondrial phylogeny

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ABSTRACT

Sea hare species within the *Aplysia* clade are distributed worldwide. Their phylogenetic and biogeographic relationships are, however, still poorly known. New molecular evidence is presented from a portion of the mitochondrial cytochrome oxidase c subunit 1 gene (cox1) that improves our understanding of the phylogeny of the group. Based on these data a preliminary discussion of the present distribution of sea hares in a biogeographic context is put forward. Our findings are consistent with only some aspects of the current taxonomy and nomenclatural changes are proposed. The first, is the use of a rank free classification for the different *Aplysia* clades and subclades as opposed to previously used genus and subgenus affiliations. The second, is the suggestion that *Aplysia brasiliana* (Rang, 1828) is a junior synonym of *Aplysia fasciata* (Poiret, 1789). The third, is the elimination of *Neaplysia* since its only member is confirmed to be part of the large *Varria* clade.

INTRODUCTION

Sea hares within the *Aplysia* clade are benthic herbivorous opisthobranch gastropods that reach large sizes compared to many other molluscs. They possess a planktonic larval stage, but have limited dispersal as adults, having only short-range crawling and swimming capabilities. Most species inhabit tidal and subtidal zones of most tropical seas, with some reaching subtropical ranges in their distributions, and one reaching polar waters (Table 1). The *Aplysia* clade has been the focus of research for many decades. Some key studies included thorough taxonomic examinations, ranging from local geographic areas (Pruvot-Fol 1929, 1934; Bebbington, 1974) to extensive worldwide revisions (Eales, 1960). Other studies involved a comprehensive appraisal of the ecology of several species (Carefoot, 1987; Johnson, 1999). A synopsis of the neurobiology of learning and behavior of several *Aplysia* species was another major contribution to the

understanding of the biology of sea hares (Kandel 1979). As Kandel (1979) pointed out, most of the research involving *Aplysia* as a model organism has been performed on *Aplysia californica* (Cooper, 1863). As studies in evolutionary neurophysiology including new species appear in the literature, however, the necessity of a phylogenetic framework for this clade has become evident. A recent attempt at using molecular data for resolving relationships among *Aplysia* species with myoglobin amino acid sequences included only three taxa (Rinaldi and Ohir, 1998). We (Medina et al., 2001) introduced the first explicit phylogenetic hypothesis for the genus (15 species included) by using partial mitochondrial DNA (mtDNA) sequence data for the small (12S) and large (16S) subunit ribosomal genes (rDNA). Although the rDNA genes clarified relationships between the early diverging lineages, relationships between species of more recent ancestry were not resolved, and in particular the placement of the model organism *A. californica* remained unclear. In this study we are opting for the use of a rank-free nomenclature (Cantino and De Queiroz, 2004) and therefore we will refer to monophyletic groups as clades as opposed to the genus or subgenus categories traditionally used in previous classifications (Table 1, Fig. 1). In her last taxonomic revision, Eales (1960) proposed a subdivision of 35 species of *Aplysia* into five subgenera. We have previously suggested that the monotypic *Neaplysia* Cooper 1863 containing *A. californica* was nested within the paraphyletic *Varria* clade (Medina et al., 2001). We confirm this relationship in the present study. Eales (1960) clearly indicated that the *Pruvotaplysia* clade (subgenus in her paper) embraced the most basal species (*A. parvula* and *A. punctata*), and pointed out similarities between *Varria*, *Neaplysia* and *Phycophilla*. Thus, although she did not present a tree, a phylogenetic hypothesis was implicit in her classification. At present there are 38 recognized valid species of *Aplysia* (Table 1). Although some are commonly found, some are rare and only known from one or a few specimens in museum collections. Three species are circumtropical: *Aplysia parvula* (Guilding in Morch, 1863), *Aplysia juliana* (Quoy and Gaymard, 1832) and *Aplysia dactylomela* (Rang, 1828). The Indo-Pacific Ocean is the most diverse basin with 23 species, one of which is also found in the Mediterranean Sea. The Atlantic Ocean contains twelve species, four of which are also found in the Mediterranean Sea. The level of endemism is higher in the Indo-Pacific, which has 16 endemic species, compared to the Atlantic (three endemic species) and the South Eastern Pacific (one endemic species, *A. inca*). The opisthobranch fauna, however, has been poorly sampled in this region (reviewed by Eales, 1960). In this study we increase phylogenetic resolution relative to our previously published topology by adding sequence data from an additional mitochondrial gene, a 658 bp portion of the cytochrome oxidase subunit 1 (cox1) gene. With an improved phylogeny we are able to address unresolved relationships within the *Varria* clade, and in particular, the placement of *A. californica* ,to suggest changes in the classification of *Aplysia*, and to postulate possible biogeographic scenarios that can be tested with additional taxon sampling in future studies.

METHODS

We obtained samples from opisthobranch specialists from around the World (listed in Medina et al., 2001). We performed cox1 amplifications in 25 µl volume of a solution containing approximately 50 ng of DNA, 1X PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 1.25 units of Taq polymerase (Perkin-Elmer/Cetus). After an initial denaturing step of 2 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C were performed, followed by a final extension step of 5 min at 72°C. We used the cox1 primers developed by (Folmer 1994): LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198: (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). We cloned purified PCR fragments into a TA vector (Invitrogen) and sequenced individual clones using the Thermo Sequenase cycle-sequencing kit (Amersham) with dye-labeled universal M13 primers in a Licor automated sequencer. We were unable to obtain cox1 products from two species (*A. depilans*, and *A. parvula*) due to degraded DNA templates and unstable primer annealing. We deposited sequences in GenBank under accession numbers AF343425-AF343432, and AY275426. We chose *Akera*, shown to be the most basal taxon in the anaspidean clade (Medina et al, 2001), as the outgroup taxon for this analysis. We performed all phylogenetic analysis using in PAUP* 4.0 (Swofford 1997). The ILD test (Farris 1995) was used to determine if different data sets from the previous and present studies (rDNA and cox1) possessed significantly conflicting phylogenetic signals. Equal weights Maximum Parsimony (MP), Mimimum Evolution (ME) and Maximum Likelihood (ML) analyses were conducted. To determine which model of sequence evolution best fit the data for ME and ML, we performed nested likelihood ratio tests (LRT) using Modeltest version 3.0 (Posada and Crandall 1998). For ME and MP we performed heuristic searches, with 1000 replicates of random stepwise addition and TBR branch swapping. For ML, we performed heuristic searches with 5 replicates of random stepwise addition and TBR branch swapping. Our Bootstrap analyses included 1000 pseudoreplicates for MP and ME, and 100 pseudoreplicates for ML.

RESULTS

All three genes exhibit base compositional bias, with A-T bias of 63.3% in cox1 (this study), 62.4% in 16S, and 65.5% in 12S (Medina et al., 2001). The nucleotide bias varied within codon positions of the cox1 gene, from 53.3% in the first-codon positions to 79% in the third-codon positions. The average nucleotide composition on the coding strand of the sequenced taxa for cox1 was 24% A, 40% T, 17% C and 19% G. No species differed by more than 2% from the average values. Third positions were the most variable sites, and accounted for 87% of the variation, with first and second positions accounting for 15% and 3% of the variable sites, respectively, in this portion of cox1. Sequence divergence values among taxa for cox1 ranged from 1.1-17.6% between *Aplysia* species, and from 17.5-20.1% for outgroup comparisons. The ILD test (Farris 1995) run with 10 random stepwise additions with TBR branch swapping and 1000 randomizations, indicated no significantly conflicting phylogenetic signal between the ribosomal and cox1 data sets. *Aplysia depilans* (Gmelin, 1791), and *A. parvula* have missing data for cox1, so

their placement is based solely on sequences from the ribosomal genes. Results were congruent for the 13 and the 15 taxon data sets. We chose, therefore, to present the results from the most comprehensive data set (Fig. 1). We obtained three shortest trees (TL=756) in the MP search (trees not shown), MP bootstrap values are depicted in Figure 1. The LRT implemented in Modeltest 3.0 (Posada and Crandall 1998) indicated that the model that best fit the data was a GTR + Γ + I. The assumed proportion of invariable sites was 0.4322, and the shape parameter (alpha) was 0.3460. Of the two model based approaches, ME was the most affected by the incomplete dataset. This is reflected in the lack of bootstrap support for the nodes in the *Aplysia* clade which are supported by the other optimality criteria. The ML tree (-Ln likelihood = 5363.12531) is depicted in Figure 1 with corresponding bootstrap values.

DISCUSSION

Previous attempts to reconstruct the phylogenetic relationships of the *Aplysia* clade were those of Fiorito (1990) and Rinaldi and Ophir (1998). Fiorito et al. (1990) attempted to test Eales's classification by means of a phenetic (UPGMA) analysis. They used 12 characters, several of which are redundant or of questionable utility. For example, four characters were redundant with respect to body size (i.e., body size, head size, foot size and size of parapodia). The ink secretion character (purple vs. white) was ambiguously coded with respect to the purple and opaline glands, both ink producing organs. Despite using characters from Eales (1960) taxonomic descriptions, Fiorito et al.'s results are incongruent with her classification. Rinaldi and Ophir (1998) used myoglobin amino acid sequence data to infer relationships among three *Aplysia* species. Their conclusion that *A. juliana* and *A. kurodai* are more closely related, and that *A. fasciata* (junior synonym *A. limacina* in Rinaldi and Ophir, 1998) is the most basal lineage of the three is not supported by our analysis. The phylogeny recovered by the rRNA dataset was in agreement with the classification proposed by Eales (1960), except for the placement of *A. californica* (Medina et al., 2001). The phylogeny inferred from the cox1 data set was also congruent with both the rRNA results and Eales's inferred phylogenetic hypothesis. The cox1 fragment was more variable than the ribosomal genes, particularly at third positions. Thus, this gene was useful in recovering relationships within the *Varria* clade (Fig. 1). Our current results, despite limited taxonomic sampling, allow us to suggest that four clades should be recognized within *Aplysia* (Table 1): *Pruvotaplysia*, *Subaplysia* (nom. nov.), *Varria,* and *Phycophilia*. It should noted however, that 1) the monophyly of *Pruvotaplysia* is not well supported, and that 2) we were unable to obtain material for *A. euchlora*, being therefore unable to address whether *Phycophilia* is a genuine clade or not. The combined analysis in this study resulted in increased overall support for many nodes. The species placed in the *Pruvotaplysia* clade were confirmed as the most basal in the *Aplysia* clade. The monophyly of this clade, however, is still poorly supported. The molecular evidence also supported the divergence of the *Subaplysia* (nom. nov.) and *Varria* clades from a *Pruvotaplysia* ancestor as suggested by the morphological data

(Eales, 1960). The monophyly of *Varria* was supported by high bootstrap values with the inclusion of *A. californica*. Eales (1960) had already pointed out that *A. californica* resembled species of the subgenus *Varria* except for the shape of the shell (the character she used to place this species in a different subgenus). Within the *Varria* clade, *A. brasiliana*, *A. fasciata* and *A. cervina* formed a monophyletic group. These three species not only have similar geographic ranges, but also share similar morphological traits. Eales (1960) pointed out the anatomical resemblance between *A. brasiliana* and *A. cervina*, which differ solely in average size and level of activity. In Eale's taxonomic descriptions, *A. fasciata* (found in the Eastern Atlantic) resembles *A. brasiliana* (Western Atlantic), in the similar opaline gland, penis and swimming behavior (Eales, 1960). In addition, breeding experiments by Susswein et al. (1993) have demonstrated that these two species freely interbreed with no apparent preferences for conspecifics. The sequence divergence between these two taxa was the lowest of all comparisons: 0.001 with the rRNA genes (Medina et al., 2001) and 0.011 with the cox1 gene (this study). This level of sequence divergence is more typical of intraspecific levels. Consequently, morphological (Eales, 1960), behavioral (Susswein et al. 1993) and molecular evidence (Medina et al. 2001; this study) suggest that *A. brasiliana* and
A. fasciata represent different geographical populations of the same species rather than separate species. Based on the congruent evidence from morphological and mtDNA character data, in combination with reproductive evidence, the name *A. brasiliana* Rang 1828 should be considered a synonym of *A. fasciata* Poiret 1789 which has nomenclatural priority. Another group of interest is the *A. dactylomela* - *A. oculifera* clade. Eales (1960) and Gosliner, 1987), noted that early taxonomic reports involving these two species should be taken with caution because they are morphologically quite similar. *A. oculifera* specimens have been mistaken as *A. dactylomela* by taxonomists in the past, however, *A. oculifera* specimens are of smaller size and the black rings on the skin are not as large as in *A. dactylomela* (Gosliner, 1987). With the exception of the *A. brasiliana*, *A. cervina* and *A. fasciata* clade, most sister taxon relationships within the genus are not of geographically adjacent populations. For example, within the *Subaplysia* clade, *A. vaccaria* (California coast) formed a well-supported clade with *A. depilans* (Mediterranean Sea). Within the *Varria* clade, *A. californica* (California coast) was closely related to the *A. cervina* clade (Atlantic), and *A. gigantea* (Western Australia coast) formed a clade with *A. morio* (Texas coast). Reconstructing the history of marine biogeographic events can be challenging (reviewed in Cunningham and Collins, 1998), and verifications of hypotheses from the fossil record are also fraught with difficulty. Although fossils of *Akera* have been found in Cretaceous-age rocks, the fossil record of *Aplysia* is sparse because of the extremely fragile shells, with the earliest record dating from the Miocene (discussed in Medina et al., 2001). Thus, the fossil record of this genus is not adequate for fossil tests of vicariance hypotheses. We observe, however, that the three circumtropical species in our topology are each a member of one of the three main clades (the three former subgenera), and the *Provotaplysia* lineage is the most basal, and sister group to the lineage that gave rise to the rest of the *Aplysia* sea hares. We

hypothesize that the members of this clade were likely present before the closure of Tethys as suggested by Bebbington (1975). Another interesting result is that the divergence of *A. californica* from its sister clade is consistent with a transisthmian vicariance. Perhaps this relationship, in combination with more intensive taxon sampling, especially of the circumtropical species and *A. parvula*, would help us to date cladogenetic events, and postulate more precise historical biogeographic scenarios for the *Aplysia* clade.

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with notes on other species. Ibid 11: 15-62.

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Figure legends Figure 1. *Aplysia* phylogenetic analysis of rRNA and cox1 mitochondrial gene fragments. Best tree obtained by ML. Major clades are depicted by numbers below branches 1) *Aplysia*, 2) *Provotaplysia*, 3) *Subaplysia*, 4) *Varria*. Values above branches correspond to bootstrap values for ML, ME and MP respectively. Symbols next to species names represent broad geographic distributions: White boxes = circumtropical, gray boxes = Atlantic, black boxes = Pacific.

Table 1. Classification and geographical distribution of species of *Aplysia* Linnaeus, 1767. Distribution ranges are based on Eales (1960), Bebbington (1974) and Marcus & Marcus (1960).

Clade (former subgenus) Species

Pruvotaplysia Engel, 1936 A. parvula Guilding in Morch, 1863
A. punctata Cuvier, 1803 Subaplysia nom. nov. A. cedroensis Bartsch & Rehder, 1939
A. depilans Gmelin, 1791
A. dura Eales, 1960
A. juliana Quoy & Gaymard, 1832
A. nigra d'Orbigny, 1837
A. reticulopoda Beeman, 1960
A. vaccaria Winkler, 1955 Varria Eales, 1960
A. brasiliana Rang, 1828
A. californica Cooper, 1863
A. cervina Dall & Simpson, 1901
A. cornigera Sowerby, 1869
A. cronullae Eales, 1960
A. dactylomela Rang, 1828

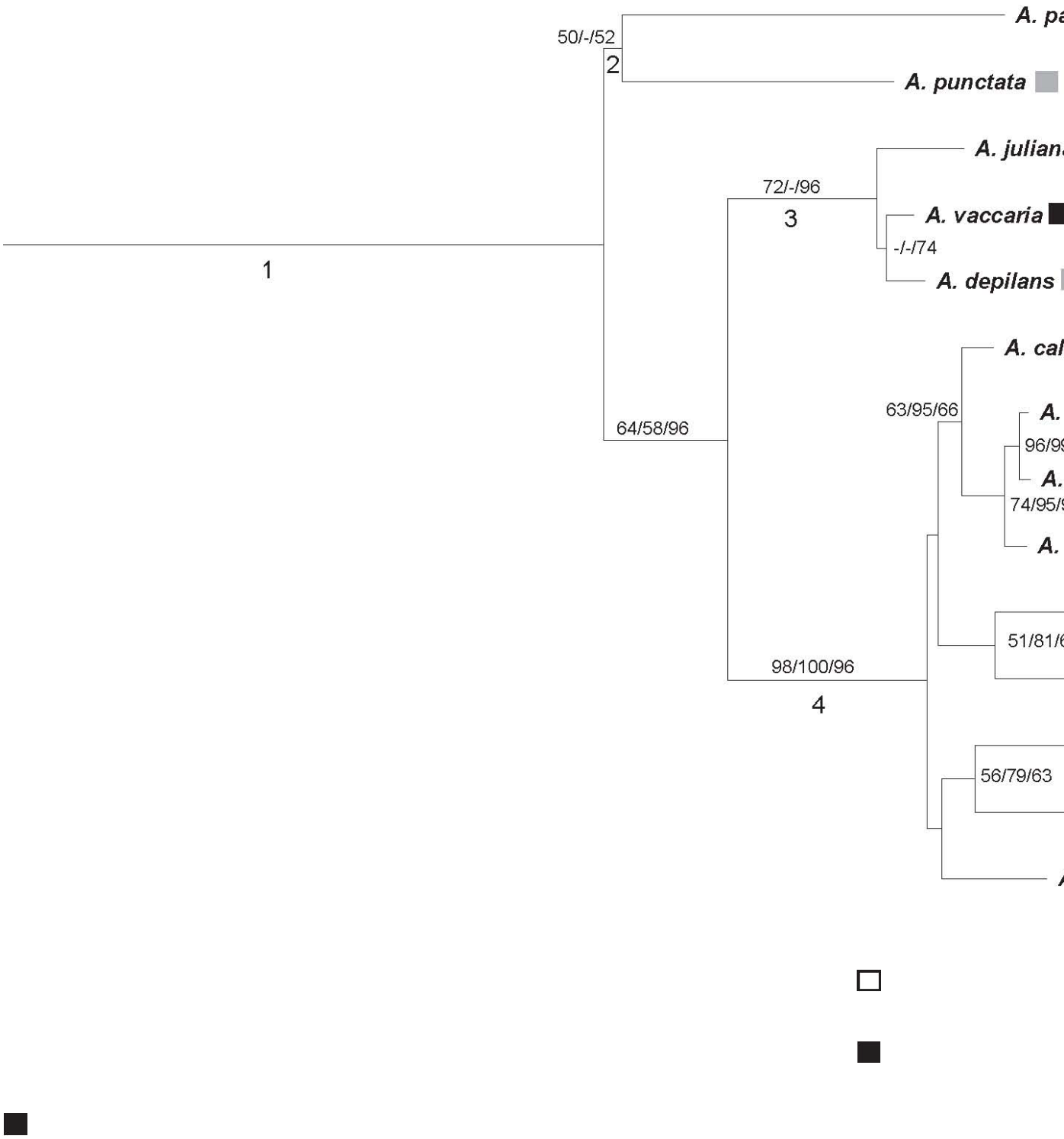
A. denisoni Smith, 1884
A. donca Marcus & Marcus, 1960
A. extraordinaria Allan, 1932
A. fasciata Poiret, 1789
A. gigantea Sowerby, 1869
A. gracilis Eales, 1960
A. inca d'Orbigny, 1837
A. keraudreni Rang, 1828

Distribution

Circumtropical North Eastern Atlantic, Mediterranean North Eastern Pacific North Eastern Atlantic, Mediterranean South Eastern Atlantic, South Western Pacific Circumtropical South Western Atlantic, South Pacific North Eastern Pacific North Eastern Pacific North Atlantic North Eastern Pacific Western Atlantic Indian, Western Pacific South Western Pacific Circumtropical Indian, Western Pacific Western Atlantic South Western Pacific South East. Atlantic, Mediterranean, Red Sea Indian, Western Pacific Red Sea South Eastern Pacific South Pacific

Table 1. (continued)

	A. kurodai Baba, 1937	North Western Pacific
	A. maculata Rang, 1828 A. morio Verrill, 1901	Western Indian North Western Atlantic
	A. oculifera Adams & Reeve, 1850	Indian, Western Pacific
	A. pulmonica Gould, 1852 A. rehderi Eales, 1960	Western Pacific North Eastern Pacific
	A. reticulata Eales, 1960	South Western Pacific
	A. robertsi Pilsbry, 1895 *A. rudmani* Bebbington, 1974 *A. sagamiana* Baba, 1949 *A. sowerbyi* Pilsbry, 1895 *A. sydneyensis* Sowerby, 1869 *A. tanzanensis* Bebbington, 1974 *A. winneba* Eales, 1957	North Eastern Pacific Indian North Western Pacific South Western Pacific South Western Pacific Indian Tropical
	A. euchlora Adams in M.E. Gray, 1850	Eastern Atlantic North Western Pacific
Phycophila Adams, 1861		


A. pa
50/-/52
2
A. punctata
A. julian
72/-/96
3
A. vaccaria
-/-/74
1
A. depilans
A. cal
63/95/66
A.
64/58/96
96/99
A.
74/95/9
A.
51/81/6
98/100/96
4
56/79/63

0.05 substitutions/site