

A centuries old problem in nephtheid taxonomy approached using DNA data (Coelenterata: Alcyonacea)

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Key words: soft corals, Octocorallia, Nephtheidae, *Dendronephthya*, *Litophyton*, *Neospongodes*, *Nephthea*, *Stereonephthya*, taxonomy, internal transcribed spacer I and II, Indo-Pacific

Abstract

The current centuries old classification of the family Nephthidae is still mostly based on colony morphology. In this family the Indo-Pacific genera *Litophyton*, *Nephthea*, *Dendronephthya* and *Stereonephthya*, and the Atlantic genus *Neospongodes* form a complex mix of closely related, poorly described species which cannot be recognized using only colony morphology. Attempts with the more modern approach of comparing skeleton composition (sclerites) have been only partly successful because of the extreme variation of sclerite forms present in these genera. The genus *Chromonephthea* Van Ofwegen, 2005, introduced for several species previously assigned to *Dendronephthya*, *Nephthea* and *Stereonephthya*, was established with sclerite morphology, but the true generic status of the majority of the nominal species of these genera remained unresolved. In an attempt to clarify the phylogenetic relationships between *Litophyton*, *Nephthea*, *Stereonephthya* and *Chromonephthea* fourteen specimens, unidentified but certainly belonging to these genera, have been used in molecular analyses. All analyses supported two clades, which could be related to the shape of the sclerites present in the polyp stalks. One clade contained the specimens with characters for *Stereonephthya* along with the *Chromonephthea* specimen as a sister group. The other clade had a 'true' *Nephthea* and *Litophyton* together with the specimens that could not be placed in any particular genus using the old classification criteria. The consequences of these results for nephtheid classification are discussed.

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Introduction

After the Alcyoniidae Lamouroux, 1812, the family Nephthidae Gray, 1862, is the most common soft coral family in tropical Indo-Pacific waters regarding numbers of genera and species. The often colourful colonies of nephtheid species can form large aggregates by asexual reproduction. Most species have tree- or bush-like colonies and can be found from low tide to the abyss.

In the tropical Indo-Pacific twelve nominal genera are currently assigned to the Nephthidae: *Litophyton* Forskål, 1775; *Nephthea* Audouin, 1828; *Lemnalia* Gray, 1868; *Capnella* Gray, 1869; *Scleronephthya* Studer, 1887; *Dendronephthya* Kükenthal, 1905; *Stereonephthya* Kükenthal, 1905; *Paralemnalia* Kükenthal, 1913; *Umbellulifera* Thomson and Dean, 1931; *Leptophyton* Van Ofwegen and Schleyer, 1997; *Pacifiphyton* Williams, 1997; and *Chromonephthea* Van Ofwegen, 2005. On the basis of a recent molecular phylogenetic study, McFadden *et al.* (2006) suggest that less genera are involved, because in their analysis *Lemnalia* and *Paralemnalia* form a clade with the alcyoniid *Rhytisma* Alderslade, 2000, and *Capnella* a clade with the paraclyoniid genera *Paralcyonium* Milne Edwards and Haime, 1850, and *Studeriotis* Thomson and Simpson, 1909.

In the Atlantic, the Antarctic and the colder waters of the Pacific an additional seven genera have been described in the nephtheids, one of which, viz. the

tropical Atlantic *Neospongodes* Kükenthal, 1903, resembles the Indo-Pacific *Stereonephthya* regarding sclerites and colony form.

To date, *Litophyton*, *Nephthea*, *Dendronephthya* and *Stereonephthya* form a complex mix of closely related but poorly described species. Hence, around 1990, the first author started a morphological revision of the genera *Litophyton*, *Nephthea*, and *Stereonephthya*, also including a number of species assigned to *Dendronephthya* and the monotypic Atlantic genus *Neospongodes*. A first outcome of this study was the description of the genus *Chromonephthya* Van Ofwegen, 2005, for several species previously assigned to *Dendronephthya*, *Nephthea* and *Stereonephthya*.

In an attempt to clarify the phylogenetic relationships between some species that are actually classified with *Litophyton*, *Nephthea*, *Stereonephthya*, and *Chromonephthya*, a few molecular markers previously tested with octocorals were used. By doing so the status of the morphological characters nowadays employed in nephtheids can be compared with the resulting phylogeny.

Short history of the genera *Litophyton*, *Nephthea*, *Stereonephthya*, *Dendronephthya* and *Neospongodes*

Forskål (1775) described the first Indo-Pacific nephtheid, *Litophyton arboreum*, from an unknown locality in the Red Sea. According to modern standards his description was inadequate. The material on which he based his description is lost.

Andouin (1828), referring to plates published by Savigny (1817), erected two additional nephtheid genera from the Red Sea, viz. *Ammothea* for *A. virescens*, and *Nephthea*, for *N. chabrolii*. As far as known, the material on which these two taxa were based has been also lost.

After this a period followed in which numerous nominal genera and subgenera were introduced or synonymized. Many new species were described, and others were moved from genus to genus.

Kükenthal (1903, 1905) made a revision of the nephtheids in which he abandoned *Ammothea* and described three more genera, *Neospongodes* Kükenthal, 1903, *Dendronephthya* Kükenthal, 1905, and *Stereonephthya* Kükenthal, 1905, all of which without assigning a type species. He presented the following classification, which is still used by most authors (for details of terminology, see Bayer *et al.* (1983)):

Polyps without a supporting bundle, polyps in catkins	<i>Litophyton</i>
Polyps with a supporting bundle, polyps in catkins	<i>Nephthea</i>
Polyps with a supporting bundle, polyps in bundles or alone, no axis ...	<i>Stereonephthya</i>
Polyps with a supporting bundle, polyps in bundles or alone, internal axis of spindles	<i>Neospongodes</i>
Polyps with a supporting bundle, polyps in bundles	<i>Dendronephthya</i>

Dendronephthya is subdivided into three groups:

Sparsely arborescent branching colonies with slender ramifications, and polyps in small insignificant bundles	Divaricata-group
Close short branching colonies where the polyp bundles are assembled, forming large, distinctly rounded bunches	Glomerata-group
Polyp bundles closely arranged at the same level on the ends of the twigs, forming numerous umbrella-like crowns. Several umbrella-like sections may combine to form hemispheres	Umbellata-group

Later on, Utinomi (1954) designated *Nephthea savignyi* Ehrenberg, 1834, as the type species of *Dendronephthya* and *Stereonephthya papyracea* Kükenthal, 1905, as the type species of *Stereonephthya*. He characterized *Stereonephthya* as follows: (1) supporting bundles of the polyps well developed, each with a strong projecting tip, (2) ventral side of the polyp stalk with small tiny rods, (3) polyp heads usually hanging down from their stalks, like a campanulate flower, (4) dorsal points strong, ventral points poorly supported or lacking.

Finally, Verseveldt (1983) re-examined *Neospongodes atlantica* and *N. bahiensis*, the two species Kükenthal (1903) referred to *Neospongodes*. Verseveldt designated a lectotype for *N. atlantica* and synonymized *N. bahiensis* with *N. atlantica*. He changed the diagnosis of *Neospongodes* to “Tree-like, very flabby nephtheids, with a slender stalk and stem. Polyps isolated and scattered on thin branches and twigs, with supporting bundles”.

The modern classification

Re-examination of most of the type specimens of the species described in *Litophyton*, *Nephthea* and *Stereonephthya* showed that the characters used by Kükenthal (1903, 1905) may be problematic:

- 1) The presence of a supporting bundle is not as straightforward a character as suggested by Kükenthal (1905). In the re-examined holotype of *Litophyton acutifolium* Kükenthal, 1913, most polyps are without a trace of such a bundle, as diagnosed for *Litophyton*, but a few polyps clearly show one. Moreover, specimens identified by Verseveldt (1966) as *L. arboreum* Forskål, 1775, have a supporting bundle, though Verseveldt reported it as missing. Re-examination of the polyps of this species brought many small, irregularly arranged rods to light, but quite a few polyps show larger sclerites at the dorsal side, which in our opinion is a clear supporting bundle.
- 2) All nephtheids have an accumulation of polyps on the end of their branches. Species of *Dendronephthya* with polyps united in small bundles clearly differ from other nephtheids with respect to this character, but the presence or absence of catkins seems to rely on the amount of contraction after collection. Species with small polyps with less sclerites simply contract more easily to form catkins.
- 3) Polyps in bundles or single. This character is very difficult to check in *Stereonephthya* and species of the Divaricata group of *Dendronephthya*, as both have very crowded polyps at the end of the branches. Using the presence of small rods in the polyp stalk, as suggested by Utinomi (1954), the type species of *Dendronephthya*, *D. savignyi* (Ehrenberg, 1834) falls within *Stereonephthya*.
- 4) The presence of small rods in the polyp stalk in species of *Stereonephthya*. Examination of *Dendronephthya* species of both the Glomerata and the Umbellata group showed that polyps never have small rods in the polyp stalk, which are present in several species of the Divaricata group. Moreover, many species assigned to *Nephthea* also show small rods in the polyp stalk.
- 5) According to Verseveldt (1983), the differences between *Neospongodes* and *Stereonephthya* are minimal, in that the latter has rigid colonies and *Neospongodes* flabby ones, which cannot be used to differentiate between genera convincingly.
- 6) Kükenthal (1903, 1905) completely ignored the sclerite characters of other parts of the colony. Using those the first author recognized *Chromonephthya* Van Ofwegen, 2005.

Short descriptions of the material used

The material used for DNA analyses is briefly characterized. Because of the above mentioned problems with the classification and the still ongoing morphological revision, the species of *Litophyton*, *Nephthea*, and *Stereonephthya* could not be identified. However, we do mention 'true' *Litophyton* and *Nephthea* following Kükenthal's classification.

- 1) RMNH Coel. 32053, MAL.05, one colony, Fauna Malesiana Maluku Expedition, 1996, Indonesia, Ambon, N coast, Manuala beach, W of Hila, 03°35'S 128°05'E, gradually sloping sandy bottom, with scattered coral heads, snorkeling and diving, depth -10 m, 7.xi.1996; 'true' *Litophyton*.

The flabby colony is 20 cm long with several stems arising from a common base (Fig. 1a). The polyps are grouped at the end of the branches, a configuration described in literature as slender catkins. Its sclerites are typical for a species of *Litophyton*. The polyps and end branches have very few sclerites (Fig. 2a). The irregularly arranged polyp sclerites are small rods, which are up to 0.07 mm long (Fig. 3a). The surface layer of the base of the colony has radiates and derivatives of these (Fig. 3b, d), and unilaterally spinose spindles with long spines (Fig. 3c). The interior of the base of the colony has spindles with sparse simple tubercles (Fig. 3e-f).

- 2) RMNH Coel. 32055, 6 colonies, Indonesia, SW Sulawesi, Spermonde Archipelago, W of Langkai Isl. (= 37 km WNW of Ujungpandang), 5°02'S 119°05'E, coral reef, scuba diving, 21 and 24.vi.1994, Buginesia Program UNHAS-NNM 1994, coll. B.W. Hoeksema; 'true' *Nephthea*.

The colony used is 10 cm long (Fig. 1b). The polyps are grouped at the end of the branches, forming conical catkins. Its sclerites are typical for a species of *Nephthea*. The polyps have a supporting bundle and eight irregular points (Fig. 2b). The supporting bundle spindles have their distal end unilaterally spinose (Fig. 4c). The spindles of the ventral points are rod-like (Fig. 4a, left), the dorsal points have unilaterally spinose spindles (Fig. 4a). The tentacles have flattened rods with a scalloped edge (Fig. 4b). Similarly shaped rods are also present at the ventral side of the polyp stalk (Fig. 2c). The surface layer of the base of the colony has radiates and derivatives of these (Fig. 4d), and unilaterally spinose spindles with long spines (Fig. 4e). The interior of the base

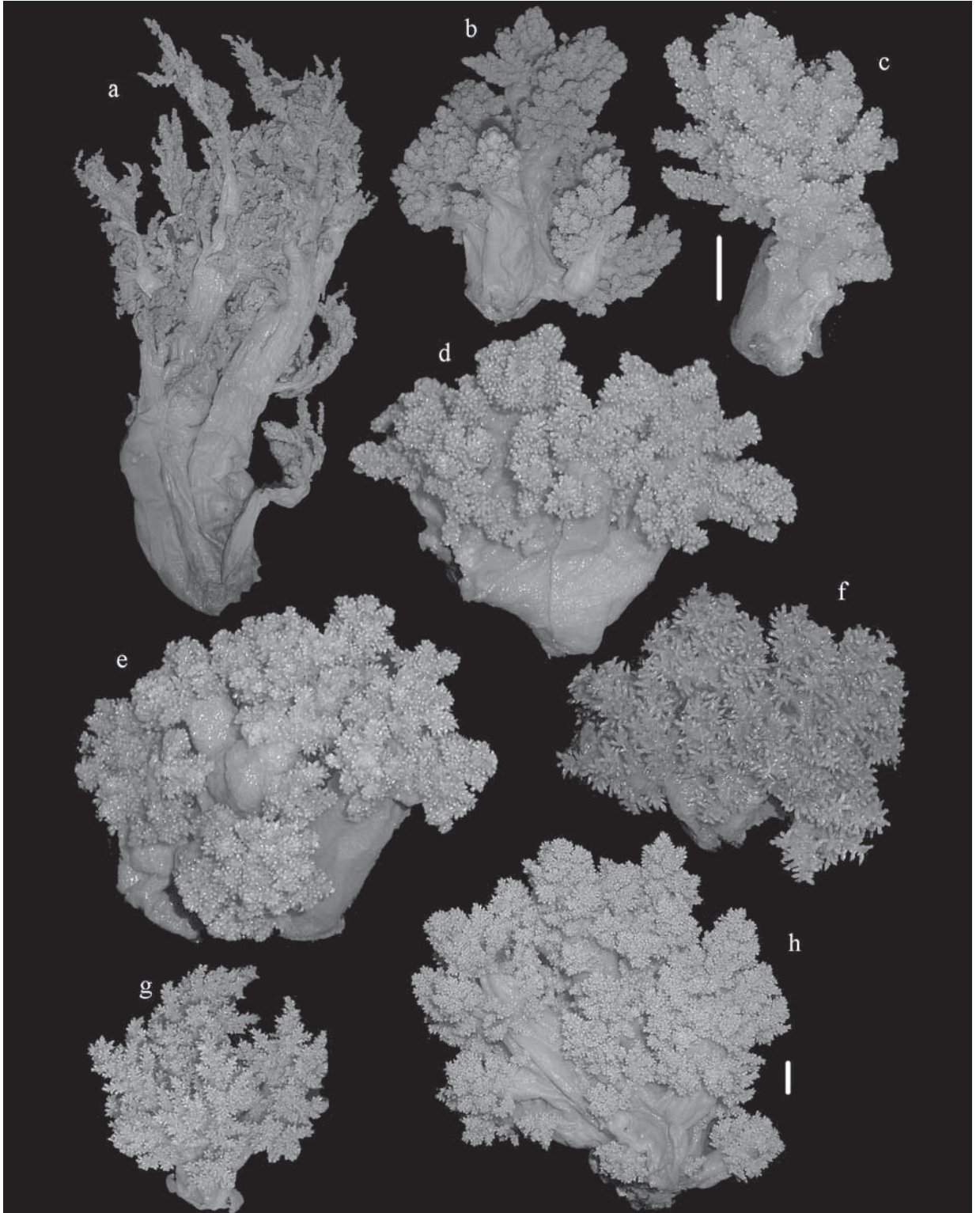


Fig. 1. Colonies; a, RMNH Coel. 32053; b, RMNH Coel. 32055; c, RMNH Coel. 34808; d, RMNH Coel. 34809; e, RMNH Coel. 34810; f, RMNH Coel. 34812; g, RMNH Coel. 34813; h, RMNH Coel. 34811. Scales 1 cm, that at c also applies to f.

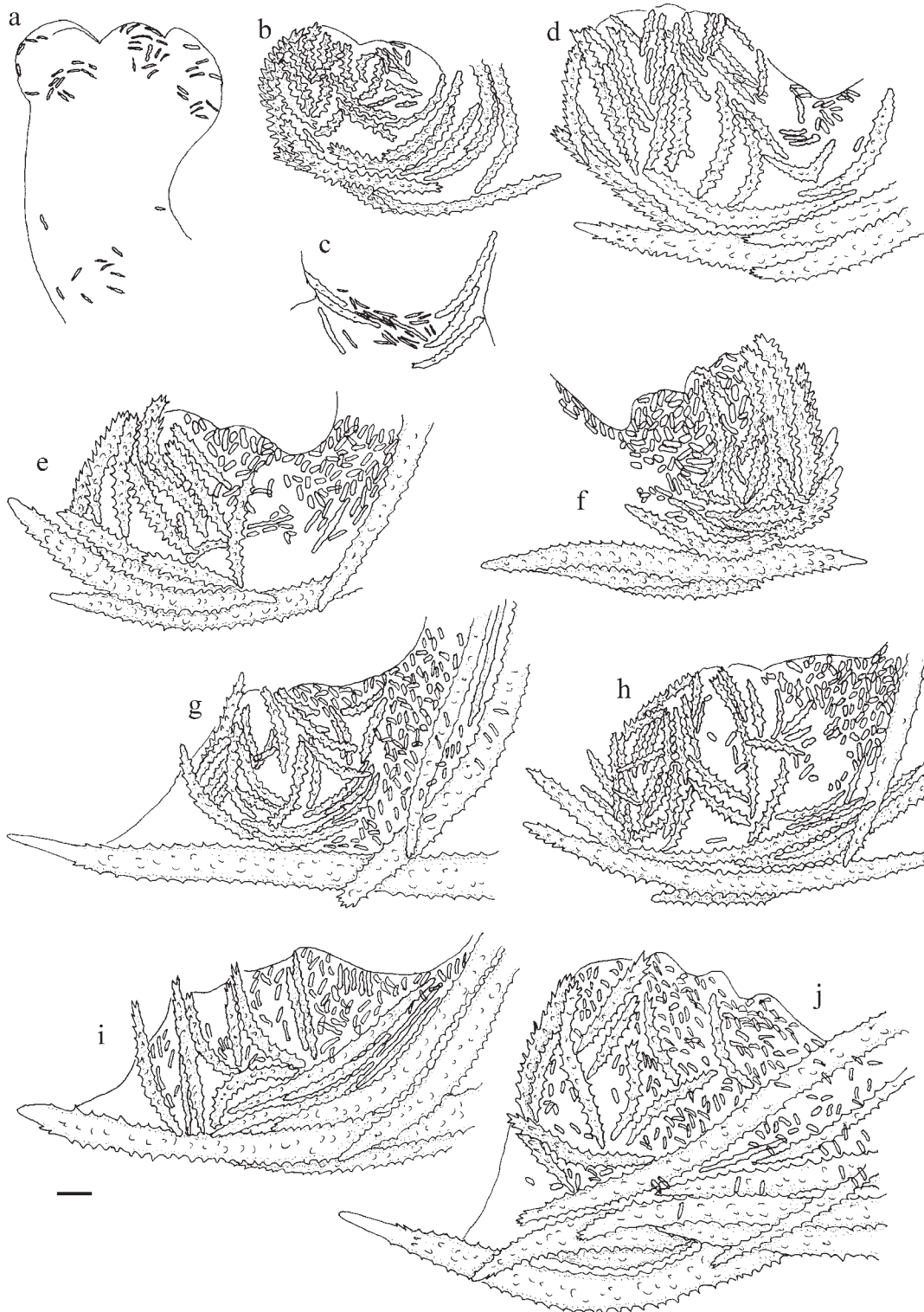


Fig. 2. Lateral views of polyps armature; a, RMNH Coel. 32053, b-c, RMNH Coel. 32055 (c, ventral side of polyp stalk); d, RMNH Coel. 34808; e-f, RMNH Coel. 34809; g, RMNH Coel. 34810; h, RMNH Coel. 34811; i, RMNH Coel. 34812; j, RMNH Coel. 34813. Scale 0.10 mm.

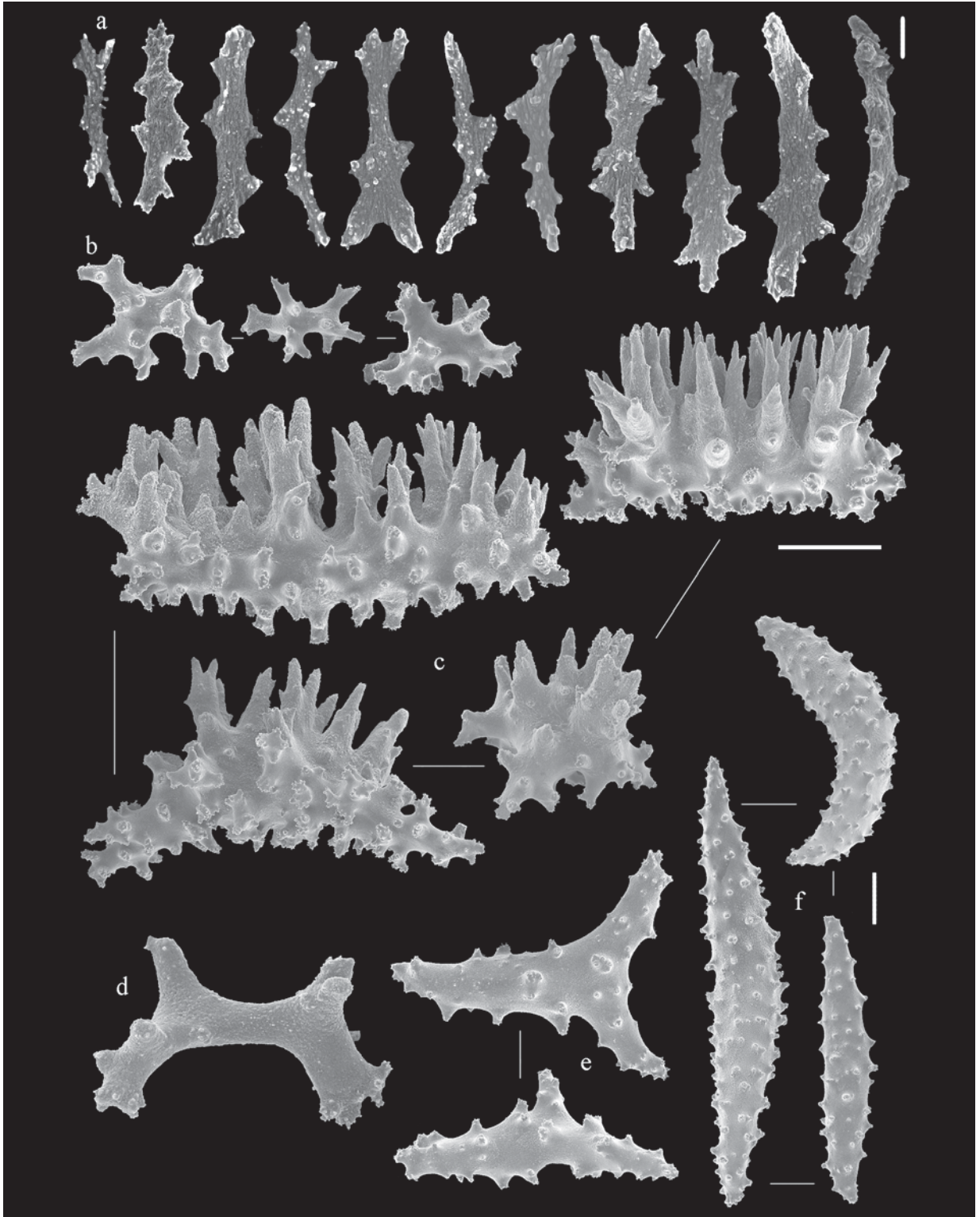


Fig. 3. RMNH Coel. 32053; a, rods of polyps; b, d, radiates, surface layer base of colony; c, unilaterally spinose spindles, surface layer base of colony; e-f, spindles, interior base of colony. Scale at a 0.01 mm, also applies to d; scale at c 0.10 mm, also applies to b, e; scale at f 0.10 mm.

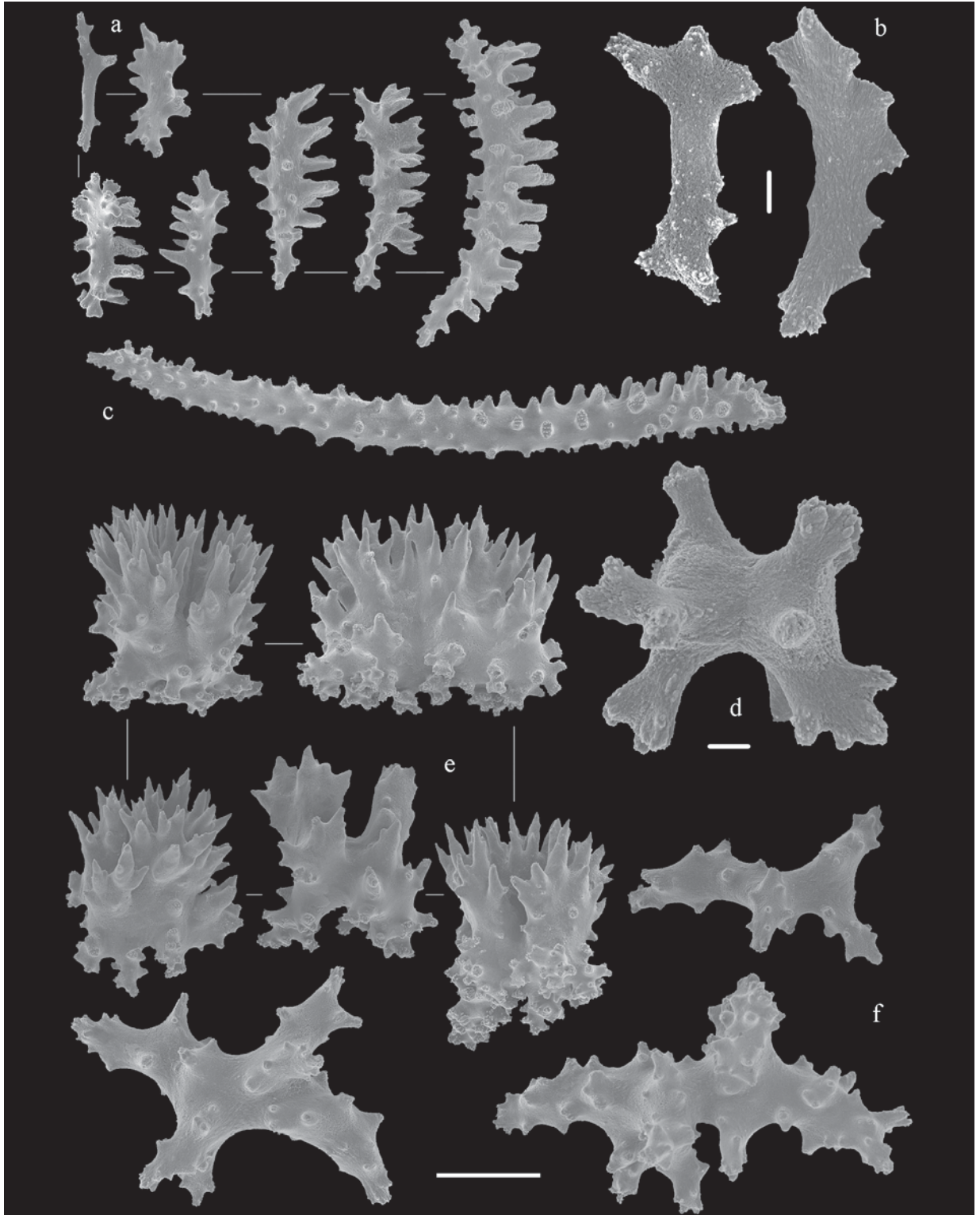


Fig. 4. RMNH Coel. 32055; a, rods and spindles of points, b, tentacular rods; c, spindle of supporting bundle; d, radiate, surface layer base of colony; e, unilaterally spinose spindles, surface layer base of colony; f, branched spindles, interior base of colony. Scale at b and d 0.01 mm, scale at f 0.10 mm, also applies to a, c, e.



Fig. 5. RMNH Coel. 34808; a, point spindles, b, tentacular rods; c, spindle of supporting bundle (part); d-e, spindles, surface layer base of colony; f, spindles, interior base of colony. Scale at b 0.01 mm, at f and e 0.10 mm; that at f also applies to a, c, d.

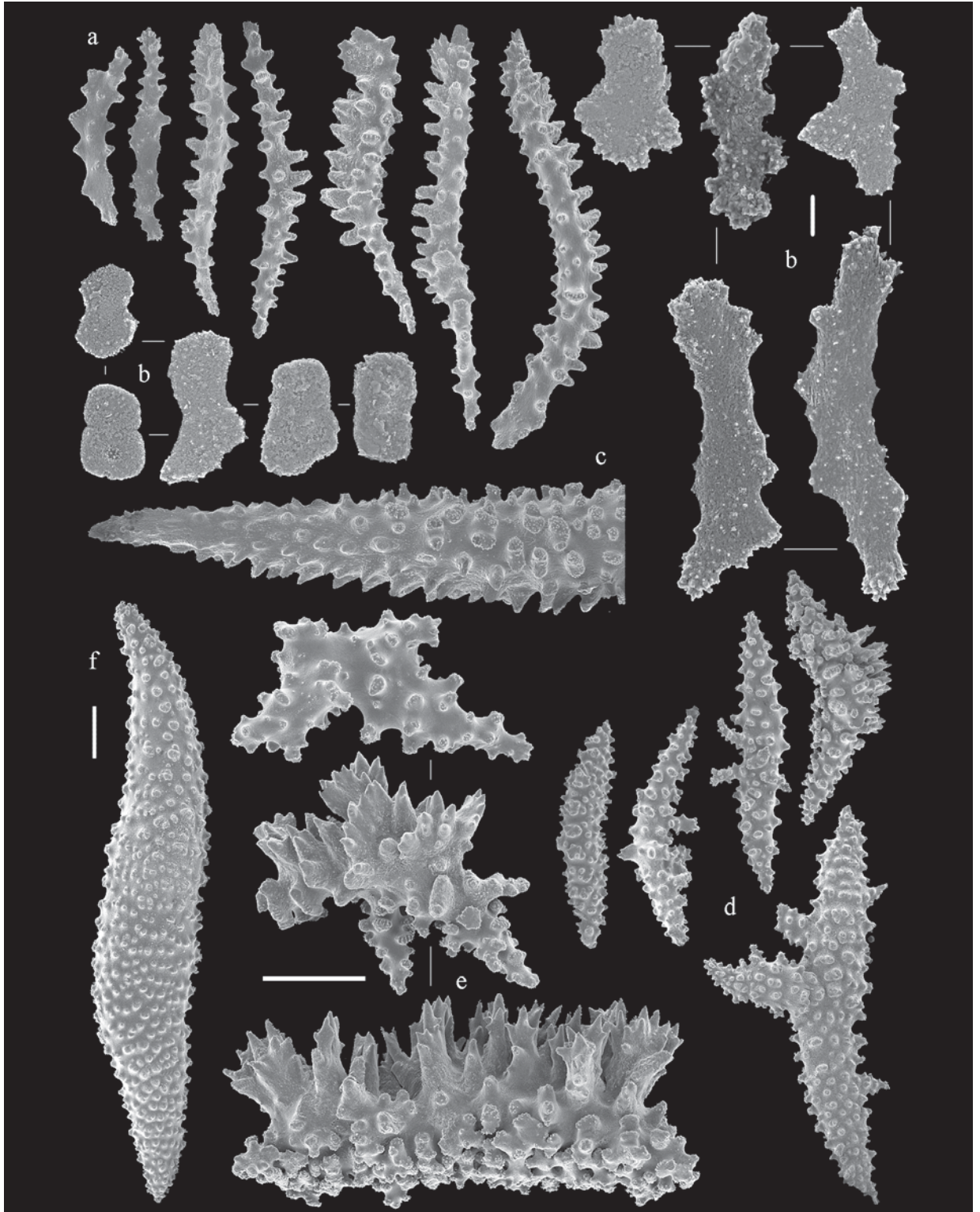


Fig. 6. RMNH Coel. 34809; a, point spindles, b, rods from tentacles, ventral points and polyp stalk; c, spindle of supporting bundle (part); d-e, spindles and unilaterally spinose spindles, surface layer base of colony; f, spindle, interior base of colony. Scale at b 0.01 mm, at f and e 0.10 mm; that at f also applies to d, that at e also to a, c.



Fig. 7. RMNH Coel. 34810; a, point spindles, b, rods from ventral points and polyp stalk; c, tentacular rods; d, spindle of supporting bundle (part); e-f, spindles and unilaterally spinose spindles, surface layer base of colony; g, spindles, interior base of colony. Scale at c 0.01 mm, also applies to b; scales at e and g 0.10 mm; that at e also applies to a, d, that at g also to f.

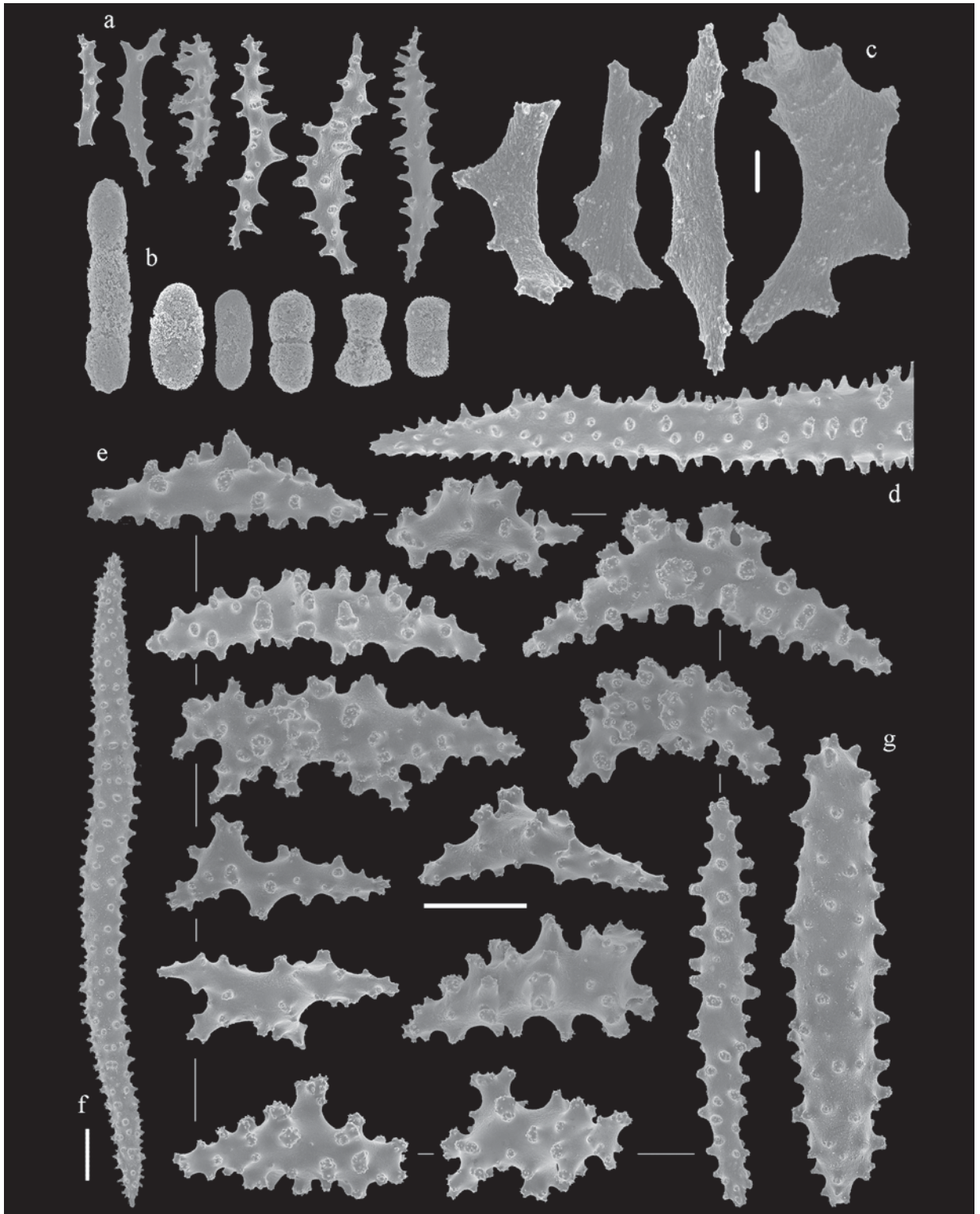


Fig. 8. RMNH Coel. 34811; a, point spindles, b, rods from ventral points and polyp stalk; c, tentacular rods; d, spindle of supporting bundle (part); e, spindles and unilaterally spinose spindles, surface layer base of colony; f-g, spindles, interior base of colony. Scale at c 0.01 mm, also applies to b; scales at e and f 0.10 mm; that at e also applies to a, d, g.



Fig. 9. RMNH Coel. 34812; a, point spindles, b, rods from tentacles, ventral points and polyp stalk; c, spindle of supporting bundle; d-e, spindles and unilaterally spinose spindles, surface layer base of colony; f-g, spindles, interior base of colony. Scale at b 0.01 mm; scales at d and e 0.10 mm; that at d also applies to a, f; that at e also to c, g.

of the colony has branched spindles with sparse simple tubercles (Fig. 4f).

- 3) RMNH Coel. 34808, KOR.09, Palau, “Toagel Mlungi Channel”, channel in western barrier reef off Babeldaob, 07°32'33.0"N 134°28'06.6"E; depth -25 m, coll. L.P. van Ofwegen, 18.v.2005.

The colony is 6 cm long (Fig. 1c). The polyps are grouped at the end of the branches, forming irregularly shaped catkins. The polyps have eight points and a slightly projecting supporting bundle (Fig. 2d). The point spindles (Fig. 5a) and supporting bundle spindles (Fig. 5c) are bigger than in the previous RMNH Coel. 32055, but basically the same types occur. The species differs from the foregoing in having more rods on the ventral side of the polyp stalk, which hardly differ from those found in the tentacles (Fig. 5b). The surface layer of the base of the colony has spindles (Fig. 5e), and weakly developed unilaterally spinose spindles (Fig. 5d), many of them with side branches. The interior of the base of the colony has spindles with sparse simple tubercles, only a few smaller ones are depicted (Fig. 5f). They can be as long as 1.7 mm, and are less tuberculate than the surface spindles.

- 4) RMNH Coel. 34809, BER.06, Indonesia, NE Kalimantan, Berau Islands, shoal between Lighthouse-2 reef and Derawan Island; 02°12'08.6"N 118°11'34.9"E, scuba diving, depth 20 m, coll. L.P. van Ofwegen and M. Slierings, 6.x.2003, E Kalimantan-Berau Exped.

The colony is 5 cm long (Fig. 1d). Like the previous two species the polyps are grouped at the end of the branches, forming irregularly shaped catkins. Also the polyp arrangement is much the same but differs in the ventral points and most of the polyp stalks having many rods (Figs. 2e-f, 6a-c). The surface layer of the base of the colony has spindles and unilaterally spinose spindles (Fig. 6d-e), many of them with side branches. The interior of the base of the colony has spindles with sparse simple tubercles (Fig. 6f), also many with side branches.

- 5) RMNH Coel. 34810, BER.26, Indonesia, NE Kalimantan, Berau Islands, Buliulin, NE-side (S of Samama Isl.); 02°07'07.2"N 118°20'31.6"E, scuba diving, depth -10 m, coll. L.P. van Ofwegen and M. Slierings, 15.x.2003, E Kalimantan-Berau Exped. The colony is 6 cm long, with several stems arising from a common base (Fig. 1e). The catkin shape

and polyp sclerite arrangement (Figs. 2g, 7b-d) is very much the same as in the previous species, however, the point sclerites are less unilaterally spinose (Fig. 7a). Also the base of the stalk has sclerites similar to those of RMNH Coel. 34809 (Fig. 7e-g), but these are also less unilaterally spinose.

- 6) RMNH Coel. 34811, BER.19a, Indonesia, NE Kalimantan, Berau Islands, off Tanjung Batu; 02°14'47.6"N 118°05'36.6"E, scuba diving, depth 12 m, coll. L.P. van Ofwegen and M. Slierings, 13.x.2003, E Kalimantan-Berau Exped.

The colony is 11 cm long, with several stems arising from a common base (Fig. 1h). The catkin shape and polyp sclerite arrangement (Fig. 2h) is very much the same as in the previous species. Also the polyp sclerites are similar (Fig. 8a-d). However, the sclerites of the surface layer of the base of the colony are only slightly unilaterally spinose (Fig. 8e), and hardly any sclerites of the base have side branches (Fig. 8f-g).

- 7) RMNH Coel. 34812, KOR.13, Palau, Koror, Uchelbeluu Reef, East side, barrier reef south-east of town of Koror, 07°16'25.2"N 134°31'27.0"E; depth -30 m, coll. L.P. van Ofwegen, 20.v.2005.

The colony is 4 cm long (Fig. 1f). The polyps appear very spiny due to strong supporting bundles (Fig. 9c), and an arrangement in catkins is not recognizable. The polyp armature is also different from the above described species, with point spindles slightly protruding beyond the polyp head (Fig. 2i). These spindles are not unilaterally spinose but have a spiny distal end (Fig. 9a). Tiny rods (Fig. 9b) are not only present in the tentacles, ventral points and ventral side of the supporting bundle, but also in between the points. The surface layer of the base has spindles and unilaterally spinose spindles (Fig. 9d-e), some of which are very large (Fig. 9e). The interior has spindles with sparse tuberculation (Fig. 9f-g).

- 8) RMNH Coel. 34813, BER.31, Indonesia, NE Kalimantan, Berau Islands, Panjang Island, NE-side; 02°25'46.0"N 118°09'49.1"E, scuba diving, depth -25 m, coll. L.P. van Ofwegen and M. Slierings, 19.x.2003, E Kalimantan-Berau Exped.

The colony is 8 cm long, but the base of the stalk is probably lost (Fig. 1g). Like in the previous species, the supporting bundles are strong (Fig. 10e), and catkins are not clearly discernable. In this species

small rods (Fig. 10b-d) are present all over the polyps (Fig. 2j), and the point spindles are unilaterally spinose (Fig. 10a). The surface layer of the base of the colony has spindles and unilaterally spinose spindles (Fig. 10f-g), the latter poorly developed. The interior has spindles with sparse tuberculation (Fig. 10h). The sclerites at the base of the colony look more like the types that are normally present higher up in the stalk of nephtheids. Hence we do not exclude the possibility that the lower part of the colony is lost.

- 9) RMNH Coel. 34814, KOR.15, Palau, Koror, Big Drop Off, east side of Ngemelis reef adjacent to island, 07°06'48.8"N 134°15'36.4"E; depth -25 m, coll. L.P. van Ofwegen, 23.v.2005.

The colony is 6 cm long with a colony shape very much alike that of RMNH Coel. 34812, but with more slender lobes; catkins are not recognizable, and the stalk is covered by sponge tissue (Fig. 11c). Also the polyp armature (Figs. 12a, 13b-d) and shape of the point spindles (Fig. 13b) are similar to those of RMNH Coel. 34812. It differs by having the point sclerites protruding more beyond the polyp body, having less small rods in the polyp (Fig. 12a), and these rods being of a different shape (Fig. 13a). The surface layer of the base of the colony has radiates and derivatives of these, spindles and poorly developed unilaterally spinose spindles (Fig. 13e). The interior of the base of the colony has spindles with sparse tuberculation (Fig. 13f).

- 10) RMNH Coel. 34815, KOR.03, Palau, Koror, Mutremdiu, west side Uchelbeluu Reef, barrier reef south-east of town of Koror, 07°16'25.2"N 134°31'26.8"E; depth -25 m, coll. L.P. van Ofwegen, 13/21.v.2005.

The colony is 10 cm high, yellow with white polyps, and a real stalk is lacking (Fig. 11b). The colony shape is typical for a species of *Stereonephthya* with the polyps making an acute angle with their polyp stalk and supporting bundles projecting considerably beyond the polyps (Figs. 12b, 14d). The points have very big spindles, some of which also project beyond the polyp with the projecting part often widened and less spiny (Fig. 14b). The polyp stalk and tentacle rods (Fig. 14a, c) are similar to those of the previous species. The surface layer of the base of the colony has radiates, derivatives of these, spindles, and poorly developed unilaterally spinose spindles (Fig. 14e-f). The interior of the

base of the colony has very few spindles, and these have sparse tuberculation (not depicted).

- 11) RMNH Coel. 34816, KOR.06, Palau, Koror, Wonder Channel (Rock Islands), NW side of Mercherchar Island, 07°10'56.5"N 134°21'38.7"E; depth -25 m, coll. L.P. van Ofwegen, 16.v.2005.

Two colonies lacking a real stalk; red with white polyps. Sclerites of this material were similar to those of RMNH Coel. 34815.

- 12) RMNH Coel. 32058, PAL.06, Palau, E off Koror I. (Oreor I.), SW off Ngeream peninsula, patch reef in KB channel, 07°20'22.2"N 134°31'04.6"E, 20.vii-6.viii.2002, coll. B.W. Hoeksema.

Several colonies, with sclerites and colony colour similar to RMNH Coel. 34815-16. For comparison the polyp armature is depicted (Fig. 12c).

- 13) RMNH Coel. 32059, 1 colony, Indonesia, SW Sulawesi, Spermonde Archipelago, S of Panikiang Isl. (= 90 km NNE of Ujungpandang, offshore NW of Sumpangbinangae) 4°22'S 119°35'E, coral reef, scuba diving, 30.viii.1994, Buginesia Program UNHAS-NNM 1994, coll. B.W. Hoeksema.

The colony is 9 cm long, white with orange polyps, with typical *Stereonephthya* colony shape (Fig. 11a). The polyp armature (Fig. 15a-d) is like the previous species but the point sclerites are much smaller (Fig. 15b) and they do not project beyond the polyp (Fig. 12d). The sclerites of the surface layer of the base of the colony are more slender than in the previous species (Fig. 15e-f). The interior of the base of the colony has spindles with sparse tuberculation (Fig. 15g).

- 14) RMNH Coel. 32050, holotype of *Chromonephthya franseni* Van Ofwegen, 2005, SUL.06, Indonesia, N Sulawesi, Selat Lembeh, Pulau Lembeh, Pantai Parigi, 01°28'N 125°14'E, small fringing reef up to 20 m wide, gently sloping from beach to 10 m, deeper slope sandy to muddy to 20 m, diving and snorkelling, 15/26.x.1994.

The colony has been described in Van Ofwegen (2005), and here the holotype (Fig. 11d) and polyp armature (Fig. 12e) are shown for comparison. The genus differs from *Nephthea* and *Litophyton* by being azooxanthellate. It differs from *Stereonephthya* and *Neospongodes* by lacking rods in the polyp stalk, and from *Dendronephthya* by having the polyps standing single instead of in small bundles.

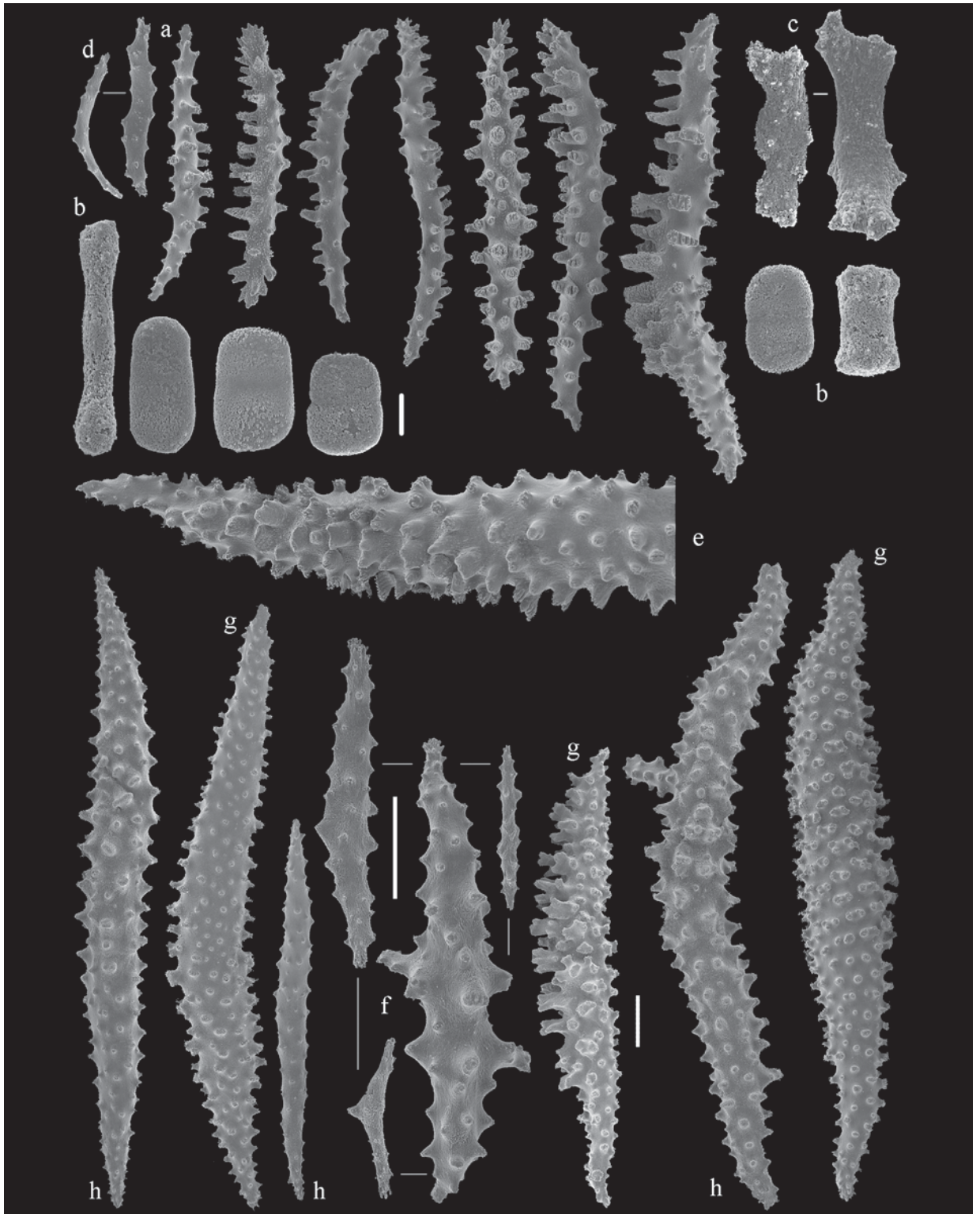


Fig. 10. RMNH Coel. 34813; a, point spindles; b, rods from polyp stalk; c-d, tentacular rods; e, spindle of supporting bundle (part); f-g, spindles and unilaterally spinose spindles, surface layer base of colony; h, spindles, interior base of colony. Scale at b 0.01 mm, also applies to c; scales at f and g 0.10 mm; that at f also applies to a, d-e; that at g also to h.

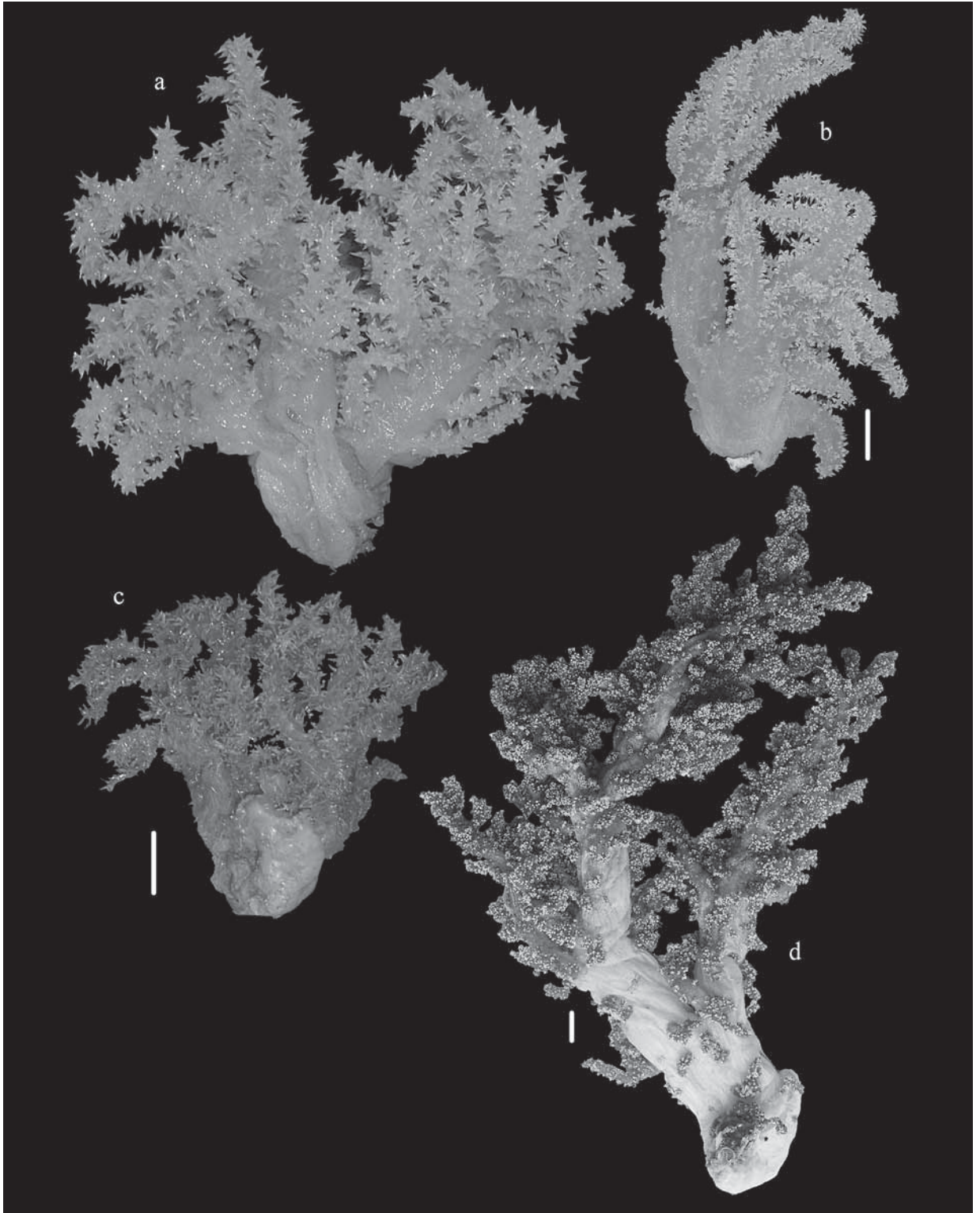


Fig. 11. Colonies; a, RMNH Coel. 32059; b, RMNH Coel. 34815; c, RMNH Coel. 34814; d, RMNH Coel. 32050, holotype of *Chromonephthea franseni* Van Ofwegen, 2005. Scales 1 cm, that at b only applies to b, that at d only to d.

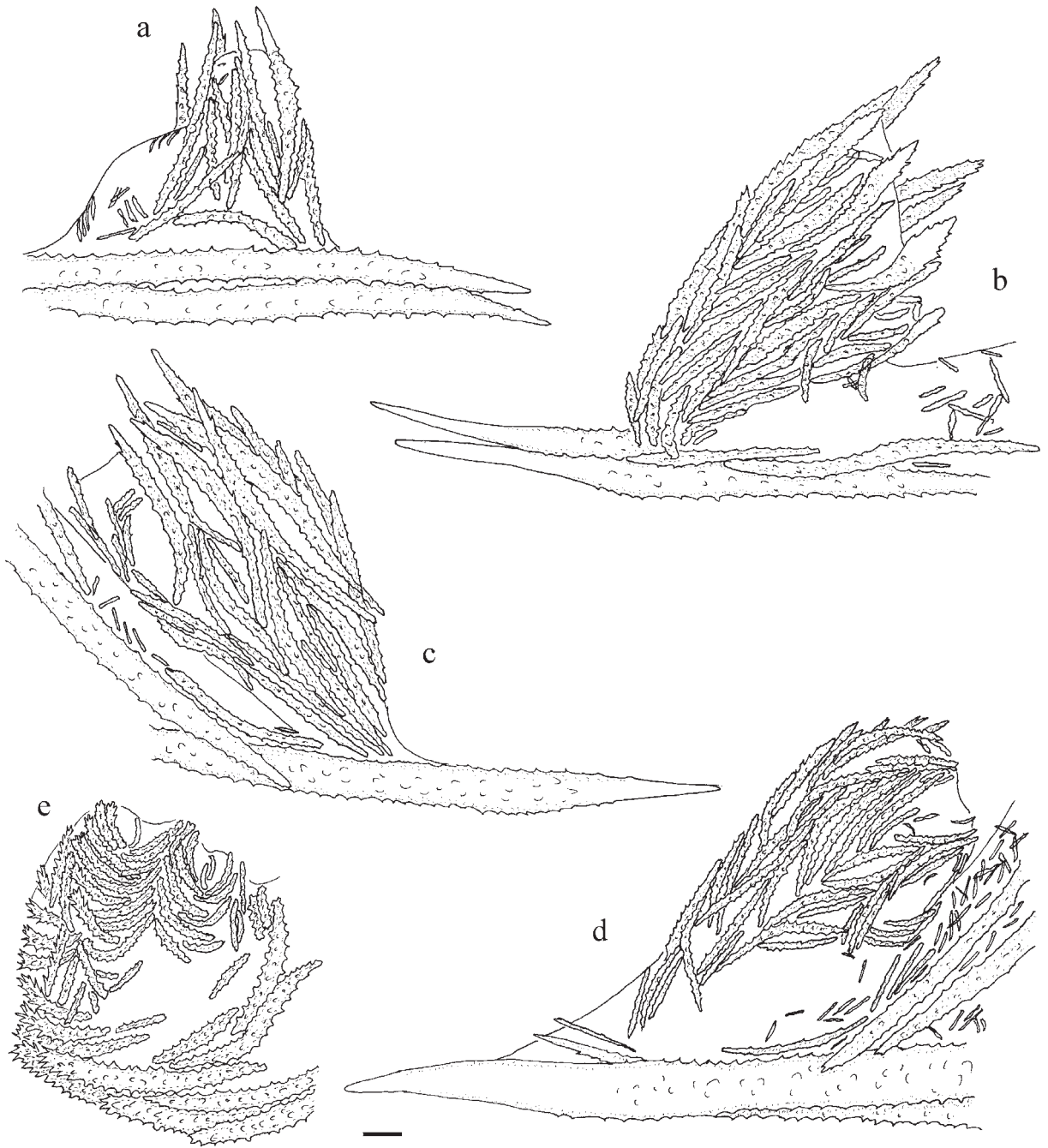


Fig. 12. Lateral views of polyps armature; a, RMNH Coel. 34814; b, RMNH Coel. 34815; c, RMNH Coel. 32058; d, RMNH Coel. 32059; e, RMNH Coel. 32050, holotype of *Chromonephthea franseni* Van Ofwegen, 2005. Scale 0.10 mm.



Fig. 13. RMNH Coel. 34814; a, rods of polyp stalk; b, point spindles; c, tentacular rods; d, spindle of supporting bundle (part); e, radiates, spindles and unilaterally spinose spindles, surface layer base of colony; f, spindle, interior base of colony. Scale at c 0.01 mm, also applies to a; scales at e and f 0.10 mm; that at e also applies to b, d.



Fig. 14. RMNH Coel. 34815; a, rods of polyp stalk; b, point spindles; c, tentacular rods; d, spindle of supporting bundle (part); e-f, radiates, spindles and unilaterally spinose spindles, surface layer base of colony; Scale at c 0.01 mm, also applies to a; scales at e and f 0.10 mm; that at e also applies to b, d.

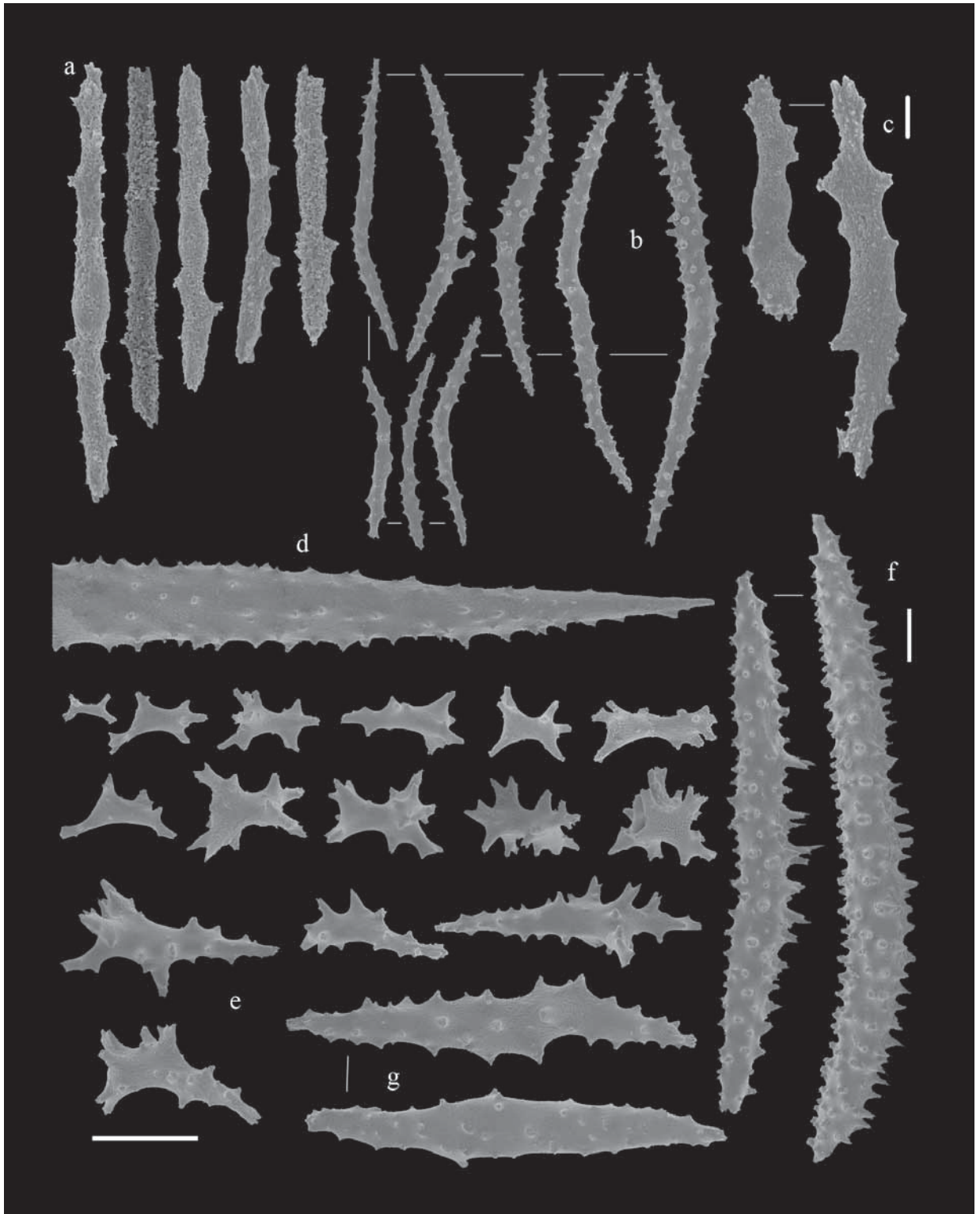


Fig. 15. RMNH Coel. 32059; a, rods of polyp stalk; b, point spindles; c, tentacular rods; d, spindle of supporting bundle (part); e-f, radiates, spindles and unilaterally spinose spindles, surface layer base of colony; g, spindles, interior layer base of colony. Scale at c 0.01 mm, also applies to a; scales at e and f 0.10 mm; that at e also applies to b, d, g.

In summary, the material used in the analyses includes one specimen referable to *Litophyton* (RMNH Coel. 32053), one to *Nephthea* (RMNH Coel. 32055), four to *Stereonephthya* (RMNH Coel. 34815-16; 32058-59), and one to *Chromonephthya* (RMNH Coel. 32050). All other specimens have intermediate characters and, according to the classification of Kükenthal, cannot be assigned to any genus or species unequivocally.

Methods

A small branch of each coral was preserved in 96% ethanol for molecular analysis. Around 0,25 cm³ of tissue was cut into small pieces for surface enlargement. Genomic DNA was extracted by use of a DN-Easy[®]Tissue Kit (Qiagen) following the manufacturer's protocol for animal tissues. Elution volume was 50-100 µl and the average DNA concentration was around 1250 ng/µl. Best PCR results were obtained using 1:10 dilutions and for most extracts, PCR's failed without the addition of Q-solution (Qiagen). Co-extraction of zooxanthellae was unavoidable and shown when we amplified the ITS-1 and ITS-2 regions.

Initial attempts to sequence mitochondrial marker MSH with primers ND42599F 5'-GCCATTATGGT-TAACTATTAC-3' and Mut-3458R 5'-TSGAG-CAAAAGCCACTCC-3' (France and Hoover, 2001, 2002) failed for most taxa. Therefore we designed internal primers MSH-Neph-F 5'-TATGAACTTT-GGCATGAGCC-3' and MSH-Neph-R 5'-TGCCC AAATTACTATTTCTCTAATACG-3' specific for these nephtheids. PCR-reactions were done using a standard Taq DNA polymerase kit from Qiagen. Reaction volume always was 25 µl and PCR conditions were 0.2 mM DNTPs, 0.4 µM of each primer, 5 µl of Q-solution, 5 units of Taq DNA polymerase (Qiagen) and no additional MgCl₂ (PCR-buffer always contains 1.5 mM MgCl₂). Thermocycling conditions were 3 min. at 94°C for initial denaturation followed by 40 cycles (15 sec. at 94°C, 1 min. 30 sec. at 50°C and 1 min. at 72°C) and final extension 5 min. at 72°C. PCR products were purified using Nucleospin[®] kit (Macherey-Nagel) and cycle sequenced in both directions with an ABI Dye Terminator Cycle Sequencing Kit (PE Biosystems). The sequenced products were purified using G-50 Fine Sephadex[®] columns and run on an ABI 377 automated sequencer. Sequences were checked and edited with Sequencer 4.2 (Genecodes inc.).

The resulting MSH sequences were hardly informative and did not give enough resolution to show rela-

tionships below the genus-level. Hence another marker, the nuclear region 18S-ITS1-5.8S-ITS2-28S for which some information was also available on Genbank was tried. This region was amplified with primers 1w - F 5'-TACCGATTGAATGGTTTAGTGAGG-3' (McFadden and Hutchinson, 2004) and 2w 5'-ATT-GCCACGTACGGGGTTGTC-3' (McFadden *et al.*, 2001). PCR conditions, as well as product purification (for both PCR and sequenced products) were identical to the procedure described above. Thermocycling conditions were 3 min. at 94°C for initial denaturation followed by 40 cycles (15 sec. at 94°C, 1 min. 30 sec. at 52°C and 1 min. 30 sec. at 72°C) and final extension 5 min. at 72°C. Next to primer 1w - F and 2w, internal primers LVO 5.8s Fwd1 5'-TTAACGGTGGATCT CTTGGCTC-3' and LVO 5.8s Rev 2 5'-TCACACTACTTATCGCAGCTGG-3' were used for cycle sequencing. This generated 4 overlapping sequences that were also assembled with Sequencer 4.2 (Genecodes inc.).

Sequence variation

The sequences we used ranged from 860 bp (RMNH Coel. 34814) to 977 bp (RMNH Coel. 32053) in length. All sequences included the 157 bp of the 5.8S gene, the 3' terminus of 18S ranged from 114 bp (most sequences) to 117 bp (RMNH Coel. 32050), but was mostly 114 bp long. The 5' end of 28S was

Table 1. Variation in length and base composition of ribosomal internal transcribed spacer regions (ITS-1, ITS-2) among species of nephtheids. *Alcyonium digitatum* is included as outgroup. bp = base pairs, CG = cytosine and guanine.

	ITS1		ITS2	
	Length bp	%CG	Length bp	%CG
1550	230	45.22	192	60.42
2028	230	45.22	192	60.42
2031	230	45.22	192	60.42
1551	217	44.24	181	59.67
2036	219	44.29	174	59.20
1542	220	45.46	208	60.10
1858	207	42.03	216	64.81
1547	208	41.83	202	63.86
2033	223	42.16	207	59.90
1861	223	44.40	212	68.87
1860	225	44.00	218	69.72
1859	207	44.93	203	66.99
2035	225	48.44	203	69.45
1545	251	43.03	260	61.92
A digi	185	40.00	197	52.79

Table 2. Genetic distances among nephtheid species (Kimura two-parameter method). Distances are given for combined 18S, ITS-1, 5.8S, ITS-2, and 28S. *Stereonephthya* data in bold.

	32058	34815	34816	32059	34814	32051	34813	34810	34811	34812	32053	34809	32055	34808	Adig
32058	-														
34815	0.00000	-													
34816	0.00227	0.00227	-												
32059	0.00587	0.00587	0.00709	-											
34814	0.00591	0.00591	0.00829	0.00938	-										
32051	0.07664	0.07664	0.07795	0.07808	0.07386	-									
34813	0.10277	0.10277	0.10420	0.09619	0.09059	0.08439	-								
34810	0.10299	0.10299	0.10442	0.09532	0.08983	0.08359	0.01349	-							
34811	0.09430	0.09430	0.09572	0.08922	0.08656	0.08477	0.02020	0.02475	-						
34812	0.09560	0.09560	0.09696	0.08626	0.08348	0.07934	0.03797	0.03906	0.03557	-					
32053	0.10068	0.10068	0.10214	0.09463	0.08900	0.09003	0.03947	0.04464	0.04739	0.04260	-				
34809	0.08804	0.08804	0.08950	0.08472	0.08047	0.07881	0.04751	0.05058	0.04502	0.04939	0.04783	-			
32055	0.08079	0.08079	0.08224	0.08341	0.07535	0.07382	0.05065	0.05374	0.05127	0.04609	0.04533	0.02233	-		
34808	0.09781	0.09781	0.09804	0.09555	0.08760	0.09056	0.05973	0.06273	0.06368	0.06775	0.06908	0.02938	0.03061	-	
Adig	0.12314	0.12314	0.12473	0.11782	0.11381	0.12163	0.11517	0.11562	0.10803	0.11560	0.12185	0.11234	0.11314	0.11556	-

194 bp long in all sequences. Both of the ITS regions varied greatly in length among species, ITS-1 ranged from 207 bp (RMNH Coel. 34809; RMNH Coel. 34811) to 251 bp (RMNH Coel. 32053), and ITS-2 ranged from 174 bp (RMNH Coel. 34814) to 260 bp (RMNH Coel. 32053) (Table 1). As the outgroup *Alcyonium digitatum* (GenBank accession number AF2623461) was used.

The long sequence length of RMNH Coel. 32053 with 977 bp is caused by its long ITS regions. In the ITS-2 region at position 547 there is a five times repeat of “GGCCCTTT”, and at position 704 a three times repeat of “TAAAGCTCTT”, which mostly explains for the extraordinary length. In the ITS-1 region such obvious repeats were not recognized.

RMNH Coel. 32058-59 and RMNH Coel. 34814-16 seem to have a shorter ITS2 region (on average 186.20) than the other nephtheids (on average 214.33).

The G+C content of ITS1 and ITS2 was calculated in GeneDoc (Table 1), the values for ITS-1 are comparable with those given for *Alcyonium* species by McFadden *et al.* (2001), the values for the ITS-2 region are on average more than 10% higher. No pattern could be recognized within the nephtheids.

Nucleotide diversity was estimated in PAUP using the Kimura two-parameter method (Table 2). Genetic distances of the combined partial sequence of the 5' end of 18S rRNA gene, the complete sequence of ITS-1, 5.8S rRNA gene and ITS-2, and a partial sequence 3' end of 28S rRNA are presented in Table 2. The distance between RMNH Coel. 32058 and RMNH Coel. 34815 is 0% and that between these two and RMNH

Coel. 34814, 16 and RMNH Coel. 32059 is also very small, 0.0023-0.0094.

Sequences were aligned using ClustalW (Thompson *et al.* 1994) with parameter GOP (Gap Opening Penalty) set to 2 and the GEP (Gap Extension Penalty) to 1. Both McFadden *et al.* (2001) and Forsman *et al.* (2006) proved that this is the best alignment setting for *Alcyonium* (soft corals) and *Porites/Siderastrea* (stony corals).

Genbank accession numbers are presented in Table 3.

Phylogenetic analyses

We used the permutation test in PAUP (version 4.0b10; Swofford, 2002) for a phylogenetic signal, the P value was 0.01.

Table 3. GenBank accession numbers.

RMNH Coel. 32050_1542	EF215838
RMNH Coel. 32053_1545	EF215839
RMNH Coel. 32055_1547	EF215840
RMNH Coel. 32058_1550	EF215841
RMNH Coel. 32059_1551	EF215842
RMNH Coel. 34809_1858	EF215843
RMNH Coel. 34811_1859	EF215844
RMNH Coel. 34810_1860	EF215845
RMNH Coel. 34813_1861	EF215846
RMNH Coel. 34815_2028	EF215847
RMNH Coel. 34816_2031	EF215848
RMNH Coel. 34808_2033	EF215849
RMNH Coel. 34812_2035	EF215850
RMNH Coel. 34814_2036	EF215851

The final alignment included 1029 nucleotide positions: 685 are constant, 123 are variable, and 221 are parsimony-informative.

PAUP was used for maximum parsimony, neighbour-joining, and the Unweighted Pair Group Method with Arithmetic mean (UPGMA). For maximum parsimony we did bootstrap analyses (100 and 1000 replicates) using the heuristic-search option with simple addition of sequences and Tree-bisection-reconnection (TBR) branch swapping. The steepest descent option was in effect. Branches having maximum length zero were collapsed to yield polytomies. Multrees' option was in effect.

For neighbour-joining we used bootstrap analyses (1000 replicates) with consensus tree option set to 50% majority-rule, for the rest we used default settings.

With MacClade (version 4.08) we examined the base pair positions used in the neighbour-joining tree

analysis. As can be seen in Fig. 16 this mostly concerned positions in the poorly aligned ITS regions. Therefore, we also did the above analyses after removing the less aligned parts of the ITS regions.

Aleyonium digitatum was used to root all trees.

Results

The different number of replicates used in the bootstrap analysis of maximum parsimony resulted in only marginal differences.

The different methods yielded similar results. In all trees two clades are supported. In all but the partial ITS parsimony analysis clade I is formed by *Chromonephthea franseni* together with RMNH Coel. 32058-59 and RMNH Coel. 34814-16 and clade II includes all other specimens. Only in the partial ITS parsimony anal-

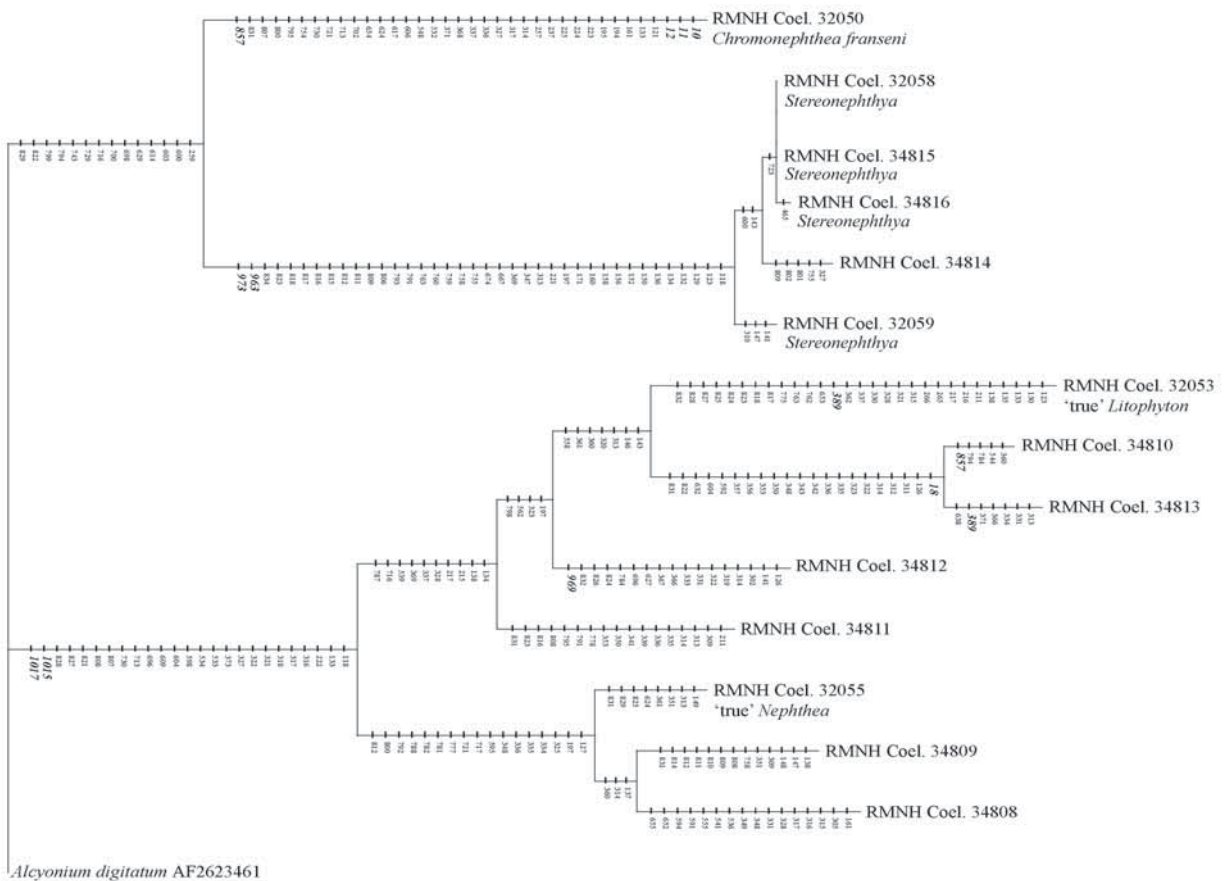


Fig. 16. Neighbour-joining bootstrap 50% majority-rule consensus tree with base pair positions used in the analysis. Positions in the 18S, 5.8S, and 28S regions are shown in bold and italics.

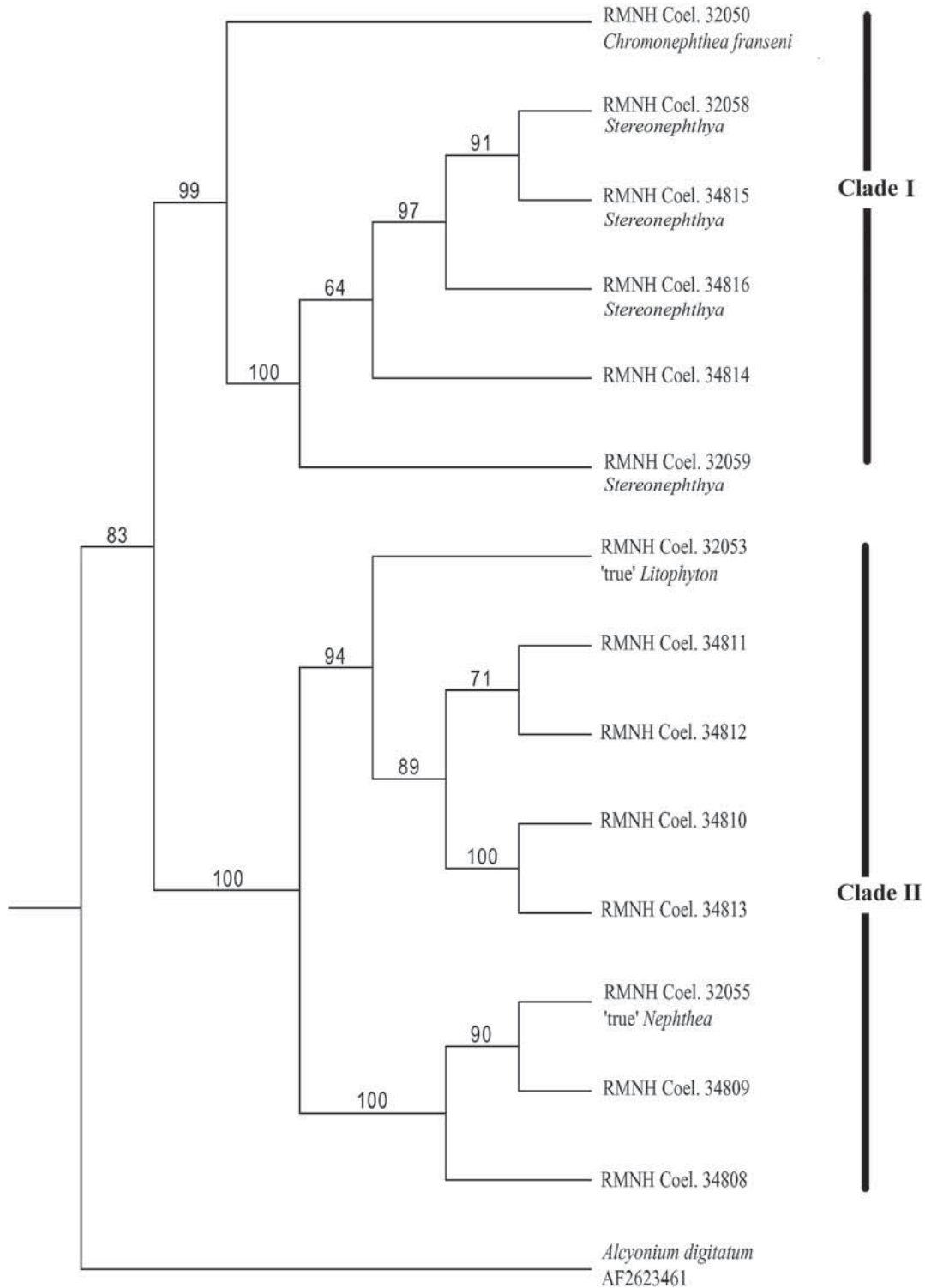


Fig. 17. Unweighted Pair Group Method with Arithmetic mean (UPGMA) bootstrap tree.

ysis *Chromonephthea franseni* clustered in clade II.

Here we only present the results of the UPGMA analysis (Fig. 17). Because of the poor alignment of the ITS regions, which are mostly used in the analyses (Fig. 16), we looked at overall sequence similarity rather than evolutionary relationships. However, the maximum parsimony and neighbour-joining bootstrap analyses gave similar results.

Discussion

The two distinct clades that show up in the analyses were compared with the morphological data. The stalk sclerites, point spindles, and colony form did not yield any clear correlation with the phylogeny found. However, specimens in Clade I, with the exception of the *Chromonephthea franseni* specimen, showed the characters for *Stereonephthya* as reported by Utinomy: 1) supporting bundles of the polyps are well developed with strong projecting tip; 2) ventral side of the polyp stalk has small tiny rods; 3) polyp heads usually hang down on their stalks, like a campanulate flower; 4) dorsal points strong, ventral points poorly supported or lacking. RMNH Coel. 34814 has both ‘campanulate-like’ polyps and ones like depicted in figure 12a. The only consistent sclerite character found for clade I is the shape of the rods in the polyp stalks, in this clade they have tiny projections, and are longer (Figs. 13a, 14a, 15a) than those in clade II.

The polytomy of RMNH Coel. 34815-16 and RMNH Coel. 32058 in most of the analyses (Fig. 16) confirms the identification of these three specimens based on sclerite characters, which showed them to belong to a single species.

The grouping of *Chromonephthea franseni* as the sister group next to the *Stereonephthya* species probably occurred because only one sequence of *Chromonephthea* was included.

Clade II is a mixture of specimens which, on the basis of the characters used in the classification of Kükenthal, could be placed in *Nephthea*, *Litophyton* or *Stereonephthya*. The *Litophyton*-like specimen (polyps in catkins, lacking supporting bundle) and the *Nephthea*-like specimen (polyps in catkins, supporting bundle) group with *Stereonephthya*-like specimens (projecting supporting bundle, catkins not obvious, tiny rods in the polyp stalk). All species in Clade II lack the long polyp stalk rods with tiny projections.

Obviously, the sclerite character found to support the two different clades is extremely small for separat-

ing different genera in nephtheids, and therefore it is not surprising that previous researchers never mentioned it as a character. However, despite the small difference in shape, the polyp stalk rods could have a different origin. In all species included in Clade II the polyp stalk rods seem to be similar to those found in the tentacles and ventral points, while in Clade I they are situated somewhat further apart from the polyp head and are clearly different from the tentacle and ventral point rods. They could represent extremely reduced polyp stalk spindles.

McFadden *et al.* (2006), in their phylogenetic analysis of octocoral genera, used the mitochondrial markers ND2 and MSH combined. They also included specimens of *Litophyton*, *Nephthea* and *Stereonephthya* in their analyses. Like we found with MSH, they also found identical sequences for *Litophyton* and *Nephthea*. In their analyses they always found a polytomy of *Litophyton/Nephthea*, *Stereonephthya* and a specimen identified as belonging to the Atlantic genus *Neospongodes*. As the ‘*Neospongodes*’ specimen was collected in Darwin harbour the first author contacted one of the authors of McFadden *et al.*, viz. Phil Alderslade (Museum and Art Gallery of the Northern Territory, Darwin, Australia), who informed us that the ‘*Neospongodes*’ specimen actually belonged to *Chromonephthea*. The *Litophyton*, *Nephthea* and *Stereonephthya* specimens used were identified with the same criteria we applied. The findings of McFadden *et al.* do not contradict ours, but apparently the 18S-ITS1-5.8S-ITS2-28S marker we used gives more resolution than the combined ND2 and MSH markers.

The results of the present molecular study have consequences for the nephtheid classification. In the morphological revisions under progress *Nephthea* should be synonymized with *Litophyton*. All species previously assigned to *Stereonephthya* and *Nephthea* should be checked with regards to the shape of the polyp stalk rods.

Moreover, *Nephthea savignyi* Ehrenberg, 1834, the type species of *Dendronephthya*, has the sclerite and colony form characters of clade II species and should therefore be transferred to *Litophyton*, making *Dendronephthya* a junior synonym of *Litophyton*.

Finally, in the Atlantic genus *Neospongodes* the polyp stalk sclerites are similar to those found in clade I (Indo-Pacific *Stereonephthya*) and, according to the code of nomenclature, this name has preference above *Stereonephthya*. Unfortunately, no recently collected material of *Neospongodes* species was available for the present DNA study.

For the moment, pending the completion of the morphological revisions in progress, we retain to the current classification.

Acknowledgements

We like to thank B.W. Hoeksema (NNM) for collecting some of the material, the Netherlands Organization for Scientific Research (NWO) and the Schure-Beijerinck-Popping Fonds for funding a journey to Palau for Leen van Ofwegen. M. Slierings (NNM) is thanked for curatorial assistance.

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Received: 21 March 2007.

Accepted: 15 May 2007.