# Frequent parasitism of *Apis mellifera* by trypanosomatids in geographically isolated areas with restricted beekeeping movements

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## Abstract

Trypanosomatids form a group of high prevalence protozoa that parasitise honey bees, with *Lotmaria passim* as the predominant species worldwide. However, the knowledge about the ecology of trypanosomatids in isolated areas is limited. The Portuguese archipelagos of Madeira and Azores provide an interesting setting to investigate these parasites because of their geographic isolation, and because they harbour honey bee populations devoid of two major enemies: *Varroa destructor* and *Nosema ceranae*. Hence, a total of 661 honey bee colonies from Madeira and the Azores were analysed using different molecular techniques, through which we found a high prevalence of trypanosomatids despite the isolation of these islands. *L. passim* was the predominant species and, in most colonies, was the only one found, even on islands free of *V. destructor* and/or *N. ceranae* with severe restrictions on colony movements to prevent the spread of them. However, islands with *V. destructor* had a significantly higher prevalence of *L. passim* and, conversely, islands with *N. ceranae* did not shown any significant correlation with the trypanosomatid. *Crithidia bombi* was detected in Madeira and on three islands of the Azores, almost always coincident with *L. passim*. By contrast, *Crithidia mellificae* was not detected in any sample. A High-Throughput Sequencing analysis distinguished two main haplotypes of *L. passim*, which accounted for 98% of the total sequence reads. This work suggests that *L. passim* and *C. bombi* are parasites that have been associated with honey bees predating the spread of *V. destructor* and *N. ceranae*.

**Keywords:** honey bee, Trypanosomatids, *Lotmaria passim*, PCR, sequencing, *Varroa destructor*

# Introduction

Trypanosomatidae are a group of parasitic protozoa that can infect a wide range of organisms, from plants to insects and vertebrates [1, 2]. In recent years, particular interest has focused on their infection of honey bees because of their possible involvement in increased colony mortality. The first trypanosomatid described in honey bees was *Crithidia mellificae* [3] and, subsequently, *Crithidia bombi* was identified in bumble bees [4]. These parasites were not given special attention until the beginning of the 21st century [5–11] and until then, the trypanosomatids found in honey bees were thought to be limited to *C. mellificae* in honey bees and to *C. bombi* in bumble bees. However, the advances in molecular techniques led to the discovery of a new species: *Lotmaria passim* [8], whose existence had previously been suggested from genetic data [12]. The re-evaluation of all the molecular data obtained in previous works showed that many sequences were incorrectly assigned to *C. mellificae* and, in fact, it became clear that *L. passim* was the predominant species worldwide [13–15]. Thus, *L. passim* seems to have been present in honey bee colonies long before its description, although there is no scientific evidence as to whether it is a novel honey bee parasite that recently spread worldwide, similar to *Varroa destructor* and *Nosema ceranae*, or whether it is an old parasite.

Due to the geographic isolation of the host populations, islands offer an ideal stage to assess these hypotheses and study the ecology and dynamics of invasive pathogen colonisation. Yet, there are very few trypanosomatid studies on islands, and these have been limited to the Pacific, including Japan [10, 16], New Zealand [15, 17, 18] and Hawaii [19]. Therefore, surveys from islands at other geographical latitudes would shed additional light as to whether these micro-organisms are recent or ancient honey bee parasites. To this regard, the Portuguese archipelagos of the Azores and Madeira are located in the Atlantic Ocean, 1,400 km and 900 km from the European mainland, Lisbon, respectively. The first introduction of honey bees on these islands dates back to the 16th century. These were taken by the Portuguese settlers, or by later inhabitants, when the islands were used as stopovers on the way to the Americas [20–22]. According to the latest report of the Portuguese Bee Health Programme [23], there are currently more than 10,000 honey bee colonies in Madeira and around 8,000 in the Azores. The severe restrictions on the importation of honey bees imposed since 2007 make the Azores archipelago epidemiologically unique, with six of the nine islands remaining free of *V. destructor*. Remarkably, in addition to being one of the few places in the world free of the mite [24], two of the Azorean islands (Flores and Santa Maria) have also escaped the worldwide spread of the microsporidium *N. ceranae* [25]. Therefore, the Azorean archipelago provides an incomparable setting for studying parasite ecology, as it is home to all kinds of parasite combinations. In light of this, we conducted a survey of trypanosomatids in the Azores as well as on Madeira, which was earlier colonized by *V. destructor*. The ultimate goal was to establish the prevailing trypanosomatid species and also to ascertain whether the queen marketing and colony movement that have been implicated in the spread of *V. destructor* have also influenced the distribution of trypanosomatids across the islands. To that end, we employed molecular methods on a cross-sectional honey bee sample that allow detection of an array of trypanosomatids, including *C. mellificae* and *L. passim*, which typically parasitize honey bees, and also species such as *C. bombi*, *Crithidia expoeki*, and *Crithidia acanthocephali*, more rarely associated with honey bees [26].

# Methods

## Survey and sample collection

This cross-sectional study is part of a larger survey conducted between 2014 and 2015 to describe the prevalence of pathogens in the Azores and Madeira archipelagos. The survey was carried out following the design described elsewhere [25, 27] and in accordance with the number of colonies on each island registered in 2013, with an expected pathogen prevalence of 15%, a precision rate of 10%, and a confidence level of 95%. About 150 adult honey bee workers were collected from 159 georeferenced apiaries of the Azores in the Summer of 2014 and 2015, and from 23 georeferenced apiaries of Madeira in the Spring of 2014. Samples were collected from three random colonies, and in a few cases from two or four colonies per apiary, resulting in a total of 483 samples for the Azores and 89 for Madeira. In addition, the islands of Faial, São Jorge, Santa Maria, and Terceira were re-sampled in the Summer of 2020 (89 colonies in 34 apiaries). In this later sampling, each sample comprised 20-30 workers. Samples from the Azores were shipped alive to the Centro de Investigação de Montanha (CIMO, Portugal) and then sent on dry ice to the Centro de Investigación Apícola y Agroambiental (CIAPA, Spain). Samples from Madeira were collected in ethanol and stored at -20 ºC until shipping to CIAPA. All samples were kept at -80 ºC for further analysis.

## DNA extraction

For each colony sampled in the Azores and Madeira in 2014-2015, a subsample of 120 workers was selected and processed as described previously [25]. In brief, the honey bees were macerated in 50% AL buffer (Qiagen®) and centrifugated at 3000 rpm for 10 minutes to obtain supernatant and sediment that were processed separately. The sediment was resuspended in 3 ml of milli-Q water and the DNA was extracted as described previously [25]. Only for the 2014-2015 Azorean samples, 400 µl of each supernatant was transferred to a 96-well plate (Deepwell, Eppendorf), treated with 15 µl of protease (Qiagen®) and incubated at 70 ºC for 10 minutes. Afterwards the DNA was extracted as indicated before [25].

The samples collected in 2020 in the Azores were processed similarly, but in this case the 20-30 honey bees were macerated in 5 ml of autoclaved milli-Q water in a Stomacher 80. In these samples, no phase separation was made so that 400 µl of each macerate was directly transferred to a 96-well Collection Microtube plate (Qiagen®) with glass beads (2 mm diameter, Sigma) and shaken (3 minutes, 30 Hz) in a TissueLyser (Qiagen®). Then, 150 µl of each sample was dispensed into a plate with 150 µl of 50% AL buffer and 15 µl of protease. After incubation, the DNA was extracted as above mentioned [25].

Extraction negative controls were included in all the processing and DNA extraction steps (one every 20 samples) and processed in parallel. All samples were stored at -80 ºC until further analysis.

## Sediment *versus* supernatant

Samples from the Azores from 2014-2015 were first processed for the detection of other pathogens in a different study [25] and, due to their value, were reused in this study. Since sediment and supernatant were available and it was not known which phase was the best for detecting these parasites after honeybee maceration, DNA obtained from the two phases was analysed separately to determine which was the best substrate for the detection of Trypanosomatidae. For this purpose, DNA was extracted from the sediment and supernatant of each sample, as above, and analysed in parallel by PCR, in order to compare the number of positive samples obtained in each phase.

## Trypanosomatidae detection

Polymerase Chain Reaction (PCR) was used to detect the Trypanosomatidae in all the samples (sediment from Madeira samples, supernatant and sediment from the 2014-2015 Azorean samples, and macerate from the 2020 Azorean samples) with the Tryp RPB1 primers described elsewhere [26] and shown in Table 1. These primers target the *DNA-dependent RNA polymerase I* (*rpb1*) gene and they amplify a fragment of 283 bp from all Trypanosomatidae species that have been detected in honey bees to date.

**Table 1** Primers and probes used for the molecular analysis of the honey bee samples.

| References | Target | Primers | Sequence 5' – 3' |
|---|---|---|---|
| [26] | Trypanosomatidae | Tryp RPB1-F1 | GTGGCTGGAYCTGTGGGAGC |
| | | Tryp RPB1-R1 | GCCRTTGATGAACTTCGCCAC |
| [28] | *C. mellificae* | qCmell_Cytb_F | TTTTGCCATGCACTATGATGTCT |
| | | qCmell_Cytb_R | AACCTATTACAGGCACAGTTGCTAAA |
| | | qCmell_Cytb_P | 6FAM-ATTGAGGATTAACAGTGTTTAGT-BHQ1 |
| | *L. passim* | qLpass_TOPII_F | GGCCATGGAAATACTCGAGTCT |
| | | qLpass_TOPII_R | ACCTTGCCTTCCTTCTTGAGATT |
| | | qLpass_TOPII_P | 6FAM-CCTCGACACGC+T+TA+GT-BHQ1 |
| | *C. bombi* | qCbom_RPB1_F | TGGTGGGTGCGATTACGAA |
| | | qCbom_RPB1_R | TCATTGAAGATGACGTGGATAAGC |
| | | qCbom_RPB1_P | 6FAM-CGTTGTCGGCGCCG-BHQ1 |

PCRs were performed in a 25 µl volume containing 13.25 µl of $H_2O$, 5 µl of 5X Phusion™ HF buffer, 0.5 µl of a 10 mM dNTP mix, 2.5 µl of each primer (5 µM), 0.25 µl of Phusion™ DNA Polymerase (ThermoFisher), and 1 µl of the DNA template. The PCR temperature profile was set according to the manufacturer's instructions, and consisted of an initial denaturation at 98 ºC for 30 s, followed by 45 cycles at 98 ºC for 10 seconds, 62.2 ºC for 30 seconds and 72 ºC for 10 seconds, and a final extension of 8 minutes at 72 ºC. PCRs were carried out in a Mastercycler® ep gradient S (Eppendorf) and the resulting amplicons were analysed in a QIAxcel Advanced System (QIAgen®), storing the remaining PCR product at -20 ºC for further processing. Extraction and PCR negative controls and a positive control were included in all the analyses and run in parallel. In the case of 2014-2015 Azorean samples, as both sediment and supernatant were analysed, a sample was considered positive if there was amplification in at least one of the phases.

## High-Throughput Sequencing

A subset of 91 positive samples that were positive for Trypanosomatidae were sequenced on the Illumina MiSeq platform together with negative controls. The PCR product of the positive samples was quantified in a NanoDrop™ 2000 Spectrophotometer (ThermoScientific™) to determine the DNA concentration and sent frozen to Universidade de Santiago de Compostela (CIMUS, Spain) for library preparation and sequencing.

Amplicons from each sample were used as input to prepare the library using the KAPA HyperPrep kit (Roche Sequencing Solutions Inc.), following the manufacturer´s protocol directly from the end-repair and A-tailing step. Library pools were normalized to a concentration of 4 nM and loaded at a concentration of 12 pM on an Illumina MiSeq instrument for 1 x 300 bp single-end sequencing (Flowcell Nano V2, 2 x 150 bp).

The reads generated in the sequencing run were de-multiplexed according to the barcodes assigned to each sample, and then processed with *fastp* [29] for adaptor removal and quality filtering. The reads were then organized

into individual files that contained the number and sequence haplotypes detected for each amplicon in a sample, as well as the number of reads in each direction (Forward and Reverse). Sequences with less than 5 reads were discarded. Subsequently, the files were converted to *fasta* format for sequence alignment with MACSE v2.05 (Multiple Alignment of Coding SEquences), a program that aligns protein-coding gene datasets without disrupting the underlying codon structure [29]. The reference sequences of the *rpb1* were obtained from GenBank for *L. passim* (MT558272.1 and LT976801. 1), *C. mellificae* (MT558227.1 and MT558204.1), *C. bombi* (MT558162.1 and MT558134.1), *C. acanthocephali* (MW28878781.1), and a new Trypanosomatidae species (Trypanosomatidae sp. MN038411.1), recently described [26]. Sequences were visualized with *BioEdit* [30] and those containing indels or stop codons were removed from the final dataset.

## Species-specific detection

All Trypanosomatidae-positive samples that were not sequenced by High-Throughput Sequencing (HTS) were analysed by real-time qPCR (qPCR) for the species-specific detection of *L. passim, C. mellificae,* and *C. bombi* using the primers described elsewhere [28] and shown in Table 1. The qPCR was performed on DNA obtained from Madeira samples, from both sediment and supernatant of the 2014-2015 Azorean samples, and from samples without phase separation from the 2020 Azorean samples. Negative and positive controls were also tested in parallel in all PCRs. As for Trypanosomatidae detection in the 2014-2015 Azorean samples, a sample was considered positive if there was amplification from either of the two phases. Amplicons from samples that gave a negative result by the specific qPCR but a positive result by the standard PCR amplification with the Tryp RPB1 primers were purified with QIAquick (Qiagen®) and Sanger sequenced in both directions (Genomic Unit, Universidad Complutense, Spain) on a 3730 Genetic Analyzer (Applied Biosystems). The sequences were checked manually with *BioEdit* [30] and compared with the sequences downloaded from GenBank using BLAST.

The geographical distribution of the colonies was compiled and plotted for each island using the *ArcGIS desktop* software [31].

## Statistical analysis

Chi-square test were performed to compare the sensitivity of Trypanosomatidae detection in the sediment and supernatant phases. Generalised linear models with a binomial family and a logit link function were used to determine whether the presence of *V. destructor* or *N. ceranae* influenced the prevalence of *L. passim* at the island scale, including island as covariable. In the latter case, only data from samples from the Azores collected in 2014-2015 were used, as this sampling was conducted and analysed homogeneously.

# Results

## Comparison between sediment and supernatant

To determine the best phase for Trypanosomatidae detection, sediment and supernatant analysis was performed on 477 Azorean samples collected in 2014-2015 (Table 2; six samples out of 483 had insufficient supernatant for this assay). The supernatant produced the highest number of positive samples (55.34%) as compared to the sediment (50.73%), although no statistically significant differences were found between both phases ($X^2$=2.04, gl=1; p=0.15). No trypanosomatid DNA was detected in any negative controls.

**Table 2** Number (and percentage) of Trypanosomatidae-positive (+) and -negative (-) samples detected on the supernatant and sediment phases obtained from the honey bee macerates.

| | Supernatant (+) | Supernatant (-) | **Total** |
|---|---|---|---|
| Sediment (+) | 161 (33.75%) | 81 (16.98%) | **242 (50.73%)** |
| Sediment (-) | 103 (21.59%) | 132 (27.67%) | **235 (49.27%)** |
| **Total** | **264 (55.34%)** | **213 (44.65%)** | **477 (100%)** |

## Prevalence of Trypanosomatidae

The prevalence of Trypanosomatidae is shown in Table 3. As the Madeira, Azores 2014-2015 and Azores 2020 samples were processed differently, no comparisons were made between those 3 groups. In Madeira the prevalence was 66.3%, whereas in the Azores was 72% in 2014-2015, with the highest number of infected colonies detected on Pico (92%) and the lowest on Graciosa (22.7%). In the later sampling period (2020), 31.5% of the colonies were positive, with the largest percentage detected on Santa Maria (42.9%) and the lowest on São Jorge (16.7%).

**Table 3** Number of colonies and apiaries sampled in each island, number of colonies positive for Trypanosomatidae and corresponding prevalence (%). [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | Apiaries (N) | Colonies (N) | Positive colonies (N) | Prevalence (%) |
|---|---|---|---|---|
| Madeira | 23 | 89 | 59 | 66.3 |
| Azores | | | | |
| Santa Maria[1, 2] | 19 | 57 | 48 | 84.2 |
| São Miguel[1] | 30 | 105 | 84 | 80.0 |
| São Jorge[1] | 17 | 37 | 19 | 51.4 |
| Faial | 20 | 60 | 43 | 71.7 |
| Pico | 25 | 75 | 69 | 92.0 |
| Graciosa[1] | 7 | 22 | 5 | 22.7 |
| Terceira[1] | 26 | 80 | 43 | 53.8 |
| Flores[2] | 15 | 47 | 37 | 78.7 |
| Total | 159 | 483 | 348 | 72.0 |
| **2020** | | | | |
| Santa Maria[1, 2] | 12 | 28 | 12 | 42.9 |
| São Jorge[1] | 10 | 30 | 5 | 16.7 |
| Faial | 2 | 8 | 2 | 25.0 |
| Terceira[1] | 10 | 23 | 9 | 39.1 |
| Total | 34 | 89 | 28 | 31.5 |
| TOTAL | 216 | 661 | 435 | |

## Species identification

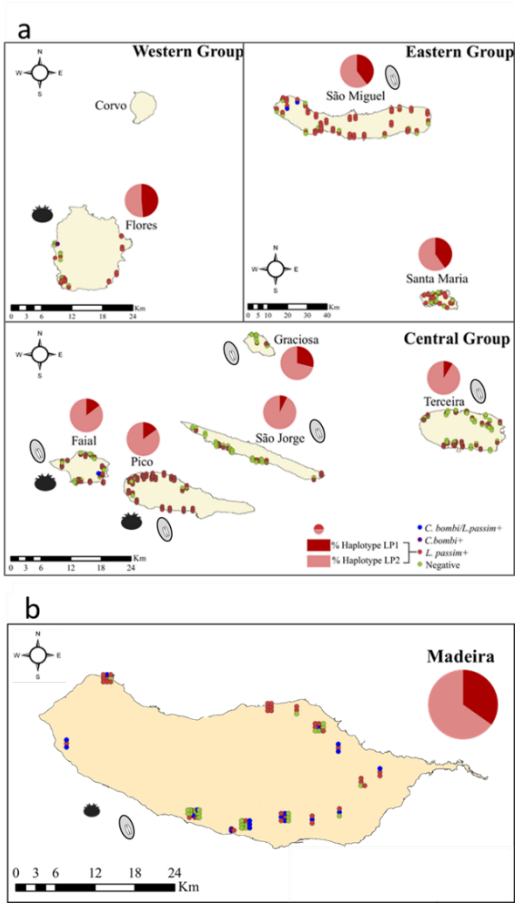
**High-Throughput Sequencing.** A total of 91 samples identified as positive to Trypanosomatidae by the PCR assay were sequenced on the Illumina MiSeq platform. The sequencing run produced 1,385,021 raw reads, which yielded 1,208,665 Trypanosomatidae sequences after filtering, representing an average of 13,282 sequences per sample. Strikingly, identification of all sequence reads revealed that *L. passim* was the only species present in the

dataset. Two main haplotypes were found in all samples and on all islands and accounted for about 98% of the sequences found (Table 4) and these matched the reference sequences MT558272.1 and LT976801.1 (hereafter referred to as Lp1 and Lp2, respectively) downloaded from GenBank. These two haplotypes were found distributed within each island without any defined geographical pattern and they differed by a single nucleotide in the amplicon at position 29, with Lp1 having a T and Lp2, a C (Supp Fig. 1). On average, 72.9% of the reads from all the islands corresponded to the Lp2 haplotype, whereas 25.3% of the sequences corresponded to the Lp1 haplotype. This difference was most notable in samples collected in Madeira and in samples collected in 2014-2015 from all islands of central group, where more than the 70% of sequences corresponded to the Lp2 haplotype (Table 4, Fig. 1). The remaining 2% of sequences were found at a very low frequency and appeared to be variants from the majority haplotypes with additional single nucleotide changes and did not match any sequence available in GenBank. The sequences of the haplotypes found (both Lp1 and Lp2 and those at low frequencies) were deposited in GenBank (accession numbers OR117383-OR117469).

**Table 4** Average number of total and valid reads (sequences with < 5 reads were removed), generated by the MiSeq platform for 91 Trypanosomatidae-positive samples, and proportion of the two main *L. passim* haplotypes (Lp1 and Lp2) detected on each island. [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | **N** | **Total reads** | **Valid reads** | **% Lp1** | **% Lp2** |
|---|---|---|---|---|---|
| Madeira | 6 | 12678 | 11509 | 34.2 | 64.1 |
| Azores | | | | | |
| Santa Maria[1, 2] | 10 | 15809 | 14547 | 39.2 | 59.0 |
| São Miguel[1] | 10 | 12940 | 11812 | 39.5 | 58.8 |
| São Jorge[1] | 7 | 12982 | 11700 | 6.5 | 91.0 |
| Faial | 10 | 12886 | 11883 | 14.5 | 83.6 |
| Pico | 10 | 12642 | 11652 | 14.9 | 83.2 |
| Graciosa[1] | 4 | 12827 | 11725 | 27.9 | 70.9 |
| Terceira[1] | 9 | 12204 | 11152 | 9.0 | 89.3 |
| Flores[2] | 10 | 13256 | 12137 | 47.6 | 50.8 |
| **2020** | | | | | |
| Santa Maria[1, 2] | 5 | 14321 | 13146 | 37.7 | 60.5 |
| São Jorge[1] | 5 | 13459 | 12490 | 19.8 | 78.4 |
| Terceira[1] | 5 | 13261 | 12116 | 12.8 | 84.7 |
| TOTAL | 91 | 13282 | 12168 | 25.3 | 72.9 |

**Fig. 1** Geographical distribution of *C. bombi* and *L. passim* (and its haplotypes) in the Azores archipelago (a) and Madeira (b). indicates the islands where *V. destructor* is present. indicates the islands where *N. ceranae* is present. The red colour in sector charts indicates the percentage of sequences that matched with Lp1 haplotype while the pink colour indicates the percentage of sequences that matched with Lp2 haplotype. ● *C. bombi* + *L. passim*; ● *C. bombi*; ● *L. passim*; ● Negative sample.

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214 **Real-time qPCR species confirmation.** The rest of the Tryp-RPB1 positive samples (n=344) were analysed
215 by qPCR to detect the presence of *L. passim, C. mellificae*, and/or *C. bombi*. At least one of these species was
216 identified in 341 samples, whereas three samples (from Madeira, Santa Maria, and São Miguel) were negative (Table
217 5). No amplification was observed in any of the negative controls.

218 **Table 5** Percentage of each Trypanosomatidae species detected in positive samples. ND: not detected. [1] *Varroa*-free
219 islands; [2] *Nosema*-free islands.

| **2014-2015** | **N** | **Only *L. passim*** | **Only *C. mellificae*** | **Only *C. bombi*** | **Co-occurrence *L. passim/C. bombi*** |
|---|---|---|---|---|---|
| Madeira | 53 | 64.2 | ND | ND | 34 |
| Azores | | | | | |
| Santa Maria[1, 2] | 38 | 97.4 | ND | ND | ND |
| São Miguel[1] | 74 | 95.9 | ND | ND | 2.7 |
| São Jorge[1] | 12 | 100 | ND | ND | ND |
| Faial | 33 | 97 | ND | ND | 3 |
| Pico | 59 | 100 | ND | ND | ND |
| Graciosa[1] | 1 | 100 | ND | ND | ND |
| Terceira[1] | 34 | 100 | ND | ND | ND |
| Flores[2] | 27 | 96.3 | ND | 3.7 | ND |
| Total | 278 | 97,8 | ND | 0.4 | 1.1 |
| **2020** | | | | | |
| Santa Maria[1, 2] | 7 | 100 | ND | ND | ND |

| | | | | | |
|---|---|---|---|---|---|
| Faial | 2 | 100 | ND | ND | ND |
| Terceira[1] | 4 | 100 | ND | ND | ND |
| Total | 13 | 100 | ND | ND | ND |

The qPCR analysis confirmed that *L. passim* was the predominant but not the only species on Madeira and in the Azores, as *C. bombi* was also found on some islands (Table 5). In the first sampling period, infection by *L. passim* was identified alone in 64.2% of the positive samples on Madeira, while *C. bombi* was found in 34% of the positive samples, always together with *L. passim* (Table 5). On São Jorge, Pico, Graciosa, Santa Maria, and Terceira, *L. passim* was the only species detected, whereas *C. bombi* was found as a mono-infection in one colony on Flores (3.7%). Yet, on São Miguel and Faial, this species was always associated with *L. passim* infection. In the samples collected in 2020, *L. passim* was the only Trypanosomatidae detected (Table 5). Finally, *C. mellificae* was not found in any of the samples analysed throughout the study.

Sanger sequencing of the Tryp RPB1 primer amplicons of the three negative samples after qPCR, showed 100% homology with the MT558162.1 sequence of *C. bombi* in the Madeira sample, in the Santa Maria sample the sequence was identical to Lp2, while in the São Miguel sample, two peaks for C/T at position 29 of the amplicon, corresponding to the Lp1 and Lp2 haplotypes, were easily distinguishable.

**Total occurrence of Trypanosomatidae species on Madeira and the Azores**. The occurrence of each Trypanosomatidae species calculated by compiling all the results obtained using the different techniques (HTS, qPCR and Sanger sequencing) is shown in Table 6 and Fig. 1. On Madeira, the proportion of colonies in which Trypanosomatidae were detected was 66.3%, with *L. passim* being the only species detected in 44.9%, *C. bombi* in 1.1%, and both species concomitantly in 20.2% of the samples analysed. In the 2014-2015 samples from the Azores, 348 colonies were positive for Trypanosomatidae (72.0%). *L. passim* was found on all the islands, and it was the only species detected in 71.2% of samples. *C. bombi* was rarely found in the Azores, being the only species detected in one colony on Flores (0.2%) and occurred concurrently with *L. passim* on São Miguel and Faial (0.6%). In the 2020 samples, all the colonies that were positive for Trypanosomatidae (31.5%) were also positive for *L. passim,* and this was the only species found in this sampling period (Table 6). Finally, as previously mentioned, *C. mellificae* was not detected in any of the samples tested.

**Table 6** Prevalence of Trypanosomatidae (%) detected either alone or simultaneously on each island grouping the data from all the tools used (conventional PCR, HTS, qPCR and Sanger sequencing). Number of samples analysed as shown in table 3. ND: not detected. [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | **Trypanosomatidae** | **Only *L. passim*** | **Only *C. mellificae*** | **Only *C. bombi*** | **Co-occurrence *L. passim*/*C. bombi*** |
|---|---|---|---|---|---|
| Madeira | **66.3** | **44.9** | **ND** | **1.1** | **20.2** |
| Azores | | | | | |
| Santa Maria[1, 2] | 84.2 | 84.2 | ND | ND | ND |
| São Miguel[1] | 80.0 | 78.1 | ND | ND | 1.9 |
| São Jorge[1] | 51.4 | 51.4 | ND | ND | ND |
| Faial | 71.7 | 70.0 | ND | ND | 1.7 |
| Pico | 92.0 | 92.0 | ND | ND | ND |
| Graciosa[1] | 22.7 | 22.7 | ND | ND | ND |

| | | | | | |
|---|---|---|---|---|---|
| Terceira[1] | 53.8 | 53.8 | ND | ND | ND |
| Flores[2] | 78.7 | 76.6 | ND | 2.1 | ND |
| Total | **72.0** | **71.2** | **ND** | **0.2** | **0.6** |
| **2020** | | | | | |
| Santa Maria[1, 2] | 42.9 | 42.9 | ND | ND | ND |
| São Jorge[1] | 16.7 | 16.7 | ND | ND | ND |
| Faial | 25.0 | 25.0 | ND | ND | ND |
| Terceira[1] | 39.1 | 39.1 | ND | ND | ND |
| Total | **31.5** | **31.5** | **ND** | **ND** | **ND** |

As *L. passim* was the main species found, the influence of *V. destructor* and *N. ceranae* was only studied for this trypanosomatid. Thus, the prevalence of *L. passim* was significantly higher on the Azores islands (2014-2015 samples) with *V. destructor* (Table 7, regression coefficient=0.77; z value=3.41; $p<0.01$) than on those without the mite. In contrast, on islands where *N. ceranae* was established, the prevalence of *L. passim* was lower (Table 7;regression coefficient= -0.56; z value=-2.03; $p<0.01$). However when island was included as covariable the effect of *N. ceranae* on the presence of *L.passim* became statistically no significant (regression coefficient= -0.29; z value=-0.66; p=0.51) while the significance was kept for *V. destructor* (regression coefficient= 1.06; z value=2.15; p=0.03).

**Table 7** Number (and percentage) of *L. passim*- positive (+) and -negative (-) samples collected in 2014-2015 in the Azores distributed according to whether they were on islands where *V. destructor* or *N. ceranae* were present (+) or not (-).

| | *L. passim* (+) | *L. passim* (-) | Total |
|---|---|---|---|
| *V. destructor* (+) | 148 (81.32%) | 34 (18.68%) | 182 (100%) |
| *V. destructor* (-) | 199 (66.11%) | 102 (33.89%) | 301 (100%) |
| *N. ceranae* (+) | 263 (69.39%) | 116 (30.61%) | 379 (100%) |
| *N. ceranae* (-) | 84 (80.77%) | 20 (19.23%) | 104 (100%) |

# Discussion

The aim of this study was to assess the status of Trypanosomatidae species in honey bee colonies in Madeira and the Azores, and to determine whether the presence of *V. destructor* and *N. ceranae* on some islands influenced their distribution. Our results show that islands with the mite had a significantly higher prevalence of *L. passim*. Other studies had reported an association between them [32] even detecting DNA of this trypanosomatid in the acari, which is still of uncertain biological significance [33]. In contrast, when the island was included as covariable no effect of *N. ceranae* on *L. passim* prevalence was observed as it was previously reported by other authors [14, 34]. However, a positive correlation between infection levels in colonies with both parasites has been also described [35].

Despite these differences related to the presence of *V. destructor* and *N. ceranae*, it is clear that trypanosomatids are well established in the Azores and Madeira, so that the strong restrictions on the introduction of honey bees on the *Varroa*-free islands of the Azores have not prevented the presence of trypanosomatids. Furthermore, *L. passim* is the main species found and the only identified in most of the colonies analysed. Interestingly, *C. mellificae*, the species associated with honey bees until the discovery of *L. passim* [8, 12] is not present in the territories analysed or is below

our detection limit. Neither *C. acanthocephali* nor *C. expoeki* were detected in this study, although they could only have been confirmed by HTS as they were not analysed by qPCR.

This scenario where *L. passim* is the main trypanosomatid species in honey bee colonies seems to be repeated worldwide [13, 14, 28, 32, 35–39]. Given the high prevalence found herein and elsewhere, it is likely that *L. passim*, rather than *C. mellificae*, was the primary species infecting honey bees in studies conducted prior to its discovery [9–12]. Indeed, the frequencies observed and the absence of *C. mellificae* in many areas of the world corroborate the hypothesis that *L. passim* is a common parasite of *Apis mellifera* and that it is currently the dominant trypanosomatid species in adult worker bees [8, 13, 28, 39, 40]. On the other hand, *C. bombi* was identified in three islands and almost always coexisted with *L. passim* although it also appeared alone in some colonies. These results further confirm previous findings [41, 42], in which *C. bombi* was detected in honey bees.

How trypanosomatids entered the Azores is still unclear since the islands are located far from the mainland, the natural arrival of bees is not possible, so it was very probably a human-made introduction. Although it could be that Pico was the point of entry, as it hosts the highest number of trypanosomatid-positive colonies and was also the point of entry of *V. destructor* into the Azores, the data from Santa Maria, where neither *V. destructor* nor *Nosema* spp. are present [25] but where *L. passim* was detected in a large proportion of the colonies, do not support this hypothesis. The widespread distribution of *L. passim* on the Azorean islands that remain free of *V. destructor* strongly suggests that this species has been associated with *A. mellifera* since before the worldwide spread of the mite. A similar scenario was found in Hawaii archipelago, in which still has some islands where the mite is not present and where *L. passim* has been detected [19]. In New Zealand (*Varroa*-free until 2000), significant colony losses began to be observed in 2014 and an analysis of the surviving colonies revealed that most were infected with *L. passim* [15, 17, 18]. On the other hand, the presence of bumblebees in Madeira [43] and in the Azores [44] could explain the presence of *C. bombi* in honey bees due to feeding on common floral resources. [45–47].

Our study supports that both sediment and supernatant phases are suitable for trypanosomatid detection. Also, the use of different technologies was key to identify all the species present in this area of Macaronesia. Thus, on the one hand, HTS technology allowed us to detect the haplotypes present but not all the species, as the cost of this tool limited the number of samples analysed (n= 91). On the other hand, the use of qPCR served to complement the analysis by allowing us to analyse the remaining Trypanosomatid-positive samples (n= 344), which served to identify *C. bombi* present in lower prevalence. There is little data available on the population genetics of the *rpb1* in the trypanosomatids that infect honey bees or bumble bees. Recently, this gene was analysed and genetic polymorphisms were observed between *C. mellificae*, *C. bombi*, and *L. passim* [26, 48, 49]. Our results are consistent with those obtained for the RPB1 gene in these three studies, indicating that there were two core haplotypes differing in only one nucleotide. It cannot be ruled out that the sequences found at low frequencies correspond to true rare haplotypes, although further experimental confirmation would be needed. Other genes like *18S rRNA*, *28S rRNA*, and *its-2* have been studied to evaluate intraspecific variation, and their sequences were almost identical between the different strains of *L. passim*, with the exception of the *virulence factor gp63*, which seems to have a variable stretch between strains [40]. Therefore, it is possible that the *rpb1* does not have sufficient variation to distinguish strains, but that this may occur in other genes.

In conclusion, despite geographical isolation, trypanosomatids frequently infect colonies in Madeira and the Azores, even when there are restrictions on honeybee movements to mite-free islands. *L. passim* is the predominant Trypanosomatidae species, occurring as the only species in many colonies and showing a higher prevalence on islands

with *V. destructor* and lower prevalence on islands with *N. ceranae*. Additionally, *C. bombi* also occurs, often co-occurring with *L. passim*. The detection of *L. passim* and *C. bombi* in areas where *V. destructor* and *N. ceranae* are not present suggests that have been associated with *A. mellifera* for much longer than originally thought.

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**Author contributions**

Conceptualization: R.M.-H., M.A.P., and M.H.; methodology: all authors; formal analysis; all authors; investigation: D.A-L., A.R.L. and S.K.S.; data curation: D.A.L.; writing - original draft preparation: D.A-L. and R.M.-H.; writing - review and editing; D.A-L., R.M.-H., C.B, X.M, M.A.P. and M.H.; supervision: R.M.-H.; project administration and funding acquisition: R.M.-H and M.A.P; All authors have read and agreed to the published version of the manuscript.

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**Competing interests**

The authors declare no competing interests.

**Figure Caption**

**Fig. 1** Geographical distribution of *C. bombi* and *L. passim* (and its haplotypes) in the Azores archipelago (a) and Madeira (b). indicates the islands where *V. destructor* is present. indicates the islands where *N. ceranae* is present. The red colour in sector charts indicates the percentage of sequences that matched with Lp1 haplotype while the pink colour indicates the percentage of sequences that matched with Lp2 haplotype. ● *C. bombi* + *L. passim*; ● *C. bombi*; ● *L. passim*; ● Negative sample.

**Table 1** Primers and probes used for the molecular analysis of the honey bee samples.

| References | Target | Primers | Sequence 5' – 3' |
|---|---|---|---|
| [26] | Trypanosomatidae | Tryp RPB1-F1 | GTGGCTGGAYCTGTGGGAGC |
| | | Tryp RPB1-R1 | GCCRTTGATGAACTTCGCCAC |
| [30] | *C. mellificae* | qCmell_Cytb_F | TTTTGCCATGCACTATGATGTCT |
| | | qCmell_Cytb_R | AACCTATTACAGGCACAGTTGCTAAA |
| | | qCmell_Cytb_P | 6FAM-ATTGAGGATTAACAGTGTTTAGT-BHQ1 |
| | *L. passim* | qLpass_TOPII_F | GGCCATGGAAATACTCGAGTCT |
| | | qLpass_TOPII_R | ACCTTGCCTTCCTTCTTGAGATT |
| | | qLpass_TOPII_P | 6FAM-CCTCGACACGC+T+TA+GT-BHQ1 |
| | *C. bombi* | qCbom_RPB1_F | TGGTGGGTGCGATTACGAA |
| | | qCbom_RPB1_R | TCATTGAAGATGACGTGGATAAGC |
| | | qCbom_RPB1_P | 6FAM-CGTTGTCGGCGCCG-BHQ1 |

**Table 2** Number (and percentage) of Trypanosomatidae-positive (+) and -negative (-) samples detected on the supernatant and sediment phases obtained from the honey bee macerates.

| | Supernatant (+) | Supernatant (-) | **Total** |
|---|---|---|---|
| Sediment (+) | 161 (33.75%) | 81 (16.98%) | **242 (50.73%)** |
| Sediment (-) | 103 (21.59%) | 132 (27.67%) | **235 (49.27%)** |
| **Total** | **264 (55.34%)** | **213 (44.65%)** | **477 (100%)** |

**Table 3** Number of colonies and apiaries sampled in each island, number of colonies positive for Trypanosomatidae and corresponding prevalence (%). [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | Apiaries (N) | Colonies (N) | Positive colonies (N) | Prevalence (%) |
|---|---|---|---|---|
| Madeira | 23 | 89 | 59 | 66.3 |
| Azores | | | | |
| Santa Maria[1, 2] | 19 | 57 | 48 | 84.2 |
| São Miguel[1] | 30 | 105 | 84 | 80.0 |
| São Jorge[1] | 17 | 37 | 19 | 51.4 |
| Faial | 20 | 60 | 43 | 71.7 |
| Pico | 25 | 75 | 69 | 92.0 |
| Graciosa[1] | 7 | 22 | 5 | 22.7 |
| Terceira[1] | 26 | 80 | 43 | 53.8 |
| Flores[2] | 15 | 47 | 37 | 78.7 |
| Total | 159 | 483 | 348 | 72.0 |
| **2020** | | | | |
| Santa Maria[1, 2] | 12 | 28 | 12 | 42.9 |
| São Jorge[1] | 10 | 30 | 5 | 16.7 |
| Faial | 2 | 8 | 2 | 25.0 |
| Terceira[1] | 10 | 23 | 9 | 39.1 |
| Total | 34 | 89 | 28 | 31.5 |
| TOTAL | 216 | 661 | 435 | |

**Table 4** Average number of total and valid reads (sequences with < 5 reads were removed), generated by the MiSeq platform for 91 Trypanosomatidae-positive samples, and proportion of the two main *L. passim* haplotypes (Lp1 and Lp2) detected on each island. [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | **N** | **Total reads** | **Valid reads** | **% Lp1** | **% Lp2** |
|---|---|---|---|---|---|
| Madeira | 6 | 12678 | 11509 | 34.2 | 64.1 |
| Azores | | | | | |
| Santa Maria[1, 2] | 10 | 15809 | 14547 | 39.2 | 59.0 |
| São Miguel[1] | 10 | 12940 | 11812 | 39.5 | 58.8 |
| São Jorge[1] | 7 | 12982 | 11700 | 6.5 | 91.0 |
| Faial | 10 | 12886 | 11883 | 14.5 | 83.6 |
| Pico | 10 | 12642 | 11652 | 14.9 | 83.2 |
| Graciosa[1] | 4 | 12827 | 11725 | 27.9 | 70.9 |
| Terceira[1] | 9 | 12204 | 11152 | 9.0 | 89.3 |
| Flores[2] | 10 | 13256 | 12137 | 47.6 | 50.8 |
| **2020** | | | | | |
| Santa Maria[1, 2] | 5 | 14321 | 13146 | 37.7 | 60.5 |
| São Jorge[1] | 5 | 13459 | 12490 | 19.8 | 78.4 |
| Terceira[1] | 5 | 13261 | 12116 | 12.8 | 84.7 |
| TOTAL | 91 | 13282 | 12168 | 25.3 | 72.9 |

**Table 5** Percentage of each Trypanosomatidae species detected in positive samples. ND: not detected. [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | **N** | **Only *L. passim*** | **Only *C. mellificae*** | **Only *C. bombi*** | **Co-occurrence *L. passim/C. bombi*** |
|---|---|---|---|---|---|
| Madeira | 53 | 64.2 | ND | ND | 34 |
| Azores | | | | | |
| Santa Maria[1, 2] | 38 | 97.4 | ND | ND | ND |
| São Miguel[1] | 74 | 95.9 | ND | ND | 2.7 |
| São Jorge[1] | 12 | 100 | ND | ND | ND |
| Faial | 33 | 97 | ND | ND | 3 |
| Pico | 59 | 100 | ND | ND | ND |
| Graciosa[1] | 1 | 100 | ND | ND | ND |
| Terceira[1] | 34 | 100 | ND | ND | ND |
| Flores[2] | 27 | 96.3 | ND | 3.7 | ND |
| Total | 278 | 97,8 | ND | 0.4 | 1.1 |
| **2020** | | | | | |
| Santa Maria[1, 2] | 7 | 100 | ND | ND | ND |
| Faial | 2 | 100 | ND | ND | ND |
| Terceira[1] | 4 | 100 | ND | ND | ND |
| Total | 13 | 100 | ND | ND | ND |

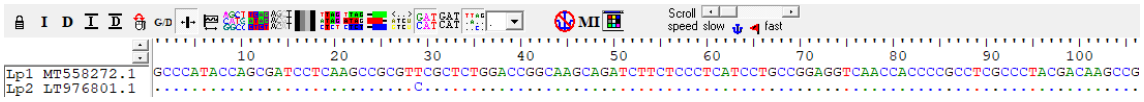
**Table 6** Prevalence of Trypanosomatidae (%) detected either alone or simultaneously on each island grouping the data from all the tools used (conventional PCR, HTS, qPCR and Sanger sequencing). Number of samples analysed as shown in table 3. ND: not detected. [1] *Varroa*-free islands; [2] *Nosema*-free islands.

| **2014-2015** | **Trypanosomatidae** | **Only *L. passim*** | **Only *C. mellificae*** | **Only *C. bombi*** | **Co-occurrence *L. passim*/*C. bombi*** |
|---|---|---|---|---|---|
| Madeira | **66.3** | **44.9** | **ND** | **1.1** | **20.2** |
| Azores | | | | | |
| Santa Maria[1, 2] | 84.2 | 84.2 | ND | ND | ND |
| São Miguel[1] | 80.0 | 78.1 | ND | ND | 1.9 |
| São Jorge[1] | 51.4 | 51.4 | ND | ND | ND |
| Faial | 71.7 | 70.0 | ND | ND | 1.7 |
| Pico | 92.0 | 92.0 | ND | ND | ND |
| Graciosa[1] | 22.7 | 22.7 | ND | ND | ND |
| Terceira[1] | 53.8 | 53.8 | ND | ND | ND |
| Flores[2] | 78.7 | 76.6 | ND | 2.1 | ND |
| Total | **72.0** | **71.2** | **ND** | **0.2** | **0.6** |
| **2020** | | | | | |
| Santa Maria[1, 2] | 42.9 | 42.9 | ND | ND | ND |
| São Jorge[1] | 16.7 | 16.7 | ND | ND | ND |
| Faial | 25.0 | 25.0 | ND | ND | ND |
| Terceira[1] | 39.1 | 39.1 | ND | ND | ND |
| Total | **31.5** | **31.5** | **ND** | **ND** | **ND** |

**Table 7** Number (and percentage) of *L. passim*- positive (+) and -negative (-) samples collected in 2014-2015 in the Azores distributed according to whether they were on islands where *V. destructor* or *N. ceranae* were present (+) or not (-).

| | *L. passim* (+) | *L. passim* (-) | Total |
|---|---|---|---|
| *V. destructor* (+) | 148 (81.32%) | 34 (18.68%) | 182 (100%) |
| *V. destructor* (-) | 199 (66.11%) | 102 (33.89%) | 301 (100%) |
| | | | |
| *N. ceranae* (+) | 263 (69.39%) | 116 (30.61%) | 379 (100%) |
| *N. ceranae* (-) | 84 (80.77%) | 20 (19.23%) | 104 (100%) |

**Supplementary Fig. 1** One nucleotide variation (T/C) in the *rpb1* gene sequence between *L. passim* haplotypes MT558272.1 and LT976801.1 at position 29. Marks and numbering indicate the position in the sequence in base pairs. A: adenine; G: guanine; C: cytosine; T: thymine.




**Supplementary Table 1** Number of total and valid reads (sequences with < 5 reads were removed), generated by the MiSeq platform for 91 Trypanosomatidae-positive samples, and number of reads and proportion of the two main *L. passim* haplotypes (Lp1 and Lp2) detected in each sample.

| Island | Sample ID | Total reads | Valid reads | Lp1 reads | Lp2 reads | % Lp1 | % Lp2 |
|---|---|---|---|---|---|---|---|
| Santa Maria | PA21-0509 | 12039 | 11019 | 5201 | 5720 | 47,20 | 51,91 |
| Santa Maria | PA21-0525 | 18735 | 16918 | 4419 | 11755 | 26,12 | 69,48 |
| Santa Maria | PA21-0527 | 12072 | 11012 | 5264 | 5674 | 47,80 | 51,53 |
| Santa Maria | PA21-0531 | 16157 | 15029 | 6943 | 7912 | 46,20 | 52,64 |
| Santa Maria | PA21-0535 | 12602 | 11754 | 2959 | 8698 | 25,17 | 74,00 |
| S. Jorge | PA21-0480 | 13958 | 13019 | 944 | 11812 | 7,25 | 90,73 |
| S. Jorge | PA21-0488 | 12718 | 11906 | 1694 | 10075 | 14,23 | 84,62 |
| S. Jorge | PA21-0490 | 15894 | 14587 | 7367 | 7008 | 50,50 | 48,04 |
| S. Jorge | PA21-0491 | 12269 | 11332 | 2242 | 8901 | 19,78 | 78,55 |
| S. Jorge | PA21-0494 | 12458 | 11607 | 125 | 11150 | 1,08 | 96,06 |
| Terceira | PA21-0537 | 10726 | 9775 | 2497 | 7105 | 25,54 | 72,69 |
| Terceira | PA21-0538 | 14870 | 13605 | 2081 | 11182 | 15,30 | 82,19 |
| Terceira | PA21-0541 | 13287 | 12163 | 1348 | 10576 | 11,08 | 86,95 |
| Terceira | PA21-0542 | 14307 | 12997 | 270 | 12173 | 2,08 | 93,66 |
| Terceira | PA21-0544 | 13117 | 12041 | 1563 | 10253 | 12,98 | 85,15 |
| Faial | PA15-0863 | 11424 | 10618 | 1518 | 8954 | 14,30 | 84,33 |
| Faial | PA15-0864 | 13783 | 12834 | 2087 | 10576 | 16,26 | 82,41 |
| Faial | PA15-0870 | 12342 | 11404 | 2191 | 9096 | 19,21 | 79,76 |
| Faial | PA15-0872 | 9894 | 9135 | 1138 | 7896 | 12,46 | 86,44 |
| Faial | PA15-0877 | 11533 | 10508 | 1378 | 9025 | 13,11 | 85,89 |
| Faial | PA15-1029 | 13727 | 12636 | 113 | 12076 | 0,89 | 95,57 |
| Faial | PA15-1042 | 13420 | 12454 | 2020 | 10285 | 16,22 | 82,58 |
| Faial | PA15-1056 | 14633 | 13581 | 2287 | 10976 | 16,84 | 80,82 |
| Faial | PA15-1057 | 14632 | 13268 | 2412 | 10465 | 18,18 | 78,87 |
| Faial | PA15-1059 | 13472 | 12387 | 2085 | 10012 | 16,83 | 80,83 |
| Flores | PA15-0920 | 12803 | 11310 | 5266 | 5585 | 46,56 | 49,38 |
| Flores | PA15-0924 | 14020 | 12888 | 5292 | 7414 | 41,06 | 57,53 |
| Flores | PA15-0929 | 12076 | 11128 | 6440 | 4579 | 57,87 | 41,15 |
| Flores | PA15-0936 | 10924 | 10004 | 4661 | 5270 | 46,59 | 52,68 |
| Flores | PA15-0938 | 14805 | 13649 | 6257 | 7244 | 45,84 | 53,07 |
| Flores | PA15-0989 | 12519 | 11581 | 5190 | 6250 | 44,81 | 53,97 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Flores | PA15-1075 | 17601 | 15947 | 7542 | 7902 | 47,29 | 49,55 |
| Flores | PA15-1078 | 12548 | 11548 | 5330 | 6140 | 46,16 | 53,17 |
| Flores | PA15-1091 | 11560 | 10658 | 5620 | 4959 | 52,73 | 46,53 |
| Flores | PA15-1093 | 13705 | 12652 | 6212 | 6297 | 49,10 | 49,77 |
| Graciosa | PA14-0567 | 10669 | 9602 | 3926 | 5583 | 40,89 | 58,14 |
| Graciosa | PA14-0580 | 12958 | 11986 | 1612 | 10149 | 13,45 | 84,67 |
| Graciosa | PA14-0596 | 13417 | 12238 | 2908 | 9220 | 23,76 | 75,34 |
| Graciosa | PA14-0598 | 14265 | 13073 | 4660 | 8276 | 35,65 | 63,31 |
| Madeira | PA14-0464 | 15603 | 14056 | 3754 | 9980 | 26,71 | 71,00 |
| Madeira | PA14-0488 | 10430 | 9419 | 3135 | 6172 | 33,28 | 65,53 |
| Madeira | PA14-0491 | 6882 | 6181 | 1857 | 4259 | 30,04 | 68,90 |
| Madeira | PA14-0495 | 13119 | 11977 | 3599 | 8224 | 30,05 | 68,66 |
| Madeira | PA14-0519 | 12436 | 11290 | 5803 | 5279 | 51,40 | 46,76 |
| Madeira | PA14-0528 | 17599 | 16132 | 5494 | 10364 | 34,06 | 64,24 |
| Pico | PA15-0875 | 11219 | 10221 | 1414 | 8722 | 13,83 | 85,33 |
| Pico | PA15-0940 | 13062 | 12195 | 2127 | 9934 | 17,44 | 81,46 |
| Pico | PA15-0960 | 13485 | 12594 | 1684 | 10693 | 13,37 | 84,91 |
| Pico | PA15-0985 | 13477 | 12553 | 1568 | 10728 | 12,49 | 85,46 |
| Pico | PA15-1028 | 11531 | 10731 | 1134 | 9451 | 10,57 | 88,07 |
| Pico | PA15-1030 | 12541 | 11637 | 1726 | 9685 | 14,83 | 83,23 |
| Pico | PA15-1031 | 13125 | 12196 | 2616 | 9436 | 21,45 | 77,37 |
| Pico | PA15-1033 | 13936 | 12285 | 1841 | 9795 | 14,99 | 79,73 |
| Pico | PA15-1034 | 11892 | 10962 | 1545 | 9266 | 14,09 | 84,53 |
| Pico | PA15-1038 | 12148 | 11141 | 1649 | 9275 | 14,80 | 83,25 |
| S. Jorge | PA14-0581 | 10102 | 9082 | 1606 | 7331 | 17,68 | 80,72 |
| S. Jorge | PA14-0587 | 14000 | 12777 | 1954 | 10594 | 15,29 | 82,91 |
| S. Jorge | PA14-0718 | 11453 | 9233 | 1103 | 7629 | 11,95 | 82,63 |
| S. Jorge | PA14-0739 | 14574 | 13495 | 198 | 12960 | 1,47 | 96,04 |
| S. Jorge | PA15-0836 | 13272 | 12015 | 243 | 11439 | 2,02 | 95,21 |
| S. Jorge | PA15-0853 | 13893 | 12721 | 112 | 12290 | 0,88 | 96,61 |
| S. Jorge | PA15-0855 | 13579 | 12577 | 102 | 12275 | 0,81 | 97,60 |
| Santa Maria | PA15-0927 | 17532 | 16271 | 6168 | 9822 | 37,91 | 60,37 |
| Santa Maria | PA15-0937 | 18502 | 17119 | 5027 | 11798 | 29,37 | 68,92 |
| Santa Maria | PA15-0979 | 14274 | 13058 | 5243 | 7562 | 40,15 | 57,91 |
| Santa Maria | PA15-0995 | 17258 | 15823 | 6266 | 9238 | 39,60 | 58,38 |
| Santa Maria | PA15-0996 | 14804 | 13658 | 6684 | 6788 | 48,94 | 49,70 |
| Santa Maria | PA15-1086 | 14047 | 12892 | 4858 | 7771 | 37,68 | 60,28 |
| Santa Maria | PA15-1088 | 11184 | 9914 | 4613 | 5027 | 46,53 | 50,71 |
| Santa Maria | PA15-1094 | 17249 | 16019 | 6792 | 8938 | 42,40 | 55,80 |
| Santa Maria | PA15-1099 | 15062 | 13975 | 4225 | 9580 | 30,23 | 68,55 |
| Santa Maria | PA15-1104 | 18180 | 16745 | 7122 | 9336 | 42,53 | 55,75 |
| São Miguel | PA14-0607 | 14082 | 12785 | 5826 | 6831 | 45,57 | 53,43 |
| São Miguel | PA14-0609 | 17975 | 16556 | 7571 | 8859 | 45,73 | 53,51 |
| São Miguel | PA14-0615 | 10814 | 9829 | 4465 | 5317 | 45,43 | 54,10 |
| São Miguel | PA14-0639 | 10711 | 9689 | 3501 | 6138 | 36,13 | 63,35 |
| São Miguel | PA14-0640 | 12565 | 11346 | 4201 | 7018 | 37,03 | 61,85 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| São Miguel | PA14-0654 | 11430 | 10471 | 4027 | 6372 | 38,46 | 60,85 |
| São Miguel | PA14-0655 | 13251 | 11990 | 5357 | 6427 | 44,68 | 53,60 |
| São Miguel | PA14-0662 | 12522 | 11597 | 5171 | 6339 | 44,59 | 54,66 |
| São Miguel | PA14-0663 | 14308 | 12970 | 4568 | 7363 | 35,22 | 56,77 |
| São Miguel | PA14-0760 | 11746 | 10884 | 1989 | 8792 | 18,27 | 80,78 |
| Terceira | PA14-0558 | 11451 | 10482 | 576 | 9757 | 5,50 | 93,08 |
| Terceira | PA14-0561 | 12345 | 11291 | 113 | 11004 | 1,00 | 97,46 |
| Terceira | PA14-0583 | 10288 | 9394 | 94 | 9136 | 1,00 | 97,25 |
| Terceira | PA14-0603 | 13116 | 11586 | 1429 | 9864 | 12,33 | 85,14 |
| Terceira | PA14-0652 | 11762 | 10781 | 2408 | 8290 | 22,34 | 76,89 |
| Terceira | PA14-0670 | 12194 | 11220 | 362 | 10668 | 3,23 | 95,08 |
| Terceira | PA14-0687 | 14193 | 13110 | 803 | 12096 | 6,13 | 92,27 |
| Terceira | PA14-0699 | 12132 | 11237 | 2225 | 8927 | 19,80 | 79,44 |
| Terceira | PA14-0720 | 12351 | 11267 | 1073 | 9884 | 9,52 | 87,73 |

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