Evidence of multiple colonizations as a driver of black fly diversification in an oceanic island

Yann Gomard1,*, Josselin Cornuault2, Séverine Licciardi3,4,5, Erwan Lagadec1, Boutaina Belqat6, Najla Dsouli7, Patrick Mavingui1,8, Pablo Tortosa1

1 Université de La Réunion, UMR PIMIT (Processus Infectieux en Milieu Insulaire Tropical), INSERM 1187, CNRS 9192, IRD 249, Plateforme Technologique CYROI, Sainte-Clotilde, La Réunion, France, 2 Department of Biodiversity and Conservation, Real Jardín Botánico, RJB-CSIC, Madrid, Spain, 3 CIRAD, UMR ASTRE, Sainte-Clotilde, La Réunion, France, 4 ASTRE, Univ Montpellier, CIRAD, INRA, Montpellier, France, 5 Groupement d’Intérêt Public Cyclotron Reunion Océan Indien (GIP CYROI), Sainte-Clotilde, La Réunion, France, 6 Department of Biology, Faculty of Sciences, University Abdelmalek Essaâdi, Tétouan, Morocco, 7 Centre de Recherche et de Veille sur les maladies émergentes dans l'Océan Indien (CRVOI), Plateforme Technologique CYROI, Sainte-Clotilde, La Réunion, France, 8 Université de Lyon, Lyon, France; Université Lyon 1, Villeurbanne, France; CNRS, UMR 5557, Ecologie Microbienne, Villeurbanne, France; INRA, UMR1418, Villeurbanne, France

* yann.gomard@gmail.com

Abstract

True oceanic islands typically host reduced species diversity together with high levels of endemism, which make these environmental set-ups ideal for the exploration of species diversification drivers. In the present study, we used black fly species (Diptera: Simuliidae) from Reunion Island as a model to highlight the main drivers of insect species diversification in this young and remote volcanic island located in the Southwestern Indian Ocean. Using local and regional (Comoros and Seychelles archipelagos) samples as well as specimens from continental Africa, we tested the likelihood of two distinct scenarios, i.e. multiple colonizations vs. in-situ diversification. For this, posterior odds were used to test whether species from Reunion did form a monophyletic group and we estimated divergence times between species. Three out of the four previously described Reunion black fly species could be sampled, namely *Simulium ruficorne*, *Simulium borbonense* and *Simulium triplex*. The phylogenies based on nuclear and mitochondrial markers showed that *S. ruficorne* and *S. borbonense* are the most closely related species. Interestingly, we report a probable mitochondrial introgression between these two species although they diverged almost six million years ago. Finally, we showed that the three Reunion species did not form a monophyletic group, and, combined with the molecular datation, the results indicated that Reunion black fly diversity resulted from multiple colonization events. Thus, multiple colonizations, rather than in-situ diversification, are likely responsible for an important part of black fly diversity found on this young Darwinian island.

Introduction

Species assemblages within a community may gain new species through immigration or in-situ diversification [1,2]. The relative importance of each of these two processes in explaining
local diversity is however subject to debate [3–7]. Islands and archipelagos represent highly favorable systems for understanding how local diversity arises and to describe evolutionary drivers at play [8–10]. Due to their geographic isolation, island systems are rather closed environments in which both species colonization and gene flow with other islands and/or continents are reduced, which is expected to favor local radiation [5,8,11,12]. Furthermore, despite their often-small size, islands generally harbor a diversity of habitats promoting sometimes exceptionally prolific diversification through adaptive radiation (e.g. spiders [13], anoles [14] and Darwin’s finches [15]). In-situ radiation may be further promoted in oceanic islands of volcanic origin, which generates topologically complex landscapes and thus varied habitats with limited inter-habitat gene flow [16,17].

Located in the Southwestern Indian Ocean (SWIO) region, Reunion Island is a young island, 2.1 million years (Myr), belonging to the Mascarene archipelago, which also includes Mauritius and Rodrigues islands (Fig 1). The SWIO basin also comprises Madagascar, Comoros and Seychelles archipelagos and forms one of the world’s 34 biodiversity hotspots [18]. These islands differ in their age, geological origin and distance to continent landmasses. As a result, biota hosted by these islands have different evolutionary histories and are appropriate for biogeographic and phylogeographic studies (see Agnarsson and Kuntner [19]). Located 800 km East of Madagascar, the Mascarene archipelago is the most remote archipelago of this insular ecosystem and is characterized by a high level of endemism [20]. In addition, Mascarene islands are young and considered as “Darwinian” islands (as defined by Gillespie and Roderick [5]) that have emerged de novo on volcanic hotspots. Hence, these islands have never been connected to continental masses and constitute suitable systems for investigating evolutionary processes involved in the assembly of species communities, as illustrated by several studies using plants [21,22], reptiles [23,24], nematodes [25], birds [26] and avian blood parasites as model organisms [27,28].

Fig 1. Geological context of the Southwestern Indian Ocean. Sample locations of black fly species are indicated by red triangles. The numbers correspond to island age in millions of years. GC: Grande Comore, MH: Mohéli, AN: Anjouan and MY: Mayotte, GLO: Glorieuses, JDN: Juan de Nova, EU: Europa, TRO: Tromelin, RE: Reunion Island, MU: Mauritius, RO: Rodrigues. Source map: ESRI World modified from Warren et al. [29].

https://doi.org/10.1371/journal.pone.0202015.g001
Black flies (Simuliidae) are insects of both medical and veterinary importance [30,31]. Some species transmit parasites such as the filarial *Onchocerca volvulus* (responsible for river blindness in human populations) or avian blood parasites of the genus *Leucocytozoon* [32–34]. The biting of adult females can also impair cattle and poultry productivity [35,36]. Furthermore, black flies play an important role in ecological processes, as their larvae constitute an important source of food for other organisms and participate to the treatment of organic materials in streams [31,37,38]. Interestingly, the Simuliidae family is highly diversified with more than 2,200 living species distributed on all continents except Antarctica [39]. Black flies are notably present on several remote islands and are hence interesting models for evolutionary studies on islands and archipelagos [40–46]. The SWIO islands house several black fly species, some of them with widespread geographic distributions whereas others are considered as endemics [39]. On Reunion Island, four species, belonging to the *ruficorne* species-group (subgenus *Nevermannia*), have been previously described based on morphological characters by Giudicelli [47]. *Simulium ruficorne* (Macquart, 1838) was originally described from Reunion Island while this species is currently known to have a large spatial distribution extending from the SWIO (Africa, Madagascar, Comoros and Mascarene archipelagos) to the Middle East and the Mediterranean sea [39,48]. Three additional species have been morphologically described using samplings carried out in 1983: *Simulium borbonense* (Giudicelli, 2008), *Simulium indoceanicum* (Giudicelli, 2008) and *Simulium triplex* (Giudicelli, 2008). These three species were considered as endemic to Reunion Island although *S. triplex* was later reported on Mauritius Island [39]. Based on morphological examinations, Giudicelli [47] suggested that the local black fly diversity results from successive colonization events from African continental *S. ruficorne* population. According to this hypothesis, each colonization has conducted to reproductive isolation before the arrival of another group of immigrants from African continental *S. ruficorne* population (multiple colonizations). This process would have taken place three times on Reunion Island.

In this study, we sequenced mitochondrial and nuclear genes from Reunion black fly species in order to investigate their phylogenetic relationships and evaluate divergence times between each of these species and African continental *S. ruficorne*. We then investigated the processes of diversification possibly involved by confronting alternative scenarios including (i) *in-situ* diversification within Reunion Island following a single colonization event of a *S. ruficorne* population and (ii) multiple independent colonizations.

**Materials and methods**

**Ethics statement**

No endangered or protected species was included in the present study. Most specimens were sampled on Reunion Island and did not require specific permits as the study did not include endangered species and did not involve sampling in protected areas. On Comoros archipelago, the samples were provided by an entomological survey carried out in the context of disease investigation for which the research protocol was approved by the Vice-Presidency of Agriculture, Fisheries and Environment of the Union of Comoros (see [49]). Samples from Morocco (Northern Africa) were provided by B. Belqat according to the permit delivered to the PhD student Y. EL Harym who was the collector.

**Sampling sites and specimens**

The main sampling was conducted on Reunion Island from January to May 2011 (Fig 1 and Table 1). Larvae were collected in all ten perennial rivers of the island. For each river, two sampling stations were set up: a downstream station located below 40 meters above sea level, and,
Table 1. Black fly specimens used in this study and GenBank accession numbers.

| Designation | Subgenus | Specific epithet | Type | Location | Collection date | Coordinates | GenBank accession number |
|-------------|----------|-----------------|------|----------|-----------------|-------------|-------------------------|
| GY018_borb  | Nevermannia | borbonense | L    | Reunion Island | 09/01/11 | 21° 18' 41.472" S, 55° 38' 29.256" E | JQ663445 JQ673499 |
| GY022_borb  | Nevermannia | borbonense | L    | Reunion Island | 10/01/11 | 21° 12' 25.524" S, 55° 27' 1.908" E | JQ663446 NA |
| GY024_borb  | Nevermannia | borbonense | L    | Reunion Island | 09/01/11 | 21° 18' 41.472" S, 55° 38' 29.256" E | JQ663447 JQ673500 |
| GY047_borb  | Nevermannia | borbonense | L    | Reunion Island | 09/01/11 | 21° 18' 41.472" S, 55° 38' 29.256" E | JQ663448 JQ673501 |
| GY048_borb  | Nevermannia | borbonense | L    | Reunion Island | 20/01/11 | 21° 2' 1.212" S, 55° 29' 37.644" E | JQ663449 JQ673502 |
| GY049_borb  | Nevermannia | borbonense | L    | Reunion Island | 20/01/11 | 21° 2' 1.212" S, 55° 29' 37.644" E | JQ663450 NA |
| GY050_borb  | Nevermannia | borbonense | L    | Reunion Island | 20/01/11 | 21° 1' 12.000" S, 55° 32' 24.000" E | JQ663451 NA |
| GY053_borb  | Nevermannia | borbonense | L    | Reunion Island | 20/01/11 | 21° 1' 12.000" S, 55° 32' 24.000" E | JQ663452 NA |
| GY062_borb  | Nevermannia | borbonense | L    | Reunion Island | 10/01/11 | 21° 12' 25.524" S, 55° 27' 1.908" E | JQ663453 NA |
| GY063_borb  | Nevermannia | borbonense | L    | Reunion Island | 10/01/11 | 21° 12' 25.524" S, 55° 27' 1.908" E | JQ663454 NA |
| GY077_borb  | Nevermannia | borbonense | A    | Reunion Island | 22/02/11 | 20° 54' 36.000" S, 55° 29' 24.000" E | JQ663455 NA |
| GY078_borb  | Nevermannia | borbonense | A    | Reunion Island | 22/02/11 | 20° 54' 36.000" S, 55° 29' 24.000" E | JQ663456 NA |
| GY079_borb  | Nevermannia | borbonense | A    | Reunion Island | 22/02/11 | 20° 54' 36.000" S, 55° 29' 24.000" E | JQ663457 NA |
| GY020_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 10/01/11 | 21° 17' 35.113" S, 55° 24' 45.623" E | JQ663458 NA |
| GY025_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 10/01/11 | 21° 15' 25.524" S, 55° 27' 32.544" E | JQ663459 NA |
| GY027_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 07/01/11 | 20° 54' 15.480" S, 55° 30' 19.800" E | JQ663460 NA |
| GY031_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 06/01/11 | 21° 2' 7.296" S, 55° 42' 52.416" E | JQ663461 NA |
| GY035_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 07/01/11 | 20° 58' 43.752" S, 55° 41' 18.492" E | JQ663462 NA |
| GY040_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 10/01/11 | 21° 15' 25.524" S, 55° 27' 32.544" E | JQ663463 JQ673503 |
| GY041_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 10/01/11 | 20° 57' 43.704" S, 55° 18' 35.352" E | JQ663464 JQ673504 |
| GY042_rufi  | Nevermannia | ruficorn | L    | Reunion Island | 10/01/11 | 20° 57' 43.704" S, 55° 18' 35.352" E | JQ663465 JQ673505 |

(Continued)
| Designation | Subgenus  | Specific epithet | Type | Location | Collection date | Coordinates | GenBank accession number |
|-------------|-----------|-----------------|------|----------|----------------|-------------|-------------------------|
| GY051_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière des Fleurs Jaunes, Salazie | 20/01/11 | 21° 1’ 12.000’ S 55° 32’ 24.000’ E | JQ663466 NA |
| GY056_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière des Fleurs Jaunes, Salazie | 20/01/11 | 21° 1’ 12.000’ S 55° 32’ 24.000’ E | JQ663467 JQ673506 |
| GY059_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière Sainte-Etienne, Saint-Louis | 10/01/11 | 21° 1’ 45.708’ S 55° 24’ 57.636’ E | JQ663468 NA |
| GY061_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière Sainte-Etienne, Saint-Louis | 10/01/11 | 21° 1’ 25.524’ S 55° 27’ 1.908’ E | JQ663469 JQ673507 |
| GY067_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière des Fleurs Jaunes, Salazie | 20/01/11 | 21° 2’ 1.212’ S 55° 29’ 37.644’ E | JQ663470 JQ673508 |
| GY068_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière des Roches, Bras-Panon | 06/01/11 | 21° 0’ 20.736’ S 55° 41’ 38.252’ E | JQ663471 NA |
| GY070_rufi  | Neumannia | ruficornis      | L    | Reunion Island Rivière des Roches, Bras-Panon | 06/01/11 | 21° 0’ 20.736’ S 55° 41’ 38.252’ E | JQ66472 JQ673509 |
| GY075_rufi  | Neumannia | ruficornis      | A    | Reunion Island Ravine du Chaudron, Sainte-Clotilde | 22/02/11 | 20° 54’ 36.000’ S 55° 29’ 24.000’ E | JQ663473 NA |
| GY076_rufi  | Neumannia | ruficornis      | A    | Reunion Island Ravine du Chaudron, Sainte-Clotilde | 22/02/11 | 20° 54’ 36.000’ S 55° 29’ 24.000’ E | JQ663474 NA |
| GY080_rufi  | Neumannia | ruficornis      | A    | Reunion Island Ravine du Chaudron, Sainte-Clotilde | 22/02/11 | 20° 54’ 36.000’ S 55° 29’ 24.000’ E | JQ663475 NA |
| GY123_rufi  | Neumannia | ruficornis      | A    | Reunion Island Riviére des Pluies, Sainte-Clotilde | 09/03/11 | 20° 55’ 12.000’ S 55° 30’ 36.000’ E | JQ663476 NA |
| GY124_rufi  | Neumannia | ruficornis      | A    | Reunion Island Riviére des Pluies, Sainte-Clotilde | 09/03/11 | 20° 55’ 12.000’ S 55° 30’ 36.000’ E | JQ663477 NA |
| GY126_rufi  | Neumannia | ruficornis      | A    | Reunion Island Sainte-Suzanne | 09/03/11 | 20° 54’ 59.8’ S 55° 36’ 30.5’ E | JQ663478 NA |
| GY133_rufi  | Neumannia | ruficornis      | A    | Reunion Island Riviére Langevin, Saint-Joseph | 12/03/11 | 21° 22’ 12.000’ S 55° 38’ 60.000’ E | JQ663479 JQ673510 |
| BB007_rufi  | Neumannia | ruficornis      | L    | Morocco (Northen Africa) Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46’48.03’ N 005° 33’42.62’ W | KY421689 KY421701 |
| BB008_rufi  | Neumannia | ruficornis      | L    | Morocco (Northen Africa) Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46’48.03’ N 005° 33’42.62’ W | KY421690 KY421702 |
| BB009_rufi  | Neumannia | ruficornis      | L    | Morocco (Northen Africa) Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46’48.03’ N 005° 33’42.62’ W | KY421691 KY421703 |
| BB010_rufi  | Neumannia | ruficornis      | L    | Morocco (Northen Africa) Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46’48.03’ N 005° 33’42.62’ W | KY421692 KY421704 |

(Continued)
Table 1. (Continued)

| Designation | Subgenus | Specific epithet | Type | Location | Collection date | Coordinates | GenBank accession number |
|-------------|----------|-----------------|------|----------|----------------|-------------|------------------------|
| BB011_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421693 KY421705 |
| BB012_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421694 KY421706 |
| BB013_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421695 KY421707 |
| BB014_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421696 KY421708 |
| BB015_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421697 KY421709 |
| BB016_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421698 KY421710 |
| BB017_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421699 KY421711 |
| BB018_rufi  | Nevermannia | ruficornis | L. | Morocco (Northen Africa) | Séguia de Hay Alt Chaib (Locality: Tazzarine, Province: Zagora) | 17/06/15 | 30° 46'48.03'' N 005° 33'42.62'' W | KY421700 KY421712 |
| GY019_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière Sainte-Etienne, Saint-Louis | 10/01/11 | 21° 17' 35.113'' S 55° 24' 45.623'' E | JQ663480 NA |
| GY029_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière des Remparts, Saint-Joseph | 08/01/11 | 21° 22' 59.196'' S 55° 37' 6.816'' E | JQ663481 NA |
| GY030_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière des Remparts, Saint-Joseph | 08/01/11 | 21° 22' 59.196'' S 55° 37' 6.816'' E | JQ663482 NA |
| GY033_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière des Roches, Bras-Panon | 06/01/11 | 21° 0' 20.736'' S 55° 41' 38.252'' E | JQ663483 NA |
| GY034_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière des Roches, Bras-Panon | 06/01/11 | 21° 1' 21.180'' S 55° 40' 9.768'' E | JQ663484 NA |
| GY036_trip  | Nevermannia | triplex | L. | Reunion Island | Rivière du Mâti, Saint-André | 07/01/11 | 20° 58' 43.752'' S 55° 41' 18.492'' E | JQ663485 NA |

(Continued)
| Designation | Subgenus | Specific epithet | Type | Location | Collection date | Coordinates | GenBank accession number | Phylogeny of Reunion black flies |
|-------------|----------|-----------------|------|----------|----------------|-------------|-------------------------|--------------------------------|
| GY038_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 08/01/11 | 21° 23’ 7.440” S | 55° 38’ 39.336” E | JQ663486 | NA |
| GY039_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 08/01/11 | 21° 23’ 7.440” S | 55° 38’ 39.336” E | JQ663487 | NA |
| GY052_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 20/01/11 | 21° 1’ 12.000” S | 55° 32’ 24.000” E | JQ663488 | JQ673511 |
| GY057_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 06/01/11 | 21° 2’ 7.296” S | 55° 42’ 52.416” E | JQ663489 | NA |
| GY058_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 06/01/11 | 21° 2’ 7.296” S | 55° 42’ 52.416” E | JQ663490 | NA |
| GY060_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 10/01/11 | 21° 17’ 45.708” S | 55° 24’ 57.636” E | JQ663491 | NA |
| GY064_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 07/01/11 | 20° 58’ 43.752” S | 55° 41’ 18.492” E | JQ663492 | NA |
| GY065_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 20/01/11 | 21° 2’ 1.212” S | 55° 29’ 37.644” E | JQ663493 | NA |
| GY069_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 06/01/11 | 21° 0’ 20.736” S | 55° 41’ 38.252” E | JQ663494 | NA |
| GY072_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 06/01/11 | 21° 0’ 20.736” S | 55° 41’ 38.252” E | JQ663495 | NA |
| GY073_trip | *Nevermannia* | *triplex* | L. | Reunion Island | 06/01/11 | 21° 1’ 21.180” S | 55° 40’ 9.768” E | JQ663496 | NA |
| GY119_trip | *Nevermannia* | *triplex* | A | Reunion Island | 08/03/11 | 20° 55’ 12.000” S | 55° 30’ 36.000” E | JQ663497 | JQ673512 |
| GY120_trip | *Nevermannia* | *triplex* | A | Reunion Island | 09/03/11 | 20° 55’ 12.000” S | 55° 30’ 36.000” E | NA | JQ673513 |
| GY121_trip | *Nevermannia* | *triplex* | A | Reunion Island | 09/03/11 | 20° 55’ 12.000” S | 55° 30’ 36.000” E | NA | JQ663498 |
| GY127_trip | *Nevermannia* | *triplex* | A | Reunion Island | 09/03/11 | 20° 54’ 59.8” S | 55° 36’ 30.5” E | JQ663499 | NA |
| SL001_como | *Nevermannia* | *triplex* | L. | Comoros archipelago | 24/02/11 | 12° 0’ 38.5” S | 44° 25’ 51.5” E | JQ663500 | NA |
| SL002_como | *Nevermannia* | *triplex* | L. | Comoros archipelago | 24/02/11 | 12° 0’ 38.5” S | 44° 25’ 51.5” E | JQ663501 | NA |
| SL003_como | *Nevermannia* | *triplex* | L. | Comoros archipelago | 24/02/11 | 12° 19’ 39.432” S | 43° 40’ 7.428” E | JQ663502 | NA |
| SL004_como | *Nevermannia* | *triplex* | L. | Comoros archipelago | 24/02/11 | 12° 19’ 39.432” S | 43° 40’ 7.428” E | JQ663503 | JQ673514 |
| GY239_seyc | *Simulium* sp. 1 | | L | Seychelles archipelago | 05/04/11 | 4° 39’ 30.444” S | 55° 24’ 45.760” E | JQ663504 | JQ673515 |
| GY240_seyc | *Simulium* sp. 1 | | L | Seychelles archipelago | 05/04/11 | 4° 39’ 30.444” S | 55° 24’ 45.760” E | JQ663505 | NA |
| GY241_seyc | *Simulium* sp. 1 | | L | Seychelles archipelago | 05/04/11 | 4° 39’ 30.444” S | 55° 24’ 45.760” E | JQ663506 | NA |
| GY242_seyc | *Simulium* sp. 1 | | L | Seychelles archipelago | 05/04/11 | 4° 39’ 30.444” S | 55° 24’ 45.760” E | JQ663507 | NA |

*S. aureohirtum* | *Nevermannia* | *aureohirtum* | Thailand | JF916830 | NA |

*S. aureohirtum* | *Nevermannia* | *aureohirtum* | Thailand | KF289401 | NA |

(Continued)
whenever possible, an upstream station located more than 500 meters above sea level. At each station, 60 larvae were sampled on submerged substrata as follows: 30 larvae were collected on submerged stones and 30 from submerged vegetation. Adults were captured using two methods. First, CDC miniature traps were mounted with UV leds and dry ice was placed in a container above the gear in order to feed the trap overnight with CO₂. These traps were placed on the sampling sites (along or at the vicinity of a river) between 5.00 and 6.00 PM and picked up the next day between 7.00 and 8.00 AM. As this method lures the flies for a blood meal, mostly unfed females were captured. In addition, adults were captured using a butterfly net held out of a car driving at 30 Km.h⁻¹ along perennial rivers. This method allowed trapping males and (fed/unfed) females. The sampling was opportunistically complemented with larvae specimens from other areas in the region (Comoros and Seychelles archipelagos) and from Africa (represented by larval specimens of *S. ruficorne* from Morocco) (Fig 1 and Table 1). All sampled specimens were immediately stored in 70˚ ethanol until laboratory analyses.

**Morphological and molecular identification**

All Reunion black fly larvae could be identified morphologically using previously published keys [47]. For larvae, three specific morphological characteristics were used: ventral and dorsal ornamentations on the head capsule together with hypostomium shape. To improve identification of larvae and adults, a potential size polymorphism in the Internal Transcribed Spacer 1 (ITS1) was investigated as this nuclear marker has been shown to be variable in size between closely related black fly species [50,51]. Using DNA from morphologically identified larvae as template; a Polymerase Chain Reaction (PCR) amplification of the ITS1 locus was carried out using previously published primers, ITS1-5'/ITS1-3' [52] (see DNA extraction, amplification

---

**Table 1. (Continued)**

| Designation | Subgenus       | Specific epithet | Type  | Location     | Collection date | Coordinates | GenBank accession number |
|-------------|----------------|------------------|-------|--------------|-----------------|-------------|------------------------|
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Larvae| China        | NA              | NA          | KP793690               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538878               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538879               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538881               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538882               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538883               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538884               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538886               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538887               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538888               |
| *S. aureohirtum* | Neverymannia | *aureohirtum*     | Adult | China        | NA              | NA          | FJ538889               |
| *S. feuerborni*  | Neverymannia | *feuerborni*      | Adult | Thailand     | JX484813        | NA          |                       |
| *S. fruticosum*  | Neverymannia | *feuerborni*      | Adult | Thailand     | KF289399        | NA          |                       |
| *S. merga*      | Montisimulium | *merga*           | Adult | Thailand     | KF289460        | NA          |                       |
| *S. pahagense*  | Davieselium | *pahagense*       | Adult | Thailand     | KF289460        | NA          |                       |
| *S. quinquestriatum* | Simulium | *quinquestriatum* | Adult | China        | JQ412151        | NA          |                       |
| *S. bidestatum* | Simulium     | *bidestatum*      | Adult | China        | DQ534947        | NA          |                       |

L: Larvae; A: Adult; NA: Not Available. The asterisks indicate species which were not sampled in the present study. For these species, only information relative to countries and GenBank accession numbers are provided.

[https://doi.org/10.1371/journal.pone.0202015.t001](https://doi.org/10.1371/journal.pone.0202015.t001)
and sequencing section). Differences in amplicon size were visualized by electrophoresis on 2% agarose gels stained with 1X GelRed™ (Biotium Inc.) and further confirmed by Sanger sequencing.

**DNA extraction, amplification and sequencing**

A sample of 14 adults and 62 larvae (comprising at least one larva per species and sampling station in Reunion) was used for molecular analyses. DNA extraction was realized with the Qia-gen EZ1 DNA Tissue kit (Qiagen Corporation, Valencia, CA) according to the manufacturer’s protocol. The mitochondrial cytochrome c oxidase subunit I encoding gene (COI) and a nuclear fragment encompassing 18S rDNA (3’ end), Internal Transcribed Spacer 1 (ITS1), 5.8S rDNA, Internal Transcribed Spacer 2 (ITS2) and 28S rDNA (5’ end) locus (thereafter referred as 18S/28S) was amplified and sequenced for phylogenetic reconstructions. COI was amplified with LCOI490/HCO2198 primers [53] and 18S/28S with specific primers described in Brockhouse et al. [54]. Amplification conditions for COI consisted of an initial denaturation step at 94˚C for 3 min followed by 30 cycles at 94˚C for 30 s, 50˚C for 30 s and 72˚C for 1 min, and by a final elongation step of 7 min at 72˚C. Ribosomal DNA, both 18S/28S and ITS1, were amplified using the following conditions: 3 min at 94˚C, followed by 40 cycles of 30 sec at 95˚C, 50 sec at 45˚C and 50 sec at 72˚C. The amplification ended with a final step of 7 min at 72˚C. Bi-directional sequencing was carried out using the amplifying primers. Whenever nuclear sequences revealed heterozygote specimens, amplicons were gel-purified and cloned into a pGEM-T Easy Vector (Promega Corporation, Fitchburg, WI). Four randomly selected transformants were then sequenced in order to get the sequences of both alleles. All COI and 18S/28S sequences were deposited in GenBank under JQ663445-JQ663507, JQ673499-JQ673515 and KY421689-KY421712 accession numbers (Table 1).

**Phylogenetic analyses**

Sequences were aligned using MAFFT implemented in Geneious pro software v.5.4 [55]. Additional black fly species sequences from GenBank were integrated in phylogenies as potential close relatives, including in particular available sequences of *Simulium aureohirtum* (Brunetti, 1911) (*ruficorne* species-group) from Asia (Table 1).

Dated phylogenetic trees were estimated with BEAST v2.3.2 [56] for nuclear and mitochondrial data independently but using the same model, described here. The substitution model used was the GTR + G + I, in which all other substitution models are nested. Relative exchangeability rates were assigned a Gamma (shape = 1, scale = 1) prior. Equilibrium base frequencies were estimated. Among-site heterogeneity in substitution rates (+ G) was accommodated with the inclusion of site-specific substitution rate multipliers. These multipliers were assigned a Gamma (shape = alpha, rate = alpha) prior, with alpha fixed to 11.1. This parametrization of the gamma prior implies a prior coefficient of variation of substitution rates among sites of ~ 0.3. Rate multipliers were integrated out of the model using a discrete approximation of the gamma prior [57] with six categories. The proportion of invariant sites (+ I) was assigned a Uniform (0,1) prior. An uncorrelated log-normal relaxed molecular clock was used, with a fixed mean (ucldMean parameter) and a free standard deviation (ucldStdev) parameter with a Gamma (shape = 0.5396, scale = 0.3819) prior. This parametrization of the ucldev prior implies that 97.5% of the prior density is in favour of a coefficient of variation of rates among branches less than 1. For the mitochondrial analysis, ucldMean was fixed to the standard mutation rate of arthropods, i.e. 0.0115 substitutions site⁻¹ Myr⁻¹ [58]. For the nuclear analysis, since we had no information about the substitution rate, we fixed ucldMean to 0.004, a value yielding a phylogenetic tree of roughly the same height as for the mitochondrial
analysis. This makes the time unit of the nuclear tree roughly similar to that of the mitochondrial DNA tree, so that the same priors for time-related parameters can be used in both analyses. However, the time-scale of the nuclear tree should not be interpreted. A birth-death tree prior was used, with a Lognormal (1,1.25) for the speciation rate (birthRate2) and a Beta (1,2) prior for the relative extinction rate (i.e. the extinction-to-speciation rates ratio, relativeDeathRate2). For both the mitochondrial and nuclear analyses, two independent MCMC chains were run for a total of 30 million generations each, of which 10% were discarded as burn-in. Samples were kept every 1,000th generation. Convergence was verified by effective sample size (ESS) values exceeding 200 for all parameters, using Tracer v1.6 for continuous parameters. For the tree topology parameter, we calculated the pseudo- and approximate ESSs, as implemented in the functions `topological.pseudo.ess` and `topological.approx.ess` of the R package `rwty` [59], based on the path distance between trees. The posterior sample of trees was summarized into a unique maximum-clade-credibility consensus tree with posterior median node ages using TreeAnnotator v2.3.2.

Sequences were available for both nuclear and mitochondrial markers only for a small number of individuals. Consequently, we chose to keep both nuclear and mitochondrial analyses separate.

**Monophyly tests**

In order to assess the likelihood of a scenario with a single colonization of Reunion by black flies, with a subsequent in-situ diversification, we assessed the likelihood of all Reunion species forming a monophyletic clade. As the Comorian specimens were identified as *S. triplex* (see the Results section), these specimens were synonymized with Reunion *S. triplex* in this monophyly analysis, effectively testing a hypothesis of in-situ diversification in the SWIO region (Hypothesis $H_1$) rather than on Reunion Island alone.

We further evaluated the monophyly of Reunion *S. ruficorne* specimens (Hypothesis $H_2$), as the mitochondrial phylogeny suggested the paraphyly of *S. ruficorne* (see the Results section).

Based on Bergsten et al. [60], we did not use Bayes Factors to evaluate clade monophyly, given the difficulty to define appropriate prior probabilities of hypotheses. Instead, each of the two hypotheses ($H_1$ and $H_2$) was assessed using its posterior odd, calculated as: $P(H|D) / (1-P(H|D))$, where $P(H|D)$ is the posterior probability of hypothesis $H$ [60]. The posterior odd of a monophyly hypothesis indicates how many times more likely monophyly is relative to non-monophyly. $P(H|D)$ was estimated as the frequency of trees in the posterior distribution that included the clade considered, when this frequency was more than 0. When the clade of interest is not comprised in any tree of the posterior sample (zero frequency), we estimated that $P(H|D)$ was less than $1/ESS_{topology}$.

**Results**

**Molecular and morphological identification**

Sampling on Reunion Island allowed the collection of three out of the four previously described species, namely *Simulium ruficorne*, *Simulium borbonense* and *Simulium triplex*. No *Simulium indoceanicum* specimen was captured. For each sampled species, the amplification of the ITS1 locus produced amplicons with different sizes: 82, 110 and 116 bp for *S. triplex*, *S. ruficorne* and *S. borbonense*, respectively (Table 2). This size polymorphism can be visualized by electrophoresis on a 2% agarose gel and is thus usable for rapid molecular identification of Reunion black flies (S1 Fig). The amplification of the ITS1 locus produced amplicons of the same size for both Reunion and continental African *S. ruficorne* specimens (Table 2). Similarly, the amplification of the ITS1 locus from all Comorian specimens and Reunion *S. triplex*
Table 2. Details on nuclear (ITS1 and 18S/28S) and mitochondrial (COI) sequences obtained from the black fly specimens of this study.

| Location | Species            | ITS1 locus size | Number of haplotypes detected for COI locus (658 bp) | Number of haplotypes detected for 18S/28S locus (576–650 bp) |
|----------|--------------------|-----------------|------------------------------------------------------|---------------------------------------------------------------|
| Reunion  | S. triflex         | 82 bp           | 1 (n = 20)                                           | 1 (n = 3)                                                     |
|          | S. ruficorne       | 110 bp          | 10 (n = 16)                                          | 4 (n = 8)                                                     |
|          | S. borbonense      | 116 bp          | 12 (n = 19)                                          | 2 (n = 4)                                                     |
| Africa   | S. ruficorne       | 110 bp          | 6 (n = 12)                                           | 5 (n = 12)                                                    |
| Comoros  | S. triflex         | 82 bp           | 1 (n = 4)                                            | 1 (n = 1)                                                     |
| Seychelles | Simulium sp. 1    | 111 bp          | 1 (n = 4)                                            | 1 (n = 1)                                                     |

For 18S/28S and COI locus the numbers in brackets correspond to the number of produced sequences according to each species.

Phylogenetic analysis

The amplification of the mitochondrial locus produced 75 sequences of 658 nucleotides without deletions or insertions. No polymorphism was found in mitochondrial sequences within Reunion (n = 20) or within Comorian (n = 4) S. triflex, nor within Seychelles specimens (n = 4) (Table 2). In contrast, mitochondrial sequences obtained from S. borbonense (n = 19) or from Reunion (n = 16) and African S. ruficorne (n = 12) showed nucleotide polymorphism comprising respectively 12, ten and six distinct haplotypes. Reunion and African S. ruficorne did not share any mitochondrial haplotype. Nuclear amplification (18S/28S) produced 29 sequences ranging from 576 to 650 bp for S. borbonense (n = 4), Reunion (n = 3) and Comorian (n = 1) S. triflex, Reunion (n = 8) and African (n = 12) S. ruficorne and only one sequence was obtained from a Seychelles specimen (Table 2). Interestingly, although S. ruficorne specimens from Reunion and continental Africa did not share any mitochondrial haplotype, they did share one nuclear sequence.

The mitochondrial phylogeny (Fig 2) confirmed the identification of Comorian specimens as S. triflex, which clustered with Reunion S. triflex (Clade A). In contrast, the specimens from Seychelles formed a clade of their own (Clade B), related to but quite divergent from S. triflex. All Reunion and African S. ruficorne grouped together. The S. aureohirtum specimens fell within the ruficorne species-group. Interestingly, a number of sequences obtained from Reunion S. ruficorne specimens (designated by stars in Fig 2) clustered with S. borbonense haplotypes in Clade C. The latter specimens were however confirmed as S. ruficorne based on morphology, ITS1 length polymorphism typing and the nuclear phylogeny (see Fig 3 and S1 Fig).
The remaining sequences from Reunion *S. ruficorne* otherwise formed a monophyletic clade (Clade D), as did sequences from African *S. ruficorne* (Clade E).

The nuclear phylogeny (Fig 3) was less resolved than the mitochondrial one but confirmed the identification of Comorian specimens as *S. triplex*. The topology further showed that the *S.

---

**Fig 2.** Phylogenetic tree based on mitochondrial sequences (COI, 658 bp) of black fly species from Reunion Island, Northern of Africa (Morocco), Comoros (Anjouan and Mohéli islands) and Seychelles archipelago (Mahé island). This analysis was carried out with BEAST v.2.3.2. Each sequence corresponds to one individual and the haplotype number is indicated between brackets. The estimated divergence times and posterior probabilities are indicated above and below the nodes, respectively. The time unit is per million year (Myr). Within the *Simulium borbonense* clade, the stars indicate specimens identified as Reunion *Simulium ruficorne* by morphology and the ITS1 diagnostic.

https://doi.org/10.1371/journal.pone.0202015.g002

**Fig 3.** Phylogenetic tree based on nuclear sequences (18S/28S, 672 bp) of black fly species from Reunion Island, Northern of Africa (Morocco), Comoros (Anjouan and Mohéli islands) and Seychelles archipelago (Mahé island). This analysis was conducted with BEAST v.2.3.2. Each sequence corresponds to one specimen. The numbers at the nodes are posterior probabilities and only posterior probabilities superior to 0.50 are indicated. The time scale is unknown for nuclear markers. Within the clade of Reunion *Simulium ruficorne*, the stars indicate specimens with *S. borbonense* mitochondrial haplotypes (see Fig 2).

https://doi.org/10.1371/journal.pone.0202015.g003
ruvicorne specimens harbouring S. borbonense haplotypes (designated by stars in Fig 3) possessed S. ruvicorne nuclear DNA. It is noteworthy that an African S. ruvicorne specimen (BB013) is embedded within S. aureohirtum clade. This topology likely results from a poor resolution of the nuclear phylogeny. Indeed, a visual inspection of the nuclear sequences further suggests that S. ruvicorne BB013 belongs to the S. ruvicorne clade with the presence of a single deletion detected only in S. ruvicorne sequences (data not shown).

Monophyly tests

The mitochondrial and nuclear phylogenies indicated that the three Reunion black fly species do not cluster together, suggesting that they originated through multiple colonizations (Figs 2 and 3). This hypothesis is corroborated by the results of monophyly tests based on mitochondrial and nuclear phylogenies, which rejected the monophyly of Reunion species (H1, posterior odd 0, Table 3).

The mitochondrial phylogeny suggested that S. ruvicorne is paraphyletic as some sequences obtained from Reunion S. ruvicorne belong to the S. borbonense clade (Fig 2). This paraphyly was further supported by the results of monophyly tests, which rejected the monophyly of Reunion S. ruvicorne (H2, posterior odd 0 for mitochondrial DNA and 0.11 for nuclear DNA, Table 3). All Reunion S. ruvicorne were unambiguously identified as S. ruvicorne based on morphology and harbored S. ruvicorne nuclear material (ITS1 diagnosis). Altogether, these results indicate that Reunion black flies identified as S. ruvicorne can also host S. borbonense haplotypes.

Estimation of divergence times

The estimation of divergence times, based on the mutation rate of arthropods for mitochondrial DNA, showed that at least two Reunion black fly species diverged several million years before the emergence of Reunion Island (estimated at 2.1 Myr). Specifically, the divergence of S. triplex and the two other species from Reunion Island is estimated at 12.87 Myr ago (95% CI: 11.11–15.75), while S. borbonense and S. ruvicorne diverged 6.46 Myr ago (95% CI: 4.71–8.30) (Fig 2). In contrast, S. triplex and specimens collected in Comoros diverged more recently: 0.67 Myr ago (95% CI: 0.31–1.11) (Fig 2). Interestingly, Reunion and African S. ruvicorne diverged 2.24 Myr ago (95% CI: 1.46–3.04), which is close to the estimated age of Reunion Island emergence (Fig 2).

Discussion

Morphological and molecular identification of black fly species from Reunion and other Southwestern Indian Ocean islands

The investigation of Reunion black fly fauna allowed sampling three out of the four species described on the island: Simulium ruvicorne, Simulium borbonense and Simulium triplex. The
fourth species, *Simulium indoceanicum*, was not observed in our samples. The sampling was carried out during the austral summer and not through the whole year, thus we cannot rule out that the absence of *S. indoceanicum* resulted from either a restricted sampling period or geographic distribution of this species. Environmental perturbations are known to have considerable impact on black fly communities. These perturbations include hydrological modifications, deforestation, tourism development, habitat destruction and pollution [61,62].

Reunion Island has experienced a rapid economical development together with a fast increase of its demography (706 300 inhabitants in 1999 and 842 767 inhabitants in 2014 [63]) that involved important environmental perturbations. Islands are known to display high rates of species extinction [64] and data accumulated in the SWIO islands actually confirm a high proportion of extinctions, sometimes associated with human activities [65]. Thus, the extinction of *S. indoceanicum*, originally described from samples collected in 1983, can be hypothesized even though it remains difficult to address.

In addition to the investigation of Reunion black flies, our study brings some original information on regional black fly diversity. We describe *S. tripexus* in the black fly fauna of Comoros archipelago. Consequently, *S. tripexus* may not be considered as endemic to Mascarene archipelago anymore. On Seychelles archipelago, only one endemic *Simulium* species has been reported so far: *Simulium speculiventre* (Enderlein, 1914) belonging to the *ruficorne* species-group (*Nevermannia*) [39]. Thus, the taxonomy is consistent with our phylogenies (Figs 2 and 3) and specimens sampled from Seychelles archipelagos could effectively correspond to *S. speculiventre*.

The identification of black fly species from Reunion Island was thus far mainly based on morphological characters of larvae, pupae and the genitalia of male adult [47]. Although these characters are helpful, they require expertise and can be time-consuming notably for the genitalia dissection. Thus, we developed a molecular identification based on ITS1 length polymorphism that allows quick determination of Reunion black fly species and may be used to solve identification problems occurring with damaged specimens (S1 Fig). Such molecular diagnosis represents an important tool for taxonomic groups sheltering species that may be hard to distinguish on a strictly morphological basis [30,51]. These results also support the use of ITS1 as a molecular marker for closely related black fly species identification [50,51,54].

**Phylogeny of Reunion black fly species reveals *S. ruficorne* paraphyly and suggests mitochondrial introgression**

Phylogenetic reconstructions allowed the description of Reunion black fly species relationships. Among the Reunion sampled species, *S. ruficorne* and *S. borbonense* are the most closely related species. Based on mitochondrial data, *S. ruficorne* appeared paraphyletic. Such paraphyly has been reported for other black fly species, complex species or species-group: *Simulium fenestratum* (Edwards, 1934) [66], *Simulium articum* (Malloch, 1914) [67] and *Simulium tuberosum* (Lundström, 1911) [68]. Interestingly, for these latter species, the paraphyly was supported by both mitochondrial and nuclear phylogenies. Here, the paraphyly of Reunion *S. ruficorne* is only supported by the mitochondrial phylogeny (Fig 2). Indeed, both the nuclear phylogeny and the ITS1 diagnostic concur to indicate that the Reunion *S. ruficorne* specimens harbouring *S. borbonense* mitotypes do possess *S. ruficorne* nuclear DNA. Such incongruence between mitochondrial and nuclear phylogenies has been previously reported within the *Simulium damnosum* complex (Theobald, 1903) [69] or other insect species [70]. Krueger and Hennings [69] proposed that such incongruence could result from mitochondrial introgression between black fly species evolving in sympatry. Interestingly, Conflitti et al. [67] also reported the paraphyly of sibling species within the *Simulium articum* complex. To explain this
situation, the authors proposed possible introgressive hybridization with the sharing of alleles between members of sympatric species. In our case, the hypothesis of mitochondrial introgression requires interspecific hybridization between *S. borbonense* and *S. ruficorne*, two sympatric species belonging to the same species-group (*ruficorne*). Interestingly, this interspecific hybridization appears to be asymmetrical, and would result from interspecific crosses between *S. ruficorne* males and *S. borbonense* females. Indeed, no introgressed specimens descending from the reciprocal hybridization (*i.e.* Reunion *S. ruficorne* females with *S. borbonense* males) were observed. Such asymmetrical introgression has been previously reported for other organisms: birds, Flycather [71], spiders, Agelenidae [72], Honey Bee [73] and mosquitoes, *Culex pipiens* [74] and has been proposed to result from distinct processes such as differences in mate choice, non-reciprocal mechanical barriers, copulation, numerical dominance of one species over the other or Wolbachia-mediated selective sweep. To test this last hypothesis, the presence of *Wolbachia wsp* gene was investigated by using previously published PCR amplification test [75] and we could not detect *Wolbachia* in any of the tested flies (data not shown). The presence of paraphyletic species feeds the controversy on the use of mitochondrial markers alone to describe the taxonomy and phylogeny of invertebrates [76,77].

In this study, we estimated a divergence time of 6.46 Myr between *S. ruficorne* and *S. borbonense*, while the times of coalescence of all mitochondrial haplotypes for each of these two species are much more recent (< 1.00 Myr). Incomplete lineage sorting would be associated with more ancient mitochondrial lineages for each species although we cannot rule out possible colonization-dependent bottlenecks that would lead to the loss of a significant part of ancestral polymorphism. Interestingly, one nuclear sequence (18S/28S) was identically recovered from both African and Reunion *S. ruficorne* populations. This pattern is suggestive of a divergence time that is not long enough for lineage sorting to complete or gene flow between Reunion and African *S. ruficorne* populations.

**Multiple colonization events are responsible of Reunion black fly diversity**

Speciation by geographical isolation following colonization is commonly accepted as a general source of endemism in oceanic island ecosystems [5,78]. The presence of black fly endemic species reported on remote oceanic islands suggests that speciation follows colonization events [41]. As far as Reunion species are concerned, a more complex scenario has been proposed according which the actual diversity results from successive colonizations from a single continental species (*i.e.* African *S. ruficorne*) [47]. Our phylogenetic reconstructions and monophyly tests support the hypothesis of multiple colonizations. *Simulium triplex* is present on different islands across the SWIO (Comoros archipelago, Mauritius Island and Reunion Island), suggesting that this species has a large spatial distribution and has potentially colonized Reunion Island from another island of the region. Altogether, we propose that the black fly diversity of Reunion Island has been built by a recurring influx of new colonists from the regional species pool, all belonging to *ruficorne* species-group.

In Pacific Ocean archipelagos, it has been shown that dispersal and speciation processes may explain the current diversity and distribution of black fly species. Within these islands, adaptive radiation represents the major mode of speciation with specialization of species to specific habitats [42,43,46,79]. Indeed, ecological adaptations has been proposed as an important factor of black fly diversification [66,68,79]. For example, on Society Islands, Joy, Craig and Conn [79] have proposed larval habitat shifts (cascades to rivers) as drivers of diversification between *Simulium oviceps* (Edwards, 1933) and *Simulium dussertorum* (Craig, 1997), two species belonging to the *oviceps* species-group (subgenus *Inseliellum*). Interestingly, Giudicelli [47] has shown that Reunion species exhibit differences in their distribution within rivers.
Indeed, *S. tripexus* is a stenothermic species, restricted to headwaters whereas *S. borbonense* and *S. ruficorne* are eurythermic species evolving within the lowland stream [47]. The implications of these ecological differences in the diversification of these black flies remain to be addressed but in any case our molecular dating does not support speciation within Reunion Island. However, we cannot rule out that *S. borbonense* and *S. tripexus* result from speciation from a hypothetical *S. ruficorne* ancestor. Such speciation process could have taken place on nearby areas such as Mauritius Island, Rodrigues, Madagascar or Africa. In Madagascar, several black fly species are present and notably species belonging to the *ruficorne* species-group [39]. Such routes of colonization are possible but additional regional sampling is required to investigate these geographical origins. Altogether the Reunion black fly model highlights the importance of dispersal as a driver of arthropod diversity on a young Darwinian Island. The dispersal can be realized by the active flight of black flies or other mechanisms such as human activities, winds and hosts [35,41,42,80]. As far as Reunion species are concerned, dispersal with bird remains possible as the species of *ruficorne* species-group are known to be ornithophilic [47,81].

**Conclusions**

In this study we showed that the Reunion black fly diversity resulted from multiple colonizations of distinct fly species rather than *in-situ* diversification, which improves our understanding of processes driving the biodiversity composition on young oceanic islands. Interestingly, we highlighted that two Reunion species, separated by approximately six million years of independent evolution, displayed an intriguing case of asymmetrical mitochondrial introgression. It is interesting to note that the evolutionary histories of some of these black flies’ hosts (i.e. *Zosterops* birds) and the blood parasites (i.e. *Leucocytozoon*) transmitted by black flies to these birds, have been investigated in the SWIO region [26–28]. The combination of these works and the present study can provide an interesting set of data to explore in detail the evolutionary histories of all partners evolving within a tripartite interaction on a young Darwinian Island.

**Supporting information**

S1 Fig. Migration results of ITS1—PCR products obtained from the different black fly species investigated in the present study. The colors red, blue, green and orange represent species from Morocco, Reunion Island, Comoros and Seychelles archipelagos, respectively. The electrophoresis was performed during 2 hours on a 2% agarose gel stained with 1X GelRed™ (Biotium Inc.) The visualization was realized under UV.

(TIF)

**Acknowledgments**

We would like to thank Younes El Harym, Jean Giudicelli, Jacques Rochat, Matthieu Roger, Louis-Clément Gouagna and the agents of Regional Health Agency of La Réunion (ARS) for providing and helping of black flies samplings. We thank Ben Warren, Olivier Florès and Steven M. Goodman for exchanges and discussions in the frame of this study. We thank David Wilkinson and Vanina Guernier for critical comments and corrections on the manuscript and Coralie Foray for preparing Fig 1. Phylogenies were performed on the University of Reunion Island supercomputer facility.

**Author Contributions**

**Conceptualization:** Séverine Licciardi, Pablo Tortosa.
**Formal analysis:** Yann Gomard, Josselin Cornuault, Najla Dsouli.

**Investigation:** Yann Gomard, Séverine Licciardi, Erwan Lagadec, Najla Dsouli, Pablo Tortosa.

**Resources:** Boutaina Belqat, Patrick Mavingui, Pablo Tortosa.

**Writing – original draft:** Yann Gomard, Josselin Cornuault, Séverine Licciardi, Erwan Lagadec, Boutaina Belqat, Najla Dsouli, Patrick Mavingui, Pablo Tortosa.

**References**

1. Goldberg EE, Roy K, Lande R, Jablonski D. Diversity, endemism, and age distributions in macroevolutionary sources and sinks. Am Nat. 2005; 165: 623–633. https://doi.org/10.1086/430012 PMID: 15937743

2. Ricklefs RE. Community Diversity: Relative Roles of Local and Regional Processes. Science. 1987; 235: 167–171. https://doi.org/10.1126/science.235.4785.167 PMID: 17778629

3. Bruun HH, Ejrnæs R. Community-level birth rate: a missing link between ecology, evolution and diversity. Oikos. 2006; 113: 185–191. https://doi.org/10.1111/j.0030-1299.2001.14174.x

4. Emerson BC, Gillespie RG. Phylogenetic analysis of community assembly and structure over space and time. Trends Ecol Evol. 2008; 23: 619–630. https://doi.org/10.1016/j.tree.2008.07.005 PMID: 18823678

5. Gillespie RG, Roderick GK. Arthropods on islands: Colonization, Speciation, and Conservation. Annu Rev Entomol. 2002; 47: 595–632. https://doi.org/10.1146/annurev.ento.47.091201.145244 PMID: 11729086

6. Mora C, Chittaro PM, Sale PF, Kritzer JP, Ludsin SA. Patterns and processes in reef fish diversity. Nature. 2003; 421: 933–936. https://doi.org/10.1038/nature01393 PMID: 12609998

7. Ricklefs RE. A comprehensive framework for global patterns in biodiversity. Ecol Lett. 2003; 7: 1–15. https://doi.org/10.1046/j.1461-0248.2003.00554.x

8. Emerson BC. Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. Mol Ecol. 2002; 11: 951–966. https://doi.org/10.1046/j.1365-294x.2002.01507.x PMID: 12030975

9. Shaw KL, Gillespie RG. Comparative phylogeography of oceanic archipelagos: Hotspots for inferences of evolutionary process. Proc Natl Acad Sci USA. 2016; 113: 7986–7993. https://doi.org/10.1073/pnas.1601078113 PMID: 27432948

10. Warren BH, Simberloff D, Ricklefs RE, Aguilée R, Condaminé FL, Gravel D, et al. Islands as model systems in ecology and evolution; prospects fifty years after MacArthur-Wilson. Ecol Lett. 2015; 18: 200–217. https://doi.org/10.1111/ele.12398 PMID: 25560882

11. Johnson KP, Adler FR, Cherry JL. Genetic and phylogenetic consequences of island biogeography. Evolution. 2000; 54: 387–396. PMID: 10937215

12. MacArthur RH, Wilson EO. The theory of island biogeography. Princeton: Princeton University Press; 1967.

13. Gillespie R. Community Assembly Through Adaptive Radiation in Hawaiian Spiders. Science. 2004; 303: 356–359. https://doi.org/10.1126/science.1091875 PMID: 14726588

14. Losos JB, Jackman TR, Larson A, de Queiroz K, Rodriguez-Schettino L. Contingency and Determinism in Replicated Adaptive Radiations of Island Lizards. Science. 1998; 279: 2115–2118. https://doi.org/10.1126/science.279.5359.2115 PMID: 9516114

15. Sato A, O’Higgins C, Figuerola F, Grant PR, Grant BR, Tichy H, et al. Phylogeny of Darwin’s finches as revealed by mtDNA sequences. Proc Natl Acad Sci USA. 1999; 96: 5101–5106. https://doi.org/10.1073/pnas.96.9.5101 PMID: 10220425

16. Juan C, Emerson BC, Oromí P, Hewitt GM. Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. Trends Ecol Evol. 2000; 15: 104–109. doi:10.1016/S0169-5347(99)01776-0 PMID: 10675925

17. Roderick GK, Gillespie RG. Speciation and phylogeography of Hawaiian terrestrial arthropods. Mol Ecol. 1998; 7: 519–531. PMID: 9528003

18. Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. Biodiversity hotspots for conservation priorities. Nature. 2000; 403: 853–858. https://doi.org/10.1038/35002501 PMID: 10706275

19. Agnarsson I, Kuntner M. The generation of a biodiversity hotspot: biogeography and phylogeography of the western Indian ocean islands. In: Anamthawat-Jonsson K, editor. Current Topics in Phylogenetics and Phylogeography of Terrestrial and Aquatic Systems. Rijeka: Intech; 2012. pp. 33–82.
20. Thébaud C, Warren BH, Cheke A, Strasberg D. Mascarene Islands, biology. In: Gillespie RG, Clague DA, editors. Encyclopedia of islands. Berkeley: University of California Press; 2009. pp. 612–619.

21. Le Péchon T, Dubuisson J-Y, Haevermans T, Cruaud C, Couloux A, Gigord LDB. Multiple colonizations from Madagascar and converged acquisition of dioecy in the Mascarene Dombeyoidea (Malvaceae) as inferred from chloroplast and nuclear DNA sequence analyses. Ann Bot. 2010; 106: 343–357. https://doi.org/10.1093/aob/mcq116 PMID: 20562131

22. Venkatasastry S, Khittoo G, Nowbuth P, Venkatastry DR. Phylogenetic relationships based on morphology among the Diospyros (Ebenaceae) species endemic to the Mascarene Islands. Biol J Linn Soc. 2006; 150: 307–313. https://doi.org/10.1111/j.1095-8339.2006.00474.x

23. Austin JJ, Arnold EN, Jones CG. Reconstructing an island radiation using ancient and recent DNA: the extinct and living day geckos (Phelsuma) of the Mascarene islands. Mol Phylogenet Evol. 2004; 31: 109–122. https://doi.org/10.1016/j.ympev.2003.07.011 PMID: 15019612

24. Harmon LJ, Melville J, Larson A, Losos JB. The Role of Geography and Ecological Opportunity in the Diversification of Day Geckos (Phelsuma). Syst Biol. 2008; 57: 562–573. https://doi.org/10.1080/10635150802304779 PMID: 18686194

25. Herrmann M, Kienle S, Rochat J, Mayer RJ, Sommer RJ. Haplotype diversity of the nematode Pristionchus pacificus on Réunion in the Indian Ocean suggests multiple independent invasions. Biol J Linn Soc. 2010; 100: 170–179. https://doi.org/10.1111/j.1095-8312.2010.01410.x

26. Warren BH, Beringham E, Prys-Jones RP, Thébaud C. Immigration, species radiation and extinction in a highly diverse songbird lineage: white-eyes on Indian Ocean islands. Mol Ecol. 2006; 15: 3769–3786. https://doi.org/10.1111/j.1365-294X.2006.03058.x PMID: 17032273

27. Cornuault J, Warren BH, Bertrand JAM, Milá B, Thébaud C, Heeb P. Timing and Number of Colonizations but Not Diversification Rates Affect Diversity Patterns in Hemisporidian Lineages on a Remote Oceanic Archipelago. Am Nat. 2013; 182: 820–833. https://doi.org/10.1086/673724 PMID: 24231541

28. Cornuault J, Bataillard A, Warren BH, Lootvoet A, Mirleau P, Duval T, et al. The role of immigration and in-situ radiation in explaining blood parasite assemblages in an island bird clade. Mol Ecol. 2012; 21: 1438–1452. https://doi.org/10.1111/j.1365-294X.2012.05483.x PMID: 22332752

29. Warren BH, Beringham E, Prys-Jones RP, Thébaud C. Tracking island colonization history and phenotypic shifts in Indian Ocean bulbuls (Hypsipetes: Pycnonotidae). Biol J Linn Soc. 2005; 85: 271–287. https://doi.org/10.1111/j.1095-8312.2005.00492.x

30. Adler PH, Cheke RA, Post RJ. Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). Infect Genet and Evol. 2010; 10: 846–865. doi:10.1016/j.meegid.2010.07.003

31. Currie DC, Adler PH. Global diversity of black flies (Diptera: Simuliidae) in freshwater. Hydrobiologia. 2007; 595: 469–475. https://doi.org/10.1007/s10750-007-9114-1

32. Smith SA. Parasites of birds of prey: Their diagnosis and treatment. Semin Avian Exot Pet. 1996; 5: 97–105. doi:10.1055/s-0022-3171

33. Hellgren O, Bensch S, Malmqvist B. Bird hosts, blood parasites and their vectors—associations uncovered by molecular analyses of blackfly blood meals. Mol Ecol. 2008; 17: 1605–1613. https://doi.org/10.1111/j.1365-294X.2007.03680.x PMID: 18266623

34. Levin II, Valkiūnas G, Santiago-Alarcon D, Cruz LL, Iezhova TA, O’Brien SL, et al. Hippoboscid-transmitted Haemoproteus parasites (Haemosporida) infect Galapagos Pelicaniform birds: Evidence from molecular and morphological studies, with a description of Haemoproteus iwa. Int J Parasitol. 2011; 41: 1019–1027. https://doi.org/10.1016/j.ijpara.2011.03.014 PMID: 21683082

35. Crosskey RW. The natural history of blackflies. London: John Wiley & Sons; 1990.

36. Adler PH, Currie DC, Wood DM. The black flies (Simuliidae) of North America. New York: Cornell University Press; 2004.

37. Malmqvist B, Wotton RS, Zhang Y. Suspension feeders transform massive amounts of seston in large northern rivers. Oikos. 2001; 92: 35–43. https://doi.org/10.1034/j.1600-0706.2001.920105.x

38. Malmqvist B, Adler PH, Kuusela K, Merritt RW, Wotton RS. Black flies in the boreal biome, key organisms in both terrestrial and aquatic environments: A review. Ecoscience. 2004; 11: 187–200. https://doi.org/10.1080/11956860.2004.11956108

39. Adler PH, Crosskey RW. World Blackflies (Diptera: Simuliidae): A comprehensive revision of the taxonomic and geographical inventory [2017].

40. Adler PH, Huang Y-T, Reeves WK, Kim SK, Otsuka Y, Takaoka H. Macrogenomic Evidence for the Origin of the Black Fly Simulium suzukii (Diptera: Simuliidae) on Okinawa Island, Japan. PLoS ONE. 2013; 8: e70765. https://doi.org/10.1371/journal.pone.0070765 PMID: 23951001

41. Adler PH, Giberson DJ, Purcell LA. Insular black flies (Diptera: Simuliidae) of North America: tests of colonization hypotheses. J Biogeogr. 2005; 32: 211–220. https://doi.org/10.1111/j.1365-2699.2004.01156.x
42. Craig DA, Currie DC, Joy DA. Geographical history of the central-western Pacific black fly subgenus Inseliellum (Diptera: Simuliidae: Simulium) based on a reconstructed phylogeny of the species, hot-spot archipelagos and hydrological considerations. J Biogeogr. 2001; 28: 1101–1127. https://doi.org/10.1046/j.1365-2699.2001.00619.x

43. Gillespie RG, Claridge EM, Goodacre SL. Biogeography of the fauna of French Polynesia: diversification within and between a series of hot spot archipelagos. Philos T Roy Soc B. 2008; 363: 3335–3346. https://doi.org/10.1098/rstb.2008.0124 PMID: 18782725

44. Hembry DH, Balukjian B. Molecular phylogeography of the Society Islands (Tahiti; South Pacific) reveals departures from hotspot archipelago models. J Biogeogr. 2016; 43: 1372–1387. https://doi.org/10.1111/jbi.12723

45. Joy DA, Conn JE. Molecular and Morphological Phylogenetic Analysis of an Insular Radiation in Pacific Black Flies (Simulium). Syst Biol. 2001; 50: 18–38. https://doi.org/10.1080/10635150120897 PMID: 12116592

46. Spironello M, Brooks DR. Dispersal and diversification: macroevolutionary implications of the MacArthur–Wilson model, illustrated by Simulium (Inseliellum) Rubstov (Diptera: Simuliidae). J Biogeogr. 2003; 30: 1563–1573. https://doi.org/10.1046/j.1365-2699.2003.00945.x

47. Giudicelli J. Les Simulies de l’île de la Réunion: présence de quatre espèces nouvelle pour la Science (Diptera, Simuliidae). Ephemeria. 2008; 9: 33–64.

48. Gibon F-M, Elouard J-M. Etude préliminaire de la distribution des insectes lotiques à Madagascar (exemple des trichoptères Philopotamidae et diptères Simuliidae). In: Lourenço WR, editor. Biogéographie de Madagascar. Paris: ORSTOM; 1996. pp. 507–516.

49. Roger M, Beral M, Licciardi S, Soule M, Faharoudine A, Foray C, et al. Evidence for Circulation of the Rift Valley Fever Virus among Livestock in the Union of Comoros. PLOS Neg Trop Dis. 2014; 8: e3045. https://doi.org/10.1371/journal.pntd.0003045 PMID: 25078616

50. Krüger A, Gelhaus A, Garms R. Molecular identification and phylogeny of East African Simulium damnosum s.l. and their relationship with West African species of the complex (Diptera: Simuliidae). Insect Mol Biol. 2000; 9: 101–108. https://doi.org/10.1046/j.1365-2583.2000.00163.x PMID: 10672077

51. LaRue B, Gaudreau C, Bagre HO, Charpentier G. Generalized structure and evolution of ITS1 and ITS2 rDNA in black flies (Diptera: Simuliidae) complex. Mol Biol Evol. 1996; 13: 244–252. https://doi.org/10.1093/molbev/msl032 PMID: 8583987

52. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, et al. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput Biol. 2014; 10: e1003537. https://doi.org/10.1371/journal.pcbi.1003537 PMID: 24722319

53. Yang Z. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J Mol Evol. 1994; 39: 306–314. https://doi.org/10.1007/BF00160154 PMID: 7932792

54. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, et al. Geneious v5.4, Available from http://www.geneious.com. 2011.

55. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu C-H, Xie D, et al. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput Biol. 2014; 10: e1003537. https://doi.org/10.1371/journal.pcbi.1003537 PMID: 24722319

56. Johnson KM, Bentley KE, Egaas B, Lindstedt SL, Sletvold NT, Skoglund P. APEX: A Tool for Rapid Population Genetic Analysis. Bioinformatics. 2014; 30: 937–939. https://doi.org/10.1093/bioinformatics/btt698 PMID: 24443475

57. Arthur–Wilson model, illustrated by

58. Lanfear R. RWTY (R We There Yet): An R Package for Examining Convergence of Bayesian Phylogenetic Analyses. Mol Biol Evol. 2017; 34: 1016–1020. https://doi.org/10.1093/molbev/msw279 PMID: 28087773

59. Bergsten J, Nilsson AN, Ronquist F. Bayesian Tests of Topology Hypotheses with an Example from Diving Beetles. Syst Biol. 2013; 62: 660–673. https://doi.org/10.1093/sysbio/syt029 PMID: 23628960

60. Lautenschläger M, Kiel E. Assessing morphological degradation in running waters using Blackfly communities (Diptera, Simuliidae): Can habitat quality be predicted from land use? Limnologica. 2005; 35: 262–273. https://doi.org/10.1016/j.limno.2005.04.003
62. Anbalagan S, Dinakaran S, Pandiarajan J, Krishnan M. Effect of tourism on the distribution of larval Blackflies (Diptera: Simulium) in Palni Hills of South India. Acta Hydrobiol Sin. 2011; 35: 688–692.

63. National Institute for Statistics and Economic Studies (INSEE). Séries historiques sur la population et le logement en 2014, Région de La Réunion (04). https://www.insee.fr/fr/statistiques/2874072?geo=REG-04 (accessed 21.07.17).

64. Kier G, Kreft H, Lee TM, Jetz W, Ibisch PL, Nowicki C, et al. A global assessment of endemism and species richness across island and mainland regions. Proc Natl Acad Sci USA. 2009; 106: 9322–9327. https://doi.org/10.1073/pnas.0810306106 PMID: 19470638

65. Thiollay J-M, Probst J-M. Ecology and conservation of a small insular bird population, the Réunion cuckoo-shrike Coracina newtoni. Biol Conserv. 1999; 87: 191–200. doi:10.1016/S0006-3207(98)00062-7

66. Pramual P, Nanork P. Phylogenetic analysis based on multiple gene sequences revealing cryptic biodiversity in Simulium multistriatum group (Diptera: Simuliidae) in Thailand. Entomol Sci. 2012; 15: 202–213. https://doi.org/10.1111/j.1479-8298.2011.00491.x

67. Confitti IM, Kratochvil MJ, Spironello M, Shields GF, Currie DC. Good species behaving badly: Non-monophyly of black fly sibling species in the Simulium arcticum complex (Diptera: Simuliidae). Mol Phylogenet Evol. 2010; 57: 245–257. https://doi.org/10.1016/j.ympev.2010.06.024 PMID: 20601001

68. Sripriphorn P, Sopoladawan PN, Wongpakam K, Pramual P. Molecular phylogeny of black flies in the Simulium tuberosum (Diptera: Simuliidae) species group in Thailand. Genome. 2014; 57: 45–55. https://doi.org/10.1139/gen-2013-0145 PMID: 24564215

69. Conflitti IM, Kratochvil MJ, Spironello M, Shields GF, Currie DC. Good species behaving badly: Non-monophyly of black fly sibling species in the Simulium arcticum complex (Diptera: Simuliidae). Mol Phylogenet Evol. 2010; 57: 245–257. https://doi.org/10.1016/j.ympev.2010.06.024 PMID: 20601001

70. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR–based classification of Wolbachia strains using wsp gene sequences. P Roy Soc Lond B Bio. 1998; 265: 505–515. https://doi.org/10.1098/rspb.1998.0324 PMID: 9569669

71. Moritz C, Cicero C. DNA Barcoding: Promise and Pitfalls. PLoS Biol. 2004; 2: e354. https://doi.org/10.1371/journal.pbio.0020354 PMID: 15486587

72. Rubinoff D. Utility of Mitochondrial DNA Barcodes in Species Conservation. Conserv Biol. 2006; 20: 1026–1033. https://doi.org/10.1111/j.1523-1739.2006.00372.x PMID: 16922219

73. Mayr E. Populations, espèces et évolution. Paris: Hermann; 1974.

74. Joy DA, Craig DA, Conn JE. Genetic variation tracks ecological segregation in Pacific island black flies. Heredity. 2007; 99: 452–459. https://doi.org/10.1038/hdy.6801023 PMID: 17611492

75. Abedraabo S, Le Pont F, Shelley AJ, Mouchet J. Introduction et acclimatation d'une simulie anthropophile dans l'île San Cristobal, archipel des Galapagos. (Diptera, Simuliidae). Bulletin de la Société Entomologique de France. 1993; 98.

76. Freeman P, Meillon B. Simuliidae of the Ethiopian region. London: British Museum Natural History; 1953.