Field biology of placozoans (*Trichoplax*): distribution, diversity, biotic interactions

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**Synopsis** The goal of this review is to highlight what little is known, and point to the bulk of what is yet to be learned, about the natural history of placozoans in the field—in order to stimulate a broader search for placozoans and a fuller exploration of their distribution, diversity, and all other aspects of their enigmatic lives. The documented geographic distribution of placozoans lies mostly in the nearshore, warm, marine waters of the tropics and subtropics. Although placozoans have long been viewed as benthic organisms, they can be more readily collected from the water column, well above the sea bottom. The full life-history of placozoans is unknown, including the nature of this abundant pelagic phase and all details of sexual reproduction and development. We note observations on the biota associated with placozoans in field collections, in particular the other regular members of the microcommunity in which placozoans occur on our collecting plates and on some factors influencing this assemblage. Among the animals found are some potential predators against which placozoans appear to be defended, although the mechanisms are still to be examined. Also yet to be uncovered is the full breadth of diversity in this phylum, certainly underrepresented by its single named species. We report here greatly expanded distributions for known haplotypes and fresh specimens that include a new haplotype, and we review the evidence that many more almost certainly await discovery. We also describe some methods for collecting and handling these small, fragile animals.

**Introduction**

As the simplest of living metazoans, *Trichoplax adhaerens* Schulze, 1883, phylum Placozoa, holds a special fascination for biologists (Fig. 1). Although its relationships with other animals remain controversial, both morphological and DNA-based phylogenetic analyses place it among the diploblasts and possibly at the very base of animal origins (e.g., Nielsen et al. 1996; Halanych 2004; Dellaporta et al. 2006; Signorovitch et al. 2007; Schierwater and DeSalle 2007). Studies on laboratory-cultured animals have provided substantial background on structure and ultrastructure (reviewed by Grell and Ruthmann 1991) and on cell and molecular biology (e.g., quantitative DNA measurements: Ruthmann 1977; evidence of a single Hox gene: Schierwater and Kuhn 1998; documentation of a circular mitochondrial (mt) genome: Ender and Schierwater 2003; mt genomes: Dellaporta et al. 2006; Signorovitch et al. 2007; expression of developmental genes: Martinelli and Spring 2003; Jakob et al. 2004; Hadrys et al. 2005). Reviews of research on *Trichoplax* (Syed and Schierwater 2002; Miller and Ball 2005; Schierwater 2005) will need frequent updating, as growing interest in these animals accelerates the rate of publications. *Trichoplax* has joined the elite list of model animals whose entire genome has been sequenced (now in the process of annotation) (Joint Genome Institute 2007).

Thus, the rocket science is well launched. In contrast, almost no one has bothered to glance down at the launch pad: little is known of the most basic biology of the living animals in the field—their biogeography, habitat, behavior, biotic interactions, or other aspects of their lives. Grell (e.g., 1981) pioneered the first outlines of their distribution by extracting placozoans from samples of seaweeds and other potential substrates collected from several tropical and subtropical sites. Decades later, we have still barely begun to document the rich taxonomic diversity that living placozoans promise (Voigt et al. 2004; Signorovitch et al. 2006). One goal of this present review is to stimulate a broader search for placozoans and fuller exploration of their distribution and diversity.
Since first working with these animals in Hawaii in 1986, V.B.P. has continued to sample for placozoans at many locales, in marine laboratory facilities and in the sea, in order to further document where they are found. Both authors have contributed samples, including a new haplotype, to the genetic and phylogenetic analysis presented here. Additional information about the field biology of placozoans, mostly fortuitous, was collected as opportunities arose. Also described are techniques for collecting and handling these minute, delicate organisms.

**Collection and observation**

V.B.P. collected placozoans on standard glass microscope slides (25 × 76 mm) placed in the sea or in laboratory seawater systems, usually in racks made from plastic slide boxes, with most of the top and bottom cut out to allow water to circulate through them. The slides were spaced ~10–15 mm apart. The top and bottom of the rack were then tied together with nylon monofilament line or cable ties, and the rack was suspended from any handy support (e.g., a coral branch, mangrove prop, or dock) (Fig. 2). Racks were oriented so that the surfaces of the slides were either parallel or perpendicular to the surface of the water. Some racks were placed directly on the substrate, with the slides roughly perpendicular to the surface of the water.

Single microscope slides were sometimes suspended in the water (weighted with a small rock by means of a rubber band slipped around the slide) or placed directly on the substrate. Being less conspicuous, these were less likely to attract tampering.

When the slides were retrieved, typically after 10–15 days, the racks of slides were placed into securely lidded plastic boxes filled with seawater in situ, so that the slides would be exposed neither to air nor to seawater from another location or depth. Loose slides were placed into racks and treated the same way. Returned to the laboratory, the slides were kept and examined in the same water, at room temperature, within hours or no more than 1–2 days.

To examine a slide for placozoans, it was quickly transferred to a Petri dish containing enough seawater to cover the slide. The slide was supported on small bits of rock, so that, while the upper surface was being examined, organisms on the lower surface were protected from rubbing against the bottom of the dish. Illumination from a mirror or light below
the dish was adjusted to optimal angle and intensity, and both sides of the slide were scanned systematically under a dissecting microscope. Placozoans were counted; other organisms were recorded as present.

Factors affecting the likelihood of success in capturing placozoans under various conditions are noted below (see section Macrohabitat and microhabitat).

**Strengths and limitations of collection methods**

The first published finding of placozoans on glass microscope slides suspended in the sea was by Sudzuki (1977) in Sagami Bay, Japan. This method was used from the start of studies by VBP in 1986, et al. have followed suit (e.g., Maruyama 2004; Signorovitch et al. 2006), because placozoans are almost invisible on any substrate except clear glass. Sampling with glass slides is also a relatively benign process, causing little or no disturbance to the benthic habitat; there is some by-catch, but it is minor on an oceanic scale.

Capture of a placozoan on a slide establishes its presence at a known location and depth, provided that the slide is kept in seawater from the original site or in adequately filtered seawater. However, because settlement may have occurred at any time after the slide was placed, the time cannot be known precisely. Likewise, the number of placozoans observed on a slide depends on where and for how long a slide is exposed and results from some combination of settlement and fission, as well as possible loss of individuals over the period of exposure and increasing difficulty in seeing placozoans as the slide becomes overgrown with other biota. Thus, only semi-quantitative sampling has been achieved.

Assuming that two or more slides bearing placozoans represent independent settlement events, reporting the number of positive slides, as well as the number of placozoans on each, adds information (e.g., Maruyama 2004). The alternative of estimating biomass (versus number) is problematic (Pearse 1989). Another method is to collect pieces of rock or shell and shake them in a plastic bag filled with seawater (Maruyama 2004). Although faster, this process further obscures the number of placozoans present, because many become broken into fragments. However, one is assured of direct sampling of the benthic phase (provided the seawater is well filtered), whereas the stage of the life-history that settles on glass slides, either suspended or placed on the bottom, is uncertain.

Also, as with any small, easily transported planktonic organism, the location of the source population on the benthos is difficult to determine. Thus, although more than one clade of placozoans can often be found together in a single sample (Voigt et al. 2004; Signorovitch et al. 2006), interpretations of sympatry are complicated by the limitations of the sampling method. Placozoans swimming or drifting in the water column settle on the suspended slides. However, two or more placozoans settling on a single slide may have originated from benthic individuals in quite separate and different microhabitats. Lacking any definition of "an ecologically relevant spatial scale" (Signorovitch et al. 2006), conclusions of sympatry may not apply to the adjacent benthic habitat, but strictly, only to sympatry in the water column.

**Distribution**

**Biogeography**

Sites where placozoans are known to have been collected are summarized on a global map (Fig. 3). The annotated list below includes published records [P], previously unpublished observations of either of this review’s authors [A], and personal
communications from other biologists [C]. Reported haplotypes of the mitochondrial 16S large subunit ribosomal RNA gene (16S rDNA) are given in bold (using the numbering of Voigt et al. 2004 and of this publication).

Mediterranean and Eastern Atlantic

(1) Gulf of Trieste, Adriatic Sea. Schulze 1883; Stiasny 1903. Schulze’s type locality for the species Trichoplax adhaerens. [P]

(2) Gulf of Naples, Tyrrhenian Sea. Monticelli 1893, 1896. Monticelli’s type locality for the species Treptoplax reptans Monticelli 1896, currently not recognized. [P]


(4) Tunisia. B. Schierwater, H. Hadrys, M. Eitel 2005 [C]

(5) Tenerife, Canary Islands. B. Schierwater, H. Hadrys 2003 [C]

Red Sea and Indian Ocean


(7) La Réunion. N. Gravier-Bonnet 2001 [C]

Pacific

(8) Presumed from Sea of Japan. Ivanov et al. 1980 [P] (Similar to H1)

(9) Oki Islands, Sea of Japan. Y.K. Maruyama [C]

(10) Shimoda, southcentral Honshu, Japan. Sudzuki 1977 [P]

(11) Shirahama, south Honshu, Japan. V. Pearse 1989 [A], Maruyama 2004 [P]

(12) Okinawa, Ryukyu Islands, Japan. V. Pearse 1989 [A], Pearse et al. 1994 [P]

(13) Iriomote, Ryukyu Islands, Japan. V. Pearse 1989 [A]

(14) Hong Kong. V. Pearse 2004 [A]

(15) Zambales, Philippines, from material transported to Monterey Bay Aquarium, Monterey, California. V. Pearse 1992 [A]


(17) Palau. V. Pearse 1988 [A]

(18) North of Manado, northeast Sulawesi, Indonesia. V. Pearse 1994 [A]


(20) Lizard Island, Great Barrier Reef, northeastern Australia. O. Voigt 2006 [A] LIZ (similar to H9)

(21) Orpheus Island, Great Barrier Reef, northeastern Australia. V. Pearse 1988 [A]

(22) Heron Island, Great Barrier Reef, northeastern Australia. A.T. Newberry 1988 [C]


(26) Presumed from southern California (see subsequently). V. Pearse 2006 [A] H11

(27) Achotines Laboratory, Azuero Peninsula, Panama. Voigt et al. 2004 [P] H4

(28) Isla Iguana, Azuero Peninsula, Panama. Voigt et al. 2004 [P] H6,8


Caribbean and Western Atlantic

(30) Southeast Atlantic coast of US: North Carolina, Grell 1980 (R.M. Rieger, personal communication); South Carolina, Klauser 1982 [P]

(31) Southeast Atlantic coast of US: Florida. V. Pearse 2006 [A]


(33) Puerto Morelos, Quintana Roo, Mexico. Grell and López-Ochoterena 1988 [P]


(35) Roatan, Honduras. V. Pearse 1985 [A]

(36) Bocas del Toro, Panama. Voigt et al. 2004; Signorovitch et al. 2006 [P] H1-4,8

(37) Galeta, Panama. V. Pearse 1997 [A]

(38) Discovery Bay, Jamaica. Signorovitch et al. 2006 [P] H1,8


(41) São Sebastião Channel, São Paulo State, Brazil. Morandini et al. 2006 [P]
Macrobenthos and microhabitat

V.B.P. found placozoans in abundance on coral reefs and in full-salinity mangroves. Results can be locally patchy, both positive and negative samples being obtained from a single site. Nonetheless, consistently negative samples or smaller yields in certain types of habitats or conditions point to factors restricting placozoan distribution. No systematic survey has been attempted but examples of findings are briefly summarized here (see also Pearse 1988).

Seasonality/Temperature

Placozoans were found on slides in samples taken at various tropical sites and all months of the year. However, no long-term surveys of placozoans at a single location have been done at tropical latitudes. At one subtropical site, Sesoko Island, Okinawa, Japan, where sea surface temperatures vary seasonally (means 20–28°C; Loya et al. 2001), collections were made at a site close to the Sesoko Marine Laboratory from March to August. In March and April, when sea temperatures at Sesoko average ~21–22°C, all samples were negative; only during May–August, when temperatures average ~24–28°C, were placozoans found on slides. Few sites from temperate latitudes have been examined; some data suggest possible seasonality in abundance at Shirahama, Japan (Maruyama 2004). A 1992 survey by V.B.P. in Monterey Bay, central California, where annual sea surface temperatures range ~11–18°C, failed to yield any specimens of placozoans. A search was made specifically for placozoans, using the same methods, but they also were not found at two sites illustrating extreme temperature conditions: −1.6°C in McMurdo Sound, Antarctica (Pearse and Pearse 1991) and ~3°C in the Monterey Canyon, Central California, ~1000–3000 m depth.

Salinity

In laboratory trials at the Christensen Research Inst. (CRI), Madang, Papua New Guinea, exposure to seawater of reduced salinity rapidly killed placozoans: exposure for 1 h to seawater diluted to 75% or 0.5 h in seawater diluted to 50% proved 100% lethal. After only 8 min or 1 min, respectively, in the test solutions, the edges of the animals began to curl, and the animals detached from the glass and became motionless and unresponsive to touch. Placed again into full salinity seawater (32.5%), some individuals showed improvement, but all died within <24 h. This intolerance for seawater of reduced salinity was evident in field collections, e.g., nearshore, shallow, previously positive sites near CRI were negative after rains. In contrast, placozoans have been found to tolerate elevated salinities in the lab, up to 40–50% (A. Signorovitch and L. Buss, personal communication, 2007).

Currents and wave surge

In a narrow channel with strong tidal flow, samples were consistently small or negative, relative to samples from the mangrove pond and fringing reef that the channel connected (Kranket Island, Madang, Papua New Guinea). Samples from reef flats with strong currents likewise failed to yield placozoans (Guam, Western Samoa), even in places where placozoans were recovered from nearby protected inlets (Cook Bay and Opunohu Bay, Moorea, French Polynesia). See also Associated biota section, subsequently.

Sandy bottoms

Almost all samples were negative in seagrass beds (Madang, Papua New Guinea; Apia, Western Samoa) and other sandy habitats. For example, at Orpheus Island, Great Barrier Reef, Australia, placozoans were found on slides placed on a fringing coral reef, but not at a site only ~200 m away where slides were suspended over a sandy bottom.

Benthos versus water column

Although placozoans have long been viewed as benthic organisms, they were regularly collected on glass slides suspended in the water column well above the bottom (Fig. 2). Moreover, when paired samples were compared (free slides or slide-racks placed on the substrate versus others hung in shallow water at the same site 20–60 cm above the bottom), those suspended in the water column bore significantly higher numbers of placozoans. For example, for 12 sets of eight slides each in Madang, Papua New Guinea, the number of placozoans was significantly greater on slides suspended above the bottom versus slides on the bottom (CRI dock, mean ± SD was 8.1 ± 3.4 for slides suspended above the bottom, 0.23 ± 0.32 for slides on the bottom, t-test, P = 0.02; Kranket Island, mean ± SD was 13.9 ± 3.1 for slides suspended above the bottom, 3.4 ± 2.0 for slides on the bottom, t-test, P < 0.01). Comparison of V.B.P.’s midsummer results in Shirahama, Japan, with those of Maruyama (2004) likewise indicate a strong effect of sampling off the bottom versus on the bottom. V.B.P. found large numbers of placozoans on slide racks suspended in the water column in July, comparable to yields during the fall peaks documented by Maruyama (2004, his Fig. 3), whereas his
slide racks, always placed on the substrate, captured very few or no specimens in July.

Substrate orientation
At the CRI dock, Madang, Papua New Guinea, placozoans were counted on two sets of eight slides each, hung in the sea for 17 days about 20 cm off the bottom so that their surfaces were horizontal (parallel to the water surface) versus vertical (perpendicular to the water surface). The number of placozoans per slide was not statistically different (mean ± SD was 5.9 ± 3.4 for a set of eight horizontal slides, 5.0 ± 3.3 for a set of eight vertical slides, t-test, \( P = 0.61 \)). In contrast, on horizontal slides, the number of placozoans was significantly greater on lower versus upper surfaces (mean ± SD was 5.5 ± 0.96 for lower surfaces, 2.0 ± 0.93 for upper surfaces for three sets of slides, t-test, \( P = 0.01 \)). Similar results were obtained from a trial at Sesoko Island, Okinawa, Japan (mean ± SD was 3.1 ± 1.6 for lower surfaces, 0.17 ± 0.13 for upper surfaces, for four sets of slides, t-test, \( P = 0.01 \)).

Remarks: biogeography and habitats
Given the limitations of collection methods, we can nonetheless draw some broad conclusions about the distribution of placozoans in terms of biogeography and at other scales. First, they have been widely documented in tropical and subtropical nearshore marine habitats, especially coral reefs and mangroves. V.B.P. seldom recovered them from sandy habitats, and this was also the most common experience of Signorovitch et al. (2006), even when hard substrates positive for placozoans were nearby. Although this might suggest a measurable limit on the distance that placozoans can travel between adjacent habitats, the number of observations is few and cannot be evaluated without information on the direction of currents and other confounding influences. Protected areas yield more placozoans than exposed sites with strong currents or wave-surge. We would expect to find fewer placozoans in areas of freshwater runoff, as near river mouths, as much because of the quantities of sediment and soft bottoms as because of their intolerance of reduced salinities.

Although whole biogeographic regions, such as the Indian Ocean, remain nearly blank on the map of placozoan distribution, sampling in those waters will almost certainly yield positive results. In contrast, our expectations of finding placozoans in deep or polar waters, or on coasts characterized by cold upwelling, are minimal. To our knowledge, no one has looked for placozoans in the open ocean. Where collections are from laboratory tanks or commercial or personal tropical aquariums rather than directly from the sea, some uncertainty about the origins of the specimens is inevitable because placozoans can easily be introduced with exotic materials.

Placozoans exhibit an abundant nearshore pelagic phase and can be efficiently collected from the water column, well above the sea bottom. In paired samples, slide racks hung suspended in the water column yielded more placozoans than did those placed directly on the substrate. The latter collect more silt and may be subject to disturbance from other benthic animals, or perhaps the pelagic phase settling on the slides is less numerous close to the bottom than within the water column. Compared to freestream flow in the middle of the water column, flow along the bottom is expected to be slower and therefore to supply fewer settlers per unit time; arriving settlers, however, probably attach more easily in slower flow. Thus, no simple conclusion emerges from the hydrodynamics.

The larger number of placozoans found on the lower surface of slides might relate to the greater amount of silt and ultraviolet radiation on the upper surface. Ultraviolet may be very important in restricting the microhabitats of these organisms and should be examined. (Placozoans have been observed to react strongly when exposed to ultraviolet radiation from laboratory sources; their sensitivity under field conditions has never been tested). Serpulids and some other organisms also appeared to settle preferentially on or migrate to the lower surface, perhaps responding to the same factors. Alternatively, placozoans might be responding directly to other biota. A study of temporal succession in this microcommunity could provide some answers. No difference was observed between upper and lower surfaces under laboratory conditions (e.g., Pearse 1989) and placozoans settled preferentially on a biofilmed surface, whether upper or lower. Thus, this differential is likely related to secondary factors present only in the field rather than directly to the orientation of the substrate.

Relations to other organisms
Associated biota
At some time or other, small representatives of most phyla of sessile invertebrates, as well as a great variety of protists and algae, have been seen on V.B.P.’s glass slides together with placozoans. Nonetheless, a few particular organisms often dominate the microcommunity that develops on glass slides and occur together with placozoans with sufficient regularity that one may almost predict placozoans by the
presence of these other associates, in particular several kinds of sessile ciliates: solitary and colonial vorticellids as well as folliculinids; spirorbid and other serpulid polychaetes; and, in smaller numbers, free-living loxosomatid kamptozoans (entoprocts). Especially remarkable was the repeated finding of this same assemblage, though not necessarily represented by the same species, together with placozoans, at a variety of sites extending across the tropical Pacific and into the Caribbean Sea. Occasionally, the typical assemblage would be present except for placozoans; even more rarely, placozoans were found in the absence of their usual associates.

The association may be roughly illustrated by two semi-quantitative vertical transects (Table 1). At an exposed, wave-swept coral pinnacle, Tripod Reef, near CRI Madang, Papua New Guinea, slides were placed at intervals from near the surface down to 20 m in depth. Along this transect, placozoans were found only at the three deepest stations, 16–20 m, largely below the influence of wave-surge, and the associated microcommunity displayed the same distribution. In another vertical transect in a quiet part of Opunohu Bay, Moorea, French Polynesia, placozoans and the associated microcommunity were present in comparable abundance throughout the range in depth sampled, down to 16 m (Table 1).

On a smaller scale, the association between serpulids and placozoans, was seen even in their distribution on each single glass slide. For example, in Madang, Papua New Guinea, data for placozoans (see section Substrate orientation, earlier) paralleled that for small spirorbids: on horizontal slides, the number of spirorbids (diameter 200–500 μm) was significantly greater on lower versus upper surfaces (mean/slide ± SD was 11.4 ± 4.1 for lower surfaces, 2.9 ± 1.6 for upper surfaces for a set of 9 slides, t-test, \( P < 0.001 \)).

**Diet**

Placozoans grew and multiplied on glass slides bearing a varied assemblage of small organisms that had settled on slides suspended in the sea; typically, we were unable to discern what they were eating. In Madang, Papua New Guinea, some slides that bore an abundant film of green algae supported unusually large placozoans. In Western Samoa, conspicuous feeding tracks were visible in the thin, red, algal film growing on aquarium glass, and at the end of each track was a pink placozoan.

**Encounters with potential predators and other organisms**

Despite the diversity of protists and animals occurring together with placozoans on the glass slides, and numerous interactions, not a single instance of predation was seen, either on or by placozoans. In several cases, however, potential predators were observed to recoil after contact with placozoans or reject them as food. At Orpheus Island, Great Barrier Reef, Australia, a small snail was watched as it headed straight for a placozoan, clearing a swath with its radula as it went. Just before reaching the placozoan, the snail extended one tentacle anteriorly, touched the placozoan, recoiled abruptly, turned, and proceeded in another direction.

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| Plac = placozoans; Vort = colonial vorticellids (Ciliata); Foll = folliculinids (Ciliata); Spir = spirorbids (Polychaeta). (-) denotes absence; (+, ++, ++++) denotes increasing abundance. |
Similarly, at Madang, Papua New Guinea, a small rhabdocoel flatworm crawling on a slide among placozoans was observed contacting a placozoan, recoiling vigorously, and changing its direction of movement only to encounter another placozoan and repeat this behavior several times. A swimming placozoan caught in the feeding current of a small sabellid tubeworm was passed down the food-groove to the mouth, where it was rejected, flicked out into the water, and then caught again; this process was repeated six times, until the placozoan finally contacted the substrate, adhered, and crept away, apparently unharmed. Various ciliates, tiny nematodes, and other small animals sometimes crawled around or under placozoans without displaying any reaction or evoking any visible response from the placozoans. Ciliates were observed to gather around wounded or degenerating placozoans (Pearse 1989).

In none of these instances did the other organisms appear to be harmed by the placozoans. In contrast, in laboratory trials carried out by L. Buss and colleagues (personal communication, 2007), placozoans that were dropped onto the tentacles of three species of hydroids caused paralysis. After transferring the placozoans from the tentacles to the mouth, the polyp became immobile and unresponsive. By 24 h after contact with a single placozoan, some polyps had recovered, but exposing a polyp to placozoans two days in a row was fatal. Only small placozoans fed to large polyps were ever swallowed; most placozoans, or fragments of them, were able to crawl away.

Remarks: relations to other organisms

Associated biota

Studies of the assemblage of microbiota that includes placozoans share many common problems, a major one being the difficulty of determining the natural substrates of these organisms that we see settling together on glass slides. Attempts to examine preferred placozoan substrates by collecting seaweeds, corals, and other possible habitats, and placing glass slides among them, typically failed because equal numbers of placozoans settled in the no-substrate controls, an early hint of the presence of placozoans in the water. Minute, solitary loxosomatids were thought to live exclusively as commensals on other organisms, almost certainly because they were observed only incidentally by biologists whose interest was in their hosts. Using glass slides as settling plates, however, Isotto (2003 and references therein) has described numerous new species of free-living loxosomatids, and in the course of his sampling has, not coincidentally, also captured placozoans (T. Isotto, personal communication 2004).

No specific interactions were ever observed by V.B.P. between placozoans and the members of the associated microcommunity, and their relationship must be presumed to consist of no more than a tendency to settle under a shared set of conditions, although it remains possible that the settlement of one positively influences that of another. Their patterns of abundance, similar among the taxa but differing between vertical transects at two quite different sites (Table 1), appear most easily explained by their common response to differences in wave action (see section Associated biota, earlier).

Diet

In the laboratory, placozoans grow on cryptomonads, other algal unicells, or heat-killed nauplii of Artemia (Grell 1983; Grell and López-Ochoterena 1988) or on commercial food for aquarium fish (Maruyama 2004, personal communication). Kept on clean glass slides versus biofilmed slides, they grew significantly only on the latter (Pearse 1989). They have been seen to capture and eat ciliates in a culture dish (Klausler 1982). Wild placozoans are probably opportunistic grazers and scavengers on organic detritus and on algae and bacteria in biofilms covering a diversity of substrates.

Potential predators

The sole published report of predation on placozoans involved the opisthobranch Rhodope (Riedl 1959). K.J. Marschall told V.B.P. of seeing a small nemertean apparently preying on placozoans in an aquarium in Western Samoa (personal communication, 1973). We have never observed any animal to prey upon healthy specimens; on the contrary, we report several instances of potential predators reacting to contact with placozoans as they would to a strongly noxious substance. We suggest that the most logical site of such a defensive substance is the so-called shiny spheres that lie throughout the upper epithelium (see, e.g., Fig. 2 of Rassat and Ruthmann 1979). The high lipid content of these osmiophilic vesicles has been confirmed; such lipid deposits normally serve for nutrient storage, and in placozoans they do become more numerous when the animals are well fed (Rassat and Ruthmann 1979). A more likely and better protected location for nutrient storage, however, would be in the lower, nutritive epithelium. Instead, these large lipid-rich vesicles are situated at the surface of the animal between the upper epithelial cells, where they are freely exposed to contact and appear to be readily expelled to the outside.
Thus, it seems more plausible that the large investment represented by the abundant shiny spheres of placozoans serves for the defense of these soft-bodied morsels. Some support for this idea has been provided by the experiments of L. Buss and colleagues (personal communication, 2007), who fed placozoans to hydroids: although intact placozoans were not usually swallowed, those previously induced to expel many of their shiny spheres were ingested far more often, and polyps that swallowed placozoans subsequently died. Analysis of the chemical contents of the shiny spheres awaits advances in microchemistry. The developmental history of these vesicles, which appear to lie within anucleate cells (Grell and Ruthmann 1991), also remains to be investigated.

**Diversity**

**Collection and sequencing**

For genetic analysis, placozoans were sampled by washing animals from the wall of a tank (containing only local reef fishes and local seawater) at Lizard Island Research Station, Australia (n = 17) and by settlement on glass slides in a holding-tank in the Tuna Research and Conservation Center at Hopkins Marine Station of Stanford University on Monterey Bay, California (n = 2). The most likely origin of the California specimens is Southern California or Northern Baja California, as the tank contained Bluefin Tuna (*Thunnus thynnus*) caught in that region. However, this tank is maintained on Central California (Monterey Bay) seawater (filtered through sand filters of nominal pore size ~20 μm), and has received in the past water from other tanks in the facility which held fishes from Hawaii; both of these eastern Pacific regions are thus other possible sources of these specimens.

Single placozoans were first transferred from the slides, through a wash of clean seawater, into 1 ml microcentrifuge tubes. Because these animals are so small, thin, and fragile, as well as extremely sticky (the species name *adhaerens* is indeed apt), they were first loosened from the glass using gentle jets from a Pasteur pipette, then drawn into the pipette and quickly expelled into a clean depression slide or watch glass, followed by a second transfer into a microcentrifuge tube, in a minimal amount of seawater. About 1 ml of ethanol was added to the tube, which was then closed and sealed with Parafilm®. Animals along with the residual precipitated salt from the seawater were pelleted by centrifugation; the pellet was air-dried at room temperature and 50 μl of sterile ultrapure water containing 5% Chelex® 100 (sodium form, Sigma–Aldrich, http://www.sigmaaldrich.com) and 20 μl of 10 mg/ml proteinase K (Sigma–Aldrich, http://www.sigmaaldrich.com) were added. Alternatively, the animals were directly transferred into a tube containing the Chelex® solution and the proteinase K.
All tubes were then treated as described by Voigt et al. (2004). They were stored at −20°C and 1–3 μl of supernatant was used in PCR to amplify a fragment of 16S rDNA [primers: 5'-GCCTGCCCARTGTTGTA-3' ; rv-5'-GGTCGCA AACATCGTCA-3'; program: 95°/5 min, 37 × (95°C/30 s; 50°C/30 s; 72°C/1 min)]. The PCR products were sequenced in both directions with our PCR primers, using chemistry and equipment as described by Dohrmann et al. (2006). For the samples from Lizard Island, cycle sequencing reactions were modified to sequence over a G–C rich partition with stable secondary structure by adding DMSO (5%) and 0.1 ml BIOTAQTM-DNA-polymerase (5 u/μl; Bioline, http://www.bioline.com) and by an initial denaturation step (96°C/10 min). New sequences have been submitted to GenBank (http://www.ncbi.nlm.nih.gov; accession numbers LIZ: EF421454, H11: EF421455).

Molecular analysis and haplotype distribution

We have chosen a fragment of 16S rDNA as a molecular marker for our phylogenetic analyses, including additional sequences from GenBank (for accession numbers, see caption of Fig. 4A). This marker is known to yield good phylogenetic resolution, but also shows considerable polymorphism in length among haplotypes (Voigt et al. 2004), hampering alignment of all sequences in three regions within our analysis. Because some differences between haplotypes (e.g., H1–H2; H7–H8) appear in these regions, we locally blocked the alignment to include the maximum of information by introducing gaps for nonalignable haplotypes. With this method, otherwise unsupported or poorly supported relationships of similar, yet not identical haplotypes could be resolved (H6, H7, H8), while the tree topology was otherwise unaffected. In all phylogenetic analyses, we applied a GTR+G model of nucleotide evolution suggested by the Akaike information criterion with the software modeltest 3.7 (Posada and Crandall 1998); likelihood values were calculated in PAUP* 4.0b10 (Swofford 1998). A maximum likelihood (ML) analysis including bootstrap resampling was carried out with PHYML (Guindon and Gascuel 2003). In addition, we conducted a Bayesian analysis with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), performing a Markov chain Monte Carlo analysis with two runs (eight chains each) for 10,000,000 generations, with sample frequency set to 100 and temperature for the heated chains set to 0.2. From the sampled trees, we discarded the first 25% (25,000 trees) as burn-in. In the absence of a suitable outgroup that would result in a well-supported rooting with our marker (Voigt et al. 2004), midpoint rooting was applied to display the tree in Fig. 4A.

Sequences from the two Californian samples were identical to each other, and sequences from the 17 samples from Lizard Island were also identical to each other. In the latter, a stretch in the middle of the fragment, flanked by several Cs on the one side and several Gs on the other side, caused problems in sequencing similar to those reported by Signorovitch et al. (2006). Even after applying the modified cycle sequencing protocol, sequence reads from both sides overlapped only slightly in the critical region. The new Australian haplotype is identical to the H9 haplotype, reported (as H4-2) from Bermuda by Signorovitch et al. (2006), in parts where information exists for H9. Our sequence, however, covers a larger part of the gene including the critical region not available for the H9 haplotype; thus, it is not clear if our new sequence is identical or shows base exchanges in these regions. Therefore, we do not use the label H9 for this haplotype, but refer to it as LIZ (Fig. 4) to avoid the introduction of another, possibly redundant haplotype number. In contrast, from the two Californian samples, we can report a new, relatively divergent haplotype, designated H11.

Our phylogenetic analyses yielded trees that differ only in the occurrence of a polytomy in the Bayesian analysis (clade H9/H10/LIZ). All other clades find high support by bootstrap or clade credibility values (Fig. 4A). According to our resulting trees and the applied rooting, the newly reported haplotype H11 is the sister group to the clade (H5, H4, H9, H10, LIZ), with high support.

Until distinguishing morphological characters are discovered, placozoan lineages can be recognized only by DNA analysis with various sequence markers. The 16S rDNA fragment we used identifies at least 11 different haplotypes, including the new one reported here. Our scant knowledge about the geographic distribution of placozoan haplotypes is summarized in Fig. 4B. The known richness of haplotypes in a region is undoubtedly correlated with sampling effort: the Caribbean and Sargasso Sea, where sampling for molecular work has been most intensive (especially by Signorovitch et al. 2006), together currently have the highest number of haplotypes (nine of the 11 known). Thus, we predict that more sampling will yield not only new haplotypes but also an increase in the documented distributions of haplotypes. Clearly, genetic
distances in Placozoa are not at all correlated with geographic distances. Several (in the case of LIZ, presumably) identical haplotypes exhibit a large distributional range: H1: Caribbean, Central Pacific (Hawaii), Red Sea; H2: Sargasso Sea (Bermuda), Caribbean, Central Pacific (Hawaii), Mediterranean; H4: Sargasso Sea, Caribbean, Eastern Pacific (Panama), Central Pacific (Hawaii) and H6: Caribbean, Eastern Pacific (Panama), Central Pacific (Hawaii); H8: Caribbean, Eastern Pacific (Panama), Central Pacific (Guam); H9/LIZ: Sargasso Sea, Western Pacific (Australia). At the same time, distantly related haplotypes can occur within the same region, as in the Caribbean (seven haplotypes), on the Pacific coast of Panama (four haplotypes), in Bermuda (four haplotypes), in Hawaii (four haplotypes), and in the Mediterranean (two haplotypes). Even the hints of geographical pattern mentioned by Voigt et al. (2004) are, with additional data, now almost entirely moot. Although Signorovitch et al. (2006) in their extensive sampling did not find haplotypes H5 and H11 in the Caribbean/Sargasso, it appears increasingly likely that all placozoan lineages may be distributed worldwide. These are minute animals with a high capacity for dispersal, easily intermingled through natural agencies—being carried in currents, on the surfaces of other animals, or on floating objects. So we may be observing species whose diversification depends on isolation in microhabitats: as mentioned, sympatry of placozoans in the plankton does not necessarily apply to the sexually reproducing forms that we assume are on the benthos. The swarvers observed by Thiemann and Ruthmann (1991) lived only about 1 week in culture before settling; however, benthic-phase placozoans probably survive for longer periods on floating debris.

An alternative, perhaps more likely hypothesis for the wide distribution of lineages is transfer by human activities, which have probably at least accelerated the mixing of lineages and contributed to the global patchwork of distributions we see today. For example, in Panama, where identical haplotypes occur on either side of the isthmus, gene flow is most parsimoniously explained by transport through the Panama Canal in ballast water, as placozoans’ sensitivity to reduced salinities would otherwise probably prevent their surviving passage through the Canal’s lakes. The ability of placozoans to proliferate rapidly through fission, fragmentation, and budding (e.g., Schulze 1891; Grell and Ruthmann 1991) would further facilitate invasive events. Thus, even if placozoan lineages were once geographically isolated, it may now be impossible to uncover their history.

**Taxonomic diversity**

The taxonomic status of clades discovered by DNA analysis has not yet been addressed. The extremely simple organization of placozoans offers few specific traits to systematists, and to date, we lack morphological or other characters that can be used to distinguish placozoan lineages. Potentially useful characters include the birefringent granules, which may or may not be present in placozoans (Pearse et al. 1994), and the conditions for culture, which differ for specimens from various sources. For example, Grell and López-Ochoterena (1988) cultured placozoans from Quintana Roo, Mexico on a green alga instead of the cryptomonad used for the Red Sea strain, and Grell (personal communication) was unable to culture a strain from the Mediterranean. Electron microscopy may provide ultrastructural characters; to date, only Grell’s strain from the Red Sea has been examined in any detail, although hints of differences have been suggested by Ivanov et al. (1980) and by Klauser and Ruppert (1981).

The question remains, how such cryptic, yet distinct, taxa are to be handled by systematists, because all evidence indicates that at least the more distant clades in Fig. 4A are indeed different biological species, if not genera or higher-level taxa; the level of divergence between placozoan lineages in the nuclear 18S rRNA gene is similar to or higher than the levels reported between species of other diploblast phyla, or even between genera or families (Voigt et al. 2004). Moreover, Signorovitch et al. (2007) have published three additional mt genomes from placozoans having the 16S rDNA haplotypes H3, H4, and H8 (Signorovitch et al. 2006). Compared to the already published mt genome of a H1 haplotype (Dellaporta et al. 2006), several gene rearrangements were observed (e.g., large inversions), accompanied by remarkable divergence in length—the mt genome size varies from 32.7 to 43.1 kb (Signorovitch et al. 2007). Such rearrangements of genes and extraordinary differences in length in the mt genome have not been reported within any metazoan species. All these findings of unexpected genetic diversity support the view that Placozoa comprises at least several deep clades that are reproductively isolated and highly divergent, comparable to species, if not genera or higher taxa of other phyla (for evidence of sexual reproduction, see Grell 1972; Signorovitch et al. 2005).

Given the lack of both biogeography and other distinguishing characters, the eventual description and naming of placozoan species will challenge the usual taxonomic conventions. Type locality will often
be meaningless, and place-based names equally so. The name *Trichoplax adhaerens* Schulze, 1883 might best attach, not to a specimen from the Adriatic Sea, but to the durable laboratory strain from the Red Sea, which K.G. Grell long ago established in culture and which survives today. This strain continues as the basis of most of our knowledge of the phylum Placozoa Grell (1971), including all of what we know about fine structure.

**Life history**

Regarded by their discoverer, F.E. Schulze, as solely benthic organisms, placozoans inspired O. Bütschli (1884) to propose the placula theory of metazoan origins from a holobenthic ancestor. Always a minority view, in the shadow of Haeckel’s powerful tradition, the idea of a benthic ancestor has most recently been reproposed and championed by Degnan and Degnan (2006), now from the perspective of sponge development and the well-described pelagobenthic life histories of sponges. Framing the processes of gametogenesis, embryogenesis, and metamorphosis as the essence of a pelagobenthic cycle, these authors argue persuasively that a pelagic phase would arise simply and inevitably from a benthic adult ancestor, practicing sex, whereas the reverse requires multiple reinvention of a sexual benthic phase. If only Bütschli had known what we now know, his placula might not have been so easily dismissed: placozoans have sex (Grell 1972; Signorovitch et al. 2005) and they also have a pelagic phase that is abundant in the water column in warm seas around the world.

The incompletely known life history of placozoans thus presents a significant gap in our understanding, not only of *Trichoplax*, but of the framework of metazoan history. For placozoans, not only do gametogenesis, embryogenesis, and metamorphosis remain undescribed, but also meiosis, sperm, and fertilization. Although field studies have revealed tropical waters teeming with pelagic placozoans, the nature of what is settling on glass slides remains a puzzle: small fragments of the benthic phase, or budded swarmers (Thiemann and Ruthmann 1991), or sexually produced larvae? Like most invertebrate larvae, they require the development of a biofilm on glass slides in order to settle (Pearse 1989), but this fact alone cannot begin to distinguish between possible larvae and various asexual products. Free-swimming placozoans of a variety of forms have been observed (Fig. 5); although the size of these seems most consistent with fragments, the shape has varied from gastrula-like to flat, very much as Thiemann and Ruthmann (1991) described and illustrated hollow swarmers preparing to settle. If V.B.P. was observing swarmers, then this stage is not an artifact of laboratory culture, an alternative judiciously discussed by Grell and Ruthmann (1991). The diameter of the smallest individuals seen on slides is ~120μm, similar to that reported for both eggs (Grell 1972) and for swarmers (Thiemann and Ruthmann 1991). Such small specimens are typically circular in outline initially, grow rapidly, and can begin to fission within a few days (Fig. 6). Eggs were never observed by V.B.P. or by A.Y. Signorovitch (personal communication, 2006) in placozoans collected on slides from the field; the field conditions required for sexual reproduction are completely unknown.

We may be able to induce sex reliably in the laboratory, however, by discovering and reproducing the conditions under which production of eggs occurs (Grell 1972), e.g., by reducing food or increasing cadmium (or other heavy metals), as has been found effective in hydrozoans such as *Laomedea* (as *Campanularia*) *flexuosa* (Stebbing 1980) and *Eleutheria dichotoma* (Schierwater and Hadrys 1998). We might then uncover suitable conditions for development, which so far has invariably ceased after a few cleavages (Grell 1972; Signorovitch et al. 2005). Given that no typical animal sperm have ever been documented in placozoans, the eggs so far observed may have been unfertilized or failed to undergo maturation; perhaps the Red Sea strain in
culture is female, as Grell once suggested (personal communication, 1992). Other missing requirements might be straightforward modifications of culture conditions, or might conceivably be as complex as a host organism in which a parasitic placozoan phase normally completes its development. This speculative possibility has been suggested by the regularity with which placozoans turn up in fish mariculture facilities (observations by V.B.P.; Tomassetti et al. 2005). As parasitism often leads to secondary reduction, it could explain the extreme simplicity of placozoans. The coincidence of placozoans with fishes, however, is far more likely a simple result of enrichment in a protected environment. Another remote possibility is that the evolution of placozoans involved mutations in their equivalent of stem cells (see Fig. 5 in Schierwater 2005), comparable to interstitial cells or archeocytes, simplifying their histology and modifying other aspects of normal development. While these ideas are purely speculative, they are put forward here to challenge the assumption that the normal cellular processes of sexual reproduction known for almost all other animals must necessarily occur in the familiar benthic phase of placozoans and have somehow merely been overlooked.

Finally, even the benthic development of placozoans is still incompletely documented. The smallest individuals are roughly circular, and they take on increasingly irregular shapes as they grow, from somewhat ameba-like (Fig. 1) to the long stringy forms (see, e.g., Fig. 1 of Schulze 1891) commonly seen in older laboratory cultures or after placozoans have been growing and multiplying on aquarium glass for some time. The occasional development of a ring-shaped form with a hole in the center has so far been recorded, to our knowledge, only in Western Samoa. As documented in film by K.J. Marschall 1970, the ring subsequently breaks through, producing a long, stringy shape, and fragments generated from both free ends crawl away as small individuals. A ring-shaped placozoan observed by V.B.P. in 1989 in W. Samoa, however, reverted to a normal form by closing up the central hole. Yet to be understood is whether the plastic spectrum of planar shapes, and of asexual proliferation (Thiemann and Ruthmann 1991), reflects developmental stages or environmental conditions or taxonomic diversity.

**Concluding remarks**

Placozoans, like sponges, lack a digestive cavity and nervous system, and like most sponges (except homoscleromorphs, see e.g., Boury-Esnault et al. 2003; Nichols et al. 2006), also lack a basal lamina. Yet the body plans and ways of life of the adults of these two groups could hardly be more different: the sessile sponges grow to large sizes and filter-feed, whereas the nearly microscopic placozoans wander freely over the benthos as active grazers or scavengers. In this respect, placozoans are more similar to acoels, which likewise lack a basal lamina.
and digestive cavity (Rieger et al. 1991), and to larval sponges. Because placozoans are such appealing models as ancestral metazoans, we are naturally avid to know where they fit into animal phylogeny. At the same time, in order to understand them as functional, living organisms, to be compared with others and understood in an ecological context, we need to learn more of the facts of their basic biology. We hope that the observations collected here might stimulate others to seek further understanding of placozoans in their natural haunts.

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O.V. carried out field work at: Lizard Island Research Station, Australian Museum, Great Barrier Reef, Northeastern Australia.

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