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Identification of ommastrephid squid paralarvae collected in northern Hawaiian waters and phylogenetic implications for the family Ommastrephidae using mtDNA analysis

TOSHIE WAKABAYASHI,[1]* NOBUAKI SUZUKI,[1] MITSUO SAKAI,[1] TARO ICHII[1] AND SEINEN CHOW[2]

[1]*National Research Institute of Far Seas Fisheries, Shizuoka 424-8633, and* [2]*National Research Institute of Fisheries Science, Yokosuka, Kanagawa 238-0316, Japan*

ABSTRACT: A total of 110 adult individuals from four ommastrephid (family Ommastrephidae) squid species (*Ommastrephes bartramii, Sthenoteuthis oualaniensis, Eucleoteuthis luminosa,* and *Hyaloteuthis pelagica*) were used to obtain diagnostic DNA markers for species identification. Restriction fragment length polymorphism (RFLP) analysis of a partial segment (855 bp) of the mitochondrial DNA cytochrome oxidase I (COI) gene amplified by polymerase chain reaction (PCR) revealed that the restriction profiles of two endonucleases (*Alu* I and *Tsp*509 I) were diagnostic for species identification. The restriction assay partially supplemented with nucleotide sequence analysis successfully assigned 69 damaged and morphologically equivocal ommastrephid paralarvae collected in northern Hawaiian waters, identifying 60 *O. bartramii*, eight *S. oualaniensis*, and one *E. luminosa*. The family Ommastrephidae appears to be monophyletic. Although the phylogenetic relationships among genera were not resolved well due to apparent homoplasy and large genetic divergence between species, COI sequence data without transitions provided support for subfamily level relationships.

KEY WORDS: **Ommastrephidae, paralarvae, PCR-RFLP, species identification.**

INTRODUCTION

The cephalopod family Ommastrephidae, which has three subfamilies (Illicinae, Todarodinae, and Ommastrephinae), is one of the largest oegopsid squid families, and it contains many commercially important species. The neon flying squid *Ommastrephes bartramii* is a large pelagic squid that is widely distributed in the Pacific, Atlantic, and Indian Oceans.[1] This species is an important fishery resource in the North Pacific,[2] and estimates of the recruited stock of this species require information about the distribution and abundance of its paralarvae. Ommastrephid paralarvae are easily discriminated from those of other families by their fused tentacles, forming a proboscis.[3] Taxonomic characters used to distinguish species of ommastrephid paralarvae are summarized in Figure 1.[4]

*Corresponding author: Tel: 81-543-36-6056.
Fax: 81-543-35-9642. Email: toshiew@fra.affrc.go.jp
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The northern Hawaiian waters are the spawning grounds of *O. bartramii*,[5,6] and three other species in the subfamily Ommastrephinae (*Sthenoteuthis oualaniensis, Eucleoteuthis luminosa,* and *Hyaloteuthis pelagica*) are also observed to distribute there.[1,7,8] A large number of ommastrephid paralarvae were collected in this area during the research cruises of the National Research Institute of Far Seas Fisheries (NRIFSF) from 2001 to 2003. The *O. bartramii* paralarva has lateral suckers that are considerably larger than the medial suckers, and no photophore is observed throughout the paralarval stage.[9] All suckers of *S. oualaniensis* paralarva are nearly equal in size, and subocular and intestinal photophores are observed in specimens larger than 4.0 mm dorsal mantle length (DML).[3] Therefore, the paralarvae of these two species may be readily identified. However, morphological identification may be very difficult when these taxonomic features are occasionally lost or damaged, as the body is relatively fragile and easily damaged. In addition, it is very difficult to identify paralarvae of *E. luminosa* and *H. pelagica* even in good condition.[3,10]

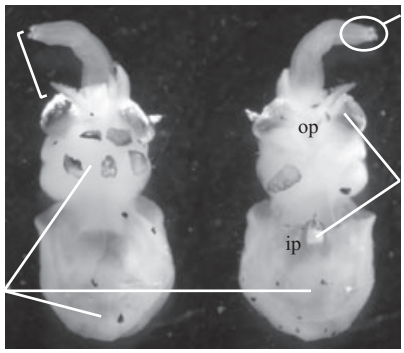

Fig. 1 Taxonomic characters used to distinguish species of ommastrephid paralarvae.

Table 1 Sampling localities and dates of standard ommastrephid samples used in this study

Species	Locality	Sampling date	DML (mm)	Number of specimens
Ommastrephes bartramii	Northern Hawaiian waters	November 2002	358–510	5
	Central North Pacific	September 2002	168–360	27
Sthenoteuthis oualaniensis	Northern Hawaiian waters	November 2002	143–260	35
Eucleoteuthis luminosa	Northern Hawaiian waters	November 2002	158–192	3
	East China Sea	May 2003	152–78	11
Hyaloteuthis pelagica	Northern Hawaiian waters	November 2002	18–51	29

Six genera are recognized in the subfamily Ommastrephinae. Four genera, *Ommastrephes, Dosidicus, Eucleoteuthis,* and *Hyaloteuthis*, are monospecific. *Dosidicus gigas* is distributed in the eastern tropical Pacific. The remaining two genera, *Sthenoteuthis* and *Ornithoteuthis,* consist of two species each, of which *S. pteropus* and *O. antillarum* are endemic in the Atlantic Ocean. The distribution of *O. volatilis* is limited to mesopelagic boundary areas of the western Pacific, and this species has never been reported from northern Hawaiian waters. Although *Nototodarus hawaiiensis* (subfamily Todarodinae) is distributed throughout Hawaiian waters, this is demersal species[11] and its paralarvae belong to an island-associated assemblage.[12] Moreover, *N. hawaiiensis* paralarvae are morphologically peculiar, having a round mantle with a distinct chromatophore pattern and a short proboscis.[3] Therefore, the four oceanic species (*O. bartramii, S. oualaniensis, E. luminosa,* and *H. pelagica*) of the subfamily Ommastrephinae were examined in this study.

Techniques based on DNA analysis, such as restriction fragment length polymorphism (RFLP) or direct nucleotide sequencing analyses based on the polymerase chain reaction (PCR), have become conventional and are used for the practical identification of fish species.[13–15] Although several studies have used mitochondrial DNA sequence analysis to investigate phylogenetic relationships and genetic population structure in neritic cephalopod species,[16–18] few attempts have been made to develop a conventional DNA protocol to identify oceanic squid paralarvae.

In this study, simple diagnostic DNA markers for identifying four oceanic ommastrephid species are presented and results of species identification of paralarvae collected from northern Hawaiian waters are reported. Further, the morphological keys used to identify the paralarvae are re-evaluated, and the phylogenetic relationships of the species in the family Ommastrephidae were investigated based on the mtDNA sequence analysis.

MATERIALS AND METHODS

Squid samples

In total, 110 adult samples representing four species were studied: 32 *O. bartramii*, 35 *S. oualaniensis*, 14 *E. luminosa*, and 29 *H. pelagica*. All specimens were collected from northern Hawaiian waters, the central North Pacific, or the East China Sea by the research vessels R/V Shunyo-Maru (NRIFSF) and Horai-Maru No. 31 using mid-water trawl nets or jigging in 2002 and 2003 (Table 1). In addition, five out-group species were used for the phylogenetic analysis. Of the three ommastrephid species from the subfamilies Illicinae and Todarodinae, *Illex illecebrosus* was collected from the North-west Atlantic, *N. hawaiiensis* from the East China Sea, and *Todarodes pacificus*

pacificus from the North-west Pacific. *Onychoteuthis* sp. (family Onychoteuthidae) was collected from northern Hawaiian waters. The nucleotide sequence for *Watasenia scintillans* (family Enoploteuthidae) was taken from Yokobori *et al.*[19]

The frozen individuals or ethanol-preserved tissue samples were transferred to the laboratory, and crude DNA was extracted from muscle tissue using a SepaGene DNA extraction kit (Sanko Junyaku, Tokyo, Japan).

A total of 513 ommastrephid paralarvae were collected from northern Hawaiian waters (24–37°N, 154–163°W) in 2002 during a cruise of the R/V Shunyo-Maru using a large larva net (2-m mouth diameter, 526-μm mesh) in surface horizontal tows at ship speeds of 1.5–2 knots. The sorted paralarval samples were preserved onboard in 99.5% ethanol, transferred to the laboratory, and identified morphologically. Of the specimens (1.14–13.18 mm DML), morphological species identification was equivocal in 69 larvae due to damage. Using the standard phenol–chloroform method, crude DNA from these samples was extracted from small pieces of dissected tissue.

PCR amplification

Three forward and two reverse primers were designed to amplify a partial segment of the mitochondrial cytochrome oxidase I (COI) gene (COI; Table 2). The sets of forward and reverse primers shared identical priming sites.

Preliminary attempts using standard adult samples indicated that the amplification efficiency differed considerably from species to species for the primer combinations used. One primer pair (LCO1495F1 and COI6786R) efficiently amplified *O. bartramii* samples, but not the other species. Primers LCO1495F1 and COI6786RIT amplified *S. oualaniensis* and *H. pelagica* samples well. Two forward primers (LCO1495F2 and LCO1495F3) with COI6786R were necessary to amplify the target segment of *E. luminosa* samples. Therefore, two different sets of primers were used to analyze the unidentified paralarval specimens. First, the primers LCO1495F1 and COI6786R plus COI6786RIT were used. If these primers did not amplify a sample well, then the primers LCO1495F2 plus LCO1495F3 and COI6786R were used.

Table 2 PCR primer sequences of a partial segment of mtCOI gene

Name	Sequence
LCO1495F1	5′-CAAATCATATTGATATTGGAAC-3′
LCO1495F2	5′-ACAAATCATAAAGATATTGG-3′
LCO1495F3	5′-ACAAATCATAAGGACATTGG-3′
COI6786R	5′-CTGTAAATATATGGTGTGCTC-3′
COI6786RIT	5′-AGTAAATATGTGATGTGCTC-3′

PCR amplification of the adult samples was carried out in 20-μL reaction mixtures containing 2 μL of 10× buffer, 1 mM $MgCl_2$, 1 mM of each deoxyribonucleoside triphosphate (dNTP), 1 μM of each primer, 1 unit of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA), and the DNA template. PCR amplification of the paralarval samples was carried out in 25-μL reaction mixtures containing 12.5 μL of 2× GC buffer, 2.5 mM of each dNTP, 1 μM of each primer, 1 unit of *LA Taq* polymerase (TaKaRa, Shiga, Japan), and the DNA template. The reaction mixtures were preheated at 95°C for 5 min, followed by 30 cycles of amplification (95°C for 40 s, 46°C for 40 s, and 72°C for 1.5 min), with a final extension at 72°C for 5 min. The PCR products were electrophoresed through a 2% agarose gel (Gibco BRL, Paisley, Scotland, UK) and stained with ethidium bromide to confirm amplification.

RFLP analysis

The PCR products were digested directly with six endonucleases (*Alu* I, *Bfa* I, *Hae* III, *Hin*f I, *Mse* I, and *Tsp*509 I). The digested PCR samples were electrophoresed through a 2.5% agarose gel (BIO101, Biogel, Regent Medical, Irlam, UK) for 3 h, stained with ethidium bromide, and photographed. Digested fragments smaller than 90 bp were not used for interpreting the restriction types because of the gel resolution.

Nucleotide sequence and phylogenetic analyses

Two individuals of each species used as standards were subjected to nucleotide sequence analysis. The PCR products were treated with ExoSAP-IT (Amersham Biosciences, Piscataway, NJ, USA) to remove the oligonucleotide primers. Sequences were generated on an automated sequencer (ABI Prism310) using the ABI Big-dye Ready Reaction kit (Applied Biosystems) following the standard cycle sequencing protocol. The nucleotide sequences of paralarval DNA samples that had restriction fragment length polymorphism (RFLP) profiles inconsistent with those of the adult standard specimens were also analyzed.

The nucleotide sequences obtained using both forward and reverse primers were edited and aligned on GENETYX ver. 6.1 (GENETYX, Tokyo,

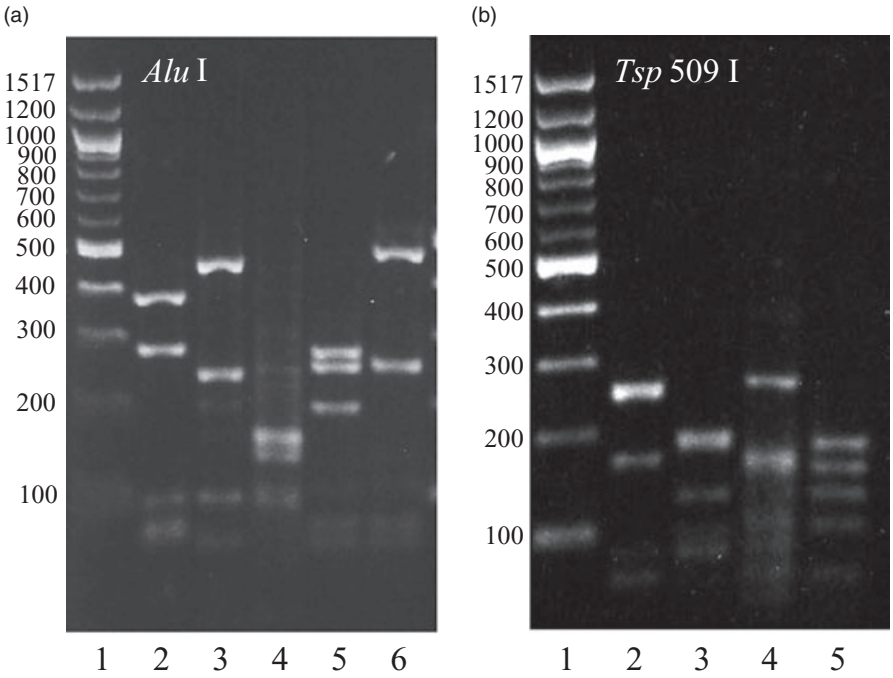

Fig. 2 Restriction profiles of the COI fragment digested by two enzymes (a) *Alu* I and (b) *Tsp*509 I. First lane is molecular maker (100b DNA ladder, NEB) and the sizes are shown on left margin. Species lined from the second lane to the right are *O. bartramii*, *S. oualaniensis*, *E. luminosa* and *H. pelagica*. The last lane of *Alu* I is also *H. pelagica*.

Japan), considering their deduced amino acid sequences, and then imported into MEGA ver. 2.1[20] and PAUP* 4.0 beta10[21] to construct neighbor-joining (NJ) and maximum parsimony (MP) phylogenetic trees, respectively. The NJ tree was inferred using Kimura's two-parameter (K2P) distance. The NJ and MP search were performed including either all substitutions or transversions only. Bootstrapping with 1000 replications was used to assess the reliability of all nodes on both trees. The maximum likelihood (ML) tree was also estimated using PAUP* based on the optimal substitution model selected by Modeltest 3.06,[22] followed by 500 bootstrap replicates using the SEQBOOT program in the PHYLIP package[23] and PHYML software.[24] The ML search was conducted under the TVM model with I + Γ corrections according to the output from Modeltest (the values of the optimized parameters are not shown). The transition and transversion (SV) ratio between species were calculated by MEGA ver. 2.1.

The nucleotide sequences reported here were deposited in DDBJ/EMBL/GENBANK under accession numbers AB199549-199561.

RESULTS

Restriction profiles of standard adult specimens

The amplified fragments were approximately 850 bp in length, and no apparent size variation was observed among the 110 individuals. Five individuals from each species were used for an initial RFLP screening. Of the six endonucleases used, *Alu* I and *Tsp*509 I produced diagnostic restriction profiles for discriminating the species used (Fig. 2). Five restriction patterns were observed in the *Alu* I digestions (Fig. 2a, lanes 2–6). The estimated sizes of the fragments were 370, 275, and 95 bp (type A) in *O. bartramii*; 445, 240, and 95 bp (type B) in *S. oualaniensis*; and 160, 150, 135, and 90 bp (type C) in *E. luminosa*. Two restriction patterns were observed in *H. pelagica*, in which the fragments of type D were 270, 250, and 195 bp, and those of type E were 470 and 250 bp. Four restriction patterns were observed with the *Tsp*509 I digestions (Fig. 2b, lanes 2–5). The estimated fragment sizes were 255, 245, and 165 bp (type A) in *O. bartramii*; 195, 180, 145, 130, and 100 bp (type B) in *S. oualaniensis*; 260, 170, 160, and 100 bp (type C) in *E. luminosa*; and 180, 150, 130, and 100 bp (type D) in *H. pelagica*. These restriction patterns are summarized in Table 3. Five composite haplotypes were observed for the 110 individuals from the four species. The composite haplotypes AA, BB, CC, DD, and ED were defined as types 1, 2, 3, 4, and 5, respectively. Intraspecific variation was not observed in the restriction profiles in 32 individuals of *O. bartramii*, 35 of *S. oualaniensis*, nor 14 of *E. luminosa*. One out of 29 *H. pelagica* individuals had a restriction profile (type 5) that differed from the others (type 4). Therefore, the four species could be discriminated by the restriction profiles

Table 3 Composite haplotypes of standard samples

Type	Species	No. Individual	Composite haplotype	
			Alu I	*Tsp*509 I
1	*O. bartramii*	32	A	A
2	*S. oualaniensis*	35	B	B
3	*E. luminosa*	14	C	C
4	*H. pelagica*	28	D	D
5	*H. pelagica*	1	E	D

Table 4 Composite haplotypes of paralarval samples

Type	No. Individual	Composite haplotype	
		Alu I	*Tsp*509 I
1	59	A	A
2	8	B	B
3	1	C	C
6	1	F	A

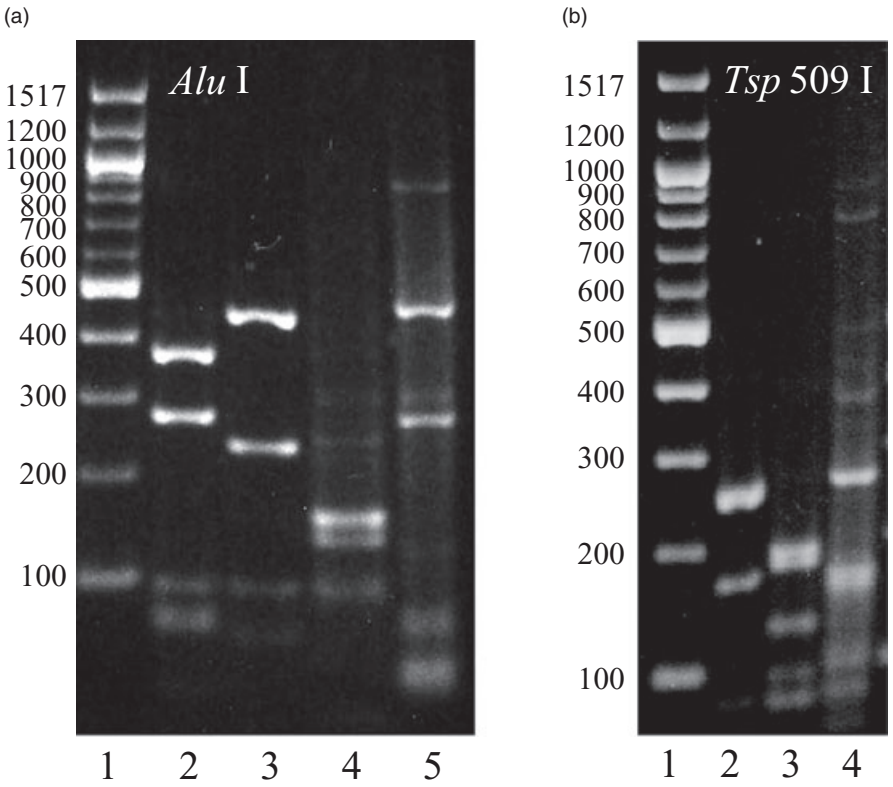

Fig. 3 Restriction profiles of the COI fragment of paralarvae by two enzymes. First lane is molecular maker (100b DNA ladder, NEB), and the sizes are shown on left margin.

using either *Alu* I or *Tsp*509 I. Nevertheless, both enzymes were used to identify paralarval specimens in this study, since the fragment sizes of some of the restriction profiles were similar between species (see Fig. 2a lanes 3 and 6 and Fig. 2b lanes 2 and 4).

Identification of paralarvae

The restriction patterns of the paralarval specimens are summarized in Table 4. Four composite haplotypes, AA, BB, CC, and FA, i.e. types 1, 2, 3, and 6, respectively, were identified. Restriction analysis of the 69 paralarvae revealed that 59, eight, and one paralarvae shared identical restriction profiles with the adult standards for *O. bartramii* (type 1), *S. oualaniensis* (type 2), and *E. luminosa* (type 3), respectively. One individual (specimen 34-8) had a novel restriction pattern in the *Alu* I digest (Fig. 3a, lane 5), but the *Tsp*509 I restriction profile was identical to that of *O. bartramii* (Fig. 3a).

Nucleotide sequence and phylogenetic analyses

Two individuals were arbitrarily selected from the adult standards, including one *H. pelagica* (specimen 07) with the variant *Alu* I restriction type (Fig. 2a, lane 6). The paralarva (specimen 34-8)

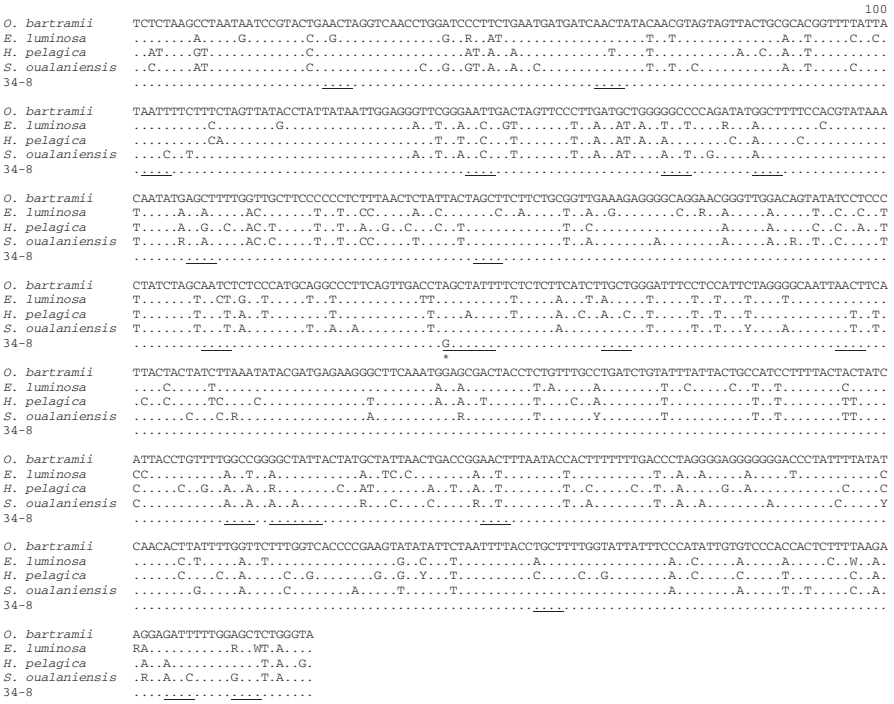

Fig. 4 Nucleotide sequences of mitochondrial COI gene of Ommastrephinae species. Identity with top reference sequence is indicated by dots. Underlined regions designate diagnostic restriction sites. Asterisks indicate substitutions responsible intraspecific polymorphism of the restricted fragment length.

with the *Alu* I digestion restriction profile that was inconsistent with the standards was also included in the nucleotide sequence analysis. The alignment of these sequences is shown in Figure 4. The diagnostic restriction sites of *Alu* I and *Tsp*509 I were substantiated by the nucleotide sequence analysis. The paralarva (34-8) appeared to be assigned to *O. bartramii*. A single nucleotide substitution difference between the *O. bartramii* standard and this paralarva (34-8) was located in a diagnostic *Alu* I restriction site (see Fig. 4, asterisk).

No insertions or deletions were evident in the aligned 724-bp fragments, and 165 variable sites were observed. The COI sequences of all the squid species studied were characteristically AT-rich (58.6–67.4%). The SV ratio between species ranged 1.416–4.009. Much lower SV ratios were observed between species used in this study from different subfamilies within the family Ommastrephidae (average 1.888, $n = 14$) and between species from different families (1.847, $n = 15$) than between species from the subfamily Ommastrephinae (3.263, $n = 7$) ($P < 0.001$, Student's t-test). The transitions among squid species of different subfamilies and families observed in our study appear to be highly saturated relative to transversions (Fig. 5). The mean nucleotide sequence diversity estimates (π) based on K2P distances are shown in Table 5. The mean nucleotide sequence diversity within species ranged from 0.001 in *O. bartramii* to 0.014 in *S. oualaniensis*, and that between species within

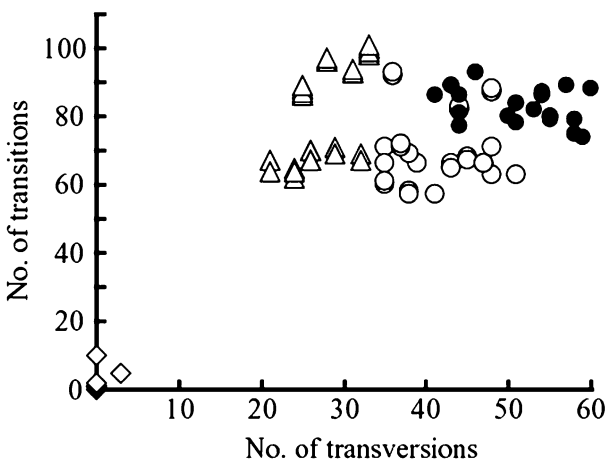

Fig. 5 Plot of the number of transitions versus number of transversions for COI of ommastrephid species. (◇) within species; (△) between species from same subfamily; (○) between species from different subfamilies; (●) between species from different families.

the subfamilies ranged from 0.133 (*S. oualaniensis* versus *E. luminosa*) to 0.217 (*O. bartramii* versus *H. pelagica*). The sequence diversity between species from different subfamilies ranged from 0.147 (*H. pelagica* versus *N. hawaiiensis*) to 0.219 (*O. bartramii* versus *I. illecebrosus*), and that between species from different families ranged from 0.196 (*S. oualaniensis* versus *Onychoteuthis*

Table 5 Estimates of mean nucleotide sequence diversity based on K2P distance between species

	OB	SO	EL	HP	NH	TP	II	O.sp.
OB								
SO	0.179							
EL	0.202	0.133						
HP	0.217	0.146	0.154					
NH	0.208	0.161	0.149	0.147				
TP	0.202	0.169	0.165	0.170	0.135			
II	0.219	0.180	0.179	0.182	0.149	0.191		
O.sp.	0.214	0.196	0.207	0.227	0.206	0.239	0.219	
WS	0.228	0.216	0.213	0.218	0.208	0.243	0.225	0.218

EL, *E. luminosa*; HP, *H. pelagica*; II, *I. illecebrosus*; NH, *N. hawaiiensis*; OB, *O. bartramii*; O.sp., *Onychoteuthis* sp.; SO, *S. oualaniensis*; TP, *T. pacificus pacificus*; WS, *W. scintillans*.

sp.) to 0.243 (*T. pacificus pacificus* versus *W. scintillans*).

Although different tree topologies were obtained in all our phylogenetic analyses, all tree topologies strongly supported the monophyly of the family Ommastrephidae (Fig. 6). Monophyly of the subfamily Ommastrephinae was not supported in all trees using both transitions and transversions (NJ and MP trees are not shown) (Fig. 6c), while NJ and MP trees using transversions only well supported monophyly of the subfamilies Ommastrephinae and Todarodinae (Fig. 6a,b). Both NJ and MP trees indicated *H. pelagica* to be the most divergent species in the Ommastrephinae (Fig. 6a,b).

DISCUSSION

The present study introduced a simple, reliable DNA analysis for identifying four ommastrephid species, and the method was powerful enough to identify ommastrephid paralarvae collected in northern Hawaiian waters. The damage to the paralarval samples varied to the extent that some were nearly identifiable, while the identification was equivocal in others. The conventional morphological characters for identifying *O. bartramii* and *S. oualaniensis* have been confirmed. If proboscis or mantle is available in damaged paralarvae, relative size between the proboscis suckers or the number and location of photophores may be keys to identify these species. It was possible to identify specimens larger than 4.0 mm DML using the intestinal and ocular photophores, even if the proboscis was broken. Intestinal photophores are much more resilient to damage than the proboscis. Most of the specimens larger than 4.0 mm DML in this study were identified using the number of intestinal photophores, and the results were consistent with the PCR–RFLP analysis. One larva identified as *E. luminosa* by PCR–RFLP analysis had a characteristic chromatophore pattern (two pairs of chromatophores on the dorsal head),[10,25] validating that character is diagnostic for distinguishing *E. luminosa* from *H. pelagica* paralarvae smaller than 3.5 mm DML.

Virtual restriction site investigation indicated that three species of the other subfamilies (Illicinae and Todarodinae) could also be discriminated using either one of the two enzymes (*Alu* I and *Tsp*509 I). Therefore, this method can be applied to other ommastrephid squid species and in other areas.

The present phylogenetic analysis strongly supported the monophyletic status of the family Ommastrephidae (Fig. 6), which had been uncertain in the previous studies using limited numbers of ommastrephid species.[26,27] Our molecular data also substantiate the inverted T-shaped funnel cartilage as a morphological character of the Ommastrephidae.

The family Ommastrephidae is traditionally divided into three subfamilies (Illicinae, Todarodinae, and Ommastrephinae) according to the structure of the funnel groove.[28] This classification based on the structure of the funnel groove corresponds to those based on other morphological characteristics[29] and allozymes[30] without intermediate forms. The trees drawn using NJ and MP method with transversions only in our study (Fig. 6a,b) also corresponded to those inferred from morphology[29] and allozymes.[30] A recent study[26] has indicated that transitions among squid species from higher taxa appear to be highly saturated relative to transversions, and increased homoplasy in transitions could mask the phylogenetic signal of transversions. Lindgren *et al.*[31] reported that the COI data supported differentiation among species well, but had very little power to resolve genus-level relationships in squid species of the family Gonatidae. However, the homoplasy in transitions was not investigated in

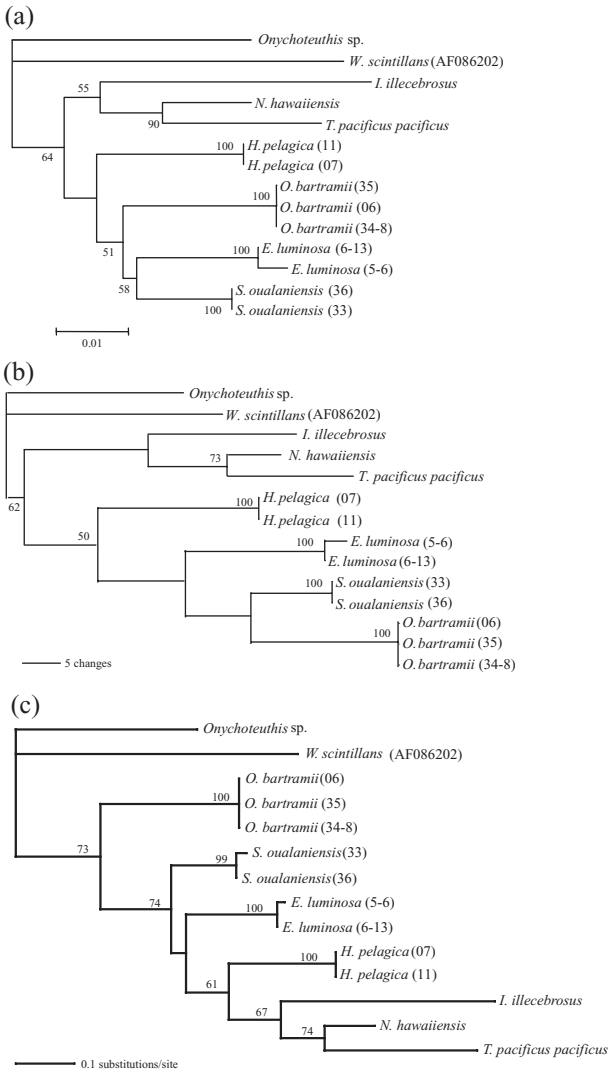

Fig. 6 COI phylogenies of standard species and paralarva having inconsistent RFLP patterns. (a) NJ method using transversions only, (b) MP method using transversions only, and (c) ML method.

their study. Taking morphological and allozyme analyses[29,30] into consideration, our phylogenetic analysis suggests that COI sequence data without transitions may infer the subfamily level relationships.

Wormuth[32] and Roeleveld[29] considered *E. luminosa* to be taxonomically close to *H. pelagica*. Conversely, *S. oualaniensis* and *E. luminosa* were once placed in the same genus, *Symplectoteuthis*,[33] because they shared a characteristic in which the funnel locking cartilages fuse with the mantle cartilages. The relationships among species within the subfamily Ommastrephinae could not be resolved by our phylogenetic analysis, possibly because the deep nodes were based on large genetic distance between species and homoplasy in transitions. Wada *et al.*[34] showed the NJ tree based on 16S rRNA sequence data of six ommastrephid species, which supported the subfamily Ommastrephinae and the relationships within the family are similar to our NJ and MP trees (transversions only). Lindgren *et al.*[31] indicated that 16S rRNA topology provides some support for genus-level group in the family Gonatidae. Since the phylogenetic relationships among cirrate octopods were successfully resolved using 16S rDNA analysis,[35] such a slowly evolving gene may be suitable for recovering the true phylogeny of ommastrephid squid species.

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