The invasive land planarian *Platydemus manokwari* (Platyhelminthes, Geoplanidae): records from five new localities, including Florida, USA

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The land planarian *Platydemus manokwari* de Beauchamp, 1963 or “New Guinea flatworm” is a highly invasive species, already reported from 16 territories in the World, mainly in the Pacific area, and recently in Europe (France). We report specimens from four additional territories: New Caledonia, Singapore, Solomon Islands and Florida, USA, and new records from French Polynesia (Tahiti Island). The external morphology and colour patterns were similar in all specimens. We analysed the COI gene (barcoding) in these specimens with two sets of primers and obtained 909 bp long sequences. In addition, specimens collected in Townsville (Australia) were also sequenced. Two variants of the COI sequence were detected: an “Australian variant”, found only in specimens from Australia, and an “Other variant”, found in specimens from France, New Caledonia, Tahiti, Singapore and Florida. The two variants differed by 5.2%. Since *P. manokwari* is now recorded from 20 territories in the world, this analysis provides only partial knowledge of the genetic structure of the invasive species. The existence of two variants suggests that two populations were at the origin of the invasion, one in Australia and one in the other territories. The new report from Florida is the first for the USA and for the American continent; specimens were found in four separate locations in Miami-Dade County since 2012, and are likely to spread widely. *P. manokwari* is a known threat for endemic terrestrial molluscs and its presence is a matter of concern.

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Introduction

The land planarian *Platydemus manokwari* de Beauchamp, 1963 or "New Guinea flatworm" is an invasive species, recorded in the wild in 15 territories in the World, and recently in France in a hothouse (Justine et al. 2014b). *Platydemus manokwari* is the only flatworm listed in the "100 world's worst invasive alien species" (Lowe et al. 2000); it is a predator of land snails and is considered a danger to endemic snails wherever it has been introduced.

We report here its presence in several additional territories: Singapore, New Caledonia, an additional island in French Polynesia, Solomon Islands and Florida, USA, the latter being the first record in the American continent. We found that barcodes of specimens comprise two variants, one found only in Australia and one found in France, New Caledonia, Tahiti, Singapore and Florida. This suggests that there are at least two different invasive populations of *P. manokwari*.

Material and methods

Origin of new reports

The new findings reported here were collected thanks to a worldwide campaign in several steps: (a) a paper published in this journal (Justine et al. 2014b); (b) its accompanying press releases; (c) a worldwide interest of the international Media, with articles in more than 15 languages (some Media enumerated here: *https://peerj.com/articles/297/#links*); (d) a wide dissemination of the paper, which was made available, thanks to open-access, from the publisher's website, and from PubMed Central, Academia and ResearchGate; (e) a "question" asked on the social networking site ResearchGate (*https://www.researchgate.net/post/Have_you_seen_this_land_planarian_an_invasive_alien_species*); (f) for France, a citizen science survey, based on a blog (Justine 2014) and a twitter account (*https://twitter.com/Plathelminthe4*).

Photographs of *P. manokwari* (Figs 1-4) were obtained from various sources (Table 1).

Fixation of worms

For fixation, two protocols were used: either (a) the living specimen was simply put into room-temperature ethanol (80-95%); or (b) the living worm was put into boiling water; after 1-2 minutes water was removed and replaced with room-temperature ethanol (80-95%). Specimens of *P. manokwari* (Table 1), collected by hand and fixed, were sent to Paris by postal service. In addition, we collected new specimens from the infested hothouse in Jardin des Plantes, Caen, France and in public and private gardens in Townsville, Australia. Specimens were deposited in the collections of the Muséum National d'Histoire Naturelle, Paris, France (MNHN), and the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS) National Malacology Laboratory, Academy of Natural Sciences, Philadelphia, USA.

Molecular sequences

For molecular analysis, a small piece of the body (1-3 mm^3) was taken from the lateral edge of ethanol-fixed individuals. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Two sets of primers were used to amplify the COI gene. A fragment of 424 bp (designated in this text as "short sequence") was amplified with the primers COI-ASmit1 (forward 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and COI-ASmit2 (reverse 5′-TAAAGAAAGAACATAATGAAAATG-3′) (Littlewood et al. 1997). The PCR reaction was performed in 20 µl, containing 1 ng of DNA, 1× CoralLoad PCR buffer, 3Mm MgCl2, 66 µM of each dNTP, 0.15µM of each primer, and 0.5 units of Taq DNA polymerase (Qiagen). The amplification protocol was: 4' at 94 °C, followed by 40 cycles of 94 °C for 30'', 48 °C for 40'', 72 °C for 50'', with a final extension at 72 °C for 7'. A fragment of 825 bp was amplified with the primers BarS (forward 5′-GTTATGCCTGTAATGATTG-3′) (Álvarez-Presas et al. 2011) and COIR (reverse 5′-CCWGTYARMCCHCCWAYAGTAAA-3′) (Lázaro et al. 2009), following (Mateos et al. 2013). PCR products were purified and sequenced in both directions on a 3730xl DNA Analyzer 96-capillary sequencer (Applied Biosystems). Results of both analyses were concatenated to obtain a COI sequence of 909 bp in length (designated in this text as "long sequence"). Sequences were edited using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA), compared to the GenBank database content using BLAST and deposited in GenBank under accession number xxxxxx. Sequences were compared using MEGA6 (Tamura et al. 2013). A newly obtained sequence of *Parakontikia ventrolineata*, collected from Brest (France), GenBank xxxxxx, was used as outgroup for the NJ tree. For several specimens only "short" sequences were obtained (Table 1). No sequence was obtained for the specimens from Guam (Table 1), which were kept in ethanol for a decade. Distances were computed with MEGA6, using Kimura 2-parameter model and pairwise deletion gap/missing data treatment.

Results

Morphology

The flatworms (Figs 1-4) presented the following morphological characteristics: body broadest in the middle, tapering evenly anteriorly but more abruptly posteriorly; two large prominent eyes back from the tip of the elongate snout-like head; dorsum a dark olive brown colour; pale cream median dorsal longitudinal stripe beginning just behind the eyes and continuing to the posterior tip; olive brown colour grading to grey at anterior tip; two thin submarginal cream stripes with fine lower greyish margin running laterally from the anterior end along the length of the body; ventral surface a pale finely mottled light brown. These features are consistent with those of *Platydemus manokwari* de Beauchamp, 1963 (Platyhelminthes, Continenticola, Geoplanidae, Rhynchodeminae) (de Beauchamp 1962) (Kawakatsu et al. 1992) (Justine et al. 2014b; Winsor 1990).

Molecular identification

COI sequences from Australia (herein designated as “Australian variant”) were identical among them, with the single difference of one nucleotide change in specimen JL178A. They showed 99% similarity (429/432 sites) to a sequence deposited in GenBank from the same location (AF178320, length 441 bp). As already mentioned (Justine et al. 2014b), specimen AF178320 was collected in Australia by one of us (LW) and the population from which it was taken had previously been confirmed histologically as *P. manokwari*. We thus consider that our new sequences from Australia correspond to *P. manokwari*.

The COI sequences we obtained from the individuals from Singapore, New Caledonia and French Polynesia were identical, and identical to sequences obtained from the specimens found in France in a hothouse in 2013. This COI sequence is herein designated as “Other variant”. For a French specimen, thanks to the use of two sets of primers, we obtained a longer sequence than the one originally published (KF887958) (Justine et al. 2014b). We also obtained a long sequence for a specimen from Singapore.

The Neighbour-Joining tree produced from all 18 sequences available (either short or long) included 317 positions. The tree showed two clades, one including all

Australian specimens, and the other including all specimens from Singapore, France, Florida and New Caledonia (Figure 5).

The distance between the two variants ("Australian" vs "Other") was 3.8% when computed on the common part of all 18 sequences (317 sites), and was 5.2% when computed on concatenated "long" sequences (907 sites).

Time of invasion and other remarks

In France, after the first finding of *P. manokwari* (October 2013), the hothouse of the Jardin des Plantes in Caen in which the species was found was closed and public was restricted. In April 2014, a thorough search was performed in the same hothouse. Several species of land planarians were found (some are still unidentified), including four adult specimens of *P. manokwari*. A citizen-based survey of land planarians in France (Thévenot et al. 2014), supported by important media involvement (list of links: https://peerj.com/articles/297/#links), yielded hundreds of records of invasive land planarians (Justine 2014; Justine et al. 2014a) but never a record of *P. manokwari* in the wild.

Invasion of Singapore was already effective in 2010, according to unpublished observations listed in Table 1, but the species is probably spreading; according to the observations of one of us (AWKH), his garden was not invaded before 2013. Observations show that *P. manokwari* predates the introduced giant African snail, *Achatina fulica* Bowdich, 1822.

Invasion of New Caledonia seems recent (2014). However, discussions with scientists involved with observation of soil species suggest that the species was already seen several years ago, but no substantiated proof could be found; since a number of endemic land planarians exist in New Caledonia (Schröder 1924; Winsor 1991), it cannot be ascertained if these earlier reports were actually *P. manokwari*, or other species. After the discovery of the first specimens in the capital city, Nouméa, in April 2014, a survey was conducted in September 2014. Specimens were found in two other sites in Nouméa, and in Mont-Dore and Païta, both located in the Southern Province. No specimens were found in various locations examined in the Northern Province and Loyalty Islands.

Invasion of Guadalcanal, Solomon Islands seems also recent (2014). Specimens of *P. manokwari* were found in fields invaded by introduced giant African snail, *Achatina fulica*.

Invasion of Guam was already recorded since 1977 (Eldredge & Smith 1995; Hopper & Smith 1992). Our records, dated 2004, confirm that the invasion was still ongoing at this date; no more recent records are available.

Invasion of Florida is probably recent, with our first specimens found in August 2012, and the species is apparently now well established in 2014, with several different locations in Miami Dade County (Table 1). No survey was done in other part of Florida or the USA.

Figure 6 shows all available records of *P. manokwari* in the world. The invasion mainly concerns territories in the Pacific Area; Miami, Florida, is the first record in the Americas.

Discussion

Identification of specimens

By their external morphological characteristics, the specimens found in the new locations, Singapore, Tahiti, New Caledonia, Solomon Islands and Florida, are similar to *Platydemus manokwari* from other locations.

Sequences of COI comprised two variants, “Australian” (with a minor variation on a single nucleotide in a single specimen among 6) and “Other” (France, New Caledonia, Singapore, Tahiti, Florida; all identical). The difference between the two variants was 3.8% (computed on short sequences) and 5.2% (computed on long sequences). In the same family as *P. manokwari*, i.e. the Geoplanidae, intraspecific and interspecific distances show variations in the literature. For *Microplana* spp., specimens considered to belong to the same species varied by up to 4%, and specimens from different species varied by 19% (Alvarez-Presas et al. 2012). In other genera, some species show high intraspecific values, up to 8.3% (Lago-Barcia et al. 2014). The difference of 5.2% between the two variants of *P. manokwari* found here could be either considered as relatively high intraspecific variation, or as evidence for the presence of two different species – one of which in need of a formal description. In the absence of information about the genetic polymorphism of the species in its land of origin (Papua-New Guinea), we adopt a conservative attitude and consider that a single species, *P. manokwari*, is involved.

We hypothesize that the world invasion by *P. manokwari*, from a putative origin in Papua-New Guinea, involved at least two populations, one whose descendants are now found in Australia (the “Australian variant”), and one whose descendants are now found in other localities investigated so far, including France, New Caledonia, Tahiti, Singapore, and Florida (the “Other variant”). All “Other variant” samples had identical sequences, suggesting that they all originate from a single population; the existence of polymorphism, although minor, in the “Australian” specimens, might indicate that the population which invaded Australia was slightly more genetically diverse than the “Other” population; but this requires verification on more numerous samples and additional genetic markers.

Significance of these new records

As far as we are informed, these records are the first records of *P. manokwari* in Singapore, New Caledonia, Solomon Islands, and North America. They thus add four territories to the list of 16 territories recently published, which includes Irian Jaya, New Guinea, Australia, Guam and Northern Mariana Islands, Philippines, Japan, Maldives, Palau, Hawaii, Federated States of Micronesia, French Polynesia, Samoa, Tonga, Vanuatu, Fiji and France (Justine et al. 2014b).

In French Polynesia, records were known only from Moorea Island (Lovenburg 2009) and Mangareva Island (Purea et al. 1998) (Justine et al. 2014b); the present records are the first in Tahiti Island, the main island of French Polynesia. However, Moorea is very close to Tahiti (15 km) with intense relations by several ferries and it is not surprising that the two islands share the species. Of interest is that the species was recorded in Tahiti Island in two stations with different altitudes, sea level and about 1000 m.

In France, the current situation is thus that *P. manokwari* is confined to a single hothouse in the Jardin des Plantes in Caen, but is not eradicated. Eradication is still an issue of concern.

Platydemus manokwari has now been found in 20 territories in the world, mainly in the Pacific area (Fig. 6). It is thus clear that the genetic results presented here represent only a small portion of these disjunct populations of the worm. A survey of the genetics of population in the area of origin (Papua New Guinea) is necessary to interpret the results, as are the use of additional genetic markers; this is out of the scope of this paper which relies on a single marker (COI) on a limited number of samples, with none from the country or origin.

Invasive land planarians, generally originating from the Southern Hemisphere or from tropical Asia, are now found in all parts of the world (Alvarez-Presas et al. 2014; Breugelmans et al. 2012; Cannon et al. 1999; Jones 1998; Jones 2005; Justine et al. 2014a; Kawakatsu et al. 2002; Mateos et al. 2013) and some of these species are a threat to local species among their prey, including earthworms (Boag et al. 1994; Boag & Yeates 2001; Jones et al. 2001; Murchie & Gordon 2013) and snails (Winsor et al. 2004).

Platydemus manokwari is a highly invasive species, and a major threat to snails and other soil invertebrates, including endemic species. This paper is voluntarily restricted to our new findings for brevity; readers are referred to a previous paper in which distribution records, reproduction, biology, prey lists, impacts, and possible control options were reviewed (Justine et al. 2014b).

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1

Platydemus manokwari in Coral Gables, Florida, USA.

Photograph by Makiri Sei.

2

Platydemus manokwari in Singapore.

Photograph by Andrew Wee Kien Han.

3

Platydemus manokwari in Nouméa, New Caledonia.

Photograph by Claire Goiran. Scale: cm and mm.

4

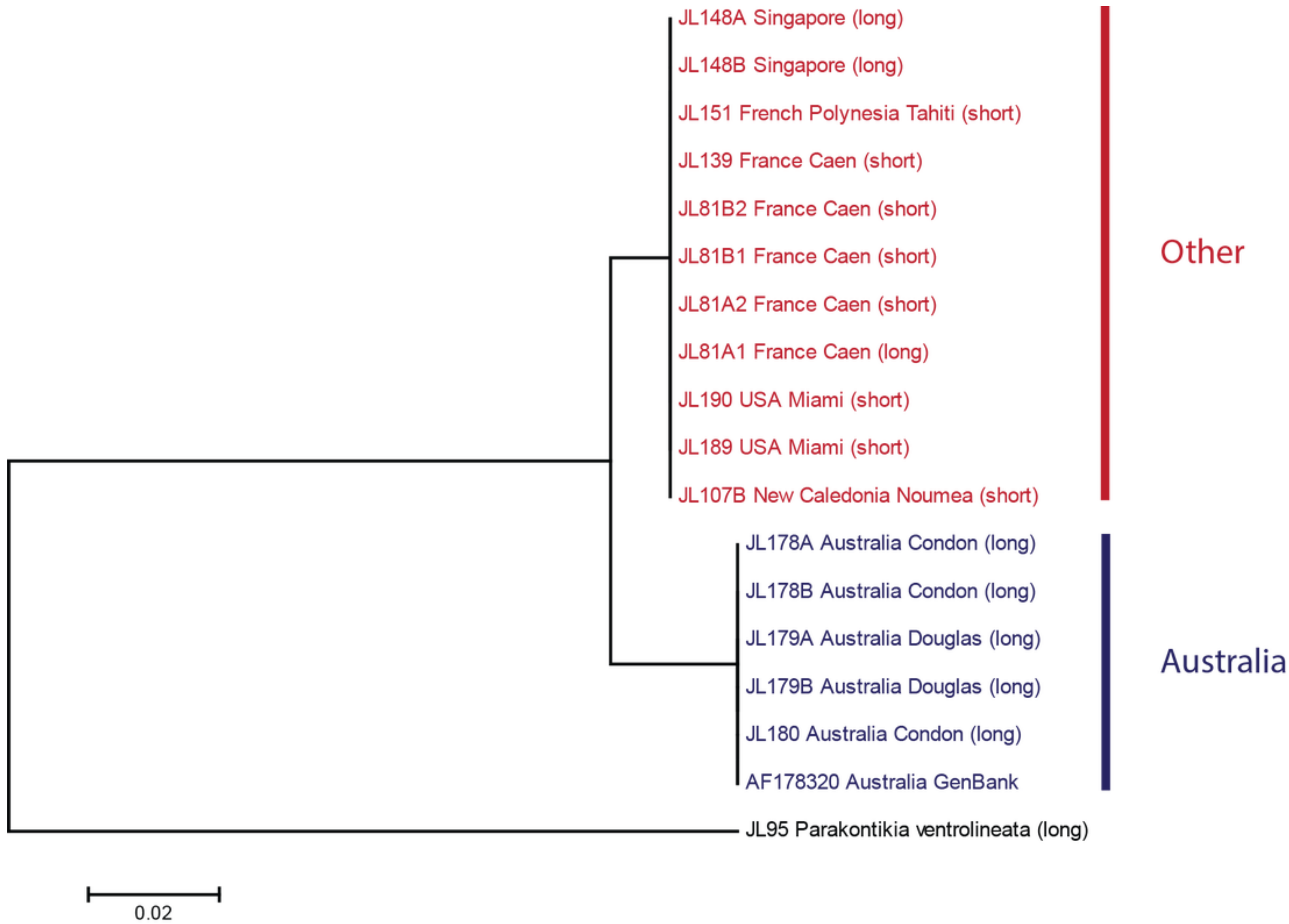
Figure 4. *Platydemus manokwari* in Fa'a'ā, Tahiti, French Polynesia.

Photograph by Jonas Fernandez. Scale: cm and mm.

5

NJ-tree of available sequences of *Platydemus manokwari* from various localities.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987) . The optimal tree with the sum of branch length = 0.21732299 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 317 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013) . *Parakontikia ventrolineata* used as outgroup.


JL148A Singapore (long)
JL148B Singapore (long)
JL151 French Polynesia Tahiti (short)
JL139 France Caen (short)
JL81B2 France Caen (short)
JL81B1 France Caen (short)
JL81A2 France Caen (short)
JL81A1 France Caen (long)
JL190 USA Miami (short)
JL189 USA Miami (short)
JL107B New Caledonia Noumea (short)
JL178A Australia Condon (long)
JL178B Australia Condon (long)
JL179A Australia Douglas (long)
JL179B Australia Douglas (long)
JL180 Australia Condon (long)
AF178320 Australia GenBank
JL95 Parakontikia ventrolineata (long)
Other
Australia
0.02

6

Platydemus manokwari, map of distribution records.

Blue: previous records (Justine et al. 2014b) ; Red: new records reported in this paper.

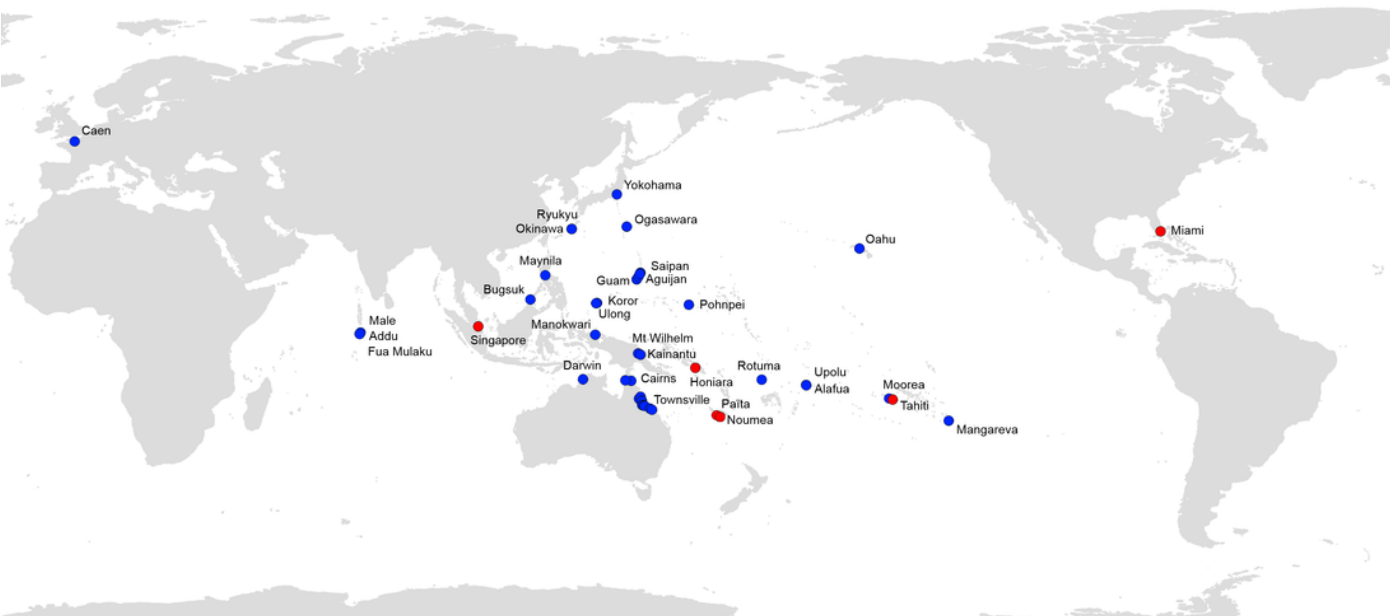


Table 1(on next page)

Origin of *Platydemus manokwari* specimens and observations

1

Table 1. Origin of *Platydemus manokwari* specimens and observations

Locality	Collector and/or photographer	Photos	Specimens	Date	Specimen deposition	COI Sequence
France						
Caen, hothouse in Jardin des Plantes	FREDON Basse Normandie		+	29/10/2013	MNHN JL81	Short: KF887958 (identical in 3 specimens) Long: new
Caen, hothouse in Jardin des Plantes	FREDON Basse Normandie		+	15/04/2014	MNHN JL139	Long: new
French Polynesia						
Fa'a'ā, Tahiti Island, altitude: sea level	Jonas Fernandez	+ (Fig. 4)	+	26/05/2014	MNHN JL151	Short: new
Moorea-Maiao, Tahiti Island, altitude 1000m	Jean-Yves Meyer *	+		19/06/2013		
New Caledonia						
Nouméa, quartier Vallée des Colons	Nicolas Rinck	+	+	09/04/2014	MNHN JL107B	Short: new
Nouméa, quartier N'Géa	Claire Goiran	+ (Fig. 3)		29/07/2014		
Nouméa, quartier Motor Pool	Vanessa Héquet			06/09/2014		
Mont-Dore, Saint Michel	Ludivine Sariman, specimen CEN#2496		+	01/10/2014	MNHN JL197	
Païta, Tontouta	Gazmira Machin-Boucher, specimen CEN#2497		+	12/10/2014	MNHN JL198	
Singapore						
Singapore, Opera Estate, Fidelio Street	Andrew Wee Kien Han	+ (Fig. 2)	+	20/03/2014	MNHN JL148A MNHN JL148B	Long: new Long: new
Singapore, Chestnut Avenue	Anonymous blog **, photos by "James", Kok Sheng, Tanah Merah	+		05/02/2011 18/04/2011		
Singapore, Admiralty Park	Anonymous blog **, photo by "James"	+		22/07/2010		
Singapore, secondary forest at the end of Sunset Way	Benjamin Paul Yi-Hann Lee	+		01/01/2014		

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Solomon Islands						
Guadalcanal, Foxwood, east of Honiara	Crispus Fanai	+		08/2014		
Guadalcanal, Henderson, east of Honiara	Crispus Fanai & Francis Tsatsia, specimen examined by LW, LW1804)		+	08/2014		
Guadalcanal, Dodo Creek, east of Honiara	B Crispus Fanai & Francis Tsatsia, specimen examined by LW, LW1805		+	09/2014		
Guam						
Quenga turnoff on Sengsong Road (28)	David G. Robinson, specimen USDA #: 04-GU-11		+	17/08/2004	MNHN JL191	Not obtained
Alongside road to Ritidian Point	David G. Robinson, specimen USDA #: 05-GUAM-15		+	22/08/2005	MNHN JL192	Not obtained
USA, Florida						
Miami, NW 5th Avenue	Mary Yong Cong, specimen DPI#: WP#2		+	10/08/2012	MNHN JL189	Short: new
Miami, SW 122 Street	Anibal Altamirano specimen DPI#: none		+	06/09/2012	MNHN JL190	Short: new
Miami, SW 192 Terrace	Mary Yong Cong & Juan Suarez, DPI#: none		+	30/10/2014	USDA 140203	
Coral Gables, Montgomery Botanical Gardens, Old Cutler Road	Makiri Sei	+ (Fig. 1)		14/08/2014		
Australia, Queensland						
Townsville, Condon	Leigh Winsor, specimen LW1794		+	28-10-2009	MNHN JL178A MNHN JL178B	Long: new – 1 difference Long: new
Townsville, Palmetum, Douglas	Leigh Winsor, specimen LW1795		+	17-09-2014	MNHN JL179A MNHN JL179B	Long: new Long: new
Townsville, Condon	Leigh Winsor, specimen LW1796		+	17-09-2014	MNHN JL180	Long: new

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* Available from: *http://www.jymeyer.com/pages/2013_12_amp_19_juin_Sorties_terrain_Fare_Rau_Ape_et_Fare_Mato_Tahiti_Nui_Societe-8698840.html*

** Available from: *http://lazy-lizard-tales.blogspot.co.uk/2011/12/ribbons-terrestrial-nemerteans-of.html*