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Morphological and morphometric appraisal of the spermatophore of the southern hermit crab *Isocheles sawayai* Forest and Saint Laurent, 1968 (Anomura: Diogenidae), with comments on gonopores in both sexes

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Abstract

The spermatophore morphology of the hermit crab *Isocheles sawayai* from southwestern Atlantic (Brazil) is described. The spermatophores show similarities with those described for other members of the family Diogenidae, especially with the recently described *Loxopagurus loxochelis*. The spermatophore is composed of three major regions: a sperm filled head or ampulla, a columnar stalk and a foot or pedestal. The spermatophores show specific morphology in having a circular ampulla, and a constriction or neck between the ampulla (100 μm) and the thin (27 μm), long stalk (500 μm). The stalk penetrates less than half way into the spermatophore head. Most spermatophores show one of the small posterior projections on the underside of the ampulla as being bigger than the other, making it asymmetrical. The size of the spermatophore is related to hermit crab size with direct relationships found between spermatophore ampulla width, total length, and peduncle length with shield length of the hermit crab. The morphological characteristics of the spermatophore of *I. sawayai* are species-specific distinguishing it from other members of the family, and are useful to infer further phylogenetic relationships.

Keywords: Crustacea; Diogenidae; Gonopores; Reproduction; Spermatophore

1. Introduction

The variability of reproductive morphology and products constitutes a good source of characters that can help to solve some of the existing taxonomic problems and help to establish phylogenetic relationships (spermiotaxonomy) between many groups of crustaceans. Recently, comparisons of the functional morphology of genitalia and subsequent sperm transfer and storage mechanisms, and the structure of spermatozoa and spermatophores have been carried out among

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crustacean taxa and provide useful information on phylogenetic relationships and evolutionary divergence, especially in the Decapoda (see Bauer 1986, 1991; Tudge, 1997; Kronenberger et al. 2004 for review).

Spermatophores play a major role in sperm transfer and storage in decapod crustaceans, especially in anomuran hermit crabs that developed a broad range of adaptations to reproduce and survive successfully in different habitats using gastropod shells and other shelters. Consequently, hermit crabs represent promising examples of study because their establishment in marine, estuarine, and semi-terrestrial habitats derives from the evolution of adaptive population strategies (Mantelatto and Sousa 2000), and particularly because they make the usual sperm transfer mechanisms complex because of shell use and external deposition and fertilization. For example, hermit crabs that are adapted to life on land, such as species of the genera *Coenobita* and *Birgus*, are faced with the problem of how to transfer sperm to the external surface of the female where it will be attached and where fertilization will subsequently occur.

In the infraorder Anomura, the hermit crabs are the best-known representatives, with more than 1200 reported species worldwide (R. Lemaitre, personal communications, 2008) and it is a morphologically diverse taxon. The family Diogenidae is comprised of 20 genera (McLaughlin 2003; Rafael Lemaitre, personal communications) and there are many for which the spermatophore morphology is unknown (*Allodardanus*, *Aniculus*, *Bathynarius*, *Cancellus*, *Ciliopagurus*, *Isocheles*, *Paguropsis*, *Petrochirus*, *Pseudopaguristes*, *Pseudopagurus*, *Stratiotes*, *Tisea*, and *Trizopagurus*), and could be very important for comparative studies. Only three of these genera (*Cancellus*, *Isocheles*, and *Petrochirus*) are reported in Brazilian marine waters (Melo 1999).

The genus *Isocheles* Stimpson, 1858 is one of the less studied diogenid hermit crab genera, with a paucity of biological information available, apart from its taxonomy. The biogeographical distribution of this genus is restricted to shallow waters of tropical and subtropical American coasts and is represented by five species: *Isocheles pilosus* (Holmes, 1900), *Isocheles pacificus* (Bouvier, 1907), and *Isocheles aequimanus* (Dana, 1852) in the eastern Pacific Ocean and *Isocheles sawayai* Forest and Saint Laurent, 1968 and *Isocheles wurdemanni* Stimpson, 1859 in the western Atlantic Ocean (Forest and Saint Laurent 1968, Mantelatto et al. 2006). Among them, however, only *I. sawayai* was previously considered endemic to Brazil with a distribution from Ceará to Santa Catarina States (Melo 1999), but its range was later "extended" to Venezuela, Antilles, and Florida (Nucci and Melo 2000) and confirmed by molecular analysis of both populations known until now (Mantelatto et al. 2006). To date, the biological aspects of *I. sawayai* have only been studied on the northern coast of the state of São Paulo, Brazil. The available data deal with the species description and adult distribution (Costa 1962; Forest and Saint Laurent 1968; Hebling and Wernick 1974), larval and juvenile development under laboratory conditions (Negreiros-Fransozo and Hebling 1983), shell occupation and distribution (Pinheiro et al. 1993; Fantucci et al. 2008; Galindo et al. 2008), influence of shell use on metabolism (Wernick 1985), zoeal morphology description (Negreiros-Fransozo and Hebling 1983), molecular analysis of the taxonomic and distributional status (Mantelatto et al. 2006) and records of intersex individuals (Fantucci et al. 2007).

Since very little information is available on the reproductive system of the western Atlantic hermit crab *I. sawayai*, we describe and illustrate the spermatophore morphology of this species and compare it with other members of the family Diogenidae, especially the closely related *Loxopagurus loxochelis*. Additionally, we furnish data and observations on gonopore morphology in both sexes of *I. sawayai* in order to enhance our knowledge of the mechanisms of reproduction in hermit crabs.

2. Material and methods

I. sawayai (Forest and Saint Laurent, 1968) were captured by hand using snorkel diving and by double rigged trawl nets, respectively, in Praia do Lázaro, Ubatuba (23°26′S and 45°02′W) and Caraguatatuba Bay (23°47′S and 45°08′W), northern coast of the state of São Paulo, Brazil, in March 2005 on sandy bottoms at depths from 1 to 10 m. They were maintained in sea water and transported alive to the University of São Paulo (USP).

In the laboratory, hermit crabs were anesthetized by chilling in a refrigerator for some minutes, and then they were carefully removed from their shells by twisting them in an anticlockwise fashion. After sexing, only males were selected for spermatophore analysis. Each individual was measured for shield length (SL) from the tip of rostrum to the V-shaped groove at the posterior edge of the dorsal shield with a caliper rule (0.1 mm) or under a stereomicroscope with a camera lucida. Each fresh crab was also weighed (W, wet weight) using an electronic microscale (0.001 g sensitivity). The voucher hermit crabs were deposited in the Crustacean Collection of the Biology Department (CCDB) of Laboratory of Bioecology and Systematics of Crustaceans at the Faculty of Philosophy, Science and Letters of Ribeirão Preto (FFCLRP), University of São Paulo (Catalogue numbers from 1687 to 1691).

In general, the methodology of analysis followed Scelzo et al. (2004). The male reproductive system was

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dissected from either freshly killed or fixed sexually mature specimens (adults) by making an incision between the fourth and fifth pair of pereopods (P4 and P5, respectively) and the abdomen was separated from the rest of the body. The thin cuticle of the abdomen was cut dorsally to access the reproductive system, comprising the testis and vas deferens (VD). The distal part of the VD attached to the basal part of P5 was specifically used for the spermatophore analysis. Spermatophores obtained near the proximal part of VD, near the testis, was also observed to compare with mature spermatophores from the distal region. The dissected materials were preserved in 3% glutaraldehyde for scanning electron microscopy (SEM) analysis.

The most distal portion of one of the two vas deferens (ejaculatory duct), near the coxa of the fifth pereopods, was isolated and fixed in 10% seawater formalin and/or 80% ethanol for morphological and morphometric analysis using light microscopy (LM). The vas deferens was dissected, liberating the spermatophore ribbon. Small portions of spermatophore ribbon were mounted on a glass slide and stained with rose Bengal and/or methylene blue in 10% formalin and examined under a Zeiss Standard 20 light microscope.

A total of 43 male crabs were dissected for spermatophore measurements. Approximately, ten straight spermatophores from each crab were selected for morphometric analysis and were drawn and measured using a camera lucida mounted on a light microscope. Measurements are given in mm or μm. Spermatophore measurements were made according to Scelzo et al. (2004), specifically, spermatophore total length (TLSp) = maximum length of spermatophore from ampulla to the base or pedestal; peduncle or stalk length (PL) = from the base or pedestal to the neck inside the ampulla; spermatophore peduncle width (PW) = size across the peduncle; spermatophore ampulla width (AW) = maximum width of the spermatophore ampulla; spermatophore ampulla height (AH) = maximum height of the spermatophore ampulla including the posterior pointing projection on the underside of the ampulla; spermatophore ampulla depth (AD)-measured across the antero-posterior (shorter) axis of the ampulla.

Since no significant differences were detected between right and left gonopore size in both sexes, the left gonopore (maximum and minimum) diameter was measured for at least two specimens of each adult male and female from every hermit crab size class analyzed (from 4.3 to 9.8 mm SL).

A regression analysis was performed to compare the dimensions of the spermatophores with the size of the crabs and the mean, standard deviation, range, coefficient of variation (CV) expressed as percentage and coefficient of determination (r^2) were performed (Sokal and Rohlf 1981).

3. Results

3.1. Reproductive morphology

Size and wet weight of hermit crabs sampled during this study ranged from 3.6 to 9.8 mm SL and 0.26 to 5.03 g, respectively (Table 1). Paired testes are located dorsal to the hepatopancreas, in the pleon or abdomen. A coiled vas deferens from each testis connects them to the base of P5.

Male and female gonopores exhibit an ovoid shape and have a gonopore cover (Gc) (Figs. 1A–D). The male gonopores are bigger than female ones in the same size class, and are surrounded by plumose (Ps) and serrate (Ss) setae (Figs. 1A and C), but the female gonopores are almost free of setation (Figs. 1B and D). The maximum mean diameter of female gonopores measured 420±93 μm (ranging from 200 to 600 μm) for crabs measuring 3.8–7.7 mm SL. The maximum mean diameter of male gonopores measured 950±220 μm (range 500–1350 μm) for crabs measuring 3.6–9.8 mm SL. In general, there was a direct relationship between the diameter of gonopores and hermit crab size (male gonopore diameter = 0.0288 $SL^{1.723}$, $r^2 = 0.61$, $N = 43$; female gonopore diameter = 0.0485 $SL^{1.2139}$, $r^2 = 0.55$, $N = 50$), and the slopes of 1.723 and 1.22 may imply that gonopore diameter grows in a positive allometric manner in both sexes. We found no significant differences among the size of gonopores between ovigerous and non-ovigerous females in the range of specimen's size analyzed. However, a direct relationship was obtained between the maximum diameter of gonopore and the size of non-ovigerous females (non-ovigerous female gonopore diameter = 0.0305 $SL^{1.4301}$, $r^2 = 0.77$, $N = 25$; SL range 4.1–7.7 mm) but no relationship between the maximum diameter of gonopore and the size of ovigerous females (ovigerous female gonopore diameter = 0.2148 $SL^{0.4289}$, $r^2 = 0.18$, $N = 25$, SL range 4.3–8.0 mm), indicating that gonopore size

Table 1. *Isocheles sawayai*: body and spermatophore dimensions of males from Ubatuba, São Paulo State, Brazil.

Measures	Minimum	Maximum	Mean±SD	CV (%)	N
SL (mm)	3.60	9.80	7.250±1.440	19.86	43
W (g)	0.26	5.03	3.030±1.740	57.43	43
TLSp (mm)	0.164	1.270	0.577±0.295	51.16	65
AW (mm)	0.055	0.151	0.098±0.027	27.57	70
PL (mm)	0.114	1.107	0.498±0.280	56.11	65
AH (mm)	0.049	0.180	0.102±0.033	32.14	70
PW (mm)	0.009	0.061	0.027±0.013	49.19	68

SL = shield length, W = wet weight, TLSp = spermatophore total length, AW = ampulla width, PL = peduncle length, AH = ampulla height, PW = peduncle width, N = number of specimens.

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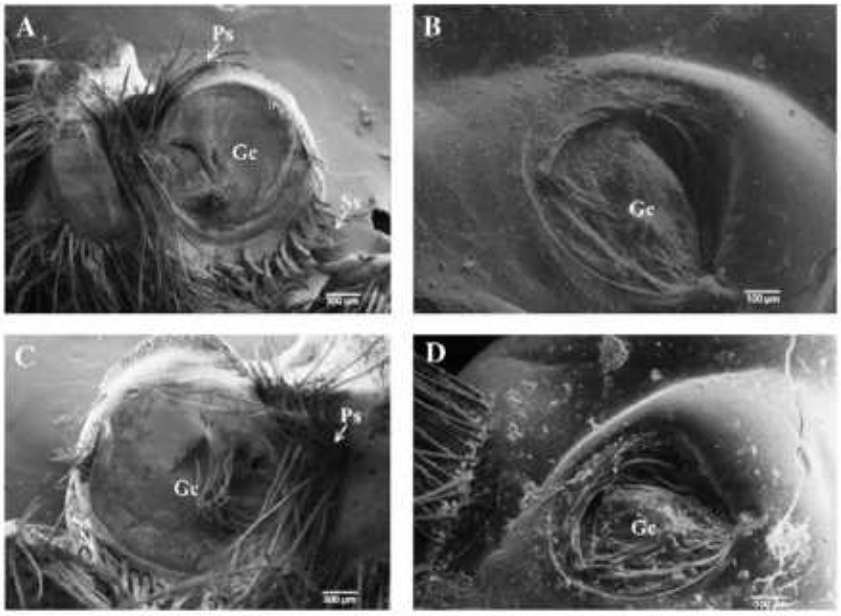

Fig. 1. *Isocheles sawayai*: scanning electron micrographs of male (A) and female (B) gonopores; details of male (C) and female (D) gonopores showing gonopore cover (Gc), plumose (Ps), and serrate (Ss) setae.

showed differences between ovigerous and non-ovigerous conditions.

3.2. Spermatophore morphology and morphometry

Spermatophores of *I. sawayai* are formed prior to ejaculation and can be observed within the lumen of the coiled vasa deferentia and ejaculatory ducts. The pedunculate spermatophores are composed of three regions: the ampulla (Am), a thin peduncle (Pe), and the base (Ba) or pedestal (Fig. 2A). The diameter of the VD is variable with crab size and stage of sexual maturation. In adult hermit crabs SL 7.0 mm, the distal portion of the VD measured 0.75 mm in diameter, meanwhile TLSp measured between 0.7 and 1.0 mm. In such cases, the spermatophores tend to be aligned inside the VD at an angle lower than 90°. The long and thin peduncle or stalk connects the ampulla to the base or pedestal. Spermatophore ampullae are generally circular in shape, and flattened antero-posteriorly (AD = 59 μm) (Figs. 2A–D). The lateral ridge structure with its suture line is a thickened border joining the two halves at the latero-dorsal edge of the ampulla and measures 2 μm in width each side of the suture line (Figs. 2C–E). Most spermatophores show one of the small posterior pointing projections (Pp) on the underside of the ampulla bigger than the other, making it asymmetrical.

Spermatozoa (Sz) fill the ampulla and can be easily observed through the thin and transparent cuticle. The external ampulla surface shows irregular shallow tubercles formed by the spermatozoa package inside (Figs. 2B, D, and F). Spermatozoa show the typical anomuran ovoid acrosome vesicle and a posterior nuclear region with three arms (Fig. 2F).

The ampulla width shows a moderate correlation with hermit crab size (AW = 0.0199 SL-0.022, $r^2 = 0.56$), to spermatophore total length (AW = 0.0684 TLSp + 0.0604, $r^2 = 0.59$), and was well correlated with ampulla length (AW = 0.7285 AH + 0.0229, $r^2 = 0.80$). The peduncle length is also directly related to spermatophore total length (PL = 0.9427 TLSp + 0.0456, $r^2 = 0.99$), as is TLSp with the size of the individuals (TLSp = 0.1833SL–0.5303, $r^2 = 0.42$). The spermatophore total length also shows a moderate correlation with gonopore diameter (TLSp = 1.042 maximum gonopore diamenter$^{0.733}$, $r^2 = 0.2$).

4. Discussion

With the present results for *I. sawayai*, the spermatophore morphology of diogenid hermit crabs is now known for 10 genera and 23 species (see Table 2 in Scelzo et al. 2004, plus Tirelli et al. 2006, 2007) including the first description of the spermatophore morphology and morphometry for hermit crabs of the genus *Isocheles*. Tudge (1991) showed that light microscope observations of spermatophores can be used successfully to distinguish hermit crab families of the Paguroidea, especially within the family Diogenidae.

I. sawayai encloses the spermatozoa in pedunculate and tripartite spermatophores that characterize most of the Diogenidae. *I. sawayai* shows a mature spermatophore with a long, thin stalk or peduncle measuring

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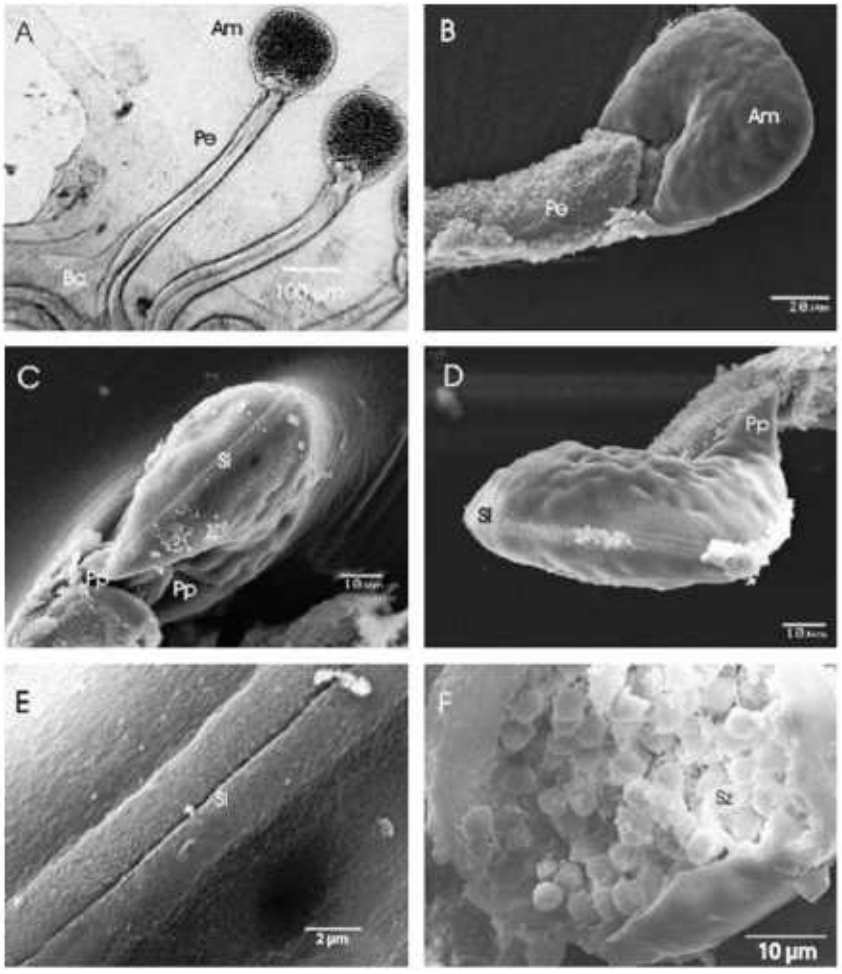

Fig. 2. *Isocheles sawayai*: light and scanning electronic micrographs of spermatophores: (A) Pedunculate spermatophores (Am: ampulla, Ba: Base or pedestal; Pe: peduncle or stalk); (B) detail of ampulla and part of the peduncle; (C,D) views of ampulla showing pointing projections (Pp) and suture line (Sl); (E) detail of lateral ridge and suture line; and (F) spermatozoa (Sz) inside opened ampulla.

27 µm in diameter and a big ampulla (about 100 µm in diameter). These dimensions are surpassed by species of *Diogenes*, *Loxopagurus*, and *Strigopagurus* (Scelzo et al. 2004). The lumps showing on the ampulla surface, due to the spermatozoa that fill the ampulla are an unusual characteristic not recorded before in other described hermit crab spermatophores. We cannot discount the possibility that the lumps are an artifact of fixation, or caused by the critical point drying process, as it looks like the ampulla surface has shrunk onto the underlying sperm.

The capsule is composed of two halves or valves meeting at a conspicuous lateral ridge (Mouchet 1930; Hamon 1937; Tudge 1991, 1999a). Ultrastructural studies (see Tudge 1999a for review) show that within the Anomura the lateral ridge is a break in the spermatophore wall structure and that there appears to be three basic types of spermatophore wall ultrastructure within the infraorder (homogeneously granular, heterogeneously granular and fibrillar). In the present study, no transmission electron micrography was carried out to determine the composition of the spermatophore wall and ridge, but future analysis will investigate this aspect.

Despite the size and possible external surface differences, the general spermatophore morphology of *I. sawayai* is similar to other diogenid species, with the pedunculate type common in the Coenobitidae and Diogenidae (Tudge 1991; Scelzo et al. 2004, for review).

Considering the variability on the morphology, the spermatophore morphometry could be one additional criterion to distinguish species of diogenid hermit crabs. Unfortunately, in many cases, no accurate measurements are available for many diogenids investigated for spermatophore morphology (e.g. Matthews 1953, 1956, 1957) and this can limit the interpretation and comparison of data. Recently, Tirelli and Pessani (2007) showed the importance of a statistical approach using both univariate and multivariate techniques as a tool to study the morphometry of paguroid spermatophores.

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Nevertheless, *Dardanus lagopodes* (Forskäl, 1775), *Strigopagurus boreonotus* (Forest, 1995) and *I. sawayai* all have spermatophore mean total lengths greater than 500 μm and can be separated from the remaining described species, which have smaller spermatophores (Scelzo et al. 2004). Also, *D. gardineri* Alcock, 1905, *S. boreonotus*, *L. loxochelis*, and *I. sawayai* all have ampullae mean sizes around or bigger than 100 μm. It is important to note that the spermatophores of *I. sawayai* fixed in 80% ethanol or 10% formalin showed irregularities in their general shape, especially some shrinkage of the total length, shape of the ampullae and peduncle width. The ampullae of spermatophores fixed in 80% ethanol were flattened while they were ovoid in fresh material. In some cases, the ampullae broke away or they opened at the dorsal suture line and the spermatozoa were liberated. This latter observation is in accordance with Hamon (1939) and Hess and Bauer (2002) who suggested that mechanical or osmotic forces might cause the ampullae to fracture and release the spermatozoa. For these reasons, we do not recommend the use of spermatophores fixed in ethanol for morphometric studies. Spermatophore total length shows a direct relationship with hermit crab size (SL), but spermatophore morphology may change in relation to degree of maturity (Tudge 1991), it means that it changes with age (size) of the male.

In agreement with Forest and Saint Laurent (1968), who first postulated the close relationship between *Isocheles* and *Loxopagurus* based on general somatic morphology of adults, we found that members of *Loxopagurus* and *Isocheles* were also similar in the morphology of their spermatophores. The most important shared feature being the constriction or neck that penetrates almost half way into the base of the ampulla (Fig. 1B). The close morphological similarity between *I. sawayai* and *L. loxochelis* spermatophores corroborates a parallel molecular analysis (Mantelatto et al. 2006) which indicates that there is no doubt that both species are phylogenetically close to one another. Also, as previously reported for *L. loxochelis* (see Scelzo et al. 2004), we found that the overall morphology of the spermatophore in *I. sawayai* is most like those reported for the genus *Clibanarius*, particularly in the small posterior pointing projections on the underside of the ampulla.

A detailed study of the composition of the population of *I. sawayai* in the Ubatuba region shows that there is regular local reproduction and recruitment (Fantucci et al. 2008). Furthermore, morphological intersex individuals, exhibiting both male and female external sexual characters such as different combinations of both genital openings at the same time (Fantucci et al. 2007) were detected in low numbers. The presence of these individuals does not affect the general structure of the population, but we have not included these specimens in the present analysis of spermatophores. The reasons for this sexual condition are not clear and the morphological organization of these intersex individuals needs future investigation. Patterns of intersex individuals in natural populations have also been reported in the hermit crabs *Paguristes tortugae* studied by Mantelatto and Sousa (2000) and *Clibanarius vittatus* by Hess and Bauer (2002), but unfortunately no information on spermatophores is available.

In summary, the spermatophore characters of *I. sawayai* are (1) the spermatophore form is pedunculate and tripartite consisting of an ampulla attached to a pedestal or base by a stalk of variable length, (2) spermatophore shows a main ampulla only and no accessory ampullae, characteristic of members of the families Paguridae, Parapaguridae (Tudge 1999b) and some members of the Lithodidae (Tudge et al. 1998), (3) ampulla has no tubular extension characteristic of some Porcellanidae, and (4) the stalk of the spermatophore is long and thin. Future TEM studies will confirm the spermatophore wall ultrastructure as either homogeneously or heterogeneously granular or fibrillar (Tudge 1999a) as well as the sperm ultrastructure.

The exact position of *I. sawayai* within the Diogenidae remains uncertain because there still remain many genera and species of this family for which spermatophore morphology is unknown. However, cladistic analysis of the data presented here in combination with recent phylogenetic analysis (Tirelli et al. 2008), along with molecular sequences and larval morphology, may make it possible to support the proposition that *I. sawayai* and *L. loxopagurus* are sister taxa and both are close to *Clibanarius* and *Calcinus*, and also allow us to determine the phylogeny and evolution of the diverse family Diogenidae.

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